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FACULTAD DE FARMACIA

DEPARTAMIENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA

Evaluation of the effects of radiation on the chemical composition and bioactivity of the plants used in the pharmaceutical and/or food industries

DOCTORAL THESIS

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Celestino Santos Buelga, Catedrático de Nutrición y Bromatología de la Universidade de Salamanca, Isabel Cristina Fernandes Rodrigues Ferreira, Professora Coordinatora con Agregación del Instituto Politécnico de Bragança (Portugal) y Albino António Bento, Professor Coordinator Principal), directores del trabajo "Evaluation of the effects of radiation on the chemical composition and bioactivity of the plants used in the pharmaceutical and/or food industries", realizado por Eliana Andreia Pires Castilho Pereira para optar al título de Mención Doctorado Internacional, AUTORIZAN la presentación del mismo al considerer que se han alcanzado los objetivos inicialmente previstos.

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Celestino Santos-Buelga

Isabel C. F. R. Ferreira

Albino António Bento

The studies developed within this PhD thesis led to the following articles, copies of wich are included as annexes in the present memory:

[1] Eliana Pereira, Lillian Barros, Isabel C.F.R. Ferreira. Chemical characterization of Ginkgo biloba L. and antioxidantproperties of its extracts and dietary supplements. Industrial Crops and Products, 2013, 51, 244– 248.

[2] Eliana Pereira, Lillian Barros, Ricardo C. Calhelha, Montserrat Dueñas, Ana Maria Carvalho, Celestino Santos-Buelga*, Isabel C.F.R. Ferreira. Bioactivity and phytochemical characterization of *Arenaria montana* L. Food & Fucntion, 2014, 5, 1848-1855.

[3] Eliana Pereira, Lillian Barros, Amilcar Antonio, Albino Bento, Isabel C.F.R. Ferreira. Analytical methods applied to assess the effects of gamma irradiation on color, chemical composition and antioxidant activity of Ginkgo biloba L. Food Analytical Methods, 2015, 8, 154-163.

[4] Eliana Pereira, Amilcar L. Antonio, João C.M. Barreira, Lillian Barros, Albino Bento, Isabel C.F.R. Ferreira. Gamma irradiation as a practical alternative to preserve the chemical and bioactive wholesomeness of widely used aromatic plants. Food Research International, 2015, 67, 338-348.

[5] Eliana Pereira, Lillian Barros, Montserrat Dueñas, Amilcar L. António, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Gamma irradiation improves the extractability of phenolic compounds in Ginkgo biloba L. Industrial Crops and Products, 2015, 74, 144–149.

[6] Eliana Pereira, Amilcar L. Antonio, Andrzej Rafalski, João C.M. Barreira, Lillian Barros, Isabel C.F.R. Ferreira. Extending the use of irradiation to preserve chemical and bioactive properties of medicinal and aromatic plants: a case study with four species submitted to electron beam. Industrial Crops and Products, 2015, 77, 972-982

[7] Eliana Pereira, Andreia I. Pimenta, Ricardo C. Calhelha, Amilcar L. Antonio, Sandra Cabo Verde, Lillian Barros, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Effects of

gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Mentha x piperita* L.. LWT - Food Science and Technology, 2016, 71, 370-377.

[8] Eliana Pereira, Lillian Barros, Amilcar L. Antonio, Sandra Cabo Verde, Celestino Santos-Buelga, Isabel C.F.R. Ferreira. Infusions from *Thymus vulgaris* L. treated at different gamma radiation doses: Effects on antioxidant activity and phenolic composition. LWT - Food Science and Technology, 2016, 74, 34-39.

[9] Eliana Pereira, Lillian Barros, João C.M. Barreira, Ana Maria Carvalho, Amilcar L. Antonio, Isabel C.F.R. Ferreira. Electron beam and gamma irradiation as feasible conservation technologies for wild *Arenaria montana* L.: effects on chemical and antioxidant parameters. Innovative Food Science & Emerging Technologies, 2016, 36, 269-276.

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[1] Eliana Pereira, Lillian Barros, Amilcar L. Antonio, Sandra Cabo Verde, Celestino Santos-Buelga, Isabel C.F.R. Ferreira, Paula Rodrigues. Is gamma radiation a suitable preservation and mycotoxin decontamination technique in aromatic plants? A case-study with *Aloysia citrodora* Paláu; Submitted to: Food Chemistry.

[2] Eliana Pereira, Amilcar L. Antonio, Andrzej Rafalski, João C.M. Barreira, Lillian Barros, M. Beatriz P.P. Oliveira, Isabel C.F.R. Ferreira. Electron-beam irradiation as an alternative to preserve nutritional, chemical and antioxidant properties of dried plants during extended storage periods; Submitted to Food Chemistry.

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“We are what we repeatedly do. Excellence, therefore, is not an act but a habit”

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Friedrich Nietzsche

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“Virtue is the first title of nobility; I do not pay much attention to the name of this or that person, but rather to their actions.”

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“To join is a beginning, staying together is a process and working together is a success.”

Henry Ford

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“The father and mother love is the fuel that enables an ordinary human being to do the impossible.”

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ABBREVIATIONS

a^*	Greenness-redness
A	Adenine
ADA	American Dietetic Association
ADIs	Acceptable Daily Intakes
AFB_1	Aflatoxin B_1
AFs	Aflatoxins
AIEA	International Atomic Energy Agency
AMA	American Medical Association
ANOVA	Analysis of variance
AOAC	Association of analytical communities
ASTM	American Society for Testing and Materials
b^*	Blueness-yellowness
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BRESA	Herbarium of the Escola Superior Agrária de Bragança
C	Cytosine
CAC	Codex Alimentarius commission
CDC	Centers for Disease Control and Prevention
CE	Catechin equivalents
CE	Collision energy
CEN	European Committee for Standardisation
CES	Collision energy spread
Co	Cobalt
CoQ10	Coenzime Q10
Cs	Cesium
DAD	Diode array detector
D_{max}	Maximum dose
DMEM	Dubelcco's modified eagle medium
D_{min}	Minimum dose
DNA	Deoxyribonucleic acid
DP	Declustering potential
DPPH	2,2-diphenil-1-picrylhydrazyl

DUR	Dose uniformity ratio
EB	Electron beam radiation
EC_{50}	Concentration with 50% of antioxidant activity
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMS	Enhanced mass spectrum
EN	European standards
EP	Enhanced potential
EPI	Enhanced product ion
ESI	Electrospray Ionization
EU	European Union
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FBS	Fetal bovine serum
FDA	Food and Drug Administration
Fe	Iron
FID	Flame ionization detector
FLD	Fluorescence detector
G	Guanine
GAE	Gallic acid equivalents
GC	Gas chromatography
GI_{50}	Concentration that inhibits 50% of cell growth
GR	Gamma radiation
HBSS	Hank’s balanced salt solution
HPLC	High Performance Liquid Chromatography
HSCCC	High Speed Countercurrent Chromatography
HSD	Honest significant difference
IAC	Immunoaffinity column
IAEA	International Atomic Energy Agency
IFT	Institute of Food Technologists
INCT	Institute of Nuclear Chemistry and Technology
IS	Internal standard

kGy	Kilogray
L^*	Lightness
LAE	Aqueous extract of dry leaves
LC	Liquid chromatography
LDA	Linear discriminant analysis
LI	Dry leaves
LME	Methanolic extract from dry leaves
LOD	Limit of detection
LOQ	Limit of quantification
LOX	Lipoxygenase
MDA	Malondialdehyde
MS	Mass spectrometer
MUFA	Monounsaturated fatty acid
nd	Not detected
NIRS	Near Infrared Spectrometry
NMR	Nuclear Magnetic Resonance
OTA	Ochratoxin A
P	Pills
PA	Proanalysis
PBS-T	Phosphate buffer saline with tween
PCA	Principal components analysis
PDA	Photo-diode array detector
PG	Propyl gallate
PUFA	Polyunsaturated fatty acid
RI	Refraction index
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RSA	Radical scavenging activity
Rt	Retention time
S	Syrup
SD	Standard deviation
SFA	Saturated fatty acid

SRB	Sulforhodamine B
ST	Storage time
T	Thymine
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tert-butylhydroquinone
TCA	Trichloroacetic acid
TI	Type of irradiation
TLC	Thin Layer Chromatography
tr	Traces
UFLC	Ultra-Fast Liquid Chromatography
UK	United Kingdom
UPLC	Ultra Performance Liquid Chromatography
USA	United States of America
UV	Ultraviolet
VIS	Visible
WB	Wide band
WHO	World Health Organization

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ABSTRACT

Medicinal plants and their extracts or isolated compounds have various applications, especially as food additives and as health promoters, as nutraceuticals and ingredients in formulations of functional foods, for pharmaceutical and food industries. However, medicinal plants may be contaminated by soil, water, air and powder, not only during the growth process but also during harvest and drying. Microbial contaminations reduce their quality and shelf life and pose a threat to public health, as they may involve the presence of pathogenic bacteria.

Irradiation is a simple, modern and clean physical non-thermal method of processing that allows significant reduction in the microbial load, which is being increasingly applied as a feasible technology for different purposes: disinfestation, decontamination, sterilization or shelf-life extension of food products. It is authorized in several countries, including the European Union (Directive 1999/2/EC).

In the present work, the feasibility of using gamma and e-beam (EB) radiation for preservation and decontamination of several aromatic and medicinal dried plants was evaluated, verifying that the main physico-chemical characteristics and relevant molecules were, in general, satisfactorily preserved, as also microbiological safety.

The nutritional value was determined by official methodologies for food analysis; free sugars were analysed by HPLC-RI, fatty acids by GC-FID, organic acids by UFLC-DAD, tocopherols by HPLC-fluorescence, and phenolic compounds by HPLC-DAD-ESI/MS. The antioxidant properties were evaluated through free radicals scavenging activity, reducing power and inhibition of lipid peroxidation in brain homogenates assays. Cytotoxic properties were tested in tumor (MCF-7, NCI-H460, HeLa and HepG2) and non-tumor (PLP2) cell lines. Mycotoxin levels were determined by HPLC-fluorescence after immunoaffinity column (IAC) cleanup.

Irradiation proved to produce heterogeneous effects on chemical, nutritional and bioactive variables, preserving or degrading molecules, and also increasing the bioavailability of some compounds. The preservation of sensible compounds over time (12 to 18 months) was not observed equally in all species submitted to irradiation, however, in general, EB stood out as the best processing option for the studied materials.

RESUMEN

Las plantas medicinales y sus extractos o compuestos aislados tienen diversas aplicaciones, especialmente como aditivos alimentarios y como promotores de salud, como nutracéuticos e ingredientes en formulaciones de alimentos funcionales, para las industrias farmacéutica y alimentaria. No obstante, estas matrices pueden contaminarse tanto durante su desarrollo, como en la cosecha o el posterior procesado por el suelo, por microorganismos presentes en el agua, el aire o el polvo. Las contaminaciones microbianas reducen la calidad y el tiempo de vida del alimento y constituyen una amenaza para la salud pública por la posible presencia de microorganismos patógenos. La irradiación es un proceso físico no térmico, simple, moderno y limpio, autorizado en varios países, incluida la Unión Europea (Directiva 1999/2/CE), que permite reducir significativamente la carga microbiana. Esta tecnología está siendo utilizada como método viable de procesamiento para la desinfestación, descontaminación, esterilización o extensión de la vida útil en productos alimenticios.

En este trabajo, se evaluó la viabilidad del uso de la irradiación por haces de electrones (EB) y rayos gamma para la preservación y descontaminación de diversas plantas secas aromáticas y medicinales. Se pudo verificar que el uso de la irradiación no afectaba de manera importante a las principales características físico-químicas y moléculas relevantes, a la vez que permitía asegurar la seguridad microbiológica.

El valor nutricional se determinó mediante métodos oficiales para el análisis de alimentos; los azúcares libres se analizaron por HPLC-RI, los ácidos grasos por GC-FID, los ácidos orgánicos por UFLC-DAD, los tocoferoles por HPLC-fluorescencia y los compuestos fenólicos por HPLC-DAD-ESI/MS. Las propiedades antioxidantes se determinaron a través de la evaluación de la actividad captadora de los radicales libres, poder reductor y la inhibición de la peroxidación lipídica en homogeneizados de cerebro. Las propiedades citotóxicas se ensayaron en líneas celulares tumorales (MCF-7, NCI-H460, HeLa y HepG2) y no tumorales (PLP2). Los niveles de micotoxinas se determinaron mediante HPLC-fluorescencia tras purificación en columna de inmunoafinidad (IAC).

La irradiación demostró ejercer efectos heterogéneos sobre parámetros químicos, nutricionales y componentes bioactivos, preservando o degradando moléculas, pero también aumentando la biodisponibilidad de algunos compuestos. La EB demostró ser, en general, la mejor opción para el procesamiento de los materiales estudiados, aunque la conservación posterior de los compuestos en el tiempo (12 a 18 meses) podía diferir según la especie sometida a irradiación.

CHAPTER 1: BACKGROUND

1.1 Food irradiation technologies

1.1.1 A brief introduction to food irradiation

Nowadays, it is essential that food and food products reach the consumer complying all security standards in order to prevent health problems. Another concern is the consumption of high quality food not only in their harvest season or after processing, but also for expanded periods of time. There are several processes used to decontaminate and preserve food that should keep the appearance and original flavor, and eliminate insects, bacteria and toxins, in order to guarantee food safety, also avoiding chemical residues from these processes and minimizing the environmental impact (Migdal and Owczarczyk, 1998). Hence, it is important to verify the maintenance of individual compounds such as fatty acids, tocopherols, organic acids or free sugars, besides ensuring that physical variables are kept unchanged in the samples submitted to the decontamination treatments. Likewise, the bioactive properties of the final products should at least maintain the effectiveness of the original products (Nagy et al., 2011).

One of the decontamination techniques used for food plants is irradiation. This method, apart from being recommended for several foods, especially dry ingredients, reduces reliance on chemical fumigants, which might be carcinogens and mutagens to humans, leave chemical residue on plants and destroy the ozone layer in the atmosphere (Migdal and Owczarczyk, 1998; Chmielewski and Migdal, 2005). It is also characterized for its efficiency in storage, reducing losses caused by natural physiological processes (budding, maturation and aging), and eliminating or reducing microorganisms, parasites and pests without causing significant changes (chemical or organoleptic), making the plants more safe for consumers (Byun et al., 1999; Molins, 2001; Villavicencio et al., 2007; Wen et al., 2010; Nagy et al., 2011). Irradiation is a non-thermal processing technique, also called “cold pasteurization”, since it does not increase significantly the temperature of the irradiated products. In this way, this technology is indicated for food components that are particular sensible to thermal treatments, like aromatic compounds in medicinal or edible plants (Alothman et al., 2009).

Despite the irradiation concept is often misunderstood by most consumers, it is a safe process that exposes food (pre-packaged or unpackaged) to a predetermined dose of radiation according to the food type to be treated, plant-derived products (such as vegetables, fruits and cereals) or even derived from animals, such as meat or fish (Sádecká 2007; Nagy et al., 2011; Kanatt et al., 2015). It is characterized as a versatile, efficient, safe, secure and highly

effective technique, *i.e.*, it is a process that fully satisfies the objective of providing stability to nutritious foods, health conditions and longer storage period (Hunter, 2000; Roberts, 2014).

The processing of irradiation for food is quite old, being used formerly the sunlight to dry and preserve fruits, herbs or spices being this process environmental-friendly and clean. This technology process is based on the physics and chemistry of radiation interactions with matter, where the radiant energy moves through space and matter in the form of electromagnetic waves (Cabo Verde et al., 2010; Khandal, 2010; Tezotto-Uliana et al., 2015).

The preserving potential of the ionizing radiation in food processing is mainly based on the effective destruction of the DNA through the radiation, in order to inactivate the living cells, and prevent the reproduction of microorganisms, insect gametes and meristems of plants. This microbial inactivation mechanism is mainly due to the damage of nucleic acids and other lesions caused by oxidative radicals originated from the water radiolysis (Farkas, 2006).

In food irradiation there are several important processing variables associated, such as type of energy, irradiated doses, dose rate and food characteristics. The dose is the unit of absorbed radiation and is given in kilogray (kGy) units (1 Gy is equal with 1 J/kg absorbed energy). The minimum dose, D_{min}, is the value to guarantee the desired effect, on the other hand, the maximum dose, D_{max}, is the value above which the food may not preserve its characteristics or just the limit imposed by the legislation; and the dose uniformity ratio (DUR) is the ratio D_{min}/D_{max} (IAEA, 2002; Farkas and Mohácsi-Farka, 2011). Thus, the amount of radiation energy needed to control microorganisms in foods varies according to the resistance of species and the number of organisms present in the food (Farkas, 2006).

Another important variable for radiation interaction with materials, principally in biological products, is the dose rate, i.e., the dose per time unit. The effects on living matter or organic material depend not only on the dose applied but also on the dose rate. The time to kill a microorganism or the effect on a chemical reaction is dependent of this factor (Cabo Verde et al., 2010). However, although the value of this variable is very important as regards the quality control, sometimes it is not considered due to the high dose rates, applied in the industrial processing.

The food characteristics such as density, temperature, pH, moisture and gases composition are also important factors that may influence the effectiveness of irradiation (Lung et al., 2015). The amount of water is another variable that interferes in this technology, influencing the radiolysis effects. The fact that the ionizing radiation passes through the food, provides primary (directly in compounds that absorb radiation energy and can bring irreversible changes) and secondary chemical transformations (which arise when the primary products

interact with themselves or with other product components, resulting in substances different from their original composition) due to the absorption of energy (Tezotto-Uliana et al., 2015). Thus, the high reactivity of free radicals and molecular ions produced form highly reactive intermediates that can undergo a variety of reactions leading to stable chemical products, is frequently referred as radiolytic products. Therefore, lower water activity of a food implies lower effect of radiolysis (EFSA, 2011; Tezotto-Uliana et al., 2015).

There are three types of ionizing radiation permitted for food processing: gamma radiation, electron beam and X-rays, having each different characteristics (EU, 1999; Kim et al., 2009b; Jung et al., 2015). Gamma radiation comes from the spontaneous emission of the isotopes of Co-60 or Cs-137; electron beam (e-beam) radiation is produced by accelerating electrons till the energy of 10 MeV (mega electron volt), and X-rays are produced by the impact of accelerated electrons on a metallic target, with the consequent emission of radiation (photons), through a physical phenomena known as "bremsstrahlung" (Farkas, 2006). The three types of radiation differ, especially, in depth of penetration; nevertheless, all can be employed for food processing using the right configuration adapted to the type, size or volume of food to be processed. The first ionizing radiation technique to be tested for food preservation was X-rays, but due to the low energy electron ray conversion efficiency, X-rays have only regained interest recently due to the development of new equipments (Farkas and Mohácsi-Farka, 2011; Antonio, 2014).

Nonetheless, irradiation is not a linear technology and it may preserve some components and degrade others. The balance of advantages and disadvantages compared to other preservation processes must be used to select or not this type of processing, so as to provide consumers products with the best quality and safety criteria.

1.1.2 Gamma radiation

Gamma radiation, a technology used over many years, has become nowadays more popular and applied to food preservation, due to its decontaminant and preservative effectiveness, while maintaining the organoleptic and nutritional quality of foods. The usefulness of this method for conservation, microbial control and disinfestation of various food products has been supported and approved by various organizations, namely by the Food and Drug Administration (FDA) (Mizani et al., 2009; Verma et al., 2016).

This procedure (below 10 kGy) is an effective alternative technology in post-harvest application due to its ability to combat pests, insects and a wide range of bacteria that cause

foodborne diseases and fungi, inhibiting consequently mycotoxin biosynthesis during storage. Gamma rays are characterized by short wavelengths, similar to X-rays but more energetic than ultraviolet light, which allows penetrating deeply into foods. This type of radiation is not only economically viable, but also possesses high antimicrobial potential besides it is physically secure (DeRuiter and Dwyer, 2002; Mexis and Kotominas, 2009).

The most suitable sources for the processing by gamma radiation are ^{60}Co and ^{137}Cs due to the high energy emitted by gamma rays and their relatively long half-life of about 30,1 and 5,27 years for ^{137}Cs and ^{60}Co, respectively. Despite the use of these two sources is considered safe, ^{137}Cs has been limited to small, self-contained dry storage irradiators, used preliminary for the irradiation of blood and for insect sterilization. But for those applications, currently all industrial installations that use this type of processing employ ^{60}Co as source of gamma radiation (IAEA, 2008).

Cobalt-60, ^{60}Co, is obtained from neutron activation of ^{59}Co, an isotope that has no reactivity and is available in large quantities in nature. This element is compressed hermetically in reduced size cylindrical capsules that are fitted in steel tubes deposited in a nuclear reactor where they are constantly bombarded with neutrons, originating the ^{60}Co. Being cobalt-60 highly unstable, it emits gamma radiation as it decays to more stable forms. The interaction of the gamma radiation with the constituents does not produce neutrons and, therefore, neither the irradiated foods nor their packages become radioactive (DeRuiter and Dwyer, 2002).

1.1.3 Electron beam radiation

The application of irradiation using electron beam is also a viable option and widely applied for decontamination and disinfection in several areas. In the pharmaceutical industry it is applied for disposable medical equipment disinfection; in chemistry it is most suitable for cross-linking and polymerization of polyethylene and polypropylene; in hospitals and medical applications it is used in radiation therapy and brachytherapy; and environmentally it is applied for the disinfection of sludge and control and monitoring pollution (Lung et al., 2015).

In food products, this type of radiation acts on microorganisms' growth leading to injury or death, due to direct or indirect damage in their metabolism, causing, essentially, interruption in DNA structure and denaturation of enzymes and proteins of the membrane. Thus, the cells are prevented of performing physiological metabolic activities normally, which leads to loss of chromosomal replication function. Decontamination efficiency depends on the microorganism's species and on the chemical composition of food (Lung et al., 2015).

In this type of ionizing radiation, accelerated electrons are produced by a machine and not from radioactive materials. Equipments accelerate electrons and concentrate them in bundles that can be bent and directed over the food on the conveyor belt. The penetration capability of the electron beam is slightly more restricted compared to other types of ionizing radiation, since its penetration is limited to only about 3.8 cm in food items for a single-sided treatment or 8.9 cm for a double-sided beam treatment. Thus, its most frequent applicability has been in the treatment of fine food packaging or fine amounts of grains or powdered samples (DeRuiter and Dwyer, 2002).

Figure 1 represents the effects of e-beam on microorganisms, targeting the genetic material (DNA and RNA) and breaking the base pairs guanine-cytosine (G-C) and thymine-adenine (T-A); and **Figure 2** represents the type of reactive oxygen species that are produced following radiolysis of water molecules when subjected to e-beam.

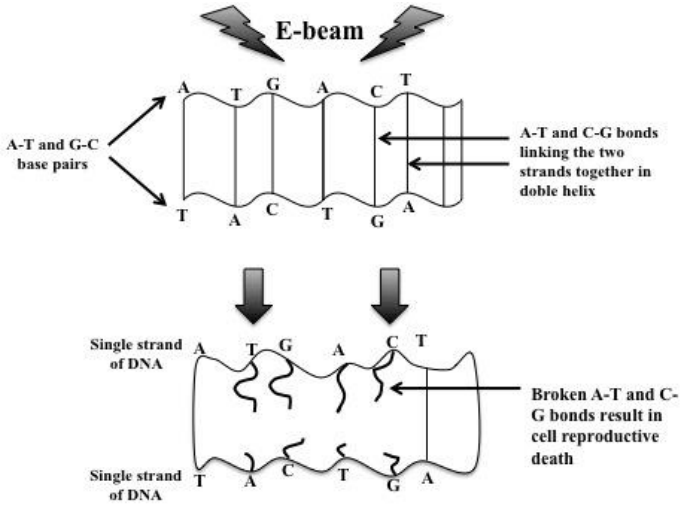


Figure 1. Microbial inactivation by e-beam radiation**.** Source: Lung et al. (2015).

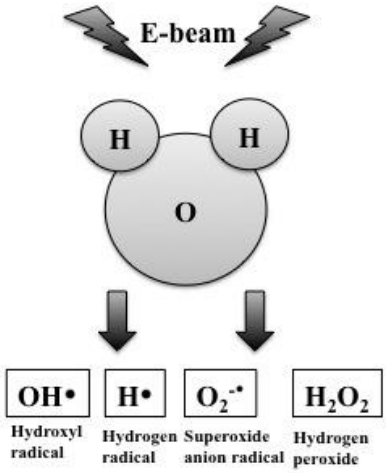


Figure 2. Reactive oxygen species generated from water by e-beam radiation**.** Source: Lung et al. (2015).

Electron beam has some advantages for food irradiation compared with other types of ionizing radiation, such as the use of non-nuclear energy for the generation of the radiation, which can be suspended in the source (machine) at any time, applicability in high-flow and high-dose irradiation, and little risk for occupational injuries (Lung et al., 2015).

1.1.4 Gamma and Electron beam radiations – Differences, applications and detection

Gamma (GR) and electron beam (EB) radiations offer potential alternatives against the conventional sterilization and decontamination of food commodities in the prevention of microbial contamination and insect infestation. These technologies do not induce radioactivity in food or its packaging, have numerous technologically and technically viable applications, and improve significantly the microbiological safety and/or food storage stability (Farkas & Mohácsi-Farka, 2011). Nevertheless, when comparing them it is notorious the disparity of attributes that characterize each one. Although both of them are indicated for industrial use, there are differences in relation to equipments, costs, applicability and the intended objectives. EB is adequate for food products with low density due to its low penetration capability, whereas GR is more suitable for higher density products, because of its higher penetration potential. Irradiation sources constitute another relevant difference. While the EB sources can easily be connected/disconnected, the sources of GR are continually decaying. Furthermore, EB has low measurable residues, small temperature rise and few variables to control, such as dose, temperature and dryness (Supriya et al., 2014). Despite GR is the most popular

technique in food processing, the electron beam employability is gradually increasing (IAEA, 2008).

Throughout time, several detection methods have been recognized by European Committee for Standardisation (CEN) as European Standards (EN), based on physical and chemical differences between irradiated or non-irradiated products (EFSA, 2011). Some detection methods are based on the analysis of including hydrocarbon analyses for lipid-containing foods by applying spin resonance spectroscopy to foods that have bones, and thermoluminescence to foods containing silicate minerals (DeRuiter and Dwyer, 2002).

1.1.5 Legislation and consumer concerns

Ionizing radiation is used as a food processing technology in different countries all over the world. All its requirements and procedures are properly defined and legislated, making it a safe and viable option.

There are several organizations that have defended and facilitated the use of irradiation throughout the world, for example, WHO (World Health Organization), FAO (Food and Agriculture Organization), IAEA (International Atomic Energy Agency), CDC (Centres for Disease Control and Prevention), the American Council on Science and Health, the Council for Agricultural Science and Technology, ADA (American Dietetic Association), AMA (American Medical Association), IFT (Institute of Food Technologists) or CAC (Codex Alimentarius Commission) (DeRuiter and Dwyer, 2002; Ihsanullah and Rashid, 2016).

In the European Union (EU), in 2011 there were 23 installations approved for this type of processing in 12 member-states: Belgium, Bulgaria, Czech Republic, France, Germany, Hungary, Italy, Netherlands, Poland, Romania, Spain and the United Kingdom, being the competent authorities in every member-state the ones which attribute the authorization in accordance with the procedure established by Directive 1999/2/EC.

In the European Union, food irradiation is regulated by Directives 1999/2/CE (European Parliament and Council, L 66/16, 1999) and 1999/3/CE (European Parliament and Council, L 66/24, 1999), which described general rules and techniques that guide the irradiation process, their conditions of use in food, the exceptions and requirements for labelling of irradiated food, and the products authorized to undergo this processing. Actually, in the EU a wide variety of products, like fruits and vegetables, cereals and rice flour, spices and condiments, fish, shellfish, fresh meats, poultry, frog legs, raw milk Camembert, arabic gum, casein/caseinates and white egg, are allowed to be irradiated (EU, 2013).

According to the established standards, all sold food submitted to this kind of processing must be clearly identified on the label with the symbol "Radura" (**Figure 3**) and with the information "Food treated by irradiation process". This symbology must be present not only in directly irradiated products but also in foods containing irradiated ingredients, presenting this statement earlier between parentheses ahead of the name of the irradiated constituent (Tezotto-Uliana et al., 2015).

Figure 3. Representative image of the symbol "Radura".

Despite all rigor, approval and control by competent organizations in the application of radiation, there are many obstacles related to the cost of this technology and also with the acceptance by consumers, which inhibit irradiated foods from reaching pioneer levels of commercialization. Specifically, on gamma radiation, there is still the scepticism that the isotopes ^{137}Cs and ^{60}Co could leave radioactive residues (Ornellas et al., 2006; Supriya et al., 2014). Thus, the lack of information available to the consumers about its benefits or harms, particularly with regard to the toxicological risks hypothetically associated, reduces confidence in the use of ionizing radiation in the processing and preservation of food.

1.2 Dried plants irradiation for nutrients and bioactives preservation

1.2.1 Common antioxidants in dried plants

The antioxidant molecules present in food are able to directly eliminate ROS, act in the regulation of antioxidant defences, and inhibit the production of these species. Furthermore, these compounds have the ability to form new radicals that are stable through intramolecular hydrogen bonding on further oxidation after radicals' scavenging (Benzie, 2003; Embuscado, 2015).

Some examples are vitamins (A, B, C, D, E and K), carotenoids (lycopene, β-carotene and xanthophylls) and polyphenols (flavonoids such as flavonols, flavones, flavanones, flavanols (flavan-3-ols), anthocyanins, and isoflavones, phenolic alcohols, phenolic acids, tannins, stilbenes and lignans) (Carocho and Ferreira, 2013). There are several foods that contain these molecules in their composition, particularly fruits, vegetables, spices, herbs, mushrooms, fish and milk products, among others (**Table 1**).

Table 1. Principal classes of compounds with antioxidant activity and their natural sources.

Antioxidant		Natural sources	References
Vitamins	Vitamin A	Milk, butter, egg, cod liver oil, fish liver.	Leboulanger, 1974
	Vitamin B	Fruit, milk, meat, rice, wheat, beans, egg, potato, sweet potato.	Leboulanger, 1974; Siró et al., 2008; Xu et al., 2015
	Vitamin C	Potato, cabbage, carrots, peas, beans, spinach, tomatoes, peach, orange, lemon, kiwifruit, apple, peach, tomato, peach, citrus, blueberry; spearmint, peppermint, tangerine, strawberry, pear, garlic, citrus peel, broccoli, bayberry, banana, apple.	Leboulanger, 1974: Isabelle et al., 2010; Liu et al., 2015; Oroian and Escriche, 2015; Tamasi et al., 2015
	Vitamin D	Cod liver oil, tuna fish liver oil, fish, butter, milk, egg, cheese.	Leboulanger, 1974; Siró et al., 2008
	Vitamin E	Green leafy vegetables, wheat germ oil, barley, egg, milk, butter, peanut oil, olive oil, soybean oil, hazelnut, green tea, olives and olive oil, pumpkin seeds, sunflower seeds and sunflower oil.	Siró et al., 2008; Oroian and Escriche, 2015; Sen et al., 2002; Do et al., 2015; Taş¸ and Gökmen, 2015; Vrolijk et al., 2015
	Vitamin K	Green vegetables, spinach, broccoli, cabbage, carrots, potatoes, peas, tomatoes, strawberries, beef liver, pork liver, milk, egg, herbs.	Leboulanger, 1974; Damon et al., 2005; Suttie and Booth, 2011; Presse et al., 2015
Carotenoids	β-carotene	Amaranth, dark green leafy vegetables, gac, olive oil, red carrots, sweet potato.	Oroian and Escriche, 2015; Vrolijk et al., 2015; Wu et al., 2008
	Lycopene	Apricots, guava, watermelon, papaya, carrots, tomatoes, gac, watermelon, pink grape fruit, guava, rosehip, dried apricots, papaya, pink grapefruit.	Oroian and Escriche, 2015; Krinsky and Johnson, 2005; Ho et al., 2015; Poojary and Passamonti, 2015
	Xanthophylls	Spinach, kale, egg, catfish, corn, spinach, meat, skin and flesh of fish (trout, salmon), carapace of crustaceans (shrimp, lobster, Antarctic krill, crawfish), egg yolks, liver.	Breithaupt, 2007; Oroian and Escriche, 2015; Tsui and Cheryan, 2007 Brulc et al., 2013; Hu et al., 2013

Polyphenols	Flavonoids	Fruits, vegetables, grape, kumquats, parsley, celery, chamomile, black rice, berry fruits (blueberry, blackberry, mulberry, raspberry, strawberry, blackcurrant), purple-fleshed sweet potato, black carrots, soybean and soybean products, tea (white, green, oolong, black), honey, orange juice.	Xu et al., 2015; Oroian and Escriche, 2015; Scalbert et al., 2005; Hostetler et al., 2013; Ivanovic et al., 2014; Li et al., 2014; Ganzera, 2015; Gras et al., 2015; Harb et al., 2015; Lee et al., 2015b; Mølmann et al., 2015; Qin et al., 2015; Ruiz et al., 2015; Ramirez et al., 2015; Pedro et al., 2016
	Phenolic acids	Plant foods, tea (white, green, oolong, black), herbs, whole grain cereals, artichoke heads, coffee, mushrooms	Qin et al., 2015; Jeszka-Skowron et al., 2015; Martini et al., 2015; Taofiq et al., 2015
	Tannins	Bean seed coats, persimmon, mangosteen, canola, green coffee beans, wine, pomegranate, strawberry, walnuts, grape, apple	Oroian and Escriche, 2015; Hosu et al., 2014; Muhacir-Güzel et al., 2014; Figueroa-Espinoza et al., 2015
	Stilbenes	Almonds, chocolate and cocoa, skins of grapes, red wine, plants, peanuts, mulberries, pistachio.	Oroian and Escriche, 2015; Grippi et al., 2008 Kasiotis et al., 2013; Silva et al., 2014; Xie and Bolling, 2014
	Lignans	Plant foods, vegetables, cereals, spices, oil seeds, fruits (particularly berries), nuts, whole grains, tea and red wine, sweet chestnut flours, wheat, oats, rye, barley, legumes.	Oroian and Escriche, 2015; Thompson et al., 2006; Durazzo et al., 2013; Edel et al., 2015

Table 1 collects information about the main classes of compounds with antioxidant activity and their natural sources. Vitamins play an important role in human health, since they are a group of essential compounds for normal growth and functioning of the organism. However, since the body does not have the capacity to synthesize vitamins, these compounds have to be obtained from food or pharmaceutical products. Most vitamins act as essential enzymes or coenzymes in the organism. The incorrect ingesting of these micronutrients triggers harmful pathological reactions that compromise the normal development of the body (Campbell and Anaesthesia, 2014). On the other hand, beyond their essential role in the organism, some vitamins or their precursors (i.e., vitamins E, C, □-carotene) can also act as antioxidants and become important for the control of oxidative processes either in food or the human body.

Other antioxidant molecules are the carotenoids that are natural pigments responsible for the yellow, orange and red colour present in many foods displaying a variety of functions in nature, since they act in deactivating a wide variety of reactive radicals in biological systems (Jomova and Valko, 2013). The natural functions of carotenoids are principally determined by their molecular properties such as size, geometry, and presence of functional groups (Saini et al., 2015). These molecules can be found in plants and microorganisms, as well as in some animal sources. In nature there are over 600 carotenoids, being classified as carotenes and xanthophylls (Jomova and Valko, 2013). β-Carotene is an abundant micronutrient that can be found in many vegetables and fruits, becoming a major source of vitamin A in the human diet. Due to its water-insolubility, this molecule's absorption process is passive and parallel to that of lipids. As negative aspect, β-carotene is prone to degradation by exposure to light, heat and/or oxygen being converted in a pro-oxidant, which might cause deleterious effects on human health (Kim and Huber, 2016). Another abundant carotenoid is lycopene that is responsible for the red colour of many common foods, such as tomatoes and tomato products. It is known for its oil solubility and sensitivity to light and heat, so that it deteriorates easily during processing and storage, and also presents low bioavailability. Diverse studies have shown that this compound beneficially intervenes in health, acting in the prevention of cancer and cardiovascular diseases (Ha et al., 2015). The xanthophylls are also carotenoids widely distributed in the different phylogenetic kingdoms and with a broad structural variety, which allows multiple functions of these organic molecules in living organisms. It has been demonstrated, that the ingestion of lutein and zeaxanthin helps in the prevention of macular degeneration associated with age and in the development of cataracts, as also in the risk reduction of several types of cancer (Breithaupt, 2007; Lee, 2008; Ruban and Johnson, 2010).

Another class of molecules with bioactive potential are polyphenols, which include a large group of biologically active compounds (flavonoids, phenolic acids, tannins, stilbenes, lignans). The interest in these compounds has been increasing gradually since they have been associated in many epidemiological studies with a decreased risk in the incidence of different chronic diseases, especially related to oxidative stress (Kalantar-Zadeh et al., 2004; Castel et al., 2014). Their antioxidant potential, largely demonstrated in *in vitro* studies, has been related with different biological effects, including anticarcinogenic, antimutagenic, antibacterial, antiviral and anti-inflammatory activities (Tapiero et al., 2002). The antioxidant activity of these molecules is also influenced by their structure, in particular by the number and position of phenolic hydroxyl groups.

Besides the above described naturally occurring antioxidants, food can also contain synthetic antioxidants that have been developed for their incorporation as additives in order to improve food stability during processing and storage, prolonging the shelf life. The principal and most usual antioxidants in the food industry are BHA, BHT, PG and TBHQ (Guan et al., 2006). BHT (butylated hydroxytoluene) and BHA (butylated hydroxyanisole) are the most widely used chemical antioxidants. The European Food Safety Authority (EFSA), between 2011 and 2012, re-evaluated all the available information about these two compounds including published information apparently contradictory. Thus, the EFSA established revised acceptable daily intakes (ADIs) of 0.25 mg/kg body weight/day for BHT and 1.0 mg/kg bw/day for BHA, and noted that the dietary exposure of adults and children was unlikely to exceed these intakes (EFSA, 2011, 2012; Carocho and Ferreira, 2013). PG (propyl gallate) has a great antioxidant activity and when used at high levels it may assume a pro-oxidant role. TBHQ (terc-butyl-hydroquinone) is basically used to stabilize and preserve freshness, nutritional value, taste and colour of food products of animal origin (Perrin and Meyer, 2002; Carocho and Ferreira, 2013). These synthetic antioxidants are effective and cheaper than natural antioxidants when added to food, which explains their wide use in food industry; notwithstanding, the demand for natural food additives over synthetic ones has been growing in an attempt to avoid the possible chemically induced carcinogenic effects (Mohamed et al., 2011). Moreover, the synergism observed between various natural antioxidants present in food extracts is an advantage that cannot be achieved with an isolated synthetic antioxidant (Erkan et al., 2008). In this field, and taking into account that lipid peroxidation is the second major cause, after microbial spoilage, of decline of food nutritional and sensory quality traits (Soyer et al., 2010), several studies have been performed in order to test the antioxidant capacity of natural antioxidants when introduced in foodstuff. This research has focused in

many occasions in animal origin products because of their specific constitution. For example, the presence of residual blood in meat products equates to retention of more haemoglobin, which is a powerful promoter of lipid oxidation and further rancidity, especially under oxidative conditions during storage or cooking (Everse and Hsia; 1997; Alvarado et al., 2007). On the other hand, fish products richness in n-3 polyunsaturated fatty acids (PUFA) makes them susceptible targets to peroxidation that lead to rancid taste and off-flavour, limiting their time of storage and processing possibilities (Medina et al., 2009). Thus, several extracts of plants have been introduced in these matrices and proved to possess preservative effects by limiting their oxidation. As an example, rosemary extracts, when employed in meat products, are able to decrease thiobarbituric acid reactive substances (TBARS), hexanal levels and protein oxidation in cooked pork patties and beef burgers (Nissen et al., 2004; Georgantelis et al., 2007; Lara et al., 2011; Doolaege et al., 2012). Furthermore, combinations of extracts from rosemary, marjoram and sage significantly decreased the lipid oxidation of ground beef (Mohamed et al., 2011), while oleoresin of rosemary reduced TBARS and hexanal in raw ground beef and cooked patties (Hygreeva et al., 2014). These plant extracts have also been tested in fish muscle, namely salmon, leading to a delay in the development of lipid oxidation secondary products (Tironi et al., 2010), as well as in flavoured cheese prepared with cream cheese base where they revealed capacity to prolong the shelf-life by preventing lipid oxidation (Olmedo et al., 2013). In 2010, the European Union authorized the use of rosemary extracts as new food additives for use in foodstuffs under Directive 95/2/EC and assigned it the E-392 number (EU, 2010a; EU, 2010b).

There are several other examples of plant extracts incorporated in meat and/or fish products for their antioxidant effects, such as extracts of clove bud and grape seeds used to retard the increase of peroxide value and TBARS in silver carp fillets during chilled storage (Shi et al., 2014), and to improve the oxidative stability of cooked beef (Ahn et al., 2002), frankfurters (Özvural and Vural, 2011), turkey patties (Lau and King, 2003), and turkey meat stored cold, both in air and under vacuum (Mielnik et al., 2006). Chinese red pepper leaf extracts were able to decrease hexanal contents, TBARS values and lipoxygenase (LOX) activity in salted silver carp dorsal and ventral muscles during processing (Li et al., 2015); water extracts of lychee seeds were added to meat paste to inhibit the adipogenesis and retard lipid oxidation (Qi et al., 2015), and lychee flower was used to delay lipid and protein oxidation of emulsified pork meatballs (Ding et al., 2015). On its turn, green tea extracts preserved both lipids and protein thiols of pork meat emulsion without jeopardizing the oxidative stability or the physico-chemical properties of the meat proteins (Jongberg et al., 2015). These extracts also

significantly reduced TBARS, putrescine, histamine and tyramine formation in Turkish dry-fermented sausage (Bozkurt, 2006), and preserved the ascorbic acid and carotenoid content of minimally processed lettuce (Martín-Diana et al., 2008). Mariutti et al. (2008) reported the positive effects (control of lipid oxidation) of sage extracts in minced chicken breast, and Fasseas et al. (2007) observed a decreased TBARS formation in raw and cooked pork in the presence of sage essential oils. Bearberry extracts were found to exhibit potent lipid antioxidant activity in raw and cooked pork (Carpenter et al., 2007), and crude cranberry extract inhibited the oxidation in mechanically separated turkey and cooked ground pork, whereas its polyphenolic fraction revealed even higher inhibition of hemoglobin-mediated lipid oxidation than the extract itself (Lee et al., 2006). Cinnamon extracts controlled warmed-over-flavour and lipid oxidation, and inhibited the release of non-haem iron in sheep, beef and pork meat (Jayathilakan et al., 2007). The addition of garlic and onion powder to pork loin and belly increased yellowness and reduced free fatty acids, peroxide, and the TBARS values (Park et al., 2008); and pomegranate rind powder reduced TBARS values in fresh chicken, and then in prepared cooked chicken patties (Naveena et al., 2008). Beyond these studies, many other extracts have been explored, as well as combinations of different plants extracts, and even combinations of natural and synthetic antioxidants.

Nevertheless, the direct use or incorporation of free bioactives in foodstuff (either as extracts, fractions of extracts, or isolated compounds) has several considerable limitations related to the loss and transformation of the antioxidant molecules, and to their possible interaction with other compounds. Moreover, the processing steps involved in the preparation of food matrices can influence the degradation or transformation of the antioxidant compounds due to factors such as endogenous enzymes action, water activity, oxygen pressure and thermal/mechanical energy (Dias et al., 2015). In connection with this, microencapsulation arises as a suitable option to stabilize the extracts to be incorporated in food.

Microencapsulation can be defined as a process through which tiny particles or droplets are surrounded by a coating or embedded in homogeneous or heterogeneous matrices with the aim of producing small capsules with many useful properties (Gharsallaoui et al., 2007). This technique was first used in the early 1930s in order to coat some flavours, and since then, ingredients such as antioxidants, preservatives, oxidation-reduction agents, enzymes, acids, bases, nutrients, cross-linking agents, buffers, flavours, sweeteners, and colours started to be worldwide produced in industrial scales (Reineccius, 1993). In food industry, microencapsulation found its application in protecting the core material from adverse environmental conditions and enhancing the shelf life of products by controlling the release of

the encapsulated materials (Shahidi and Han, 1993). A great deal of studies have been performed in this field and there is a significant use of plant extracts, polyphenols, essential oils, vitamins, proteins and fat extracts applied to food.

Dairy products are among the most usually foodstuffs to which microencapsulated preparations have been added. Thus, microencapsulated isoflavones added to milk improved their absorption in the intestine without affecting the taste (Seok et al., 2009); phenolic extracts from pomegranate peels incorporated in ice cream proved to enhance antioxidant and α-glucosidase inhibitory properties (Çam et al., 2014); phenolic extracts from elm leaf and blackberry flowers increased the antioxidant activity of yogurt compared to the same product containing the extract in the free form (Martins et al., 2014); yogurt added encapsulated extracts from pomegranate fruit showed higher content of phenolic compounds and anthocyanins than the non-encapsulated control (Robert et al., 2012). Cheese was enriched with Vitamins E, A and CoQ10 to inhibit lipid peroxidation (rancidity) (Stratulat et al., 2014). Also, cottage cheese enriched with mushrooms (*Suillus luteus* (L.: Fries) and *Coprinopsis atramentaria* (Bull.)) extracts revealed a clear tendency for better antioxidant preservation over the time of storage when microencapsulated forms were applied (Ribeiro et al., 2015). Furthermore, microencapsulated aqueous extracts of wild strawberry revealed ability to preserve the antioxidant properties of *k*-carrageenan gelatin when compared to the product added with the free form (Dias et al., 2015). Pasrija et al. (2015) introduced microencapsulated green tea polyphenols in bread to retain the quality characteristics along with its functionality. In order to promote health properties, citric acid was incorporated to chewing gum (Abbasi et al., 2009), whereas its derivative (-)-hydroxycitric acid was added to bread (Ezhilarasi et al., 2013a,b) and pasta (Pillai et al., 2012), maintaining good sensory and quality attributes. A soup powder was enriched with microencapsulated linseed oil (rich in omega-3 fatty acids) to allow a controlled release of the lipophilic compounds (Rubilar et al., 2012), etc.

Beyond microencapsulation, the incorporation of bioactive extracts or isolated compounds in packaging films has been assessed in different studies (Moore et al., 2000; Barbosa-Pereira et al., 2013) and represents a new packaging concept with great interest in the food industry to preserve food characteristics and delay oxidative processes. In some cases, the incorporation of antioxidants to the film seems to be more efficient than the direct use of additives on meat surface and, thereby, some packaging systems containing natural extracts (such as cocoa, rosemary, oregano, green tea, oolong tea, and black tea, among others) have been developed to increase the stability of meat products and extend their shelf-life (Moore et al., 2000; Nerín

et al., 2006; Camo et al., 2011; Calatayud et al., 2013; Yang et al., 2016). This technique has also been applied to extend cheese shelf life (Jalilzadeh et al., 2015), as well as salmon muscle, using natural antioxidant products containing tocopherols in the development of active packaging (Barbosa-Pereira et al., 2013).

1.2.2 Detection and determination of antioxidant molecules

Natural antioxidants have become a topic of great interest in the last decades. Thus, the full characterization of these compounds is mandatory for the understanding of their behaviour and to establish relationships between their chemical structure and the displayed activity. Commonly used techniques for the detection, quantification and structure elucidation of antioxidant molecules in foodstuffs are schematized in **Figure 4** and discussed below.

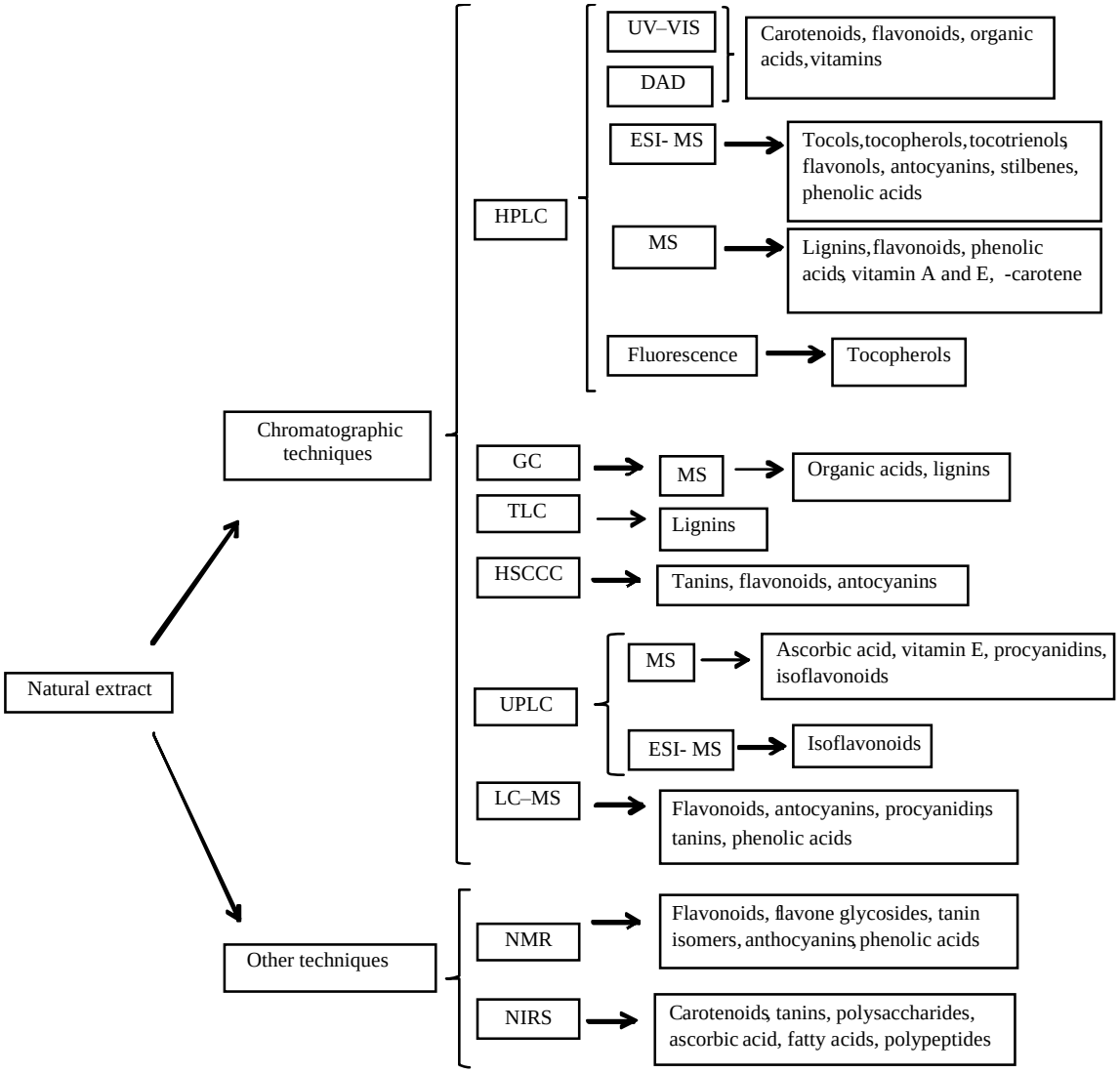


Figure 4. Techniques used for the detection, quantification and structure elucidation of antioxidant molecules in foodstuffs.

In the last decades, different techniques have been developed for the analysis of antioxidant molecules in different matrices. An initial approach can be made through colorimetric assays such as the Folin-Ciocalteu (FC) method that is commonly used to determine "total phenolic compounds". The FC reagent is a mixture of tungsten and molybdenum oxides that present a yellow colour, under alkaline conditions it can react with the antioxidants, forming a complex that present a blue colour and can be monitorized photometrically at 765 nm. However, the method is not specific for polyphenols as all reducing substances present in the sample can react with the FC reagent, such as sugars or ascorbic acid, but also other enediols and reductones, proteins, aromatic amines, sulfur dioxide, or organic acids. Thus, the FC method is being mostly used to measure the reducing capacity rather than to determine the phenolic

content (Huang et al., 2005; Carocho and Ferreira, 2013; García-Guzmán et al., 2015). Similarly, the 2,6-dichlorophenol indophenol method, commonly employed to determine ascorbic acid is not specific for this antioxidant either, since that oxidized dye can also be reduced by other substances (Highet and West, 1942). Additionally, there are a large number of methods to evaluate the antioxidant activity, either in foodstuffs or biological systems, based on different mechanisms and employing different techniques for measurement, e.g., spectrophotometric, electrochemical and chemiluminescent methods. They allow measuring the total antioxidant activity conferred by the different compounds present in the foodstuff, but do not allow identifying the individual molecules responsible for such activity and the results vary depending on the mechanism of action of the selected assay (Carocho and Ferreira, 2013). These methods are usually performed as an initial screening, but they are not precise and suffer the interference of other molecules present in the mixture, thus specific technologies for the determination of each molecule individually have been optimized.

The most common techniques to analyse individual molecules are High Performance Liquid Chromatography (HPLC) and Gas Chromatography (GC) coupled to different detection devices (**Figure 4**) (Barros et al., 2009; Carocho and Ferreira, 2013; Gouveia-Figueira and Castilho, 2015).

HPLC is the predominant and more versatile technique for compound separation. In most cases, the separation is performed on reversed-phase C18 columns, using binary solvent systems consisting on an aqueous polar solvent and a less polar organic solvent, more often acetonitrile or methanol. As for compound determination, classical methods are based on UV-vis detection at one or multiple wavelengths, although other techniques such as fluorometry, electrochemical, refractometry, MS or NMR are also employed, depending on the chemical features of the target molecules and the purpose of the analysis. Thus, whereas UV-Vis or diode array detection are usually employed for the determination of carotenoids and phenolic compounds, respectively (Barros et al., 2009; Zhang et al., 2013; Oroian and Escriche, 2015), fluorescence detection offers higher sensitivity for the determination of tocopherols (Barros et al., 2008). A reproducible and accurate method for the analysis of organic acids using ultra-fast HPLC coupled to diode array detection and verified that allowed the separation of compounds in a time as short as 8 min was optimized by Barros et al. (2012).

Gas chromatography (GC) is a separation technique that offers excellent results for the resolution of complex mixtures. However, its applicability is limited as only volatile compounds can be analyzed, either in their natural form or after derivatization (Wang et al., 2003; Pereira et al., 2013a).

Another chromatographic technique is Thin Layer Chromatography (TLC), known for its simplicity, low cost and ability to easily screen a large number of samples. This technique has been applied, for instance, to the separation and detection of lignins (Waksmundzka-Hajnos et al., 2008; Simões-Pires et al., 2009; Oroian and Escriche, 2015). Fractions separated by TLC, can further be analysed by HPLC-MS or GC-MS for compound identification (Willför et al., 2005; Oroian and Escriche, 2015).

High Speed Counter Current Chromatography (HSCCC) is one more separation technique that works without any solid stationary phase. This technology uses two immiscible liquid phases, one acts as the stationary phase and the other as the mobile phase allowing soft separation conditions (Köhler et al., 2008). It has been particularly used for the isolation of bioactive natural products (Rodríguez-Rivera et al., 2014), such as tannins (Liu et al., 2010), anthocyanins (Watson and Sparkman, 2007) and other flavonoids (Liang et al., 2011). As the use of a solid support is eliminated, losses by irreversible interactions with the solid stationary phase are avoided, thus facilitating the complete of compounds.

In recent years, Mass Spectrometry coupled to Liquid Chromatography (LC-MS) has become the most common technique for natural compounds identification. Using this technique several phenolic compounds have been identified in natural matrices, such as anthocyanins, proanthocyanidins and other flavonoids (Flamini, 2003) or phenolic acids (Bursal et al., 2013). Tandem MS in connection with HPLC (HPLC-MS/MS) can also be used for quantification of compounds that are present in very low amounts in the sample (Plozza et al., 2012; Oroian and Escriche, 2015). Higher sensitivity is achieved when MS is coupled to Ultra-High Performance Liquid Chromatography (UHPLC), which provides faster separations and is less time- and solvent consuming. UHPLC-MS constitutes a powerful technique that have been applied to the analysis of different antioxidants, such as ascorbic acid (Wong et al., 2014), vitamin E (Klimczak and Gliszczyńska-Świgło, 2015), tocols, tocopherols and tocotrienols (Hejtmánková et al., 2010), flavonols (Liu et al., 2014), procyanidins (Ortega et al., 2010), isoflavonoids (Prokudina et al., 2012) or complex polyphenols mixtures (Stalmach et al., 2011).

Near Infrared Spectrometry (NIRS) is another sensitive technology to help in the antioxidants analysis. This technique has been described as a fast, non-invasive, low cost, non-polluting and non-destructive in the analysis of food matrices (Cen and He, 2007; Oroian and Escriche, 2015). It is a spectroscopic technique that uses the near-infrared region of the electromagnetic spectrum within the wavelength range 700 nm to 2500 nm. It has been extensively used in the determination of the quality of grains, fruits, vegetables, milk and dairy products or meat,

among other agricultural products. Several studies are described in the literature reporting the use of NIRS in the determination of natural antioxidants, such as total (Atienza et al., 2005) and individual carotenoids like lycopene and lutein (Pedro and Ferreira, 2005; Chen et al., 2009) or β-carotene (Chen et al., 2009); tannins and anthocyanins (Cozzolino et al., 2008), polysaccharides (Wu et al., 2015), ascorbic acid (Pissard et al., 2013; Blanco-Díaz et al., 2014), or polypeptides, fatty acids, esters and acids (Mushtaq et al., 2015).

1.2.3 Structure elucidation of individual antioxidant compounds

Natural matrices are very complex and contain a lot of unknown compounds that require structural identification using adequate techniques In this respect, Nuclear Magnetic Resonance (NMR) is the most important technique used for natural products structure analysis. ^{1}H-NMR and ^{13}C-NMR spectroscopy, respectively based on proton and carbon nuclear magnetic resonance, allow a precise characterization of all the hydrogen and carbon bonds established between themselves (Kazuma et al., 2003; Francis et al., 2004; Grotewold, 2006). It is a non-destructive technique that can be performed without the use of an internal standard and permits the sample recovery (Soininen et al., 2012; Liu et al., 2015). In the presence of a magnetic field, the NMR active nuclei (^{1}H or ^{13}C) absorb electromagnetic radiation at a frequency characteristic of the isotope. The resonant frequency, energy absorption and the signal intensity are proportional to the magnetic field strength. The information obtained by the signals is related to the chemical and physical environments surrounding the spins, obtaining different information from each signal (Otero and Préstamo, 2009). Another important advantage of this technology is that the signals obtained are proportional to the molar metabolite concentration, avoiding the need of calibration curves (Kim et al., 2010; Oroian and Escriche, 2015).

Several studies describe the use of NMR in the identification of different compounds in natural matrices: epimeric forms of flavone glycosides (Maltese et al., 2009); tannin isomers (Chai et al., 2012); anthocyanins (Lee et al., 2013); rosmarinic acid (Jun et al., 2014); para-hydroxy methyl benzoate glucoside, cycloeucalenol *cis*-ferulate, cycloeucalenol trans-ferulate, trans-ferulic acid, trans-ferulic acid methyl ester, *cis*-ferulic acid, *cis*-ferulic acid methyl ester, methyl caffeate, vanillic aldehyde and para-hydroxy benzaldehyde (Wang et al., 2015); 9-*O*-(3-carboxymethyl-4-(*p*-formylstyryl))hydroxybutanoic acid, 2-hydroxy-3-methoxycaffeic acid 5-*O*-β-D-glucopyranoside and 3′-*O*-methyl-4′-*O*-(4-*O*-galloyl-α-L-rhamnopyranosyl) ellagic acid (Chen et al., 2015), etc.

NMR can also coupled to liquid chromatography (LC-NMR), which constitutes a powerful tool for the separation and structural elucidation of unknown compounds in complex mixtures without requiring previous isolation. However, this technique still presents drawbacks and despite the advances made in instrumentation in recent years, it has not yet become popular, among others due to the high price of the equipments, so that few papers have been published using this technique (Silva Elipe, 2003; Wolfender et al., 2003; Bino et al., 2004; Frank et al., 2013).

1.2.4 Dried plants irradiation

From the most ancient times the dried plants have been used not only as food but also for therapeutic applications, both in developed and in developing countries. Some of these plants have medicinal properties with great potential in the treatment of various diseases (infectious and non-infectious), since they are a natural source of bioactive compounds such as polyphenols, vitamins, carotenoids or unsaturated fatty acids. This diversity in biomolecules enables their use in various areas, *e.g.*, as food additives, health promoters or ingredients in the formulation of functional foods and nutraceuticals (Ramarathnam et al., 1995; Skerget et al., 2005; Pal et al., 2010). In recent years, great interest in medicinal plants has increased around the world, due to their potential beneficial properties, triggering several scientific studies. This is due to the fact that these products are considered to have no side effects, and because sometimes it is easier and more convenient to extract the biologically active substances from plants than to do their synthesis (Marques and Farah, 2009; Yordanov et al., 2009).

In addition to the wild plants, also spices and condiments not only contribute to the taste and flavour of food, but they also are commonly used as natural preservatives in the food industry and for therapeutic purposes in the pharmaceutical industry (Gahukar, 2012; Pal et al., 2010). Their putative beneficial effects on lipid metabolism, antidiabetic efficacy, ability to stimulate digestion and antioxidant and anti-inflammatory potential confer to plant bioactive compounds the character of nutraceuticals (Srinivasan, 2005). In the pharmaceutical industry, the use of raw materials of good microbiological quality is one of the indispensable requirements, since contaminating microorganisms can lead to product deterioration and disease (Rosa et al., 1995). The same is true in the food industry, where the microbiological decontamination to completely inactivate or inhibit microbial growth provides the product greater shelf life and safety while maintaining its quality (Kamat et al., 2003).

The plants are contaminated from the soil, water, air and dust not only during their growth but also during harvest and drying (Shim et al., 2009). The harvesting of herbs, air-drying and storage over a long period of time is a common practice in many parts of the world. However, microbial deterioration or insect infestation during storage and transportation can reduce the quality and shelf life of herbs and also represents a threat to public health, since they are exposed to a high level of natural contamination; thus, microorganisms of great relevance to public health, such as Salmonella, *Escherichia coli*, *Clostridium perfringens*, *Bacillus cereus* or toxinogenic moulds may be present (Sádecká, 2007; Pal et al., 2010). There are more than 400 compounds classified as mycotoxins and, among them, aflatoxins (AFs) and ochratoxin A (OTA) have been the most studied. AFs are produced by *Aspergillus flavus* and some close relatives. AFB_1 is the most common aflatoxin contaminating food products; it is reported as the most toxic and carcinogenic compound naturally produced, being classified as a Group 1 carcinogen (IARC, 2002; Rodrigues et al., 2012). The mutagenic and carcinogenic effects of AFB_1 in various animals have been documented, and different epidemiological studies have shown the existence of a correlation between human liver cancer and the levels of this mycotoxin in the diet (Romagnoli, et al., 2007; Lee et al., 2015a). OTA is produced by several *Aspergillus* and *Penicillium* species and known as a nephrotoxic, hepatotoxic, neurotoxic, teratogenic and immunotoxic agent. Its presence in the diet has been associated with a fatal human kidney disease, referred to as Balkan Endemic Nephropathy (BEN), and with an increased incidence of tumors of the upper urinary tract (Harris and Mantle, 2001; Majeed et al., 2013; Waśkiewicz et al., 2013). It is classified as Group 2B carcinogen (IARC, 1993).

The natural occurrence of mycotoxins in plants has been frequently reported, some examples being traditional medicinal and aromatic herbs from different Asian and African countries reported to contain exceeding levels of aflatoxins and ochratoxin A (Ashiq et al., 2014; Santos et al., 2009; Waśkiewicz et al., 2013). There are various techniques for the decontamination of dried plants such as fumigation with ethylene oxide or methyl bromide, heat treatment and irradiation (Pal et al., 2010). Ethylene oxide is commonly used to decontaminate spices, with varying degrees of success. However, despite the effectiveness of this chemical agent for decontamination, its use is banned in several countries, including Japan, the EU, or Brazil, because it is considered a human carcinogen and a mutagen when inhaled, and may leave chemical residues in plant (Migdal & Owczarczyk, 1998; Chmielewski & Migdal, 2005). Irradiation is an alternative decontamination process for spices and dried plants. It is characterized by being efficient in conservation reducing losses caused by natural physiological processes, by eliminating or reducing microorganisms, making it safer for the

consumer, and also by reducing dependence on chemical fumigants and preservatives used by the food industry (Byun et al., 1999; Nagy et al., 2011).

1.2.5 Effects on dried plants nutrients and bioactives

The high interest in irradiation has triggered several studies aiming to test the effects of this processing in several physico-chemical variables. **Table 2** refers to various studies in this area, taking into account the analyzed plant species, its origin, the technique used and the corresponding irradiation dose.

Table 2. Studies regarding irradiation of dried plants in relation to nutrients and bioactives.

Compounds	Species	Origin	Radiation technology	Doses (kGy)	References
Organic acids	*Capsicum frutescens* (Chilli) Five-spice powder	China	Electron beam	9.94	Lianzhong et al., 1998
Proteins (amino acids)	*Camellia sinensis:* Green tea Black tea Oolong tea	Korea Sri Lanka China	Gamma rays	5, 10	Kausar et al., 2013
Sugars	*Camellia sinensis:* Green tea Black tea Oolong tea	Korea Sri Lanka China	Gamma rays	5, 10	Kausar et al., 2013
Total phenolics	*Glycyrrhiza glabra* –root (Licorice)	Pakistan	Gamma rays	5, 10, 15, 20, 25	Khattak and Simpson, 2010
	Olea europaea (Olive) leaves	Tunisia	Gamma rays	5, 10, 15, 20, 25	Aouidi et al., 2011
	Nelumbo nucifera (Lotus)	Korea	Gamma rays	10, 20, 50	Jeong et al., 2009
	Rosmarinus officinalis (Rosemary) *Nasturtium officinale* (Watercress) *Cynara scolymus* (Artichoke) *Ocimum basilicum* (Sweet basil)	Brazil	Gamma rays	10, 20, 30	Koseki et al., 2002
	Folium Salviae officinalis (Sage) *Folium thyme* (Thyme) *Folium origani cretici* (Oregano)	Albania Poland Turkey	Gamma rays	10	Brandstetter et al., 2009 Nagy et al., 2011
	Nigella sativa (Black cumin)	Germany	Gamma rays	2, 4, 8, 10, 12, 16	Khattak and Simpson, 2008
	Polygonum multiflorum (Fleeceflower) *root*	China	Gamma rays	2, 4, 6, 8, 10	Chiang et al., 2011
	Ginkgo biloba (Ginkgo) *Foeniculum vulgare* (Fennel) *Piper methysticum* (Kawa-kawa)	---	Gamma rays	5, 10, 15, 20, 25	Dall'Agnol, 2001
Vitamins	*Folium Salviae officinalis* (Sage) *Folium thyme* (Thyme) *Folium origani cretici* (Oregano)	Albania Poland Turkey	Gamma rays	10	Brandstetter et al. 2009

Studies regarding the effects of irradiation on macronutrients of dried plants are scarce. However, there is some information concerning phenolics, flavonoids and antioxidant activity, as well as on amino acids, vitamins and organic acids. Similarly, there are studies on the impact of irradiation in volatile compounds and organoleptic properties using different techniques, such as gas chromatography coupled to flame ionization detection (GC/FID) or mass spectrometry (GC/MS), high performance liquid chromatography coupled to refraction index (HPLC/RI) or fluorescence detection (HPLC-FL) and sensory evaluation (Khan and Abrahem, 2010; Fernandes et al., 2012b; Carocho et al., 2013).

Kausar et al. (2013) studied the effects of irradiation in amino acids and sugars, and reported that the content in amino acids such as leucine, alanine, and glutamic acid increased in irradiated samples. Otherwise, histidine content decreased as the irradiation dose increased. However, the contents of total free amino acids were not significantly changed. Regarding sugars, sucrose, glucose and fructose contents increased significantly after treatment with gamma radiation. In general, gamma radiation had no unfavourable effects on reducing sugars and major carbohydrate components. Lianzhong et al. (1998) did not find significant differences in organic acids content and profile in irradiated samples of spices in relation to non-treated ones. Brandstetter et al. (2009) examined the changes produced by gamma radiation on tocopherols and total polyphenol content of various plant species, without observing significant differences in the composition of total phenolics and antioxidant activity between irradiated and non-irradiated samples. Other authors have also evaluated the phenolic/flavonoid composition and antioxidant activity concluding that, in general, gamma radiation at the tested doses does not affect significantly the total phenolics content and does not show significant effects on the antioxidant capacity, in comparison with non-irradiated samples (e.g., Dall'Agnol, 2001; Koseki et al., 2002; Khattak and Simpson, 2008, 2010; Jeong et al., 2009; Nagy et al., 2011; Aouidi et al., 2011; Chiang et al., 2011).

1.3 The particular case of *Aloysia citrodora* Paláu, *Arenaria Montana* L., *Ginkgo biloba* L., *Melissa officinalis* L., *Melittis melissophyllum* L., *Mentha pipperita* L. and *Thymus vulgaris* L.

1.3.1 Botanical characterization and medicinal uses

Aloysia citrodora Paláu (lemon verbena; **Figure 5**) is an herb belonging to the Verbenaceae family. It is originated from South America, although its cultivation has already spread to North Africa and Southern Europe (Ragone et al., 2007). This plant is grown in gardens and vegetable gardens; it shows upright stalks of cylindrical configuration with a height from 2 to 6 m, and green and lanceolate leaves with an acute apex and attenuated base measure of approximately 45-110 mm (López-González, 1991). The leaves are used with culinary purposes, giving a lemon flavour to many dishes, such as fish, poultry, vegetable marinades, salad dressings, jams, puddings and drinks; it is also used to make teas and ice cream. However, this plant beyond its organoleptic properties also has therapeutic potential, with effects on gastrointestinal, nervous and respiratory systems. It has been reported to possess anti-inflammatory, antimicrobial, antitumor, digestive, antispasmodic, antipyretic and sedative activities, being indicated in the treatment of asthma, cold, fever, flatulence, colic, diarrhea and indigestion. Its consumption is done by infusion, decoction and condiment. In addition to its use as fresh or dry plant, the essential oil is also widely employed for its medicinal properties (Argyropoulou el al., 2007; Ragone et al., 2007; Funes et al., 2009).

Figure 5. *Aloysia citrodora* L. Source: PRO Quinta de Corujas, by www.Flickr.com.

Arenaria Montana L. (Mountain sandwort; **Figure 6**) is a flowering plant belonging to the Caryophyllaceae family. It is native and cultivated in mountainous regions of South-Western Europe (Timité et al., 2011; Carvalho and Morales, 2013). Its growth is wild and happens in forests and thickets, sometimes in rocky outcrops and slopes of roads, preferably in acid substrates. April and June are the months when flowering occurs (Flora-On, 2014). It is a low evergreen perennial, forming a loose mat of 30 cm in width, and possesses small, green, elliptic leaves, with erect stems of approximately 10 cm in height, each carrying a few white flowers approximately 1.5-2 cm in width in early summer (Royal Society of Horticulture, 2016/ https://www.rhs.org.uk/plants/details?plantid=162). It is used in the Portuguese traditional medicine as an anti-inflammatory and diuretic, being mainly ingested in the form of infusion, prepared from the leaves, stems and flowers (Timité et al., 2011; Carvalho and Morales, 2013).

Figure 6. *Arenaria montana* L. flowers. Source: *k59, by www.Flickr.com.

Ginkgo biloba L. (ginkgo; **Figure 7**) is a long-lived deciduous tree belonging to the Ginkgoaceae family. Its existence dates back millions of years ago, and although it was previously disseminated worldwide, particularly in Europe, North America and Asia, it gradually disappeared till being only present in Asia. However, its high environmental adaptability and unprecedented tolerance to environmental stress make this tree suitable for planting in high temperate and subtropical climates (Gong et al., 2008). It is a slow growing and deciduous tree that loses all the leaves in winter and reaches an average height of 20 to 35 m (Singh et al., 2008). Leaves, light green and between 4-7 cm of length and 4-10 cm of

width, are flat and fan-shaped with dichotomous rib; those born in the long shoots usually have notches or lobules (Santos et al., 2015).

Despite its quite old existence, its therapeutic recognition is more recent, giving it the applicability, at an initial stage, in traditional medicine and later in scientific medicine, occupying a leading position among herbal drugs (Singh et al., 2008; Wohlmuth et al., 2014). It is used not only in traditional medicine but also by professionals in the medical field to assist in treating problems typically associated with aging, such as intermittent claudication, decreased mental vitality in old age (mental confusion, memory loss, dementia praecox, concentration problems), poor circulation and tinnitus. EGb 761 is a standardized extract of *Ginkgo biloba* leaves, that contains approximately 24% flavone/ol glycosides (primarily quercetin, kaempferol and isorhamnetin) and 6% terpene lactones (2.8-3.4% ginkgolides A, B and C, and 2.6-3.2% bilobalide), and has been used experimentally as natural therapeutic agent in the treatment of Alzheimer's disease (Smith et al. 1996; Diamond et al. 2000; Annaházi et al. 2010). It can also be consumed in the form of infusion or incorporating its extract in drugs (Santos et al., 2015).

Figure 7. *Ginkgo biloba* L. leaves. Source: Landahlauts, by www.Flickr.com.

Melissa officinalis L. (lemon balm; **Figure 8**) is an herb belonging to the Lamiaceae family. It is a perennial herb with lemon flavor that is renewed every year, blooming in spring and summer. It is cultivated worldwide, varying their growth from 30 to 125 cm. Leaves are petiolate, ovate, 6 cm long, 3 cm broad, the upper cuneate, the lower cordate at base, crenate-toothed, subglabrous, sometimes with glandular hairs or punctuate glands beneath. Its uses

have been described over the time, namely to add flavor to food, and in traditional medicine in the treatment of disorders related to the central nervous system, cardiovascular, gastrointestinal and respiratory system and various types of cancer, acting also as a digestive, analgesic, sedative, spasmolytic and hypotensive. It is usually consumed in the form of infusion (Quer, 1999; Duda et al., 2015; Shakeri et al., 2016).

Figure 8. *Melissa officinalis* L. leaves. Source: Wendell Smith, by www.Flickr.com.

Melittis melissophyllum L. (bastard balm; **Figure 9**) is a perennial herb belonging to the Lamiaceae family. Its flowering occurs in spring from May to July and grows commonly in woods and forests and inhabiting shady places in Western, Southern and Central Europe. It shows ovate leaves of about 5-7 cm and has big axillary flowers whose origination is almost always in pairs on each node of the plant; the corolla has about 3 cm long and usually an intense pink colour. Its use in folk medicine is an old practice that has been maintained through time, particularly in the countries of central Europe. It is consumed in the form of infusion by its effects on the nervous and gastrointestinal systems (as anti-spasmodic), against insomnia and eye inflammation, and for treatment of cough and sore throat (Quer, 1999; Skrzypczak-Maggy et al., 2009; Pietraszek and Pietraszek, 2012).

Figure 9. *Melittis melissophyllum* L. Source: Werner_Schmutz, by www.Flickr.com.

Mentha x Piperita L. (peppermint; **Figure 10**) is a perennial herb belonging to the Lamiaceae family. It is a hybrid of two species: *Mentha aquatica* L. and *Mentha spicata* L.. Its flowering occurs in summer and its cultivation is done easily in temperate climates. It originated from Europe and the Middle East, although it extended to American and Asia continents. It is a green plant, with no pubescence, approximately 30-100 cm long and pleasant and intense aroma of mint (Quer, 1999). It is commonly used in traditional medicine and has been associated to antioxidant, antitumor, antimicrobial, hypoallergenic, antiviral, anti-inflammatory, mildly anaesthetic, antispasmodic, antiulcer, cytoprotective, hepatoprotective, immunomodulatory properties, and beneficial effects in the gastrointestinal system (Riachi and Maria, 2015; Singh et al., 2015; Uribe et al., 2016). Its essential oil is also well known and widely used in food, pharmaceutical and cosmetic industries, because it presents biological activity against several organisms (Moghaddam et al., 2013; Sharma and Sharma, 2013). Due to its organoleptic characteristics, it is used as a single infusion not only for therapeutic purposes, but just to be taken as a drink, as well as employed as a spice in food (Riachi and Maria, 2015).

Figure 10. *Mentha x Piperita* L. leaves. Source: Hidetsugu Tonomura, by www.Flickr.com.

Thymus vulgaris L. (thyme; **Figure 11**) is a popular spice also belonging to the Lamiaceae family. It is a perennial herb whose flowering occurs in spring, from March, and that grows abundantly in Central and Southern Europe, Africa and Asia (Quer et al., 1999; Nicolic et al., 2014). This plant forms a mantle of about 30 cm tall and very thick; it has dark green leaves with maximum length of 1 cm, lanceolate and narrow, and light mauve-pink flowers in early Summer (Quer, 1999; Nezhadali et al., 2014).

Thyme is a plant widely used in folk medicine, in the treatment of convulsions, respiratory diseases, smooth muscle spasm and swelling, and its essential oil is recognized by its composition in bioactive metabolites (flavonoid glycosides, hydroquinone derivatives, terpenoids and biphenyls) and bioactive properties, such as antimicrobial, anti-inflammatory, expectorant, spasmolytic, antioxidant and hepatoprotective activities (Fecka and Turek, 2008; Nikolić et al., 2014; Gavarić et al., 2015; Martins et al., 2015). It is one of the most cultivated herbs of *Thymus* genus, being used in food, pharmaceutical and cosmetic industries; thyme oils are listed in the Pharmacopoeia of Europe, and used as natural preservatives in the food industry (Nezhadali et al., 2014; Gavaric et al., 2015). Thyme is commonly used as an herbal tea, condiment and spice.

Figure 11. *Thymus vulgaris* L. Source: A.M. Carvalho.

1.3.2 Phenolic compounds as bioactive substances

In recent years there has been an increasing interest in plant products as a source of beneficial compounds for health. This interest is related to their natural origin, the possibility to be ingested through the diet, easy acquisition and their perception as safe products with reduced adverse effects (Dillard and German, 2000). Indeed, the use of medicinal plants for the treatment of different diseases dates back to ancient times, many of them revealing satisfactory results as anti-inflammatory, antimicrobial, antimutagenic, anti-cancer and/or antioxidant agents (Wojdyło et al., 2007). For this reason, more and more species are being explored for their bioactive components, so as to identify the most active chemical compounds, establish adequate amounts for their incorporation in drugs or nutraceuticals, and get to know their potential side effects. Phenolic compounds are one of the classes of bioactive compounds that are highly reported in the plants dealt with in the present work (**Table 3**).

Table 3. Representative bioactive compounds reported in the plants studied in the present work.

Plant	Bioactive compounds	References
Aloysia citrodora P.	Acacetin-7-diglucuronide; Apigenin; Chrysoeriol-7-diglucuronide; Cirsilineol; Cirsimaritin; Cistanoside F; Diosmetin; Eukovoside or isomer; Eupatorin; Forsythoside A; Gardoside; Hastatoside; Hispidulin; Isoverbascoside; Jaceosidin; Luteolin; Martinoside; Nepetin; Theveside; Verbascoside; Verbenalin.	Andrade et al., 2016; Valentão et al., 1999; Quirantes-Piné, 2009; Bilia et al., 2008.
Arenaria montana L.	-	-
Ginkgo biloba L.	3,4-dihydroxybenzoic acid; 4-*O*-methylpyridoxine; 5-caffeoylshikimic acid; 6-hydroxykynurenic acid; protocatechuic acid, quinic acid, alkylphenols; biflavones; bilobalides; carboxylic acids; ginkolides; flavone glycosides; flavonoids (apigenin; catechin; isorhamnetin; kaempferol; myricetin; patuletin, quercetin, rutin, shekanin, syringetin, yinxingensin); proanthocyanidins; terpene trilactones; sterols.	Ismail and El-Sorbaty, 2016; López-Gutiérrez et al., 2016; Yan et al., 2015; Guo et al., 2015
Melissa officinalis L.	apigenin; caffeic acid; catechin; chlorogenic acid; coumaric acid; ellagic acid; eriodictyol; ferulic acid; gallic acid; hesperidin; isoquercitrin; lithospermic acid; luteolin; melitric acid; naringin; naringenin; rhamnocitrin; rosmarinic acid; rutin; sagerinic acid; salvianolic acid; sinapic acid; vanillin; yunnaneic acid.	Duda et al., 2015; Shakeri et al., 2016; Barros et al., 2013c;
Melittis melissophyllum L.	caffeic acid; chlorogenic acid; cinnamic acid; coumaric acid; coumarin; ferulic acid; hydroxybenzoic acid; methyl salicylate; protocatechuic acid; syringic acid; sinapic acid; vanillic acid.	Skrzypczak-Pietraszek and Pietraszek, 2012; Maggy et al., 2009;

Mentha x Piperita L.	apigenin; caffeic acid; catechin; cinnamic acid; citric acid; chlorogenic acid; coumaric acid; diosmin; diosmetin; eriocitrin; eriodictyol; ferulic acid; gallic acid; gallocatechin gallate; gardenin; hesperetin; isorhoifolin; luteolin; malic acid; myricetin; naringenin; narirutin; pebrellin; protocatechuic acid; quercetin; resveratrol; rosmarinic acid; rutin; salvianolic acid; sinapic acid; syringic acid; vanillic acid.	Andrade et al., 2016; Areias et al., 2001; Kapp et al., 2013; Riachi and Maria, 2015
Thymus vulgaris L.	apigenin; arbutin; caffeic acid; caffeoylquinic acid; carnosic acid; chlorogenic acid; cinnamic acid; coumaric acid; dicaffeoylquinic acid; eriocitrin; eriodictyol; ferulic acid; gallic acid; hesperetin; hydroxybenzoic acid; isorhamnetin; kaempferol; luteolin; lithospermic acid; methyl rosmarinate; narirutin; naringenin; neochlorogenic acid; protocatechuic acid; quercetin; quinic acid; rosmarinic acid; rutin; syringic acid; sinapic acid; vanillic acid.	Martins et al., 2015; Fecka and Turek, 2008; Boros et al., 2010; Roby et al., 2013; Vallverdú-Queralt et al., 2014

1.4 Objectives and working plan

Medicinal and aromatic plants, due to their composition in bioactive compounds, have been used as auxiliaries or even as therapeutic promoters being incorporated in the pharmaceutical, cosmetic and food industries, in the form of different formulations. However, in order to be employed in the industry fulfilling all health and safety standards, it is necessary to proceed by effective decontamination and safe processes. Irradiation is a technology applied in various countries to different types of food that is considered safe not only for consumers but also for the environment. Several studies have been performed evaluating the effects of irradiation in food products, as also spices and medicinal and aromatic plants.

Thus, in this context, the aim of the present study was to evaluate chemical and nutritional characteristics of seven plant species, their infusions and different dietary supplements (syrup and pills) based on some of these species and used in traditional medicine, as well as to test the effects of irradiation in physical (color), nutritional (proteins, ash, fat, carbohydrates and energy), chemical (sugars, tocopherols, fatty acids, organic acids), toxicological (mycotoxins) and bioactive (antioxidant activity, free radicals scavenging activity, reducing power, inhibition of lipid peroxidation, phenolic compounds and cytotoxicity) variables.

The effects of irradiation were evaluated using:

i) different plant species: *Aloysia citrodora* P. (Verbenaceae; lemon verbena), *Arenaria montana* L. (Caryophyllaceae, mountain sandwort), *Ginkgo biloba* L. (Ginkgoaceae; ginkgo), *Melissa officinalis* L. (Lamiaceae; lemon balm), *Melittis melissophyllum* L. (Lamiaceae; bastard balm), *Mentha x piperita* L. (Lamiaceae; peppermint) and *Thymus vulgaris* L. (Lamiaceae; thyme);
ii) different types of ionizing radiation: gamma radiation and electron beam;
iii) different doses of radiation: 0, 1, 2, 5 and 10 kGy;
iv) different storage periods: 0, 12 and 18 months;

Different statistical tools (analysis of variance (one-way ANOVA and 2-way ANOVA), principal components analysis and linear discriminant analysis) were used to treat the results.

A scheme of the working plan is shown in **Figure 12**.

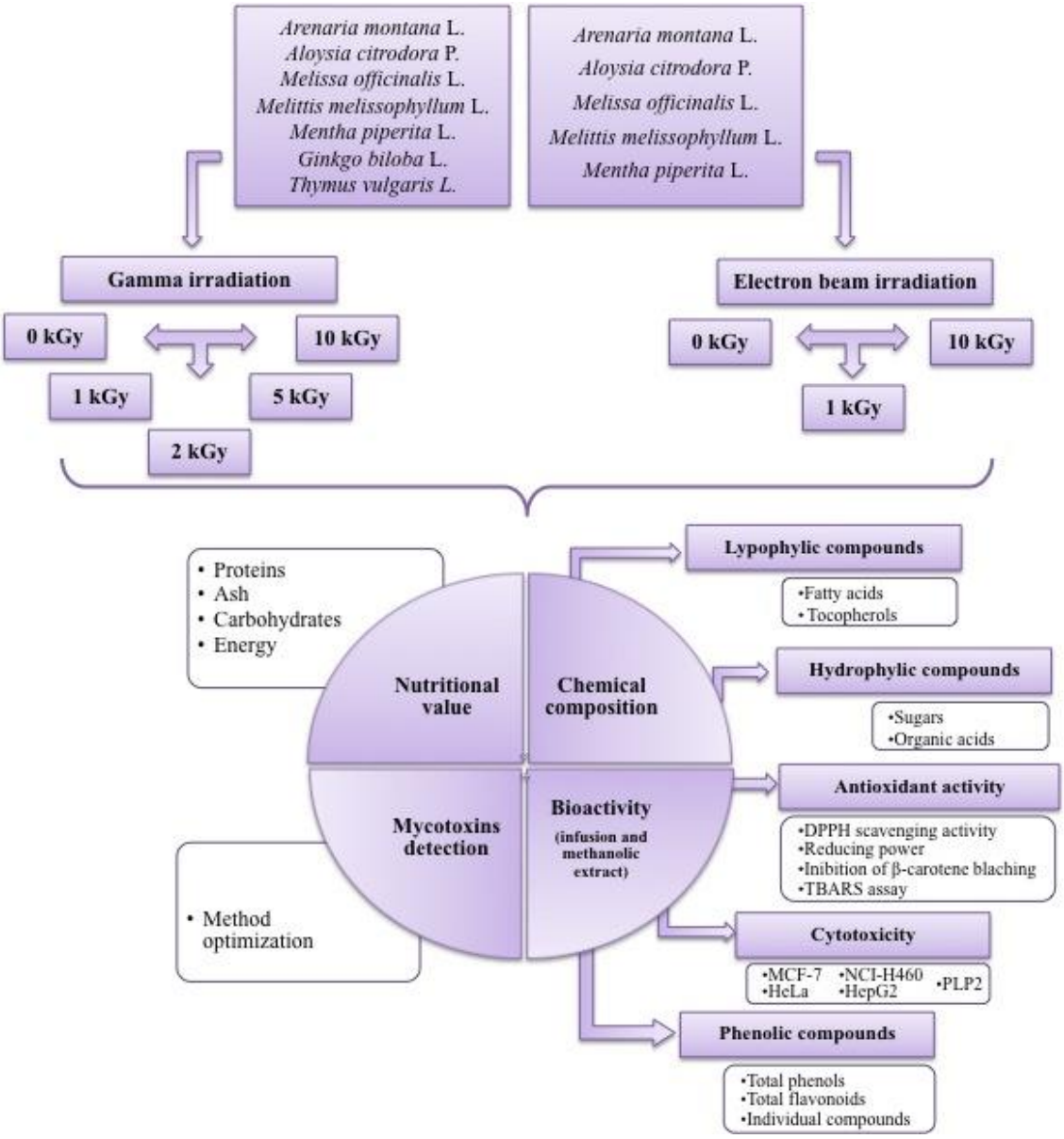


Figure 12. Scheme of working plan.

CHAPTER 2: MATERIAL AND METHODS

2.1 Standards and reagents

2.1.1 For irradiation

A chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Company, UK) were used to estimate the dose and dose rate of irradiation. The acid aqueous Fricke dosimeter solution was prepared using the following reagents: ferrous ammonium sulfate (II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA).

2.1.2 For chemical analyses

Acetonitrile (99.9%), n-hexane (95%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, tocopherols (α-, β-, λ-, and δ-isoforms), sugars ((D(-)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose and D(+)-raffinose pentahydrate) and organic acid standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.1.3 For bioactivity evaluation

Cytotoxicity analysis: Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma. Water was treated in Milli-Q water purification system.

Antioxidant activity: Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) was obtained from Alfa

Aesar (Ward Hill, MA, USA). β-carotene and linoleic acid were acquired from Sigma and Tween 80 from Panreac. All other solvents and reagents were purchased from scientific retailers.

2.1.4 For mycotoxins extraction and detection

Aflatoxin B_1 standard solution was obtained from Biopure (Tulln, Austria). Ochratoxin A (OTA) standard was purchased from Sigma. Methanol, water and acetonitrile were of HPLC grade from Fisher Scientific (Lisbon, Portugal). For aflatoxins and OTA extracts purification, AflaTest WB and OchraTest WB immunoaffinity columns (IACs) were obtained from VICAM (Watertown, MA, USA). Phosphate Buffer Saline with Tween (PBS-T) was prepared as follows: NaCl (8.0 g), Na_2HPO_4 (1.2 g), KH_2PO_4 (0.2 g), KCl (0.2 g), Tween 20 (0.1 mL) were made up to 1 L with deionized water and the pH value was adjusted to 7.0 with NaOH aquouse solution (1 mL).

2.2 Plant material

Samples of *Ginkgo biloba* L. (Ginkgoaceae; Ginkgo) dry leaves were provided by Américo Duarte Paixão Lda., in Alcanede (Portugal), imported from China as material for infusion preparation. Dietary supplements and other *G. biloba* samples (syrup and different pills based on leaves standardized extract containing 24% glycosides and 6% terpenes) were obtained from a Pharmacy and a Herbalist shop, respectively, located in Bragança, Portugal. A description of the different *G. biloba* studied samples is collected in **Table 4**.

Table 4. Information about the studied *Ginkgo biloba* samples.

Code	Sample	Composition	Recommended mode of consumption (minimal dose/day)	Concentration of the prepared solution
LI	Plant	Dry leaves	Infusion (lyophilized infusions)	20 mg/mL
LME		Methanolic extract from dry leaves	- (dried extracts)	20 mg/mL
LAE		Aqueous extract of dry leaves	- (dried extracts)	20 mg/mL
S	Syrup	40 mg of *Ginkgo biloba* standardized extract[a]/mL	1 mL diluted in a glass of water (200 mL); 2 or 3 times	200 μg/mL
P1	Pills	40 mg of *Ginkgo biloba* standardized extract[a]/pill	1 pill dissolved in ½ glass of water (100 mL); 2 or 3 times	400 μg/mL
P2		60 mg of *Ginkgo biloba* standardized extract[a]/pill	1 pill dissolved in a glass of water (200 mL); 2 times	300 μg/mL
P3		100 mg of *Ginkgo biloba* standardized extract[a]/pill	1 pill dissolved in a large glass of water (350 mL); 1 time	286 μg/mL

[a] Corresponds to *G. biloba* leaves extract containing 24% glycosides and 6% terpenes (according to the information available in the label). Indicated therapeutic properties: antioxidant, antiasthmatic, radicals scavenging, wound healing and neuroprotective properties, as well as it improves mental capacities in Alzheimer's patients.

Samples of *Aloysia citrodora* P. (Verbenaceae; lemon verbena), *Melissa officinalis* L. (Lamiaceae; lemon balm), *Melittis melissophyllum* L. (Lamiaceae; bastard balm), *Mentha x piperita* L. (Lamiaceae; peppermint) and *Thymus vulgaris* (Lamiaceae; thyme) were provided

as dry leaves by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal), under the scope of a joint research project (PRODER nº 53514, AROMAP).

Arenaria montana L. (Caryophyllaceae; mountain sandwort) flowers and leafy stems (approximately the upper 15 cm of the dense clumps produced in spring) are commonly wild gathered in Bragança (Northeastern Portugal). The samples was collected in full bloom, in spring along paths through the oak trees, in Oleiros, Bragança. A sample for analysis was prepared by putting together the material from different specimens. Voucher specimens are deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA).

The samples were analyzed immediately after irradiation (0 months) and after further storage in a dry place protected from light for 12 and 18 months. In all cases, the analyses were also performed in non-irradiated samples (control).

2.3 Irradiation of dry material

2.3.1 Dosimeters reading and calibration

Fricke dosimeters were used to obtain the dose rate for the positions inside the chamber where the samples were irradiated. This a chemical radiation sensitive solution, where the ions Fe^{2+} change to Fe^{3+} due to ionizing radiation induced oxidation, that changes the absorbance in the region of 300 nm.

The dose is estimated based in the equation (ASTM, 1992):

$$D_F = \frac{\Delta A}{\rho \varepsilon G d}$$

Where:

D_F - dose absorbed by the solution of Fricke (Gy);

ΔA - absorbance difference between irradiated and non-irradiated solution;

ρ - density of the dosimetric solution (kg m^{-3});

ε - Linear molar absorption coefficient (m^2 mol^{-1});

G - chemical yield of ferrous ions (mol J^{-1});

d - optical path in the solution and in the reading cell (m);

To estimate the dose rate, these dosimeters are irradiated at several times, establishing a first order relation of "Dose *versus* Time", that is then used to predict the doses before an irradiation.

During gamma irradiations were used the routine dosimeters Amber Perspex (a trademark of Harwell Company, Oxfordshire, UK). These dosimeters are made of polymethylmethacrylate (PMMA), a transparent material impregnated with a radiation sensitive pigment.

The dosimeters were previous calibrated, based on the dose rates obtained with Fricke dosimeter. For that, a "Dose *versus* Absorbance" curve fitting was obtained, where the Absorbance was measured by spectrophotometry (at 603 nm for Amber Perspex), after irradiation at several times.

And then perform a fitting "Dose *versus* Absorbance", considering the lowest polynomial order that represents the data, using the residuals plot and R-squared value to check the quality of adjusted function (Sharpe, 2009).

The fitting equation obtained for Amber Perspex dosimeter was:

$$D = 0.347 + 2.0281 \times Abs + 0.1378 \times Abs^2$$

Equation 1. Equation for D values determination in calibration dosimeters procedure.

Where:

- D: dose (kGy)
- Abs: specific absorbance (cm^{-1})

Gammachrome YR dosimeters (Harwell Company, Oxfordshire, UK) were calibrated at the electron-beam irradiation facility, using a Calorimeter as a reference dosimeter.

A Calorimeter used in radiation processing is a wafer of a material, that changes the temperature during irradiation. A curve of Dose *versu*s Temperature is then established, to be used as a reference for other dosimeters calibration.

The absorbed energy, E, is estimated based on the Thermodynamic equation:

$$E = m\, C\, \Delta T$$

Where:

m: mass of the material

C: specific heat

ΔT: is the temperature variation, increase after irradiation.

And the absorbed dose, *D*, is obtained from its definition:

$$D = E / m$$

Where:

E: energy

m: mass.

These dosimeters were then attached to the samples, measuring their absorbance after irradiation to estimate the dose (in kGy).

With that, it was possible to know:

- the dose rate, dose per unit of time (kGy h^{-1});
- the maximum dose (registering the maximum value, in the dosimeters that were read);
- the minimum dose (registering the minimum value, in the dosimeters that were read);
- the dose uniformity ratio (DUR), the ratio between the maximum and minimum value;
- and the average dose (in kGy).

2.3.2 Gamma radiation

After confirmation of the taxonomical identification, the samples were divided into groups (**Figure 13**) to be submitted to different irradiation treatments depending on the sample and type of assay. The irradiation was performed in a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with total activity 177 TBq (4.78 kCi) (**Figure 14**), in September 2013. The estimated dose rate for the irradiation position was obtained with Fricke dosimeter, and during the irradiation process, the dose was determined using Amber Perspex routine dosimeters (batch V, from Harwell Company, U.K.), following the procedure previously described by Fernandes et al. (2013).

Figure 13. Samples prepared for gamma radiation. Author: Eliana Pereira.

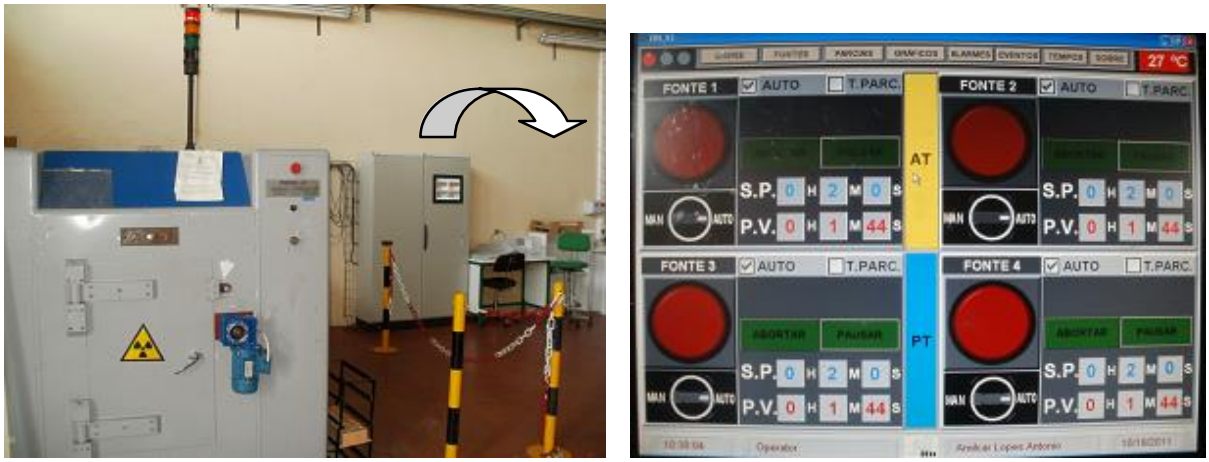


Figure 14. (A) Experimental gamma irradiation chamber, and (B) sources touch control panel. Author: Amilcar Antonio.

In order to evaluate the effects on chemical components and antioxidant properties, the samples were submitted to irradiation doses of 1 and 10 kGy and compared with the respective non-irradiated controls (0 kGy). In those assays, the applied estimated doses, dose rates and dose uniformity ratios (D_{max}/D_{min}) were respectively: 1.20 ± 0.07 kGy, 2.57 ± 0.15 kGy h^{-1} and 1.20, for the treatment at the predicted dose of 1 kGy; and 8.93 ± 0.14 kGy, 1.91 ± 0.03 kGy h^{-1} and 1.02, for the treatment at 10 kGy.

In the cytotoxicity assays perfomed on the samples of thyme and peppermint theoretical doses of 0, 2, 5 and 10 kGy were applied, being the estimated doses: 2.4 ± 0.1 kGy, 5.5 ± 0.2 kGy and 10.4 ± 0.5 kGy, for thyme, and 2.2 ± 0.3 kGy, 5.7 ± 0.2 kGy and 10.3 ± 0.4 kGy, for peppermint samples; dose rates and dose uniformity ratios (D_{max}/D_{min}) were, 1.2 kGy h^{-1} and 1.1, respectively.

Finally, in the assays for mycotoxins detection in lemon verbena samples, theoretical doses of 0, 1, 5 and 10 kGy were applied, being the estimated absorbed gamma radiation 1.2 ± 0.1 kGy, 5.2 ± 0.2 kGy and 10.4 ± 0.4 kGy, and the estimated dose rate and the dose uniformity ratio 1.7 kGy h^{-1} and 1.2, respectively.

After irradiation, the samples were reduced to powder and mixed to obtain homogenized samples for subsequent analysis. For simplicity, in the text and tables the theoretical values of 0, 1, 2, 5 and 10 kGy were considered.

Table 5. Information about the studies performed.

Sample	Type of assay	Predicted dose	Estimated dose (kGy)	Estimated dose rate (kGy h^{-1})	Dose uniformity ratio (D_{max}/D_{min})
All samples	Chemical and antioxidant studies	1 kGy	1.20 ± 0.07	2.57 ± 0.15	1.20
		10 kGy	8.93 ± 0.14	1.91 ± 0.03	1.02
Thyme	Cytotoxity	2 kGy	2.4 ± 0.1	1.2	1.1
		5 kGy	5.5 ± 0.2		
		10 kGy	10.4 ± 0.5		
Peppermint	Cytotoxity	2 kGy	2.2 ± 0.3	1.2	1.1
		5 kGy	5.7 ± 0.2		
		10 kGy	10.3 ± 0.4		
Lemon verbena	Mycotoxin detection	1 kGy	1.2 ± 0.1	1.7	1.2
		5 kGy	5.2 ± 0.2		
		10 kGy	10.4 ± 0.4		

2.3.3 Electron-beam radiation

After confirmation of the taxonomical identification, the samples were divided into three groups: control (non-irradiated, 0 kGy), group 1, submitted to 1 kGy, and group 2, submitted to 10 kGy, as predicted doses of irradiation (**Figure 15**). The irradiation was performed at the INCT- Institute of Nuclear Chemistry and Technology, in Warsaw, Poland (**Figure 16**), in February, 2014. To estimate the dose during the irradiation process three types of dosimeters were used: a standard dosimeter, a graphite calorimeter, and two routine Gammachrome YR

and Amber Perspex dosimeters, from Harwell Company (UK). The irradiation took place in an e-beam irradiator of 10 MeV of energy with pulse duration of 5.5 ms, pulse frequency of 440 Hz and average beam current of 1.1 mA; the scan width was 68 cm, the conveyer speed was settled to the range 20-100 cm/min and the scan frequency was 5 Hz. The estimated absorbed dose for irradiated samples was 0.83 for group 1 and 10.09 kGy for group 2, with a maximum uncertainty of 20%. In the Amber Perspex and Gammachrome YR dosimeters, the irradiation dose was estimated by spectrophotometric measurement at 603 nm and 530 nm, respectively, by comparison with a calibration curve. For the graphite calorimeter dosimeter the electrical resistance was read and converted in dose according to a calibration curve, obtained following the standards during the Quality Control procedures of the irradiation equipment and facility.

Figure 15. Picture of samples submitted to electron beam radiation. Author: Eliana Pereira

Figure 16. Conveyor of electron beam radiation. Author: Amilcar Antonio.

2.4 Color evaluation

A colorimeter (model CR-400; Konica Minolta Sensing, Inc., Japan), with an adapter for granular materials (model CRA50) was used to measure the color of the samples (**Figure 17**). Measurements were made in the CIE L*a*b* color space (**Figure 18**) using the illuminant C and a diaphragm aperture of 8 mm; data were processed with the "Spectra Magic Nx" (version CM-S100W 2.03.0006) software, from Konica Minolta. Prior to the measurements the instrument was calibrated against a standard white tile (Fernandes et al. 2012a). The color of three samples from each batch was measured in three different points, for each dose and at each time point, being considered the average value. The color difference or total color change for each sample was determined using the three-dimensional color space coordinates with the equation 1:

$$\Delta E = \sqrt{(L^*)^2 + (a^*)^2 + (b^*)^2}$$

Equation 2. Equation for total color determination.

Where:

- L^*: Lightness
- a^*: Redness/greennes value
- b^*: Blueness/Yellowness

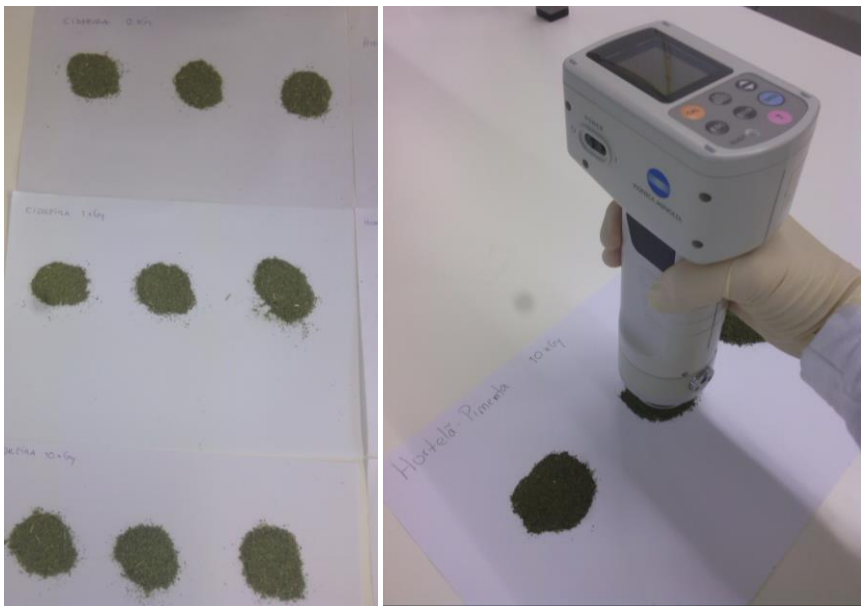


Figure 17. Color measurement procedure. Author: Eliana Pereira.

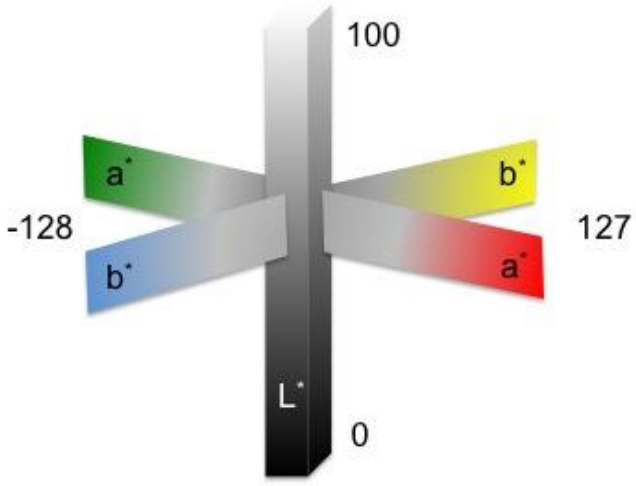


Figure 18. CIE L*a*b* model.

(L* = 0 yields black and L* = 100 indicates diffuse white; specular white may be higher; a*, negative values indicate green while positive values indicate magenta; b*, negative values indicate blue and positive values indicate yellow).

2.5 Evaluation of nutritional and chemical variables

2.5.1 Nutritional value

Protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 2002). Crude protein content (N×6.25) was estimated by the macro-Kjeldahl method. This method is based on the amount of nitrogen present in a sample and relies on the destruction of all organic matter by addition of a strong acid (sulphuric acid) that retains nitrogen under the form of $(NH_4)_2SO_4$. Further addition of NaOH releases the nitrogen as NH_3 that is collected by steam distillation on a solution of 0.1N H_2SO_4; afterwards a titration with 0.1N NaOH using methyl red as an indicator is made to calculate the amount of nitrogen. Crude fat was determined using a Soxhlet apparatus by extracting a known weight of sample with petroleum ether. The ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference and total energy was calculated according to the equation 2:

$$\text{Energy (kcal)} = 4 \times (g_{protein} + g_{carbohydrates}) + 9 \times (g_{fat})$$

Equation 3. Equation for energy determination.

2.5.2 Chemical composition

2.5.2.1 Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI). Dried sample powder (1.0 g) was spiked with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000 *g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm Whatman nylon filters (Pinela et al., 2011). The HPLC equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.5.2.2 Organic acids

Organic acids were determined using ultra-fast liquid chromatography coupled to a photodiode array detector (UFLC–PDA). Samples (approximately 2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 μm nylon filters (Barros et al., 2013a). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C18 column (5 μm, 250 mm· 4.6mm i.d.) thermostatted at 35 ºC. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by

comparison of the area of their peaks recorded at 215 nm (245 nm for ascorbic acid) with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

2.5.2.3 Tocopherols

Tocopherols were determined by HPLC (equipment described in section 1.5.2.1) coupled to a fluorescence detector (FP-2020; Jasco). BHT solution in hexane (10 mg/mL; 100 μL) and IS solution in hexane (tocol; 50 μg/mL; 400 μL) were added to the sample prior to the extraction procedure. The samples (approximately 500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000 *g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of n-hexane, dehydrated with anhydrous sodium sulphate, filtered through 0.2 μm nylon filters from Whatman, and transferred into a dark injection vial for the analysis (Pinela et al., 2011). The fluorescence detector was programmed for excitation (λ_{ex}) at 290 nm and emission (λ_{em}) at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm x 4.6 mm i.d.) normal-phase column from YMC Waters operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on calibration curves obtained from commercial standards of each compound using the IS methodology. The results were expressed in mg/100 g of dry weight (dw).

2.5.2.4 Fatty acids

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously (Pereira et al., 2013b). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey–Nagel column (30 m x 0.32 mm ID x 0.25 μm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC,

20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

2.6 Mycotoxins extraction and analysis

Due to the toxicity of AFs and OTA, all the necessary safety considerations were taken into account when handling these substances, as recommended (Castegnaro et al., 1980). Solutions were handled with protective gear; all disposable materials were decontaminated by autoclaving before being disposed; reusable materials were decontaminated by immersion in 10% bleach overnight, immersion in 5% acetone for one hour and washed with distilled water several times.

2.6.1 Spiking

For determination of the effect of irradiation on mycotoxin reduction, three hundred and sixty grams of powdered material were spiked with AFB_1 and OTA at 30 ng/g, thoroughly homogenized and divided in 72 aliquots of 5 g each, packaged in appropriate bags (polyethylene, 63 μm thickness). Samples were irradiated at 1, 5, and 10 kGy. Each irradiation dose was applied to 3 aliquots in two independent treatments, for a total of 6 replicates. Non-irradiated samples (n = 6) were used as control (0 kGy).

2.6.2 Aflatoxins extraction and quantification

For AFB_1 extraction, 2.5 g of each irradiated sub-sample were extracted by stirring (25 ºC at 150 rpm) with 0.5 g sodium chloride and 20 mL of methanol/water (80:20, v/v) for 30 minutes. The mixture was then filtered by gravity through a Whatman no. 4 (Sigma Aldrich Co., St. Louis, MO, USA) fluted filter paper and a 10 mL aliquot of the filtrate was diluted with 40 mL of water. The extract was homogenized and further filtered through a Whatman glass microfibre filter (934-AH). Subsequent cleanup was made by passing 20 mL of the

filtered extract through an immunoaffinity column (IAC) by gravity, at a rate of approximately 1-2 drops/s. The column was washed with 10 mL of PBS-T followed by 10 mL of ultra-pure water. AFB_1 was eluted with 2 mL of methanol, collected in a glass vial, filtered through 0.2 µm nylon filters (Whatman) and analysed by reversed-phase HPLC with fluorescence detection (FLD). The HPLC equipment (described in section 1.5.2.1) was coupled to a photochemical post-column derivatization reactor (PHRED unit - Aura Industries, USA) and a fluorescence detector (FP-2020, Jasco) set to λ_{ex} 365 nm and λ_{em} 435 nm. Data were analysed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). The chromatographic separation was achieved using an isocratic elution with a reverse-phase C_{18} column (100 x 4.6 mm, Merck Chromolith Performance, Darmstadt, Germany) operating at 35 ºC (7971 R Grace oven). The mobile phase was a mixture of acetonitrile/methanol/water (10:30:60, v/v/v) at a flow rate of 1 mL/min, and the injection volume was 10 µL. AFB_1 was identified by chromatographic comparison with the standard. Quantification was based on the fluorescence signal response.

2.6.3 Ochratoxin A extraction and determination

OTA extraction followed the procedure described by Zhao et al. (2014) with some modifications. Briefly, 2.5 g of each irradiated sample were extracted by stirring (25 ºC at 150 rpm) in 12.5 mL MeOH / 1% $NaHCO_3$ solution (70:30, v/v) for 30 minutes and subsequently filtered through Whatman No. 4 fluted filter paper. Afterwards, 10 mL of the extract was diluted with 40 mL of PBS-T and further filtered through a Whatman glass microfibre filter (934-AH). Subsequently, 20 mL of the filtered extract were purified through an Ochratest WB IAC. The column was washed with 10 mL of PBS-T followed by 10 mL of ultra-water. OTA was eluted with 2 mL of methanol, collected in a glass vial, filtered through 0.2 µm nylon filters (Whatman) and analysed by HPLC-FLD. The HPLC equipment and column used for OTA analysis were the same as described for aflatoxin analysis, but without derivatization. The fluorescence detector was set to λ_{ex} 330 nm and λ_{em} 463 nm. The mobile phase was a mixture of acetonitrile/water /acetic acid (70:29.5:0.5, v/v/v) at a flow rate of 0.8 mL/min, and the injection volume was 10 µL. The compound was identified by chromatographic comparison with the standard. Quantification was based on the fluorescence signal response.

2.6.4 In-House method validation

Stock solutions of 5 µg/mL of AFB_1 and 1 mg/mL of OTA were prepared and stored at -20 ºC. Working standard solutions with a concentration of 100 ng/mL of each mycotoxin were prepared from the stock solutions daily. Precision and recovery were performed by spiking the blank sample with two different mycotoxin concentrations: 10 ng/g and 30 ng/g of AFB_1 and OTA. One set of unspiked sample was used as blank. Each sample set was composed of six replicates and tested in two different days (three replicates each day). The recovery rates were determined from the 6 replicates of the two spiking levels, by calculation of the ratio of recovered AF concentration relative to the known spiked concentration. Precision was calculated in terms of intraday repeatability (n=3) and intermediate precision (interday within laboratory reproducibility; 2 different days) for each AF at the two contamination levels in spiked samples.

Linearity, limit of detection (LOD), and limit of quantification (LOQ) were determined by three series of analyses, using 11 standard solutions with concentrations ranging from 0.05 ng/mL to 20 ng/mL of AFB1 and OTA. LOD and LOQ were calculated according to the equations 3 and 4 (Taverniers et al., 2004):

$$LOD = 3 \times \frac{sa}{b}$$

Equation 4. Equation for limit of detection determination.

$$LOQ = 10 \times \frac{sa}{b}$$

Equation 5. Equation for limit of quantification determination.

Where:

sa*:* standard deviation of the intercept of the regression line obtained from the calibration curve

b: slope of the line.

2.7 Bioactive variables evaluation

2.7.1 Extracts preparation

2.7.1.1 Infusions

The infusions were obtained from the dried plant material. The sample (2 g) was added to 100 mL of boiling distilled water (after being taken out from the heating source) and left to stand at room temperature for 5 min, and then filtered under reduced pressure, lyophilized (FreeZone 4.5, Labconco, Kansas, USA) and redissolved in water.

The lyophilized infusions were tested for bioactive properties after preparation of several dilutions, according with the described in section 2.7.4. For the analysis of individual phenolic compounds, the lyophilized infusions were redissolved in water at 5 mg/mL (Section 2.7.3).

2.7.1.2 Aqueous and methanolic extracts

The extracts were obtained from the dried plant material. The sample (1 g) was extracted by stirring with 25 mL of water or methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of water or methanol (25 ºC at 150 rpm) for 1 h. The combined aqueous or methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness and re-dissolved in the corresponding solvent at 20 mg/mL (for *G. biloba* non-irradiated) or 10 mg/mL (for *A. citrodora, M. officinalis, M. melissophyllum; A. montana, M. pipperita* and *G. biloba* irradiated samples). The extracts were tested for bioactive properties after preparation of several dilutions, according with the described in section 2.7.4. For the analysis of individual phenolic compounds, the dried methanolic extracts were redissolved in water methanol:water (20:80, v/v) at 5 mg/mL (section 2.7.3).

2.7.2 Phenolic compounds

2.7.2.1 Total phenolic compounds

Total phenolics were determined by the Folin-Ciocalteu (FC) method, as previously described by Wolfe et al. (2003). The extract solution (0.5 mL) was mixed with the FC reagent (2.5 mL,

previously diluted with water 1:10, v/v) and sodium carbonate (75 g/L, 4 mL). The tubes were vortex mixed for 15 s and allowed to stand for 30 min at 40 ºC for colour development (**Figure 19**). Absorbance was then measured at 765 nm. Gallic acid was used to obtain the standard curve and the results were expressed as mg of gallic acid equivalents (GAE) per g of extract (lyophilized infusion or dried extract).

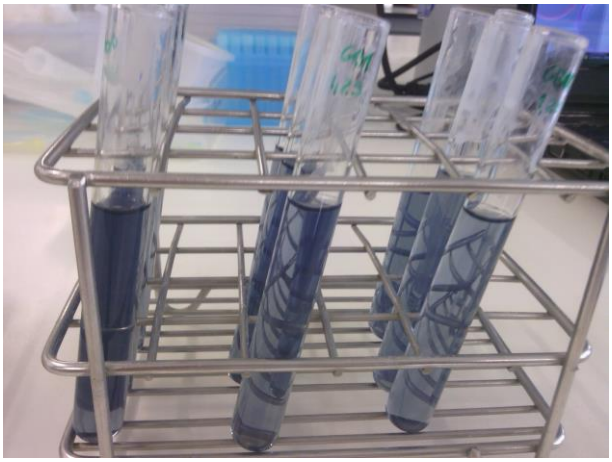


Figure 19. Test tubes containing solutions after treatment with the FC reagent for total phenolics determination. Author: Eliana Pereira.

2.7.2.2 Total Flavonoids

Total flavonoids were determined by a method previously described by Jia et al. (1999). The extract sample at a concentration of 2.5 mg/mL (0.5 mL) was mixed with distilled water (2 mL) and $NaNO_2$ solution (5%, 0.15 mL). After 6 min, $AlCl_3$ solution (10%, 0.15 mL) was added and allowed to stand further 6 min. NaOH solution (4%, 2 mL) was added to the mixture, followed by distilled water until a final volume of 5 mL. The mixture was properly mixed and allowed to stand for 15 min. The intensity of pink colour was measured at 510 nm (**Figure 20**). (+)-Catechin was used to prepare the standard curve (0.015-1.0 mM) and the results were expressed as mg of (+)-catechin equivalents (CE) per g of extract (lyophilized infusion or dried extract).

Figure 20. Test tubes containing solutions for total flavonoids determination. Author: Eliana Pereira

2.7.2.3 Individual phenolic compounds

The extracts obtained above were analysed using an HPLC chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, US) with a double online detection using a diode array detector (DAD) and 280, 330 and 370 nm as preference wavelengths, and a mass spectrometer (MS) equipped with an ESI source and a triple quadrupole-ion trap mass analyser, which was connected to the HPLC system via the DAD cell outlet (Barros et al., 2013b). The DAD was coupled to an HP Chem Station (rev. A.05.04) and the MS was controlled by Analyst 5.1 software. The separation was achieved using a Spherisorb S3 ODS-2 C_{18} column (3 μm, 4.6 × 150 mm) thermostatted at 35 °C, using a gradient elution with the following solvents: 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient established was 15% B for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% B for 10 min, and re-equilibration of the column (10 min), using a flow rate of 0.5 mL/min. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS), employed to show full scan spectra, and enhanced product ion (EPI) analysis, to obtain the fragmentation pattern of the parent ion(s) in the previous scan. Air (zero graded) was used as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen functioned as the curtain (20 psi) and collision gas (medium). The ion spray voltage was -4500 V and spectra were recorded in negative ion mode between *m/z* 100 and 1700. The settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI analysis was performed using the following variables: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Compounds were tentatively identified from the obtained information (retention times, and UV-vis and mass spectra) by comparison with data reported in the literature and standard compounds, when available. For quantification, calibration curves from phenolic standards (apigenin-6-*C*-glucoside, caffeic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside,

naringenin, quercetin-3-*O*-rutinoside, rosmarinic acid) were obtained based on the UV signal at maximum wavelength of absorption of each standard compound. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of another compound from the same phenolic group. The results were expressed as mg per g of extract.

2.7.3 Antioxidant activity

2.7.3.1 General

The antioxidant activity was evaluated using different methods as below described. In all cases, the lyophilized infusions or dried extracts were redissolved in the corresponding solvent at a concentration of 10 mg/mL and sequential dilutions were made from this stock solution. The results were expressed in EC_{50} values, which correspond to the concentration of the extract that is able to inhibit 50% of the oxidative process, involving free radicals or lipid peroxidation (Sánchez-Moreno et al., 1998). EC_{50} values were calculated using the equation 5:

$$EC_{50} = \frac{(x_2 - x_1)}{(y_2 - y_1)} \times (50 - y_1) + x_1$$

Equation 6. Equation for EC_{50} values determination.

Where:

- x_1 is the concentration of the extract whose inhibition percentage is under 50%
- x_2 is the concentration of the extract whose inhibition percentage is over 50%
- y_1 inhibitory percentage under 50%
- y_2 inhibitory percentage over 50%

2.7.3.2 DPPH scavenging activity

This method is based on the reduction of the DPPH radical by hydrogen donation from an antioxidant (**Figure 21**). The DPPH radical is a nitrogen compound that is stable due to the relocation of the free electron and that possesses a purple colour. It reacts easily with molecules that can donate hydrogen atoms, changing to yellow when it accepts them (**Figure 22**). This assay

is widely used as a preliminary antioxidant study (Antolovich et al., 2002; Amarowicz et al., 2004; Moon and Shibamoto, 2009).

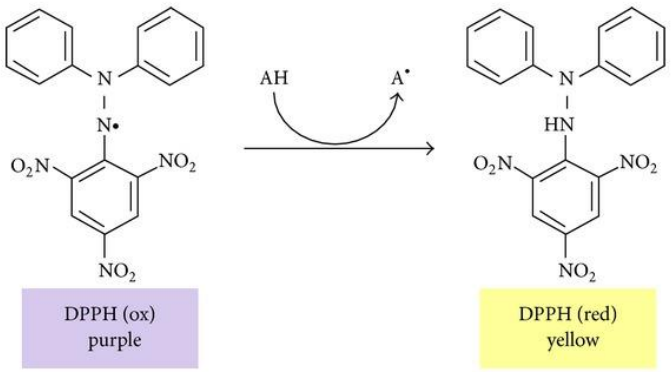


Figure 21. Reduction of the DPPH radical. Source: Teixeira et al. (2013).

Analyses were performed using an ELX800 Microplate Reader (Bio-Tek). The reaction mixture in each one of the plate wells consisted of one of the different concentrations of the extracts (30 µL) and aqueous methanolic solution (80:20 v/v, 270 µL) containing DPPH radicals (6 x 10^{-5} mol/L). The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation 6:

$$\% \text{ RSA} = \frac{(A_{DPPH} - A_S)}{A_{DPPH}} \times 100$$

Equation 7. Equation for RSA determination in the DPPH method.

where:

A_S: absorbance of the solution when the sample extract has been added at a particular level

A_{DPPH}: absorbance of the DPPH solution.

The extract concentration providing 50% of the radicals scavenging activity (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. Trolox was used as the standard.

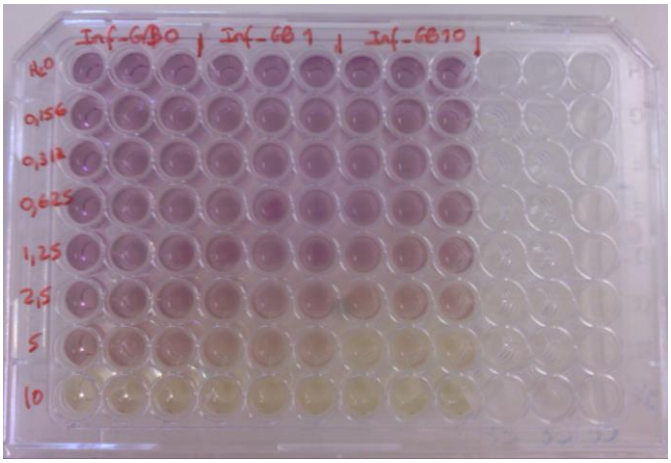


Figure 22. Example of a test plate with different solutions for determination of the DPPH scavenging activity. Author: Eliana Pereira.

2.7.3.3 Reducing Power

This method measures the ability of antioxidants to reduce Fe(III) to Fe(II) (Berker, 2007). The chemistry of the assay can be summarized in the following reactions:

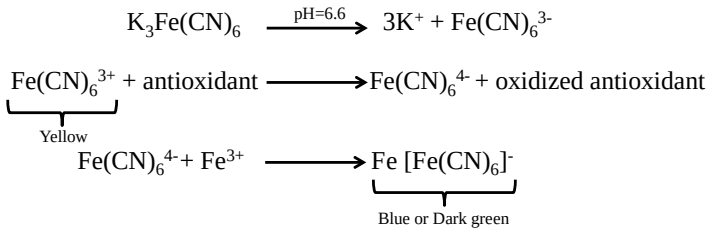


Figure 23. Chemical equations of the reducing power assay.

Depending on the reducing power of the compounds, the yellow color of the test solution changes to different shades of blue or green (**Figure 24**), and can be monitored spectrophotometrically at 700 nm. The assay was performed using the Microplate Reader described above. The different concentrations of the extracts (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixtures were incubated at 50 ºC for 20 min, and afterwards trichloroacetic acid (10% w/v, 0.5 mL) was added. A 0.8 mL of each solution was poured into a well of the microplate, together with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm. The extract concentration providing 0.5 of

absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as the standard.

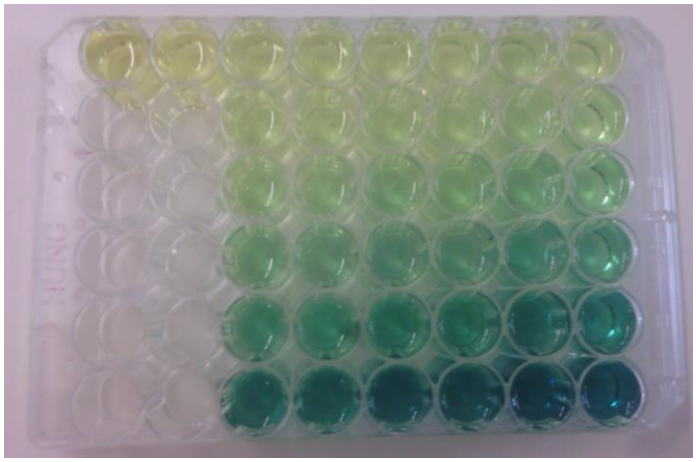


Figure 24. Example of a test plate for measurement of the reducing power. Author: Eliana Pereira.

2.7.3.4 β-carotene bleaching inhibition

This assay is based on the discolouration of β-carotene (**Figure 25**) produced by free radicals generated during peroxidation of linoleic acid, which can be followed by measurement of the absorbance at 470 nm (**Figure 27**). This discolouration can be inhibited or reduced by the antioxidants contained in the sample according with the following reactions (Amarowicz et al., 2004; Kaur and Geetha, 2006):

β-carotene - H (orange colour) + ROO· ⟶ β-carotene· (discoloured) + ROO

β-carotene - H (orange) + ROO· + AH ⟶ β-carotene – H (orange) + ROOH + A·

Figure 25. Chemical equations of β-carotene assay.

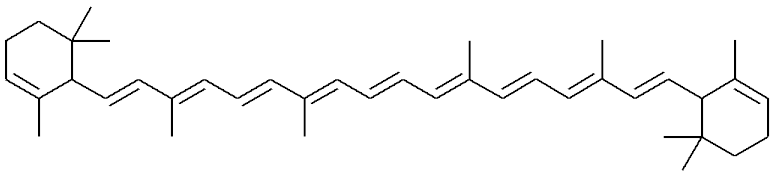


Figure 26. Chemical structure of β-carotene.

A solution was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two millilitres of this solution were pipetted into a round-bottom flask. After the chloroform was removed at 40 ºC under vacuum, linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing different concentrations of the extracts (0.2 mL). The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm. β-carotene bleaching inhibition was calculated using the equation 7:

$$\text{β-carotene bleaching inhibition} = \frac{\text{β-carotene content after 2 h of assay}}{\text{initial β-carotene content}} \text{ x } 100$$

Equation 7. Equation of the β-carotene bleaching inhibition assay.

The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

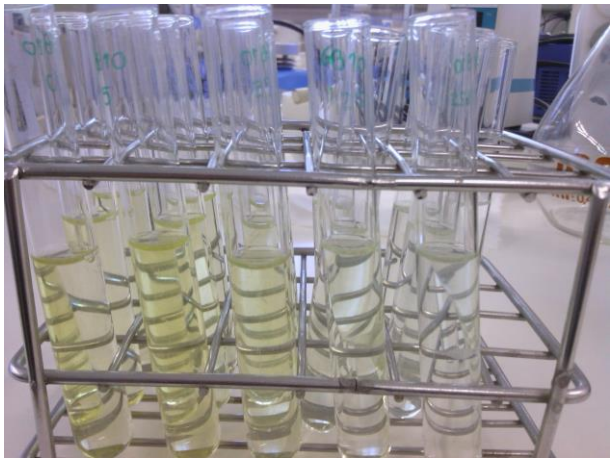


Figure 27. Examples of test tubes in the β-carotene bleaching inhibition assay. Author: Eliana Pereira.

2.7.3.5 TBARS formation inhibition

Lipid peroxidation can be determined by the products of the oxidation that react with thiobarbituric acid (TBA) giving rise to pink compounds that are known as thiobarbituric acid reactive species (TBARS). One of the products commonly used as a biomarker of lipid

peroxidation is malodialdehyde (MDA) that associated with TBA in the presence of H^+ ions to form a chromogen (MDA-TBA) according to the reaction shown in **Figure 28**. In this methodology, the oxidation of a lipid-rich preparation is induced by addition of a metallic ion (iron or copper), and the extension of the reaction with thiobarbituric acid is determined by the ability of the antioxidants present in the sample that to stop the oxidation process, thus inhibiting the formation of the chromogen (less pink) (**Figure 29**) (Gutteridge, 1995; Ng et al., 2000; Kaur and Geetha, 2006).

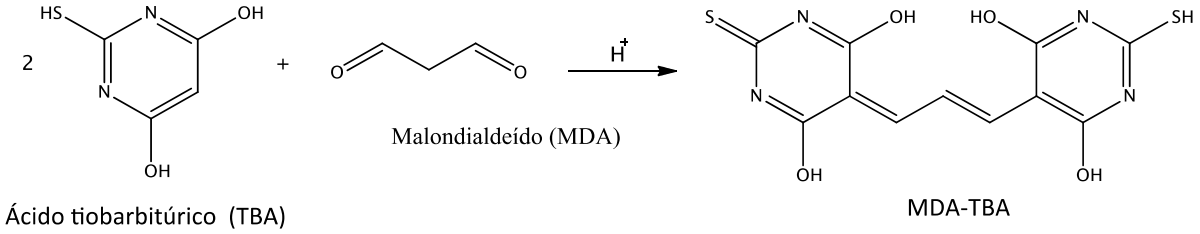


Figure 28. Formation of the complex MDA-TBA.

Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenised with a Polytron in ice-cold Tris-HCl buffer (20 mM, pH 7.4), to produce a 1:2 (w/v) brain tissue homogenate that was centrifuged at 3000 *g* for 10 min. An aliquot (0.1 mL) of the supernatant was incubated with the different solution concentrations (0.2 mL) in the presence of $FeSO_4$ (10 μM; 0.1 mL) and ascorbic acid (0.1 mM; 0.1 mL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 mL), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 mL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000 *g* for 10 min to remove the precipitated protein, the colour intensity of the MDA-TBA complex in the supernatant was measured by its absorbance at 532 nm. The inhibition ratio (%) was calculated using the equation 8:

$$\text{Inhibition ratio (\%)} = \frac{(A-B)}{A} \times 100$$

Equation 8. Equation of the inhibition ratio determination in TBARS assay.

Where:

A: absorbance of the control and

B: absorbance of the compound solution

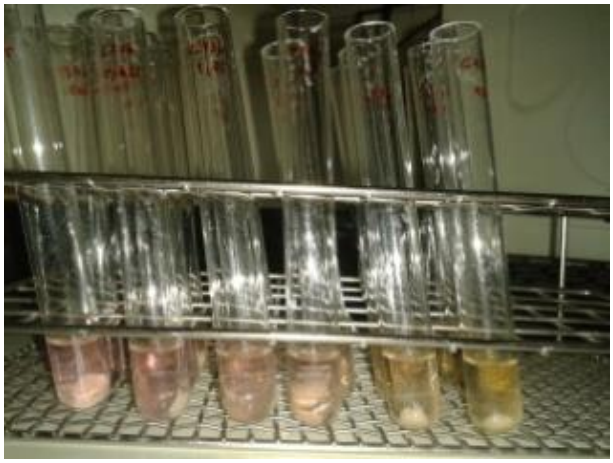


Figure 29. Examples of test tubes in the TBARS assay. Author: Eliana Pereira

2.7.4 Cytotoxicity

2.7.4.1 General

For cytotoxicity evaluation, lyophilized infusions or dried extracts were redissolved in water to a final concentration of 8 mg/mL, from which further dilutions were prepared for the assays; ellipticine was used as positive control. The results were calculated as GI_{50} values (sample concentration that inhibited 50% of the net cell growth).

2.7.4.2 Assays in tumor cell lines

Four human tumor cell lines were used: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines were plated in a 96-well plate, at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460, and 1.0×10^4 cells/well for HeLa and HepG2) and allowed to attach for 24 h. The cells were then incubated in the presence of different extract concentrations during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100 μL) was added in order to bind the adherent cells and further incubated for 60 min at 4 ºC. After the incubation period, the plates were washed with deionised water and dried, and

sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 µL) was incorporated to each plate well and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 µL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Guimarães et al., 2013). **Figure 30** shows an example of a microplate ready for citotoxicty measurement.

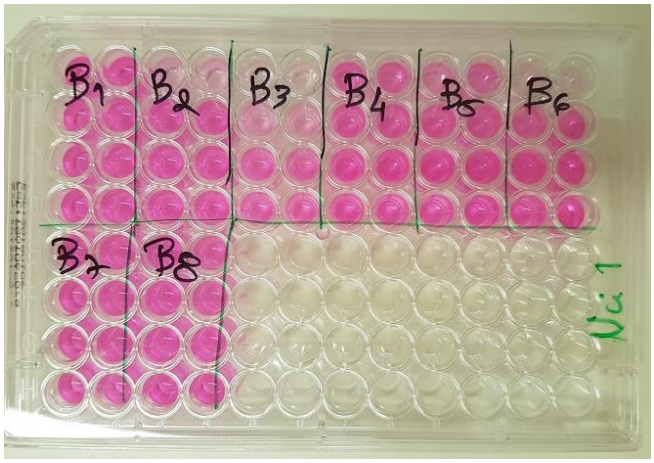


Figure 30. Example of a test plate prepared in a cytotoxicity assay. Author: Ricardo Calhelha.

2.7.4.3 Assays in non-tumor cells

A freshly harvested porcine liver, obtained from a local slaughter house, was used in order to obtain the cell culture, designated as PLP2. The liver tissues were rinsed in Hank's balanced salt solution containing penicillin (100 U/mL) and streptomycin (100 µg/mL) and divided into 1×1 mm^3 explants. A few of these explants were transferred to tissue flasks (25 cm^2) containing DMEM medium supplemented with fetal bovine serum (FBS, 10%), nonessential amino acids (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL), and incubated at 37 ºC with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cell cultivation was continuously monitored using a phase contrast microscope. When confluence was reached, the cells were sub-cultured and plated in 96-well plate (density of 1.0×10^4 cells/well) containing DMEM medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Guimarães et al., 2013). The growth inhibition was evaluated using the SRB assay described in the previous section.

2.8 Statistical analysis

The results obtained throughout the different evaluation studies were analysed by applying different statistical tools, selected according to the degree of complexity of the results and considering the defined research purposes. Besides using some common comparative methodologies, such as one-way ANOVA and 2-way ANOVA (specifically the generalized linear model), advanced classification tools such as principal components analysis (PCA) and linear discriminant analysis (LDA) were also performed.

For each level of the studied factors, three samples were prepared, and each sample was analysed in triplicate. The results were expressed as mean values±standard deviation (SD). All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0 (IBM Corp., Armonk, NY, USA).

2.8.1 One-way ANOVA

Before the analysis, the fulfilling of some specific requirements was verified. The normal distribution of the residuals and the homogeneity of variance were tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

This analysis was performed in the evaluation of the results obtained in the studies of:

a) *Ginkgo biloba* (section 3.1.1), specifically to compare:

(i) Chemical, nutritional and bioactive variables in infusion, methanolic extracts and dietary supplements (article: Chemical characterization of *Ginkgo biloba* L. and antioxidant properties of its extracts and dietary supplements - annex 1).

(ii) The effects of different doses of gamma radiation on nutritional, chemical and antioxidant properties (article: Analytical methods applied to assess the effects of gamma irradiation on color, chemical composition and antioxidant activity of *Ginkgo biloba* L. - annex 2).

(iii) The effects of different doses of gamma radiation on individual phenolic compounds profile (article: Gamma irradiation improves the extractability of phenolic compounds in *Ginkgo biloba* L - annex 3)

b) *Thymus vulgaris* L. and *Mentha x piperita* L. (section 3.1.4), in order to compare the effects of different doses of gamma radiation in the phenolic composition and cytotoxicity potential (article: Effects of gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Mentha x piperita* L. - annex 5).

c) *Thymus vulgaris* L. (section 3.1.5), particularly to analyse the effects of different doses of gamma radiation in phenolic composition and antioxidant potential (article: Infusions from *Thymus vulgaris* L. treated at different gamma radiation doses: effects on antioxidant activity and phenolic composition - annex 6).

d) *Aloysia citrodora* L. and *Mentha x piperita* L. (section 3.1.6), aiming to evaluate the effects of different doses of gamma radiation in phenolic composition and cytotoxicity potential (unpublished results).

e) *Aloysia citrodora* L. (section 3.1.7), to evaluate the effects of different doses of gamma radiation (0, 1, 5 and 10 kGy) on decontaminant capacity, *i.e.*, elimination of toxins (OTA and AFB_1) (article: Is gamma irradiation a suitable preservation and mycotoxin decontamination technique in aromatic plants? A case-study with *Aloysia citrodora* Paláu - submitted).

f) *Arenaria montana* L. (section 3.3.1), particularly to compare the:

(i) Chemical, nutritional and bioactive variables (article: Bioactivity and phytochemical characterization of *Arenaria montana* L. - annex 8).

(ii) Effects of gamma and electron beam radiation on individual properties (nutritional, chemical and antioxidant) (article: Electron beam and gamma irradiation as feasible conservation technologies for wild *Arenaria montana* L.: effects on chemical and antioxidant parameters - annex 9).

g) *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x pipperita* L. (section 3.1.1), in order to evaluate the effects of gamma radiation on individual properties (nutritional, chemical and antioxidant) (article: Gamma irradiation as a practical alternative to preserve the chemical and bioactive wholesomeness of food plants - annex 4).

2.8.2 2-way ANOVA

An analysis of variance with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software version 22.0 (IBM Corp., Armonk, NY: USA). When a statistically significant interaction among factors was detected, the two factors were evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction was verified, means within each factor were compared using appropriate tests.

This analysis was generally used to study the effects of irradiation doses, irradiation types and storage times in several variables, in different plants (section 3.3.3, 3.2.2 and 3.3.2). Specifically it was used to:

(i) analyse the variation induced by gamma radiation throughout storage time in the nutritional, chemical and antioxidant variables (article: Chemometric study of multi-parameter variations affecting dried plants submitted to gamma radiation and extended storage periods - submitted).

(ii) analyse the variation induced by electron beam radiation over the same variables as in the previous point (article: Electron-beam irradiation as an alternative to preserve nutritional, chemical and antioxidant properties of dried plants during extended storage periods – submitted).

(iii) Analyse the variation induced by irradiation doses and irradiation technology in phenolic composition of *Melissa officinalis* L. and *Melittis melissophyllum* L. (article: Gamma and electron beam irradiated *Melissa officinalis* L. and *Melittis melissoffillum* L. overexpress individual phenolics – submitted)

2.8.3 Linear Discriminant Analysis (LDA)

A stepwise technique, using the Wilks' λ method with the usual probabilities of *F* (3.84 to enter and 2.71 to remove) was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable, it is verified whether all variables previously selected remain significant (Palacios-Morillo et al., 2013). With this approach, it is also possible to identify the significant variables that contribute most to the possible discrimination according to the factor in analysis. To verify which canonical discriminant functions were significant, the

Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

This analysis was performed in the evaluation of the results obtained in:

a) *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x pipperita* L. (section 3.2.1), particularly to evaluate the results considering data for all irradiation conditions and evaluated variables simultaneously (article: Extending the use of irradiation to preserve chemical and bioactive properties of aromatic plants: a case study with four species submitted to electron beam - annex 7).

b) *Arenaria montana* L. (section 3.3.1), to evaluate the overall significance of the detected differences in the preliminary one way-ANOVA, by verifying which statistically significant differences maintained their relevance when compared globally (article: Electron beam and gamma irradiation as feasible conservation technologies for wild *Arenaria montana* L.: effects on chemical and antioxidant variables - annex 9).

c) *Melissa officinalis* L. and *Melittis melissoffillum* L. infusions (section 3.3.2) principally to evaluate the effect of irradiation over the individual phenolic compounds (article: Gamma and electron beam irradiated *Melissa officinalis* L. and *Melittis melissoffillum* L. overexpress individual phenolics – submitted).

2.8.4 Principal component analysis (PCA)

PCA was applied as pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by the Cronbach's alpha variable (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

PCA was applied in the following sections:

a) *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x pipperita* L. (section 3.1.1), to identify overall trends for a better characterization of the effects of gamma irradiation (article: Gamma irradiation as a practical alternative to preserve the chemical and bioactive wholesomeness of food plants - annex 4).

b) *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x pipperita* L. (section 3.1.3), to achieve a comprehensive characterization of the variations measured in all variables simultaneously in response to different doses of gamma radiation or storage times (12 and 18 months) (article: Chemometric study of multi-parameter variations affecting dried plants submitted to gamma radiation and extended storage periods - submitted)

c) *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x pipperita* L. (section 3.2.2) to obtain correlations among all evaluated variables and each storage time (12 and 18 months) or electron beam irradiation dose (article: Electron-beam irradiation as an alternative to preserve nutritional, chemical and antioxidant properties of dried plants during extended storage periods - submitted).

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Studies with gamma radiation

3.1.1 Ginkgo biloba L.

3.1.1.1 Chemical characterization of *Ginkgo biloba*

The results obtained in the analysis of the proximate composition of *G. biloba* leaves are shown in **Table 6**. Carbohydrates, calculated by difference, were the most abundant macronutrients (72.98 g/100 g dw). Otherwise, fat was the macronutrient present in lower amount (4.75 g/100 g dw). The levels of proteins and ash were 12.27 and 10.01 g/100 g dw, respectively.

Table 6. Proximate composition of *Ginkgo biloba* dry leaves.

Variable	Amount
Ash	10.01 ± 0.06 g/100 g dw
Proteins	12.27 ± 0.24 g/100 g dw
Fat	4.75 ± 0.22 g/100 g dw
Carbohydrates	72.98 ± 0.20 g/100 g dw

Chemical composition in fatty acids, sugars, organic acids and tocopherols was also assessed and the results are shown in **Table 7.**

Palmitic (C16:0), α-linolenic (C18:3n3) and oleic (C18:1n9) acids were the most abundant fatty acids (35.90, 18.03 and 11.18%, respectively). The latter one is considered as a preferred fatty acid for edible purposes, because it combines a hypocholesterolemic effect and a high oxidative stability (Mensink and Katan, 1989). By groups, saturated fatty acids appeared in higher proportion (59.15%), followed by polyunsaturated (28.85%) and lastly monounsaturated fatty acids (12%).

The sugars found were fructose, glucose and sucrose, being fructose (1.42 g/100 g dw) the main one and sucrose the least abundant (0.23 g/100 g dw).

Among organic acids, oxalic, quinic, malic and shikimic acids were identified and quantified, being quinic acid the most abundant one (2.26 g/100 g dw). This acid is a very useful and versatile chiral pool starting material for natural product synthesis and many groups have developed elegant syntheses based on stereoselective reactions of quinic acid derivatives (Murray et al., 2004). Shikimic acid was present in similar levels (2.24 g/100 g dw); it is also

interesting as key starting material for the synthesis of the neuramidase inhibitor GS4104 for treatment of antiviral infections (Krämer et al., 2003). On the other hand, malic acid was the organic acid found in lower quantity.

Regarding tocopherols, the isoforms α-, β-, γ- and δ-tocopherol were all detected. α-Tocopherol was, by far, the most abundant vitamer (124.88 mg/100 g dw from a total of 126.23 mg/100 g dw). Considering its antioxidant potential and various functions at the molecular level (eliminating reactive oxygen species, inhibiting lipid peroxidation and attenuating inflammatory reactions), this vitamer has been associated with a reduced risk of cardiovascular diseases and neurodegenerative disorders, particularly Alzheimer's disease (Burton, 1994; Berman and Brodaty, 2004; Kontush and Schekatolina, 2004).

As far as we know, the present study is pioneer regarding nutritional characterization of *G. biloba* leaves, which is important considering that they are widely consumed as infusions and incorporated in dietary supplements.

Table 7. Composition of individual fatty acids, sugars, organic acids and tocopherols in *Ginkgo biloba* dry leaves.

Fatty acids (relative percentage)		**Free sugars** (g/100 g dw)	
C6:0	0.24 ± 0.01	Fructose	1.42 ± 0.05
C8:0	0.27 ± 0.04	Glucose	0.78 ± 0.01
C10:0	0.24 ± 0.01	Sucrose	0.23 ± 0.02
C12:0	0.61 ± 0.09	Total	2.43 ± 0.04
C14:0	6.13 ± 0.47	**Organic acids** (g/100 g dw)	
C15:0	0.68 ± 0.03	Oxalic acid	0.90 ± 0.00
C16:0	35.90 ± 0.97	Quinic acid	2.26 ± 0.09
C16:1	0.82 ± 0.12	Malic acid	0.58 ± 0.01
C17:0	1.28 ± 0.02	Shikimic acid	2.24 ± 0.01
C18:0	4.17 ± 0.24	Total	5.98 ± 0.10
C18:1n9	11.18 ± 1.23	**Tocopherols** (mg/100 g dw)	
C18:2n6c	10.53 ± 0.09	α-Tocopherol	124.88 ± 0.37
C18:3n3	18.03 ± 0.06	β-Tocopherol	0.36 ± 0.03
C20:0	2.70 ± 0.05	γ-Tocopherol	0.72 ± 0.06
C20:3n6	0.11 ± 0.07	δ-Tocopherol	0.28 ± 0.01
C20:3n3+C21:0	0.19 ± 0.08	Total	126.23 ± 0.47
C22:0	2.19 ± 0.01		
C23:0	0.92 ± 0.00		
C24:0	3.82 ± 0.04		
SFA	59.15 ± 1.39		
MUFA	12.00 ± 1.35		
PUFA	28.85 ± 0.04		

SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty acids; dw- dry weight.

3.1.1.2 Antioxidant properties of *Ginkgo biloba,* extracts and dietary supplements

The antioxidant properties of different dietary supplements based on *G. biloba* (syrup and pills) and of extracts obtained from the leaves (infusion, methanolic and aqueous extracts) were analysed and the results are included in the **Table 8**. The preparation of the samples for these analyses is described in the chapter of Materials & Methods (section 2.2, Table 4, and section 2.7.1)

Dietary supplements showed higher antioxidant activity than the different extracts prepared from the dry leaves (**Table 8**). Among dietary supplements, pills gave higher antioxidant activity than syrup. Furthermore, as it can be expected, it was observed an increase of antioxidant properties with the increase of *G. biloba* extract concentration in the pills. Thus, sample P3 (containing 100 mg of *G. biloba* standardized extract) was better than P2 (60 mg *G. biloba* standardized extract), and this one better than P1 (40 mg *G. biloba* standardized extract) in all the performed assays. This was observed despite the final concentrations of the preparations followed an opposite trend (**Table 5**), which correspond to the recommended mode of consumption. The same behaviour was observed for bioactive components, namely phenolics and flavonoids; the samples with highest antioxidant activity also showed the highest contents of those compounds (**Table 8**), suggesting that they were involved in the observed activity.

In previous studies of our research group (Pereira et al., 2013b), higher antioxidant activity was found in infusions than in dietary supplements prepared from *Cynara scolymus* L. (artichoke), *Silybum marianum* (L.) Gaertn (milk thistle) and *Cochlospermum angolensis* Welw. (borututu), although in those cases the supplements contained much lower amounts of phenolics (3.35 to 30.70 mg GAE/g) than the ones found in *G. biloba* syrup and pills (396.98 to 553.41 mg GAE/g) herein.

Regarding the extracts prepared from the dry leaves, better antioxidant values in all the performed assays were found in the methanolic extract (LME), which also showed higher phenolics and flavonoids contents than the infusion or aqueous extract; the decrease in bioactive compounds observed in infusion in comparison with aqueous extracts could be probably related to a degradation caused by heat. According to other authors, phenolic compounds are unstable and easily become non-antioxidative under heating and in the presence of other antioxidants (Yen and Hung, 2000).

Table 8. Total phenolics and flavonoids, and antioxidant properties of *Ginkgo biloba* extracts and dietary supplements.

	LI	LME	LAE	S	P1	P2	P3
Total phenolics (mg GAE/g lyophilized infusion or dried extract)	37.71 ± 0.04^{g}	129.5 ± 5.30^{e}	61.58 ± 0.53^{f}	461.45 ± 5.75^{c}	396.98 ± 4.84^{d}	501.86 ± 4.24^{b}	553.41 ± 1.19^{a}
Total flavonoids (mg CE/g lyophilized infusion or dried extract)	1.53 ± 0.01^{f}	14.87 ± 0.84^{d}	1.39 ± 0.32^{f}	5.89 ± 0.90^{e}	28.30 ± 1.61^{c}	47.89 ± 0.55^{b}	53.21 ± 0.59^{a}
DPPH scavenging activity (EC_{50}, mg/mL)	1.52 ± 0.17^{a}	0.74 ± 0.04^{b}	1.58 ± 0.05^{a}	0.14 ± 0.00^{cd}	0.18 ± 0.01^{c}	0.11 ± 0.01^{cd}	0.07 ± 0.01^{d}
Reducing power (EC_{50}, mg/mL)	0.83 ± 0.02^{a}	0.36 ± 0.01^{c}	0.73 ± 0.00^{b}	0.14 ± 0.00^{d}	0.13 ± 0.00^{e}	0.08 ± 0.00^{f}	0.06 ± 0.00^{g}
β-carotene bleaching inhibition (EC_{50}, mg/mL)	4.71 ± 0.35^{a}	4.47 ± 0.08^{b}	4.85 ± 0.10^{a}	0.47 ± 0.05^{c}	0.56 ± 0.07^{c}	0.43 ± 0.03^{c}	0.22 ± 0.02^{d}
TBARS inhibition (EC_{50}, mg/mL)	1.29 ± 0.18^{a}	0.13 ± 0.01^{cd}	0.82 ± 0.09^{b}	0.24 ± 0.06^{c}	0.12 ± 0.01^{de}	0.03 ± 0.00^{de}	0.02 ± 0.01^{e}

LI- infusion prepared from dry leaves; LME- methanolic extract prepared from dry leaves; LAE- aqueous extract prepared from dry leaves; S- syrup; P- pills (**Table 4**).
EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GAE- gallic acid equivalents; CE- catechin equivalents. In each row different letters mean significant differences ($p<0.05$).

3.1.1.3 Color of irradiated samples of *G. biloba*

The results of the chromatic analyses in the CIE L*a*b* color space of non-irradiated and irradiated *G. biloba* samples are presented in **Table 9**. It seems that for the highest irradiation dose, 10 kGy, there was a tendency for the samples to loose lightness (L* value diminishes), when compared to non-irradiated (0 kGy) and irradiated samples at 1 kGy. For the b* value, which represents the yellowness-blueness tendency, it was not observed any significant difference with irradiation dose, although the samples irradiated at 10 kGy showed lower b* value. The results obtained for a* variable (greenness-redness) were close to zero (data not shown). Color is an attribute of great importance not only for plants but also for other foods. For example, the cosmetics industry has a very stringent selection of the plants color; the dark color of some natural matrices such as green tea or persimmon leaf makes very difficult their application in food or cosmetic products, and the process to remove color is a difficult, time-consuming and costly procedure (Jo et al., 2003a,b).

Table 9. CIE color L* (lightness), a* (redness) and b* (yellowness) of non-irradiated and irradiated G. biloba samples. The results are presented as mean ± SD.

	0 kGy	1 kGy	10 kGy
L*	46.43 ± 1.42^{a}	46.15 ± 2.71^{a}	42.94 ± 1.58^{b}
b*	22.58 ± 2.17^{a}	22.18 ± 1.64^{a}	20.52 ± 2.03^{a}
ΔE	51.90 ± 1.55^{a}	51.37 ± 2.75^{a}	49.14 ± 1.92^{b}

In each row different letters mean significant differences, $p < 0.05$.

3.1.1.4 Nutritional profile of irradiated samples of *G. biloba*

The macronutrients profile of non-irradiated and irradiated *G. biloba* samples is shown in **Table 10**. The control sample showed values very similar to the ones previously obtained in a sample from different commercial origin (**Table 6**, section 3.1.1.1). Gamma radiation did not alter significantly the profile of the samples regarding ash, fat and energy, for which similar values were obtained in the control and irradiated samples, either at 1 or 10 kGy, which is in agreement with previous studies in other foods, such as hazelnuts, walnuts, almonds, or pistachios (Gecgel et al., 2011). Nevertheless, the dose of 10 kGy conducted to a decrease in proteins content and, consequently, to an increase in carbohydrates level (as they were determined by

difference). Protein values have been previously reported as having no significant changes after irradiation treatments (Fernandes et al. 2012a, 2014; Kasera et al. 2012), although in a study on wild mushrooms by Fernandes et al. (2013) a decrease was also observed. The decrease produced in protein levels at 10 kGy could be explained by a possible degradation due to the relatively high intensity applied. Actually, proteins are considered reliable irradiation indicators, especially due to degradation reactions, such as scission of the C–N bonds in the backbone of the polypeptide chain or splitting of the disulfide bonds, and physical changes like unfolding and aggregation (Molins, 2001). Nevertheless, the fact that irradiation induces alterations in the protein content does not mean a significant problem from the nutritional point of view, since protected amino acids within the structure of the protein complex generally resists to this method (Kausar et al. 2013).

Table 10. Macronutrients and energetic value of non-irradiated and irradiated *G. biloba* samples (mean ± SD).

	0 kGy	1 kGy	10 kGy
Ash (g/100 g dw)	12.91 ± 0.20[a]	12.74 ± 0.65[a]	12.34 ± 1.59[a]
Proteins (g/100 g dw)	15.32 ± 0.16[a]	15.19 ± 0.59[a]	12.79 ± 0.15[b]
Fat (g/100 g dw)	4.42 ± 0.13[a]	4.32 ± 0.24[a]	4.56 ± 0.42[a]
Carbohydrates (g/100 g dw)	67.36 ± 0.28[b]	67.75 ± 0.74[b]	70.31 ± 0.88[a]
Energy (kcal/ 100 g dw)	370.44 ± 0.09[a]	370.64 ± 2.69[a]	373.44 ± 5.98[a]

dw- dry weight. In each row different letters mean significant differences ($p<0.05$).

3.1.1.5 Composition in lipophilic compounds of irradiated samples of *G. biloba*

Twenty-two different fatty acids were identified (**Table 11**), in accordance with a previous analysis on another *G. biloba* sample (**Table 7**, section 3.1.1.1). Irradiated and control samples revealed similar fatty acids profile, with α-linolenic acid as main compound, followed by palmitic acid. Some studies showed that the lack of α-linolenic acid in the diet compromises the brain and heart function (Taha et al. 2006; Nguemeni et al. 2013), and therefore, it is important to preserve this and other compounds in irradiated samples. In all the samples, saturated fatty acids appeared as the best represented group, followed by polyunsaturated and lastly monounsaturated fatty acids. No significant differences were observed between the control and the irradiated samples at two different doses (**Table 11**; **Figure 31A**). Similar results were reported for

irradiated samples of cashew nuts (Mexis and Kontominas, 2009) and lamb meat (Alfaia et al. 2007), where no significant changes were observed in the concentration of SFA, MUFA and PUFA.

Data concerning tocopherols are also given in **Table 11** and representative chromatograms are shown in **Figure 31B**. The four vitamers were found in all the analyzed samples of *G. biloba*, with α-tocopherol as predominant form, as also previously stated in a sample from different commercial (Table 6, section 3.1.1.1), despite significant differences observed in the concentrations reported. In fact, tocopherols are very sensitive molecules that suffer rapid variation due to oxidation processes (Birringer et al. 2001; Luo et al. 2011). α-Tocopherol was the most susceptible isoform to irradiation process, decreasing with 10 kGy (**Table 11, Figure 31B**). Nevertheless, it should be pointed out that 1 kGy of irradiation dose did not significantly affected this vitamer, as also observed in a study with *Carya illinoensis* (Taipina et al. 2009). This is important as α-tocopherol, beyond being a vitamin, holds several beneficial functions for humans, including antioxidant, anti-inflammatory, anti-carcinogenic and antiatherogenic properties (Manosso et al. 2013). In other studies performed in plants (sage, thyme, and oregano) irradiated with 10 kGy, there were no significant differences in the content of α-and γ-tocopherol between control and irradiated samples (Brandstetter et al. 2009).

Table 11. Lipophilic compounds (fatty acids and tocopherols) in non-irradiated and irradiated *G. biloba* samples (mean ± SD).

	0 kGy	1 kGy	10 kGy
Fatty acids (relative percentage)			
C6:0	0.11 ± 0.01	0.17 ± 0.01	0.14 ± 0.03
C8:0	0.14 ± 0.02	0.32 ± 0.01	0.19 ± 0.04
C10:0	0.15 ± 0.03	0.13 ± 0.02	0.18 ± 0.02
C12:0	0.95 ± 0.06	0.88 ± 0.06	1.09 ± 0.14
C13:0	0.21 ± 0.01	0.25 ± 0.01	0.25 ± 0.03
C14:0	9.58 ± 0.08	9.10 ± 0.48	10.19 ± 1.00
C14:1	3.34 ± 0.21	3.17 ± 0.21	3.41 ± 0.19
C15:0	0.52 ± 0.05	0.67 ± 0.03	1.01 ± 0.10
C15:1	0.09 ± 0.00	0.07 ± 0.00	0.10 ± 0.00
C16:0	24.84 ± 0.51	23.50 ± 0.66	25.15 ± 0.57
C16:1	0.90 ± 0.05	1.00 ± 0.03	0.92 ± 0.09
C17:0	0.85 ± 0.03	0.85 ± 0.02	0.90 ± 0.03
C18:0	2.66 ± 0.06	2.41 ± 0.05	2.45 ± 0.04
C18:1n9	7.03 ± 0.06	6.74 ± 0.06	6.66 ± 0.23
C18:2n6	7.94 ± 0.54	8.21 ± 0.47	7.73 ± 0.21
C18:3n3	28.64 ± 2.12	31.63 ± 0.87	28.85 ± 2.31
C20:0	3.63 ± 0.07	2.56 ± 0.04	2.65 ± 0.12
C20:1	0.19 ± 0.02	0.17 ± 0.01	0.27 ± 0.03
C20:3n3+C21:0	1.21 ± 0.01	1.48 ± 0.06	1.29 ± 0.23
C22:0	2.25 ± 0.26	2.34 ± 0.03	2.22 ± 0.11
C23:0	0.87 ± 0.01	0.82 ± 0.06	0.71 ± 0.02
Total SFA (relative %)	50.65 ± 2.83^{a}	47.54 ± 1.13^{a}	50.79 ± 2.24^{a}
Total MUFA (relative %)	11.56 ± 0.16^{a}	11.16 ± 0.15^{a}	11.35 ± 0.51^{a}
Total PUFA (relative %)	37.79 ± 2.67^{a}	41.31 ± 1.29^{a}	37.86 ± 2.74^{a}
Tocopherols (mg/100 g dw**)**			
α-tocopherol	58.77 ± 0.74^{b}	61.18 ± 0.15^{a}	52.64 ± 0.92^{c}
β-tocopherol	28.96 ± 0.74^{b}	29.59 ± 0.62^{ab}	30.30 ± 0.59^{a}
γ-tocopherol	0.92 ± 0.02^{a}	0.98 ± 0.13^{a}	0.95 ± 0.01^{a}
δ-tocopherol	0.60 ± 0.04^{a}	0.54 ± 0.03^{a}	0.54 ± 0.04^{a}
Total tocopherols (mg/100 g)	89.25 ± 1.53^{b}	92.29 ± 0.86^{a}	84.43 ± 1.56^{c}

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecanoic acid (C12:0); Tridecanoic acid (C13:0); Myristic acid (C14:0); Myristoleic acid (C14:1); Pentadecanoic acid (C15:0); cis-10-Pentadecenoic acid (C15:1); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); α-Linolenic acid (C18:3n3); Stearic acid (20:0); Eicosenoic acid (C20:1); cis-11,14,17-Eicosatrienoic acid and Heneicosanoic acid (C20:3n3+C21:0); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). In each row different letters mean significant differences ($p<0.05$).

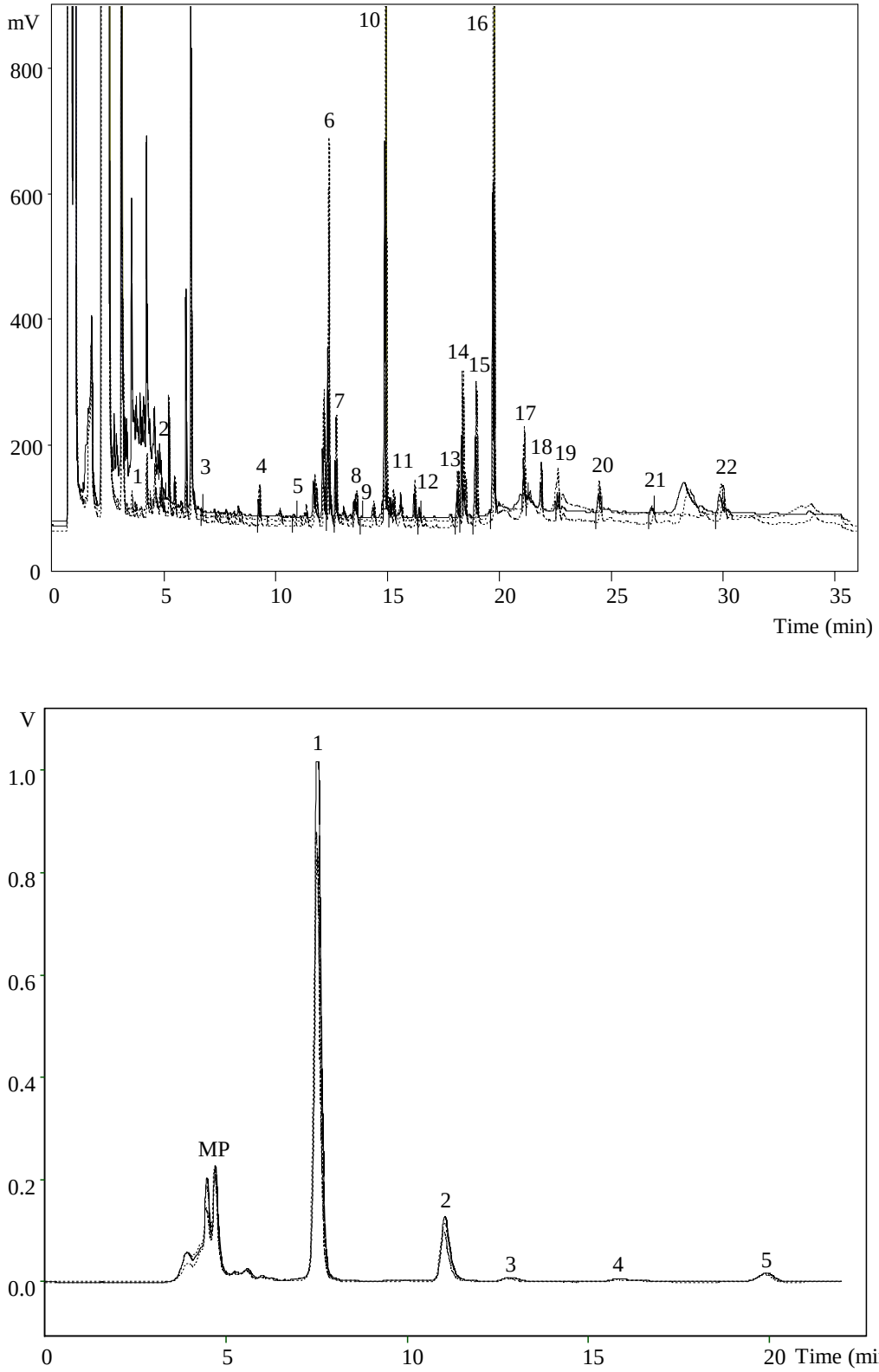


Figure 31. Profiles of fatty acids (A) and tocopherols (B) in *G. biloba* samples irradiated at 0 kGy (⋯⋯), 1 kGy (—) and 10 kGy (-----). Fatty acids: 1- C6:0; 2- C8:0; 3- C10:0; 4- C12:0; 5- C13:0; 6- C14:0; 7- C14:1; 8- C15:0; 9- C15:1; 10- C16:0; 11- C16:1; 12- C17:0; 13- C18:0; 14- C18:1n9; 15- C18:2n6; 16- C18:3n3; 17- 20:0; 18- C20:1; 19- C20:3n3+C21:0; 20- C22:0; 21- C23; 22- C24:0. Tocopherols: 1- α-tocopherol; 2- β-tocopherol; 3- γ-tocopherol; 4- δ-tocopherol; 5- tocol (IS). MP- mobile phase.

3.1.1.6 Composition in hydrophilic compounds of irradiated samples of *G. biloba*

The composition in sugars and organic acids was also assessed and the results are shown in **Table 12**. Fructose, glucose, sucrose and trehalose were identified and quantified in the samples. The observed sugar profile showed differences with the one obtained for the previously analysed *G. biloba* sample, in which trehalose was not detected and much lower amounts of sucrose were determined (**Table 7**, section 3.1.1.1). This might be explained since the concentration of sugars depends on the maturity stage of the leaves and other environmental factors that influence the use of these primary metabolites for energy production (Apone et al. 2010). A decrease in the levels of glucose was produced at both irradiation doses, while no significant differences were found regarding fructose and trehalose levels, and sucrose (the most abundant sugar) decreased only with 10 kGy of gamma radiation dose (**Table 12**, **Figure 32**). Kausar et al. (2013) in a study on tea samples observed an increase in sugar levels with the application of gamma radiation, which attributed to a degradation of polysaccharides, a process that did not seem to occur in the present study.

Regarding organic acids, oxalic, quinic, malic and shikimic acids were identified and quantified in all the analyzed samples (**Table 12**), in agreement with previous results (**Table 7**, section 3.1.1.1). Quinic and shikimic acids concentration s were not significantly affected by the applied irradiation doses, whereas oxalic and malic acids levels slightly increased at 1 kGy and decreased at 10 kGy (**Table 12, Figure 33**). Wen et al. (2006) did not find significant changes in the concentrations of malic and oxalic acids in irradiated lycium fruits.

Table 12. Hydrophilic compounds (sugars and organic acids) in non-irradiated and irradiated *G. biloba* samples (mean ± SD).

Free sugars (g/100 g dw)	0 kGy	1 kGy	10 kGy
Fructose	1.86 ± 0.12^{a}	1.87 ± 0.17^{a}	1.81 ± 0.01^{a}
Glucose	0.98 ± 0.07^{a}	0.73 ± 0.03^{b}	0.79 ± 0.04^{b}
Sucrose	3.78 ± 0.09^{a}	3.83 ± 0.11^{a}	3.60 ± 0.07^{b}
Trehalose	0.38 ± 0.04^{a}	0.41 ± 0.00^{a}	0.40 ± 0.02^{a}
Unknown	0.55 ± 0.03^{a}	0.51 ± 0.04^{a}	0.50 ± 0.05^{a}
Total	7.55 ± 0.07^{a}	7.35 ± 0.34^{ab}	7.10 ± 0.04^{b}
Organic acids (g/100 g dw)	0 kGy	1 kGy	10 kGy
Oxalic	0.82 ± 0.00^{b}	0.89 ± 0.00^{a}	0.80 ± 0.00^{c}
Quinic	1.37 ± 0.09^{a}	1.31 ± 0.01^{a}	1.33 ± 0.01^{a}
Malic	1.09 ± 0.00^{b}	1.21 ± 0.02^{a}	1.05 ± 0.01^{c}
Shikimic	1.49 ± 0.09^{a}	1.43 ± 0.00^{a}	1.42 ± 0.01^{a}
Total	4.78 ± 0.17^{ab}	4.83 ± 0.01^{a}	4.60 ± 0.03^{b}

dw- dry weight. In each row different letters mean significant differences (p<0.05).

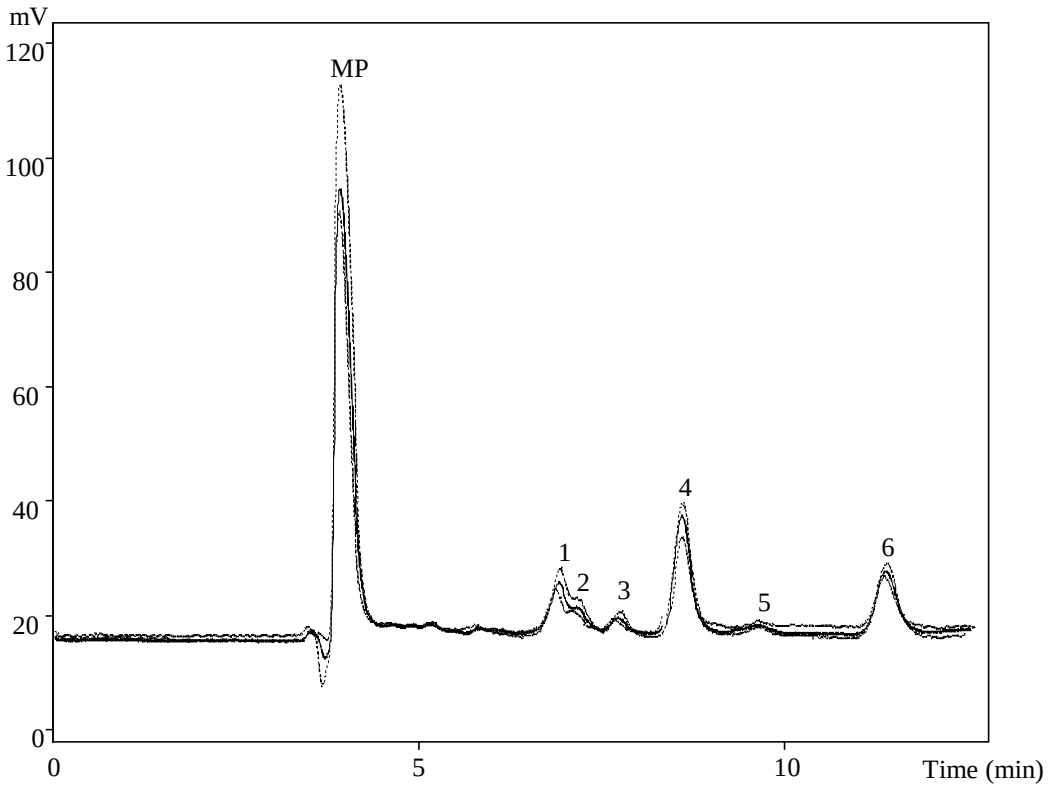


Figure 32. Sugar profiles in *G. biloba* samples irradiated at 0 kGy (⋯⋯), 1 kGy (—) and 10 kGy (-----). 1- Fructose; 2- glucose; 3- unknown; 4- sucrose; 5- trehalose; 6- melezitose (IS). MP- mobile phase.

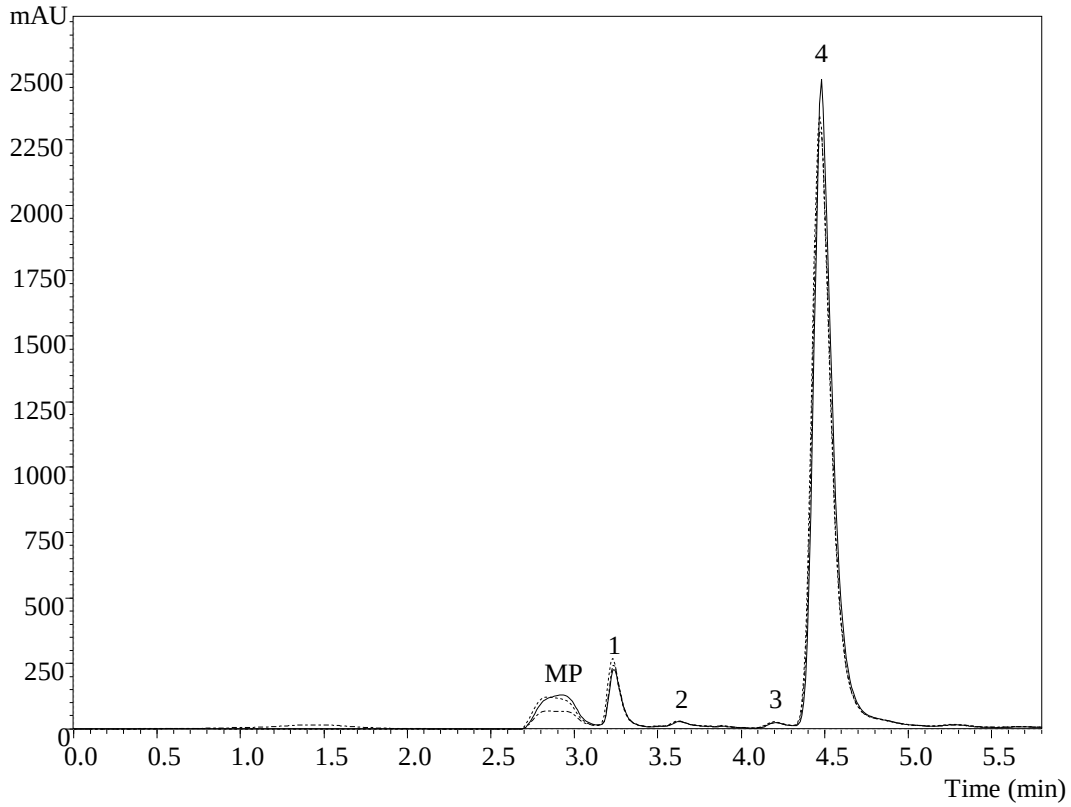


Figure 33. Profiles of organic acids in *G. biloba* samples irradiated at 0 kGy (·····), 1 kGy (—) and 10 kGy (-----). 1- Oxalic acid; 2- quinic acid; 3- malic acid; 4- shikimic acid. MP- mobile phase.

3.1.1.7 Antioxidant properties of irradiated samples of *G. biloba*

The results of antioxidant activity of infusions and methanolic extracts prepared form non-irradiated and irradiated samples, measured by four *in vitro* assays, are shown in **Table 13**. In general, methanolic extracts gave higher antioxidant activity (lower EC_{50} values) than the corresponding infusions (EC_{50} values ranging from 0.24 and 4.48 mg/mL when compared to the infusion 0.13 - 9.04 mg/mL), in agreement with previous observations (**Table 8**, section 3.1.1.2). These results are also consistent with a previous study, where alcoholic extracts showed better results than the corresponding infusions prepared from irradiated Korean medicinal plants (Byun et al. 1999).

For both infusion and methanolic extracts, gamma radiation at both doses increased DPPH scavenging activity, reducing power, β-carotene bleaching and lipid peroxidation inhibition of Ginkgo samples. In general, gamma radiation at 10 kGy led to more the antioxidant potential in *G. biloba* samples. This is in agreement with the results reported by the research group in a previous study with *Castanea sativa* fruits and skins (Antonio

et al. 2011). Khattak and Simpson (2008) also reported an increase in DPPH scavenging properties of *Nigella sativa* seeds irradiated at 16 kGy.

The increase in the antioxidant activity with the increasing irradiation dose may due to an effect of improved extractability of certain compounds (Alothman et al., 2009).

Table 13. Antioxidant activity (EC_{50} values, mg/mL) of infusions and methanolic extracts obtained from non-irradiated and irradiated *G. biloba* samples (mean ± SD).

Antioxidant activity	Infusion			Methanolic extract		
	0 kGy	1 kGy	10 kGy	0 kGy	1 kGy	10 kGy
DPPH scavenging activity	5.80±0.24^{a}	4.09±0.07^{b}	2.88±0.23^{c}	1.64±0.02^{a}	1.54±0.05^{ab}	1.49±0.16^{b}
Reducing Power	4.58±0.06^{a}	3.41±0.01^{b}	2.37±0.02^{c}	0.65±0.00^{a}	0.63±0.00^{b}	0.49±0.00^{c}
β-Carotene bleaching inhibition	11.09±0.54^{a}	9.04±0.35^{b}	8.79±0.23^{b}	10.39±0.66^{a}	5.26±0.18^{b}	4.48±0.17^{c}
TBARS inhibition	0.15±0.01^{a}	0.13±0.01^{b}	0.10±0.01^{c}	0.24±0.01^{a}	0.16±0.03^{b}	0.08±0.00^{c}

3.1.1.8 Phenolic compounds of irradiated samples of *G. biloba*

The chromatographic profile of *G. biloba* samples non-irradiated and irradiated at 10 kGy, and recorded at 370 nm is shown in **Figure 34**; compound characteristics and tentative identities are presented in **Table 14**. Twenty-five compounds were detected, eighteen of which were flavonoids, one phenolic acid, five terpene lactones and one unknown compound.

Protocatechuic acid (compound 3), myricetin-3-*O*-rutinoside (compound 11), quercetin-3-*O*-rutinoside (compound 15), quercetin-3-*O*-glucoside (compound 17), kaempferol-3-*O*-rutinoside (compound 19), isorhamnetin-3-*O*-rutinoside (compound 20) and isorhamnetin-3-*O*-glucoside (compound 22) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. All the compounds mentioned above, with the exception of protocatechuic acid were previously reported in leaves of *G. biloba* (Tang et al., 2001; Zhang et al., 2007; Ding et al., 2008; Lin et al., 2008).

Peaks 1, 2, 4-6 and 12 were associated to terpene trilactones. That type of compounds have low UV absorption and coexisting substances present in the complex matrix of *G. biloba* extracts make it difficult their detection and quantification using UV detection (Sloley et al., 2003; Mesbah et al., 2005). Nonetheless, they could be detected in the analysed extracts and tentatively identified based on their MS characteristics and comparison with data reported in the bibliography (Bedir et al., 2002; van Beek, 2002; Ding et al., 2008; Lin et al., 2008; van Beek and Montoro, 2009; Rossi et al., 2011; Liu et al., 2014); in particular, bilobalide (compound 4), ginkgolide A (compound 5), ginkgolide B (compound 6) and ginkgolide C isomer (compound 12). The latter compound was assigned to an isomer of ginkgolide C, since ginkgolide C would be expected to elute earlier than ginkgolides A and B (Ding et al., 2008; Lin et al., 2008; van Beek and Montoro, 2009). No identification could be assigned to compound 1 ($[M-H]^-$ at *m/z* 453), although the main *m/z* fragment at 407 might point to it could be a derivative of ginkgolide A. No conclusions could be drawn about the identity and nature of compound 2 ($[M-H]^-$ at *m/z* 449). Three compounds with the same pseudomolecular ion were also detected by Ding et al. (2008) in *G. biloba* supplements, which were assigned as unknown glycosyl flavonoids. However, the flavonoid nature of the compound was not clear in our samples, but the presence of MS^2 fragments at *m/z* 179 and 113 also observed for compounds 1 and 6 rather suggested that it might correspond to a ginkgolide that remains unknown.

The rest of compounds showed UV and mass characteristics coherent with flavonol glycosides. Compounds 9, 19 and 23 ($[M-H]^-$ at *m/z* 593) presented characteristics that match a structure of kaempferol bearing deoxyhexosyl and hexosyl residues. Ding et al. (2008) also

detected three compounds with the same mass in *G. biloba* supplements, all of them identified as kaempferol-*O*-rhamnosyl-glucoside. Zhang et al. (2007) and Lin et al. (2008) also found two compounds with similar characteristics in *G. biloba* leaves, one of them kaempferol-3-*O*-rutinoside (positively identified as peak 19 in our samples) and the other one as kaempferol-3-*O*-glucosyl-(1,2)-rhamnoside. This latter identity was assumed for peak 23 owing to its delayed elution in relation to peak 19, as reported by those authors. As no further information was available for compound 9 it was just assigned as a kaempferol-*O*-rhamnosyl-glucoside. Similar reasoning was applied for the identification of compounds 15 and 18 showing the same pseudomolecular ion $[M-H]^-$ at *m/z* 609, which were respectively identified as quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucosyl-(1,2)-rhamnoside, as also reported Zhang et al. (2007) and Lin et al. (2008).

Compounds 7, 10 and 24 presented the same pseudomolecular ion $[M-H]^-$ at *m/z* 755. Their MS^2 spectra pointed to they are derived from different aglycones, i.e., kaempferol (7) and quercetin (10 and 24), owing to the observation of fragments at m/z 285 and 301, respectively. Different compounds with similar UV and mass characteristics were reported by Lin et al. (2008) and Ding et al. (2008) in *G. biloba* leaves and supplements. Compound 7 would correspond to a kaempferol derivative bearing one deoxyhexosyl and two hexosyl residues; the observation of a main MS^2 fragment at *m/z* 593 from the lost of the hexosyl residue suggested that this latter was located at a different position of the other two glycosyl moieties that could be constituting a disaccharide. Although no information about the actual nature and position of the sugar substituents could be obtained from the available data, based on the previous comments assumption and the identification made by Lin et al. (2008) of a similar compound, peak 7 was tentatively assigned as kaempferol-3-*O*-rhamnosylhexoside-7-*O*-glucoside. Characteristics of compound 10 were consistent with a quercetin derivative possessing two deoxyhexosyl and one hexosyl residues. The fact that only one MS^2 fragment was released corresponding to the aglycone (i.e., *m/z* at 301, quercetin) would suggest that the three sugars constituted a trisaccharide; based on this assumption the compound was tentatively identified as quercetin 3-*O*-2’’,6’’-dirhamnosylglucoside also reported in *G. biloba* leaves by Lin et al. (2008). Compound 24 showed different UV and MS^2 spectra than compound 10, presenting an additional maximum wavelength at 316 nm (characteristic of hydroxycinnamoyl derivatives) and a minor MS^2 fragment at *m/z* 609 (loss of 146 mu that matches either a rhamnosyl or a *p*-coumaroyl moiety). These features allowed its tentatively assignment as quercetin-3-*O*-*p*-coumaroyl-rhamnosylhexoside, which was also coherent with its delayed retention time. Similar compounds were also reported by Lin et al. (2008) and

Ding et al. (2008). The same reasoning was applied for assigning compounds 13 and 25, both showing pseudomolecular ions $[M-H]^-$ at *m/z* 739, as kaempferol-3-*O*-dirhamnosylglucoside and kaempferol-3-*O*-*p*-coumaroyl-rhamnosylhexoside, respectively, as also proposed by Lin et al. (2008).

Mass spectra characteristics of compounds 8 ($[M-H]^-$ at *m/z* 785) and 14 ($[M-H]^-$ at *m/z* 769) were similar to those of compounds 7 and 13 respectively, but derived from an isorhamnetin aglycone as revealed by the MS^2 fragment produced at *m/z* 315. Based on this observation and previous identifications by Lin et al. (2008), these compounds were respectively assigned as isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside and isorhamnetin-3-*O*-dirhamnosylglucoside. Compounds 16 ($[M-H]^-$ at *m/z* 639) and 21 ($[M-H]^-$ at *m/z* 447) were assigned as patuletin-3-*O*-rutinoside and quercetin-3-*O*-rhamnoside, based on their UV spectra and mass characteristics and previous identification in *G. biloba* leaves by Lin et al. (2008) and the latter one also by Yao et al. (2013).

Among the twenty-five compounds detected, compounds 1, 2, 4-6 and 12, associated to ginkgolides, were not quantified due their low UV absorption and possible interferences in the complex matrix of *G. biloba* extracts, as well as the unavailability of commercial gingkolide standards. Flavonoids were the main group present, being two kaempferol derivatives the majority compounds found (**Table 15**). Thus, kaempferol-3-*O*-dirhamnosylglucoside (compound 13) was the most abundant compound in all the infusion preparations and in the methanol/water extract irradiated at 1 kGy, whereas kaempferol-3-*O*-rutinoside (compound 19) was the most abundant one in the control and irradiated at 10 kGy methanol/water extracts. Protocatechuic acid was the only phenolic acid identified and the quantities present were in the same range as the main flavonoids.

One of the objectives of this study was to evaluate whether irradiation could improve the extractability of phenolic compounds in *G. biloba* samples (methanol extracts and lyophilized infusions) and what dose would be the most efficient one. Infusions presented lower quantities than the methanolic extracts, which might be explained by a lower efficiency of water as solvent for the extraction of polyphenols compared with methanol, but also due to the lower extraction time and the high temperatures applied that could destroy some thermal sensitive compounds. With both of solvents, higher amounts of phenolic compounds were extracted from the samples irradiated at 10 kGy compared with those non-irradiated or irradiated at 1 kGy (Table 14). Although it is considered that during irradiation some compound degradation occurs by complex mechanisms that involve the breakdown of molecular bonds resulting in smaller products (Stewart, 2001), the obtained results suggest

that the use of high irradiation doses might also improve compound extractability. This might be due to the release of compounds linked to matrix structures that may also be degraded in some extent. Low doses up to 1 kGy are used for preservation of fresh samples (Molins, 2001), although they are more sensitive than dried food, they should not have effects or produce only slight changes in food main characteristics. The dose of 10 kGy is enough to guarantee product disinfestation and microbial decontamination (Molins, 2001), and might also contribute to an increase in the phenolic compounds extractability, both in methanolic extracts and infusion preparations. The use of irradiation to improve bioactive properties as a result of an increase in the levels of phenolic compounds was also suggested by other authors in extracts obtained from cooked and derived plant products (Zhu et al., 2010; Aouidi et al., 2011; Lee et al., 2013; Wanyo et al., 2014).

To our knowledge, this is the first study on the effects of irradiation on the phenolic composition in *G. biloba* samples using different radiation doses. Nevertheless, further research is needed to understand the mechanisms involved in the effects of the irradiation processing in plant constituents.

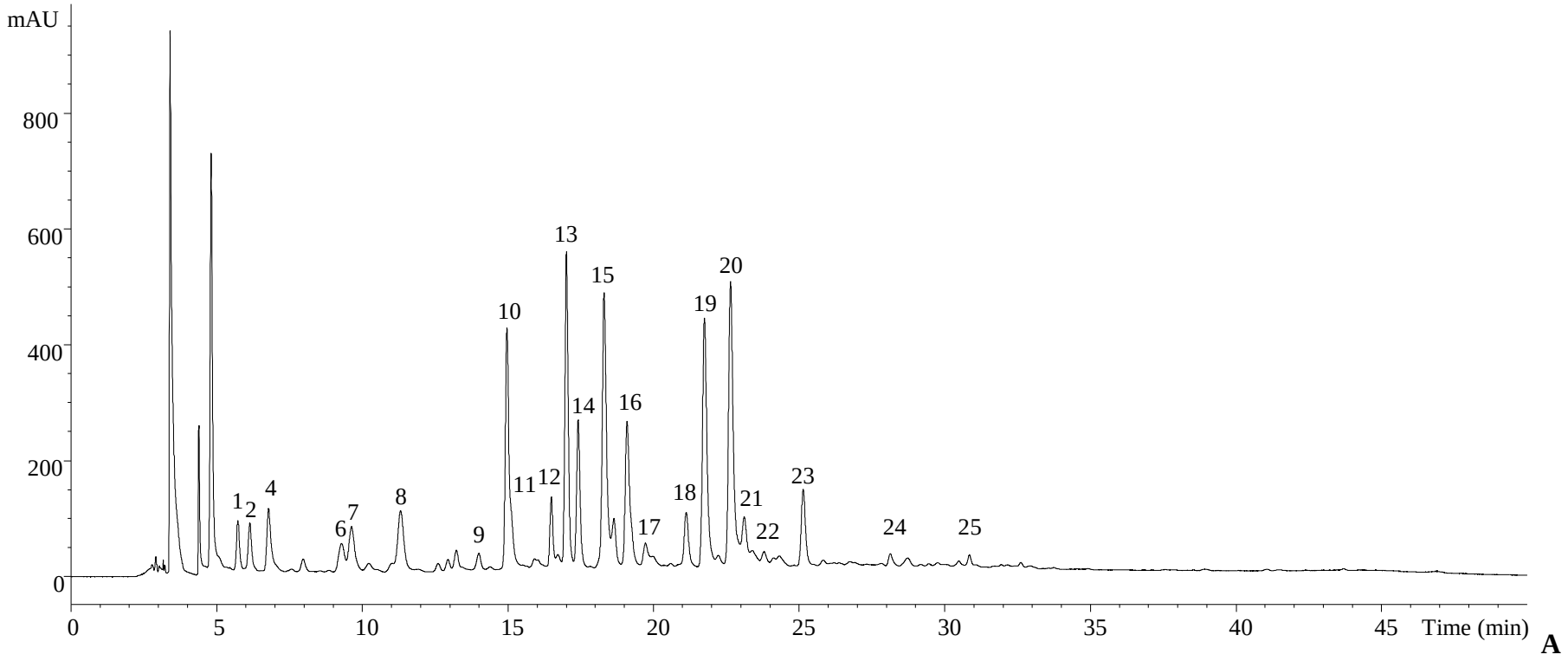
Table 14. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and compound identification in *G. biloba* samples.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1	5.7	260,294,350	453	407(100),245(3),179(7),161(3),113(5)	Ginkgolide A derivative
2	6.1	358	449	403(16),269(4),205(8),179(5),113(4)	Unknown ginkgolide
3	6.2	262sh294	153	109(100)	Protocatechuic acid
4	6.8	252,356	325	163(100),119(87)	Bilobalide
5	7.1	274	407	245(100)	Ginkgolide A
6	9.3	282sh336	423	221(15),179(13),161(22),131(21),113(32)	Ginkgolide B
7	9.6	348	755	593(100),285(22)	Kaempferol-3-*O*-rhamnosylhexoside-7-*O*-glucoside
8	11.3	358	785	623(100),315(17)	Isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside
9	14.0	348	593	447(23),285(58)	Kaempferol-*O*-rhamnosyl-glucoside
10	15.0	354	755	301(100)	Quercetin 3-*O*-2’’,6’’-dirhamnosylglucoside
11	15.1	350	625	317(100)	Myricetin-3-*O*-rutinoside
12	16.5	318	439	411(18),383(93),365(12),322(26),304(7),277(7),259(8)	Ginkgolide C isomer
13	17.0	348	739	285(100)	Kaempferol-3-*O*-dirhamnosylglucoside
14	17.4	356	769	315(100)	Isorhamnetin-3-*O*-dirhamnosylglucoside
15	18.3	356	609	301(100)	Quercetin-3-*O*-rutinoside
16	19.1	360	639	331(100)	Patuletin-3-*O*-rutinoside
17	19.7	358	463	301(100)	Quercetin-3-*O*-glucoside
18	21.1	352	609	301(100)	Quercetin-3-*O*-glucosyl-(1,2)-rhamnoside
19	21.7	348	593	285(100)	Kaempferol-3-*O*-rutinoside
20	22.6	356	623	315(100)	Isorhamnetin-3-*O*-rutinoside
21	23.1	352	447	301(100)	Quercetin-3-*O*-rhamnoside
22	23.8	350	477	315(100)	Isorhamnetin-3-*O*-glucoside
23	25.1	348	593	285(100)	Kaempferol-3-*O*-glucosyl-(1,2)-rhamnoside
24	28.1	268,316	755	609(46),301(21)	Quercetin-3-*O*-*p*-coumaroyl-rhamnosylhexoside
25	30.8	266,316	739	593(51),285(21)	Kaempferol-3-*O*-*p*-coumaroyl-rhamnosylhexoside

Table 15. Quantification of phenolic compounds (mg/g of dried extract/lyophilized infusion) identified in methanolic extracts and infusions of *G. biloba* non-irradiated and irradiated samples.

Compounds	Methanol/water extracts			Infusions		
	0 kGy	1 kGy	10 kGy	0 kGy	1 kGy	10 kGy
Protocatechuic acid	1.39 ± 0.08	1.22 ± 0.04	4.49 ± 0.05	0.43 ± 0.04	0.38 ± 0.01	1.95 ± 0.05
Kaempferol-3-*O*-rhamnosylhexoside-7-*O*-glucoside	0.34 ± 0.02	0.49 ± 0.01	1.16 ± 0.06	0.24 ± 0.02	0.25 ± 0.01	0.34 ± 0.05
Isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside	0.32 ± 0.04	0.45 ± 0.04	1.09 ± 0.14	0.22 ± 0.02	0.24 ± 0.02	0.35 ± 0.05
Kaempferol-*O*-rhamnosyl-glucoside	0.12 ± 0.02	0.16 ± 0.01	0.53 ± 0.11	0.03 ± 0.01	0.04 ± 0.00	0.11 ± 0.02
Quercetin 3-*O*-2’’,6’’-dirhamnosylglucoside	0.55 ± 0.01	0.66± 0.04	1.67 ± 0.07	0.17 ± 0.01	0.19 ± 0.01	0.56 ± 0.03
Myricetin-3-*O*-rutinoside	0.11 ± 0.01	0.07 ± 0.01	0.59 ± 0.08	nd	nd	0.12 ± 0.03
Kaempferol-3-*O*-dirhamnosylglucoside	1.26 ± 0.01	1.48 ± 0.02	3.57 ± 0.03	0.44 ± 0.02	0.40 ± 0.02	1.29 ± 0.01
Isorhamnetin-3-*O*-dirhamnosylglucoside	0.42 ± 0.01	0.51 ± 0.03	1.30 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.45 ± 0.01
Quercetin-3-*O*-rutinoside	0.84 ± 0.06	0.34 ± 0.02	2.74 ± 0.07	tr	0.07 ± 0.01	0.74 ± 0.03
Patuletin-3-*O*-rutinoside	0.52 ± 0.01	0.25 ± 0.01	1.89 ± 0.04	tr	0.04 ± 0.00	0.51 ± 0.03
Quercetin-3-*O*-glucoside	0.08 ± 0.01	tr	0.56 ± 0.05	nd	nd	0.05 ± 0.02
Quercetin-3-*O*-glucosyl-(1,2)-rhamnoside	0.20 ± 0.02	0.04 ± 0.01	0.78 ± 0.06	nd	tr	0.17 ± 0.01
Kaempferol-3-*O*-rutinoside	1.38 ± 0.04	0.57 ± 0.03	4.21 ± 0.02	0.02 ± 0.00	0.16 ± 0.02	1.15 ± 0.04
Isorhamnetin-3-*O*-rutinoside	1.06 ± 0.02	0.49 ± 0.03	3.05 ± 0.06	0.03 ± 0.00	0.16 ± 0.01	0.95 ± 0.01
Quercetin-3-*O*-rhamnoside	0.16 ± 0.01	0.09 ± 0.01	0.67 ± 0.05	nd	tr	0.17 ± 0.02
Isorhamnetin-3-*O*-glucoside	0.15 ± 0.04	0.12 ± 0.01	0.46 ± 0.05	nd	0.04 ± 0.00	0.16 ± 0.01
Kaempferol-3-*O*-glucosyl-(1,2)-rhamnoside	0.49 ± 0.02	0.15 ± 0.03	1.81 ± 0.15	0.02 ± 0.00	0.02 ± 0.00	0.38 ± 0.01
Quercetin-3-*O*-*p*-coumaroyl-rhamnosylhexoside	0.06 ± 0.01	0.04 ± 0.00	1.30 ± 0.08	tr	tr	0.10 ± 0.02
Kaempferol-3-*O*-*p*-coumaroyl-rhamnosylhexoside	0.11 ± 0.01	0.09 ± 0.01	1.83 ± 0.04	0.02 ± 0.00	0.01 ± 0.00	0.15 ± 0.01
Total phenolic acids	1.40 ± 0.08b	1.22 ± 0.04c	4.49 ± 0.05a	0.43 ± 0.04b	0.38 ± 0.01b	1.95 ± 0.05a
Total flavonoids	8.16 ± 0.04b	6.00 ± 0.18c	29.20 ± 0.74a	1.35 ± 0.01c	1.75 ± 0.06b	7.76 ± 0.16a
Total phenolic compounds	9.56 ± 0.06b	7.22 ± 0.22c	33.69 ± 0.69a	1.78 ± 0.06c	2.13 ± 0.04b	9.70 ± 0.11a

nd-not detected; nq- not quantified; tr-traces. In each row and for each extract (methanolic extracts or infusions) different letters mean significant differences among total compounds ($p<0.05$).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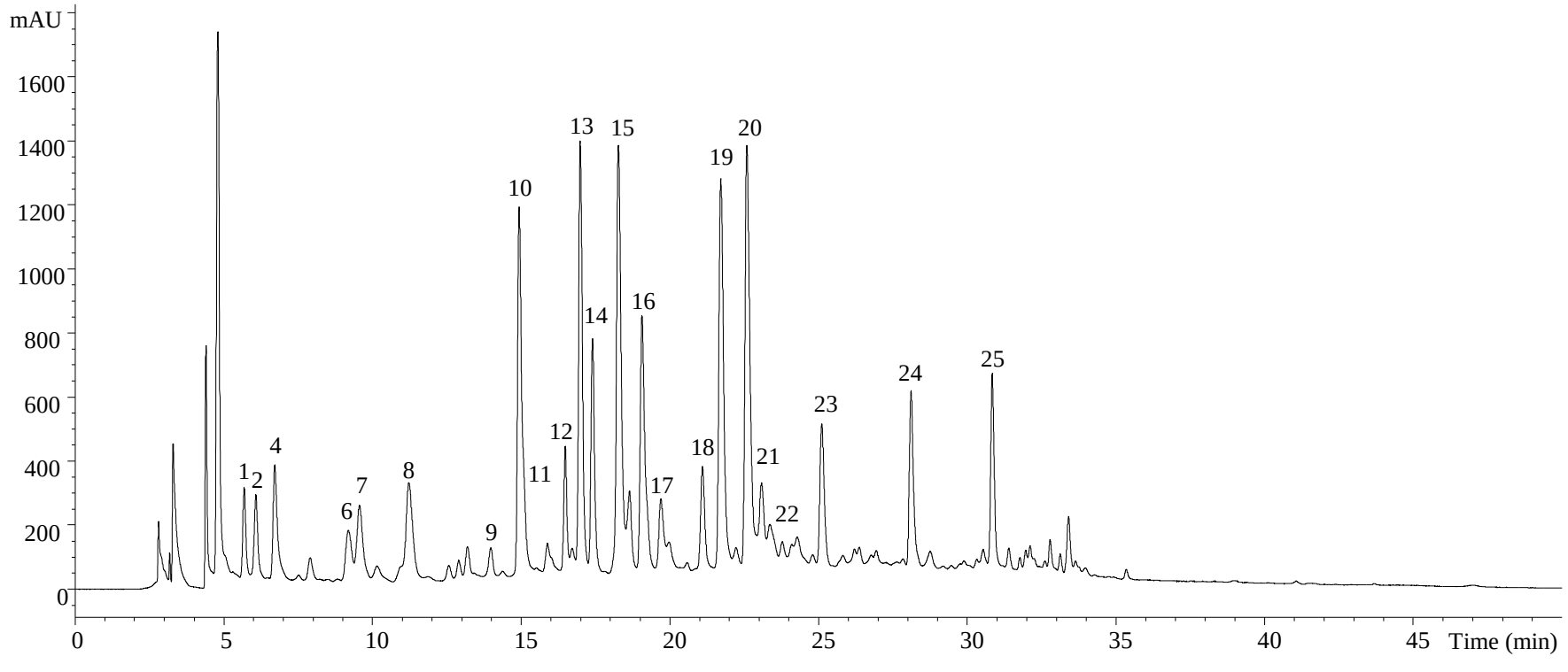
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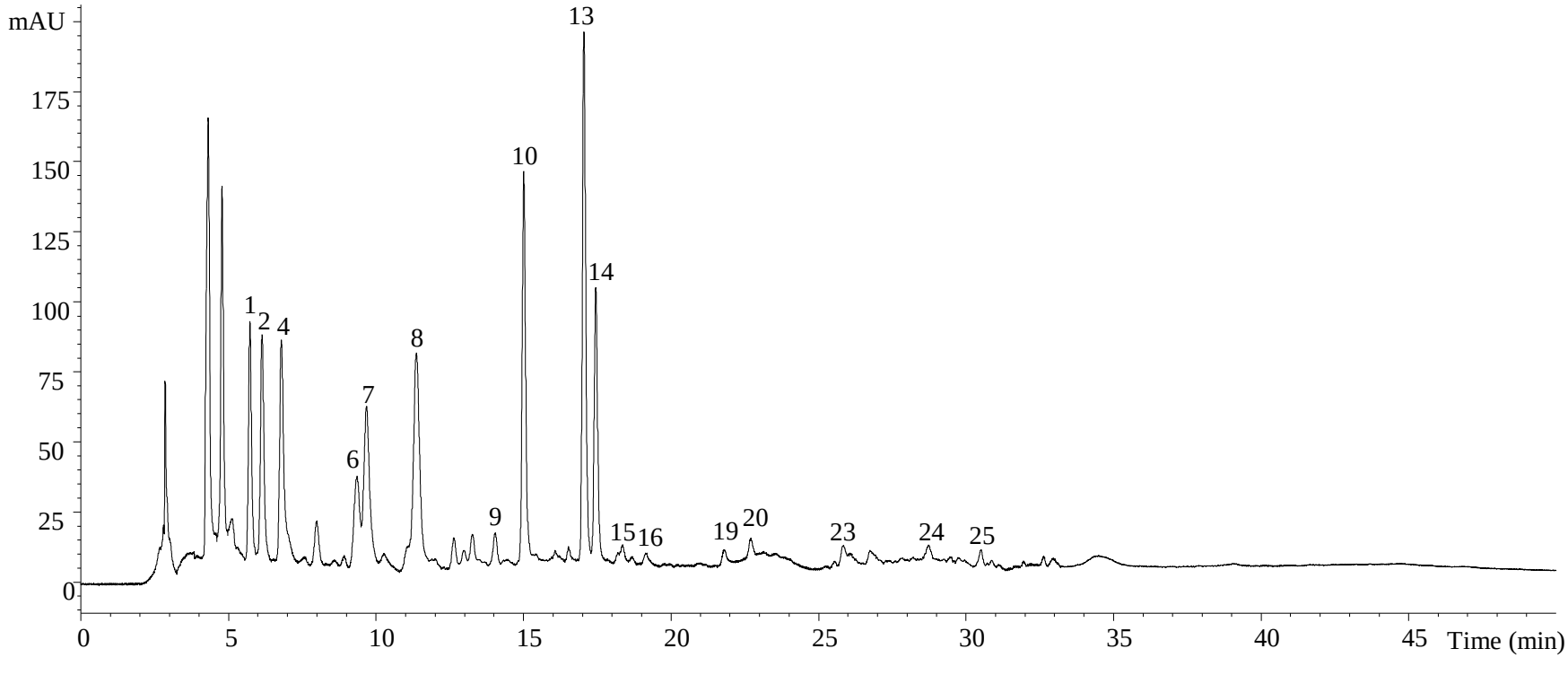
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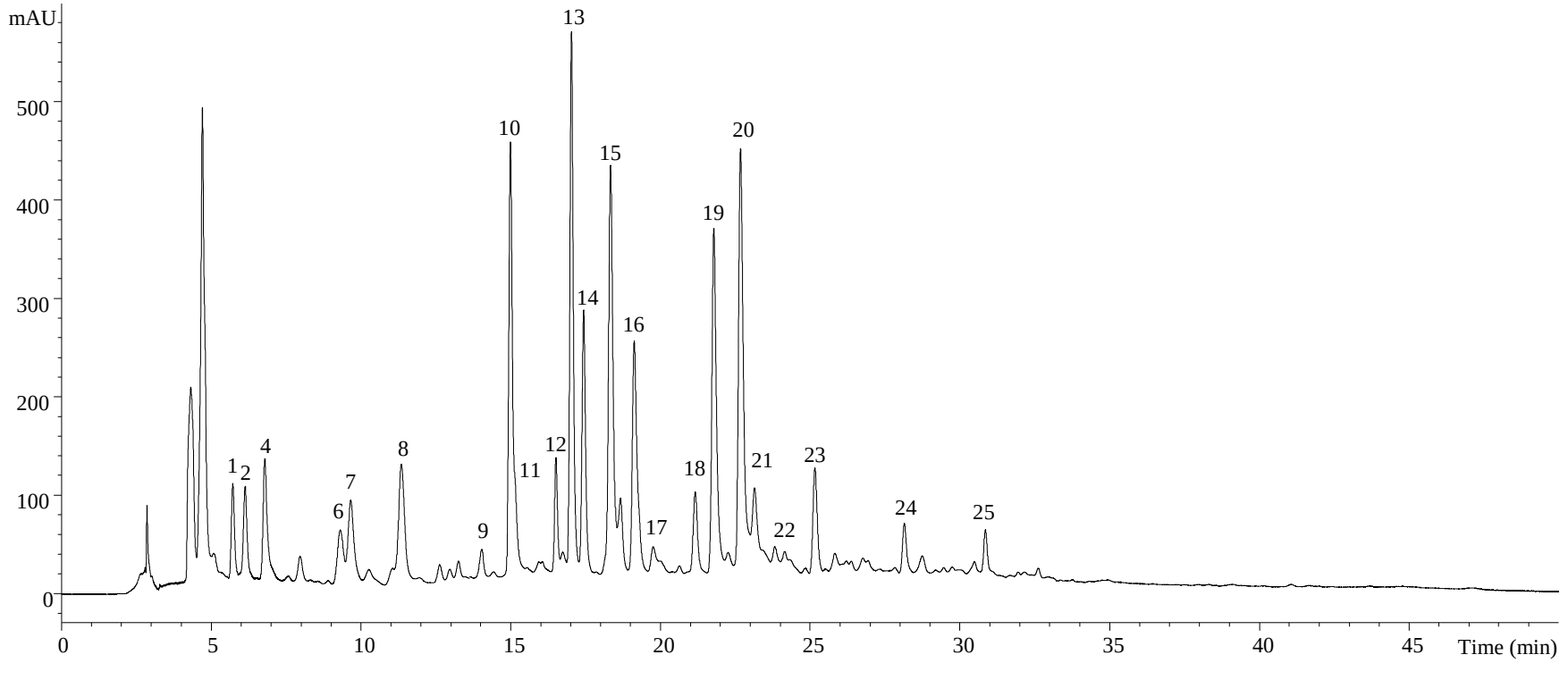
Time (min)

C



D

Figure 34. Chromatographic profiles recorded at 370 nm of methanolic extracts from G. biloba samples: non-irradiated (A) and irradiated at 10 kGy (B), and of lyophilized infusions from G. biloba: non-irradiated (C) and irradiated at 10 kGy (D).

3.1.2 Aloysia citrodora Paláu, Melissa officinalis L., Melittis melissophyllum L. and Mentha x pipperita L.

3.1.2.1 Effects of irradiation on chemical variables

The proximate composition and color variables of *A. citrodora* (lemon verbena), *M. officinalis* (lemon balm), *M. melissophyllum* (bastard balm) and *M. piperita* (peppermint) are shown in **Table 16**. In all cases, carbohydrates are the predominant component, followed by ash, protein and fat contents. In our knowledge, except for lemon balm, the proximate composition of these species is described for the first time. The nutritional profile detected for lemon balm is coherent with that reported in a previous work (Dias, et al., 2012). Regarding the effect of gamma radiation (GR), all proximate composition variables showed sensitivity, except ash content in lemon balm (p=0.072). Despite the detected variations, it was not possible to identify marked tendencies, with the exception of proteins, which tended to be higher in samples irradiated at 10 kGy for all species. As above commented, the increase in proteins might be related to scission of the C-N bonds in the backbone of the polypeptide chain or splitting of the disulphide bonds, or physical changes like unfolding, which are commonly associated to irradiation treatments (Molins, 2001).

Color variables are used in the quality control of post-harvest preservation processes (Hsu et al., 2010). Herein, these variables were also similar, with higher lightness values in lemon verbena (≈49) and lemon balm (≈49), lower redness in lemon verbena (≈-8.4) and bastard balm (≈-8.2) and higher yellowness (≈27) in lemon verbena. Color variables proved to be less susceptible to irradiation than macronutrients, since the detected differences had no statistical significance (p>0.050) in most cases. Considering the cases where a statistically significant difference was found, it might be said that lightness, redness and yellowness leaned toward lower values in samples irradiated at 10 kGy. Similar decrease of a* and b* was observed in gamma irradiated green tea extracts (Jo et al., 2003b). The results for peppermint are in agreement with those reported in North American samples, showing no variation in color variables when irradiated at low doses (Hsu et al., 2010).

Table 16. Proximate composition and color variables (L*: lightness, a*: redness, b*: yellowness) of the four assayed species submitted to gamma radiation (GR).[1]

		Fat (g/100 g fw)	Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)	*L**	*a**	*b**
					Aloysia citrodora				
GR	0 kGy	1.6±0.1^{b}	3.0±0.1^{a}	8.2±0.1^{b}	87.1±0.1^{b}	375±1^{b}	49±1^{b}	-8.4±0.2	27.2±0.3^{b}
	1 kGy	2.1±0.1^{a}	1.8±0.1^{b}	8.5±0.3^{a}	87.6±0.4^{a}	377±1^{a}	50±1^{a}	-8.8±0.3	28.0±0.4^{a}
	10 kGy	1.7±0.1^{b}	3.0±0.2^{a}	8.6±0.2^{a}	86.7±0.1^{c}	374±1^{c}	48±1^{b}	-8±1	26.4±0.4^{c}
p-values	Homoscedasticity[2]	0.471	0.323	0.001	0.003	0.074	0.495	0.031	0.951
	Normal distribution[3]	0.001	<0.001	0.016	0.033	0.125	0.110	<0.001	0.612
	1-way ANOVA[4]	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	0.100	<0.001
					Melissa officinalis				
GR	0 kGy	1.2±0.1^{b}	2.5±0.3^{b}	8.4±0.4	88±1^{a}	372±2^{c}	48±1	-5.1±0.5	20.9±0.4^{a}
	1 kGy	1.9±0.1^{a}	7±1^{a}	8.1±0.3	83±1^{b}	377±1^{a}	48±1	-5.1±0.5	20.9±0.4^{a}
	10 kGy	1.8±0.1^{a}	6±1^{a}	8.4±0.2	83±1^{b}	376±1^{b}	47±1	-5.0±0.5	20.3±0.5^{b}
p-values	Homoscedasticity[2]	0.113	0.003	0.054	0.002	0.004	0.191	0.926	0.412
	Normal distribution[3]	<0.001	0.005	0.145	0.002	0.037	0.346	0.703	0.096
	1-way ANOVA[4]	<0.001	<0.001	0.072	<0.001	<0.001	0.269	0.926	0.022
					Melittis melissophyllum				
GR	0 kGy	1.8±0.1^{a}	4.6±0.2^{b}	7.6±0.1^{c}	86.0±0.4^{b}	378±1^{a}	42±2	-8.4±0.5	18±3
	1 kGy	1.6±0.1^{b}	2.6±0.1^{c}	8.1±0.1^{b}	87.7±0.2^{a}	376±1^{b}	44±2	-8.2±0.5	17±1
	10 kGy	1.5±0.1^{b}	5.6±0.5^{a}	8.6±0.2^{a}	84±1^{c}	373±1^{c}	41±2	-8.0±0.5	16±1
p-values	Homoscedasticity[2]	0.007	<0.001	0.108	<0.001	0.002	0.811	0.555	0.053
	Normal distribution[3]	0.056	0.004	0.124	0.057	0.291	0.090	0.588	<0.001
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	0.055	0.311	0.381
					Mentha x piperita				
GR	0 kGy	2.4±0.1^{b}	5.1±0.3^{b}	9.2±0.2^{a}	83.3±0.5^{b}	375±1^{b}	40±1^{a}	-5.9±0.1^{a}	23.9±0.3^{a}
	1 kGy	2.7±0.2^{a}	3.1±0.1^{c}	8.4±0.1^{c}	85.8±0.3^{a}	380±1^{a}	39±1^{a}	-5.7±0.2^{a}	23.2±0.5^{a}
	10 kGy	2.0±0.2^{c}	10.5±0.3^{a}	8.6±0.1^{b}	78.9±0.4^{c}	375±1^{b}	37±1^{b}	-4.8±0.4^{b}	20.7±0.5^{b}
p-values	Homoscedasticity[2]	0.169	<0.001	<0.001	0.379	0.006	0.515	0.072	0.036
	Normal distribution[3]	0.448	<0.001	0.010	0.001	<0.001	0.406	0.008	0.005
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among GR doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one GR dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p<0.05$).

Concerning free sugars composition (**Table 17**), fructose, glucose, sucrose and trehalose were quantified in all species. A fifth sugar, whose identity could not be established, was also detected in relevant amount in bastard balm. Sucrose was the main sugar in lemon verbena (≈6.7 g/100 g dw) and lemon balm (≈5.3 g/100 g dw), while the unidentified sugar (≈2.7 g/100 g dw) and trehalose (≈0.9 g/100 g dw) were the most abundant ones in bastard balm and peppermint, respectively. Lemon verbena showed the highest content (≈10.2 g/100 g dw) in total sugars. The 10 kGy dose seemed to increase sugars content in lemon balm and bastard balm, while lemon verbena and peppermint tended to present higher values in non-irradiated samples. The increase in free sugars as a result of gamma radiation was previously reported in soybean (Byun et al., 1996), ginseng (Byun et al., 1997), green, black and oolong teas (Kausar, et al 2013), and plant waste materials (Tissot et al., 2013), and it might be due to the shortening or depolymerization of polysaccharide molecules. Other verified changes that might also explain the variations in sugar levels are the changes in the optical rotation of sugars, which is a common effect within the radiation treatments (Molins, 2001).

Peppermint gave the highest contents in organic acids (**Table 17**), mainly due to citric acid concentrations (≈7.6 g/100 g dw). Malic acid was the predominant form in bastard balm (≈5.5 g/100 g dw), while shikimic acid (≈4.1 g/100 g dw) and citric acid (≈1.7 g/100 g dw) were the organic acids quantified in highest levels in lemon balm and lemon verbena, respectively. Oxalic acid and quinic acid were also detected in all species, except for quinic acid in lemon verbena. In the samples of *M. melissophyllum* and *M. piperita* greater changes were detected in the samples irradiated at 1 kGy dose, suggesting a certain protective effect of higher irradiation doses that might prevent acid degradation by decreasing the molecular oxygen availability inside the polyethylene bags.

Table 17. Hydrophilic compounds (free sugars and organic acids) composition (g/100 g dw) in the four assayed species submitted to gamma radiation (GR). The results are presented as mean±SD[1].

		Fructose	Glucose	Sucrose	Trehalose	Unknown	Total sugars	Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Citric acid	Total organic acids
						Aloysia citrodora							
GR	0 kGy	1.0±0.1	1.3±0.1	7.1±0.3^{a}	1.2±0.1	nd	10.7±0.4^{a}	1.1±0.1	nd	0.14±0.03^{b}	1.4±0.1^{c}	1.4±0.1^{c}	4.1±0.1^{c}
	1 kGy	1.0±0.1	1.2±0.1	6.4±0.3^{b}	1.2±0.1	nd	9.8±0.4^{b}	1.1±0.1	nd	0.17±0.02^{a}	1.8±0.1^{a}	2.0±0.2^{a}	5.1±0.3^{a}
	10 kGy	1.0±0.1	1.2±0.1	6.6±0.3^{b}	1.2±0.1	nd	10.0±0.5^{b}	1.1±0.1	nd	0.13±0.02^{b}	1.6±0.1^{b}	1.7±0.1^{b}	4.6±0.3^{b}
p-values	Homoscedasticity[2]	0.115	0.072	0.818	0.011	-	0.944	0.401	-	0.190	0.625	0.034	0.154
	Normal distribution[3]	0.672	0.333	0.308	0.319	-	0.799	0.288	-	0.481	0.281	0.184	0.140
	1-way ANOVA[4]	0.882	0.065	<0.001	0.843	-	0.001	0.233	-	0.007	<0.001	<0.001	<0.001
						Melissa officinalis							
GR	0 kGy	1.2±0.1^{b}	1.0±0.1	4.8±0.2^{c}	0.49±0.05^{c}	nd	7.5±0.2^{c}	0.5±0.1	0.26±0.04	0.4±0.1	4.1±0.2	Nd	5.3±0.3
	1 kGy	1.4±0.1^{a}	1.0±0.1	5.4±0.2^{b}	0.67±0.03^{b}	nd	8.4±0.3^{b}	0.5±0.1	0.23±0.03	0.4±0.1	4.1±0.4	Nd	5.3±0.4
	10 kGy	1.3±0.1^{ab}	1.0±0.1	5.6±0.2^{a}	0.85±0.05^{a}	nd	8.8±0.4^{a}	0.5±0.1	0.24±0.04	0.4±0.1	4.1±0.4	Nd	5.3±0.4
p-values	Homoscedasticity[2]	0.045	0.051	0.931	0.009	-	0.680	0.836	0.745	0.393	0.059	-	0.540
	Normal distribution[3]	0.357	0.167	0.361	0.440	-	0.684	0.179	0.140	0.121	0.115	-	0.073
	1-way ANOVA[4]	0.004	0.832	<0.001	<0.001	-	<0.001	0.818	0.185	0.540	0.986	-	0.929
						Melittis melissophyllum							
GR	0 kGy	1.0±0.1	0.8±0.1	0.9±0.1	0.28±0.03^{c}	2.5±0.1^{b}	5.5±0.3^{b}	1.4±0.1^{a}	0.17±0.01^{ab}	6.0±0.3^{a}	0.97±0.05^{a}	0.022±0.001^{b}	8.6±0.4^{a}
	1 kGy	0.9±0.1	0.8±0.1	0.9±0.1	0.53±0.05^{b}	2.7±0.1^{a}	5.9±0.4^{b}	1.2±0.1^{b}	0.15±0.02^{b}	4.5±0.2^{b}	0.86±0.05^{b}	0.019±0.001^{c}	6.6±0.3^{b}
	10 kGy	1.0±0.1	0.9±0.1	1.0±0.1	0.63±0.05^{a}	2.8±0.1^{a}	6.3±0.3^{a}	1.4±0.1^{a}	0.19±0.01^{a}	5.9±0.3^{a}	0.95±0.05^{a}	0.026±0.002^{a}	8.5±0.4^{a}
p-values	Homoscedasticity[2]	0.495	0.954	0.040	<0.001	0.709	0.431	0.921	0.630	0.269	0.902	0.058	0.378
	Normal distribution[3]	0.270	0.759	0.005	0.012	0.799	0.681	0.054	0.839	0.002	0.998	0.113	0.005
	1-way ANOVA[4]	0.052	0.055	0.072	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001
						Mentha x piperita							
GR	0 kGy	0.47±0.05^{a}	0.30±0.05	0.7±0.1	1.0±0.1^{a}	Nd	2.4±0.2	1.1±0.1^{a}	0.040±0.003^{a}	0.9±0.1^{a}	nd	8.5±0.2^{a}	10.6±0.3^{a}
	1 kGy	0.42±0.03^{b}	0.29±0.03	0.8±0.1	1.0±0.1^{a}	Nd	2.5±0.2	1.2±0.1^{a}	0.036±0.004^{ab}	0.9±0.1^{a}	nd	6.5±0.2^{c}	8.7±0.2^{c}
	10 kGy	0.47±0.04^{ab}	0.31±0.03	0.7±0.1	0.8±0.1^{b}	Nd	2.3±0.2	1.0±0.1^{b}	0.035±0.003^{b}	0.7±0.1^{b}	nd	7.7±0.2^{b}	9.5±0.2^{b}
p-values	Homoscedasticity[2]	0.665	0.061	0.131	0.320	-	0.573	0.934	0.880	0.880	-	0.559	0.039
	Normal distribution[3]	0.767	0.240	0.818	0.626	-	0.681	0.178	0.196	0.016	-	0.046	<0.001
	1-way ANOVA[4]	0.030	0.507	0.060	<0.001	-	0.094	<0.001	0.013	<0.001	-	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among GR doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one GR dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p<0.05$).

The four tocopherols isoforms (α, β, γ and δ) were detected in the four analyzed species, except for δ-tocopherol in lemon verbena (**Table 18**). α-Tocopherol was the main isoform in lemon balm (≈30.3 mg/100 g dw), lemon verbena (≈15.4 mg/100 g dw) and peppermint (≈15.1 mg/100 g dw), while β-tocopherol predominated in bastard balm (≈18.5 mg/100 g dw). In line with previous results (Taipina et al., 2009), tocopherol contents were significantly changed in response to the irradiation treatment (especially at the 10 kGy dose for *M. melissophyllum* and at the 1 kGy dose for the remaining species) in all the assayed samples, except for γ-tocopherol in peppermint ($p = 0.797$). Except for *M. melissophyllum*, the obtained differences are mainly linked to α-and β-tocopherol contents, which are not as stable to irradiation as γ-tocopherol, recognized as having higher oxidative stability (Warner et al., 2008)

Table 18. Tocopherols composition (mg/100 g dw) in the four assayed species submitted to gamma radiation (GR). The results are presented as mean±SD[1].

		α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
			Aloysia citrodora			
GR	0 kGy	15.3±0.4^{b}	0.41±0.04^{a}	1.8±0.1^{ab}	nd	17.5±0.4^{b}
	1 kGy	17.5±0.4^{a}	0.44±0.05^{a}	1.9±0.1^{a}	nd	19.8±0.4^{a}
	10 kGy	13.4±0.3^{c}	0.29±0.04^{b}	1.7±0.1^{b}	nd	15.4±0.3^{c}
p-values	Homoscedasticity[2]	0.831	0.012	0.341	-	0.412
	Normal distribution[3]	0.024	0.378	0.352	-	0.020
	1-way ANOVA[4]	<0.001	<0.001	0.002	-	<0.001
			Melissa officinalis			
GR	0 kGy	29±1^{b}	1.3±0.1^{a}	1.5±0.1^{b}	0.37±0.05^{b}	32±1^{b}
	1 kGy	33±1^{a}	1.1±0.1^{b}	1.8±0.1^{a}	0.38±0.05^{b}	37±1^{a}
	10 kGy	29±1^{b}	0.9±0.1^{c}	1.7±0.1^{a}	0.49±0.05^{a}	33±1^{b}
p-values	Homoscedasticity[2]	0.646	0.017	0.264	0.215	0.671
	Normal distribution[3]	0.001	0.139	0.553	0.151	0.003
	1-way ANOVA[4]	<0.001	<0.001	<0.001	0.001	<0.001
			Melittis melissophyllum			
GR	0 kGy	0.88±0.05^{a}	13.4±0.3^{b}	0.18±0.02^{a}	0.14±0.02^{a}	14.6±0.4^{b}
	1 kGy	0.81±0.05^{b}	13.2±0.2^{b}	0.16±0.02^{a}	0.14±0.02^{a}	14.3±0.2^{b}
	10 kGy	0.46±0.04^{c}	28.9±0.3^{a}	0.11±0.02^{b}	0.08±0.01^{b}	29.5±0.2^{a}
p-values	Homoscedasticity[2]	0.073	0.501	0.423	0.245	0.481
	Normal distribution[3]	0.001	<0.001	0.386	0.180	<0.001
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001
			Mentha x piperita			
GR	0 kGy	16.5±0.4^{a}	1.1±0.1^{a}	1.8±0.1	0.23±0.03^{b}	19.7±0.5^{a}
	1 kGy	15.7±0.2^{b}	0.8±0.1^{b}	1.8±0.1	0.28±0.04^{a}	18.6±0.2^{b}
	10 kGy	13.2±0.2^{c}	0.9±0.1^{b}	1.8±0.1	0.30±0.03^{a}	16.2±0.4^{c}
p-values	Homoscedasticity[2]	0.002	0.064	0.778	0.427	0.001
	Normal distribution[3]	0.001	0.012	0.187	0.559	0.021
	1-way ANOVA[4]	<0.001	<0.001	0.797	0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among GR doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one GR dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p<0.05$).

Tables 19 and 20 show the distribution of individual fatty acids (FA) divided as those present in a percentage below 1% in all the analysed samples (**Table 19**) and those quantified above 1% at least in one of them (**Table 20**). The FA detected in highest amounts in the four species was linolenic acid (C18:3n3), followed by palmitic (C16:0) and linoleic (C18:2n6) acids in lemon verbena and lemon balm, linoleic and palmitic acids in bastard balm, and arachidic and palmitic acids in peppermint. The profile detected for lemon balm is similar to that reported previously in the same species (Dias et al., 2012). Despite the detected differences, polyunsaturated fatty acids (PUFA) were predominant in all species (52.6 to 69.5%), followed by monounsaturated fatty acids (MUFA, 2.07 to 16.6%) and saturated fatty acids (SFA, 28.1 to 41.2%). The FA distribution percentages significantly changed by the irradiation treatment with the exceptions of C23:0 in lemon balm (p = 0.110), C17:0 (p = 0.507), C24:0 (p = 0.124) and SFA (p = 0.214) in bastard balm and C15:1 (p = 0.135) and C16:0 (p = 0.313) in peppermint. The changes in FA levels induced by irradiation have been explained as due to processes of lipid radiolysis, involving primary ionization, followed by migration of the positive charge either toward the carboxyl carbonyl group or double bonds (Molins, 2001).

Table 19. Distribution of minority fatty acids (values < 1% in all species) in the four assayed species submitted to gamma radiation (GR). The results are presented in relative percentage as mean±SD[1].

		C6:0	C8:0	C11:0	C12:0	C13:0	C15:0	C15:1	C17:0	C20:1n9	C20:2n6	C20:3n3 + C21:0	C22:1n9
							Aloysia citrodora						
GR	0 kGy	0.30±0.01^{a}	0.11±0.01^{b}	0.26±0.02^{a}	0.26±0.02^{b}	0.32±0.01^{c}	0.58±0.02^{b}	0.10±0.01^{a}	0.22±0.01^{c}	0.25±0.03^{b}	0.21±0.01^{b}	0.30±0.01^{a}	0.27±0.02^{b}
	1 kGy	0.28±0.04^{a}	0.10±0.01^{b}	0.21±0.01^{b}	0.29±0.02^{b}	0.46±0.03^{a}	0.61±0.05^{b}	0.09±0.01^{b}	0.24±0.01^{b}	0.39±0.04^{a}	0.17±0.01^{c}	0.27±0.01^{c}	0.37±0.01^{a}
	10 kGy	0.23±0.02^{b}	0.13±0.01^{a}	0.24±0.03^{a}	0.37±0.03^{a}	0.35±0.02^{b}	0.71±0.02^{a}	0.10±0.01^{a}	0.27±0.01^{a}	0.22±0.02^{b}	0.27±0.01^{a}	0.28±0.01^{b}	0.19±0.01^{c}
p-values	Homoscedasticity[2]	<0.001	0.008	0.008	0.100	0.004	0.003	0.002	0.038	0.001	0.008	<0.001	<0.001
	Normal distribution[3]	0.015	0.163	0.210	0.071	0.003	0.010	0.038	0.002	0.001	0.001	0.001	0.001
	1-way ANOVA[4]	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
							Melissa officinalis						
GR	0 kGy	0.22±0.01^{a}	0.40±0.02^{a}	0.13±0.01^{b}	0.46±0.01^{a}	0.14±0.01^{b}	0.44±0.03^{a}	0.55±0.01^{a}	0.81±0.01^{b}	0.18±0.02^{a}	nd	0.28±0.01^{c}	nd
	1 kGy	0.15±0.01^{b}	0.30±0.02^{b}	0.13±0.01^{b}	0.34±0.01^{b}	0.16±0.01^{a}	0.42±0.01^{a}	0.49±0.01^{c}	0.87±0.01^{a}	0.15±0.01^{b}	nd	0.35±0.01^{b}	nd
	10 kGy	0.14±0.01^{c}	0.29±0.01^{b}	0.17±0.01^{a}	0.30±0.01^{c}	0.14±0.01^{b}	0.36±0.01^{b}	0.51±0.01^{b}	0.80±0.01^{c}	0.12±0.03^{b}	nd	0.36±0.01^{a}	nd
p-values	Homoscedasticity[2]	0.002	0.672	0.089	0.002	<0.001	<0.001	<0.001	0.007	0.039	-	<0.001	-
	Normal distribution[3]	0.001	0.001	<0.001	<0.001	0.058	0.006	0.001	<0.001	0.500	-	<0.001	-
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-	<0.001	-
							Melittis melissophyllum						
GR	0 kGy	0.18±0.01^{a}	0.07±0.01^{b}	0.04±0.01^{b}	0.18±0.01^{b}	0.05±0.01^{c}	0.90±0.02^{b}	0.09±0.01^{b}	0.24±0.02	0.16±0.01^{c}	0.09±0.02^{c}	0.24±0.01^{b}	nd
	1 kGy	0.06±0.01^{c}	0.07±0.01^{b}	0.04±0.01^{b}	0.24±0.02^{a}	0.06±0.01^{b}	0.83±0.03^{c}	0.08±0.01^{c}	0.24±0.01	0.20±0.01^{a}	0.15±0.01^{b}	0.27±0.01^{a}	nd
	10 kGy	0.08±0.01^{b}	0.09±0.01^{a}	0.08±0.01^{a}	0.25±0.01^{a}	0.07±0.01^{a}	0.96±0.02^{a}	0.10±0.01^{a}	0.24±0.01	0.18±0.01^{b}	0.17±0.01^{a}	0.24±0.01^{b}	nd
p-values	Homoscedasticity[2]	0.025	0.004	<0.001	<0.001	0.034	0.828	<0.001	0.005	0.001	0.001	0.003	-
	Normal distribution[3]	<0.001	0.117	<0.001	<0.001	0.005	0.547	0.037	0.277	0.024	0.002	<0.001	-
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.507	<0.001	<0.001	<0.001	-
							Mentha x piperita						
GR	0 kGy	0.15±0.02^{a}	1.0±0.1^{a}	0.12±0.01^{b}	0.14±0.01^{b}	0.15±0.01^{a}	0.59±0.05^{a}	0.04±0.01	0.44±0.01^{b}	0.25±0.01^{b}	0.19±0.01^{a}	0.45±0.04^{b}	0.11±0.01^{c}
	1 kGy	0.16±0.02^{a}	1.0±0.1^{a}	0.17±0.02^{a}	0.15±0.02^{b}	0.12±0.01^{b}	0.48±0.01^{b}	0.05±0.01	0.47±0.01^{a}	0.28±0.05^{b}	0.18±0.01^{b}	0.47±0.02^{b}	0.21±0.04^{b}
	10 kGy	0.10±0.03^{b}	0.9±0.1^{b}	0.11±0.01^{b}	0.20±0.01^{a}	0.09±0.01^{c}	0.53±0.04^{b}	0.04±0.01	0.45±0.02^{b}	0.52±0.02^{a}	0.16±0.01^{c}	0.54±0.02^{a}	0.28±0.02^{a}
p-values	Homoscedasticity[2]	0.437	0.002	0.021	0.992	<0.001	<0.001	0.260	<0.001	<0.001	0.207	0.036	0.016
	Normal distribution[3]	0.118	0.022	<0.001	0.035	0.011	<0.001	0.218	0.084	<0.001	0.885	0.604	0.006
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.135	0.001	<0.001	<0.001	<0.001	<0.001

Table 20. Distribution of majority fatty acids (values > 1%, at least in one species) in the four assayed species submitted to gamma radiation (GR). The results are presented in relative percentage as mean±SD[1].

		C10:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n6	C18:3n3	C20:0	C20:5n3	C22:0	C23:0	C22:6n3	C24:0	SFA	MUFA	PUFA
Aloysia citrodora																				
GR	0 kGy	nd	1.1±0.1^{b}	nd	15.7±0.2^{b}	0.50±0.02^{b}	1.17±0.01^{b}	0.95±0.02^{b}	12.6±0.1^{a}	nd	56.2±0.3^{a}	0.87±0.02^{b}	nd	1.00±0.02^{a}	5.4±0.1^{b}	nd	1.4±0.1^{c}	28.6±0.2^{b}	2.07±0.03^{c}	69.3±0.3^{a}
	1 kGy	nd	1.3±0.1^{a}	nd	15.8±0.4^{b}	0.62±0.01^{a}	1.10±0.01^{c}	0.95±0.02^{b}	12.4±0.1^{b}	nd	56.6±0.5^{a}	0.99±0.03^{a}	nd	0.82±0.01^{c}	4.2±0.1^{c}	nd	1.7±0.1^{b}	28.1±0.5^{c}	2.42±0.03^{a}	69.5±0.5^{a}
	10 kGy	nd	0.9±0.1^{c}	nd	16.6±0.5^{a}	0.64±0.03^{a}	1.31±0.01^{a}	1.13±0.03^{a}	12.6±0.1^{a}	nd	54.3±0.4^{b}	0.59±0.04^{c}	nd	0.93±0.04^{b}	5.9±0.4^{a}	nd	1.8±0.1^{a}	30.3±0.5^{a}	2.27±0.03^{b}	67.4±0.5^{b}
p-values	Homoscedasticity[2]	-	0.273	-	0.071	0.008	0.002	0.225	<0.001	-	0.259	0.265	-	0.001	<0.001	-	<0.001	0.158	0.742	0.231
	Normal distribution[3]	-	0.080	-	0.025	0.001	<0.001	<0.001	<0.001	-	0.007	0.001	-	0.004	0.001	-	0.003	0.045	0.033	0.005
	1-way ANOVA[4]	-	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	<0.001	<0.001	-	<0.001	<0.001	<0.001	<0.001
Melissa officinalis																				
GR	0 kGy	0.29±0.02^{a}	2.9±0.1^{a}	0.53±0.01^{b}	22.7±0.3^{a}	nd	3.6±0.1^{a}	4.9±0.2^{a}	15.3±0.4^{ab}	nd	33.2±0.5^{c}	3.4±0.1^{c}	3.9±0.1^{b}	1.3±0.1^{b}	3.3±0.2	nd	1.2±0.2^{ab}	41.2±0.5^{a}	6.2±0.2^{a}	52.6±0.5^{c}
	1 kGy	0.25±0.01^{b}	2.6±0.1^{b}	0.52±0.01^{b}	20.9±0.1^{c}	nd	3.6±0.1^{a}	4.8±0.1^{a}	15.0±0.1^{b}	nd	34.4±0.1^{b}	3.9±0.1^{a}	4.5±0.1^{a}	1.5±0.1^{a}	3.2±0.1	nd	1.3±0.1^{a}	39.7±0.2^{b}	6.0±0.1^{b}	54.3±0.1^{b}
	10 kGy	0.22±0.01^{c}	2.4±0.1^{c}	0.62±0.02^{a}	21.5±0.1^{b}	nd	3.2±0.1^{b}	4.3±0.1^{b}	15.5±0.1^{a}	nd	36.3±0.2^{a}	3.5±0.1^{b}	3.5±0.1^{c}	1.5±0.1^{a}	3.1±0.1	nd	1.1±0.1^{b}	38.7±0.2^{c}	5.6±0.1^{c}	55.7±0.2^{a}
p-values	Homoscedasticity[2]	0.001	<0.001	<0.001	<0.001	-	0.048	<0.001	<0.001	-	0.003	0.002	0.437	<0.001	0.005	-	0.107	0.005	<0.001	0.007
	Normal distribution[3]	0.061	0.002	<0.001	0.002	-	0.002	0.001	0.062	-	0.012	<0.001	0.002	<0.001	0.033	-	0.411	0.041	0.020	0.029
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	0.001	-	<0.001	<0.001	<0.001	<0.001	0.110	-	0.004	<0.001	<0.001	<0.001
Melittis melissophyllum																				
GR	0 kGy	nd	0.58±0.03^{c}	nd	14.3±0.2^{b}	1.29±0.05^{a}	2.41±0.05^{b}	11.5±0.3^{c}	14.8±0.4^{c}	5.8±0.1^{b}	36±1^{a}	0.88±0.02^{b}	nd	1.3±0.1^{b}	6.2±0.2^{a}	nd	3.0±0.1	30.4±0.2	13.1±0.2^{c}	56.5±0.2^{a}
	1 kGy	nd	0.81±0.05^{b}	nd	14.2±0.5^{b}	1.14±0.03^{b}	2.43±0.01^{b}	13.0±0.4^{b}	16.2±0.4^{b}	5.8±0.1^{b}	33±1^{b}	0.96±0.02^{a}	nd	1.3±0.1^{b}	5.9±0.4^{a}	nd	2.9±0.2	30.1±0.4	14.4±0.3^{b}	55.5±0.5^{b}
	10 kGy	nd	0.92±0.03^{a}	nd	15.1±0.1^{a}	1.25±0.04^{a}	2.76±0.01^{a}	15.1±0.5^{a}	18.2±0.4^{a}	6.3±0.1^{a}	28±1^{c}	0.97±0.03^{a}	nd	1.4±0.1^{a}	4.1±0.1^{b}	nd	3.1±0.2	30.2±0.3	16.6±0.5^{a}	53.2±0.5^{c}
p-values	Homoscedasticity[2]	-	0.022	-	<0.001	0.005	0.004	<0.001	0.964	0.009	0.010	0.497	-	<0.001	<0.001	-	0.002	0.186	<0.001	0.001
	Normal distribution[3]	-	0.004	-	0.006	0.214	<0.001	0.029	0.049	<0.001	0.003	0.454	-	0.001	<0.001	-	0.491	0.532	0.013	0.005
	1-way ANOVA[4]	-	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-	0.003	<0.001	-	0.124	0.214	<0.001	<0.001
Mentha x piperita																				
GR	0 kGy	0.07±0.01^{a}	1.4±0.1^{b}	1.2±0.1^{a}	10.4±0.3	0.88±0.05^{b}	2.47±0.03^{b}	1.62±0.05^{b}	7.3±0.1^{b}	nd	46±1^{a}	15.8±0.5^{c}	2.8±0.2^{c}	2.6±0.1^{b}	0.24±0.0^{b}	1.4±0.1^{c}	2.1±0.1^{a}	38±1^{c}	4.1±0.1^{c}	58±1^{a}
	1 kGy	0.04±0.01^{b}	1.5±0.1^{a}	1.2±0.1^{a}	10.4±0.3	0.97±0.01^{a}	2.55±0.01^{a}	1.61±0.01^{b}	7.5±0.1^{a}	nd	44±1^{b}	16.7±0.5^{b}	3.0±0.1^{b}	2.8±0.1^{a}	0.21±0.0^{c}	1.5±0.1^{a}	1.9±0.1^{b}	39±1^{b}	4.3±0.1^{b}	57±1^{b}
	10 kGy	0.02±0.01^{c}	1.6±0.1^{a}	1.0±0.1^{b}	10.1±0.5	0.81±0.05^{b}	2.60±0.05^{a}	1.91±0.05^{a}	7.2±0.1^{c}	nd	43±1^{c}	17.9±0.1^{a}	3.3±0.1^{a}	2.9±0.1^{a}	0.26±0.0^{a}	1.6±0.1^{a}	1.9±0.1^{b}	40±1^{a}	4.6±0.2^{a}	56±1^{c}
p-values	Homoscedasticity[2]	0.160	0.062	0.001	0.036	0.001	0.005	0.001	0.001	-	0.151	0.001	<0.001	0.237	<0.001	<0.001	0.058	0.134	0.361	0.050
	Normal distribution[3]	0.008	0.660	0.179	0.103	0.017	0.509	<0.001	0.006	-	0.246	0.012	0.057	0.904	0.002	<0.001	0.262	0.381	0.815	0.247
	1-way ANOVA[4]	<0.001	<0.001	<0.001	0.313	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among GR doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one GR dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p<0.05$).

3.1.2.2 Effects of irradiation on antioxidant variables

In order to compare the effects of the irradiation on the antioxidant activity, three *in vitro* assays were applied: scavenging effects on DPPH radicals, reducing power and inhibition of β-carotene bleaching. Moreover, a preliminary quantification of total phenolics and total flavonoids was also performed; the results are collected in **Table 21**. In all cases, the methanolic extracts possessed higher antioxidant activities and concentrations of phenolic compounds than the corresponding infusions. Among the analysed plants, lemon balm showed the highest antioxidant activity in all the assays, difference that was especially outstanding in the case of infusions, presenting EC_{50} values close to those published by other authors (Dastmalchi et al., 2008; Kamdem et al., 2013). Even though the infusions prepared in this study revealed lower amounts of phenolic compounds than previously reported (Dias et al., 2012). On the other hand, bastard balm proved to be the less effective in terms of antioxidant activity, and also showed lower total phenolics and flavonoids contents. Changes induced by gamma radiation proved to be statistically significant in almost all cases, except for DPPH scavenging activity in the methanolic extracts (p = 0.996) of bastard balm. Likewise, changes in the concentrations of phenolic compounds were always significant except for total phenolics in the infusions of bastard balm (p = 0.474). Despite the significant changes found within these variables, it was not possible to identify unequivocal tendencies regarding the influence of the irradiation common to all assays and/or plant species.

Table 21. Antioxidant properties of extracts from the four species submitted to gamma radiation (GR).[1] EC_{50} values (μg/mL) are presented for all assays except for total phenolics and flavonoids contents, expressed as mg GAE/g extract and mg CE/g extract, respectively.

		DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		Phenols		Flavonoids	
		Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
						Aloysia citrodora					
GR	0 kGy	232±8[a]	39±4[c]	169±1[b]	22.8±0.3[c]	580±31[c]	208±9[b]	134±8[c]	665±13[a]	92±1[a]	369±5[a]
	1 kGy	237±5[a]	90±6[b]	184±2[a]	49.2±0.4[b]	1004±23[a]	235±5[a]	188±2[b]	531±34[b]	60±2[c]	359±9[b]
	10 kGy	205±16[b]	109±4[a]	170±1[b]	62±1[a]	829±36[b]	198±6[c]	205±3[a]	455±12[c]	76±3[b]	277±2[c]
p-values	Homoscedasticity[b]	0.002	0.238	0.031	0.005	0.340	0.200	0.002	<0.001	<0.001	<0.001
	Normal distribution[c]	0.002	<0.001	<0.001	<0.001	0.005	0.033	<0.001	0.002	0.001	<0.001
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
						Melissa officinalis					
GR	0 kGy	101±3[b]	67±1[b]	80±1[b]	44±1[c]	165±4[a]	125±3[a]	100±1[c]	829±6[a]	63±1[c]	448±4[b]
	1 kGy	101±1[b]	73±3[a]	75±1[c]	48±1[b]	130±5[c]	113±2[b]	108±2[a]	786±22[b]	69±1[a]	498±11[a]
	10 kGy	107±2[a]	73±2[a]	103±1[a]	55±1[a]	135±2[b]	109±2[c]	104±2[b]	742±8[c]	65±1[b]	417±4[c]
p-values	Homoscedasticity[b]	<0.001	0.010	0.037	0.397	0.028	0.224	<0.001	<0.001	<0.001	0.023
	Normal distribution[c]	0.097	0.029	<0.001	<0.001	<0.001	0.008	0.029	0.002	0.016	0.006
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
						Melittis melissophyllum					
GR	0 kGy	583±24[c]	354±39	512±16[b]	249±2[b]	1648±154[c]	447±66[b]	70±4	160±3[a]	29±2[a]	108±4[a]
	1 kGy	696±92[b]	355±19	605±29[a]	198±3[c]	2105±139[b]	538±61[a]	73±5	100±3[c]	16±1[b]	73±1[c]
	10 kGy	843±28[a]	354±23	457±12[c]	290±2[a]	2299±187[a]	595±37[a]	70±3	135±2[b]	15±1[b]	83±5[b]
p-values	Homoscedasticity[b]	0.171	0.005	0.017	0.300	0.359	0.082	0.233	0.199	<0.001	<0.001
	Normal distribution[c]	0.008	0.007	0.054	0.001	0.286	0.060	0.007	0.001	<0.001	<0.001
	1-way ANOVA[d]	<0.001	0.996	<0.001	<0.001	<0.001	<0.001	0.474	<0.001	<0.001	<0.001
						Mentha x piperita					
GR	0 kGy	184±5[b]	83±7[b]	119±2[c]	52±2[a]	597±44[b]	184±5[a]	218±2[c]	591±19[a]	117±2[a]	319±6[b]
	1 kGy	192±6[b]	98±5[a]	136±2[b]	43±1[b]	465±5[c]	137±2[b]	276±4[a]	572±25[a]	95±3[b]	354±3[a]
	10 kGy	225±9[a]	86±3[b]	146±4[a]	53±1[a]	715±67[a]	95±4[c]	242±4[b]	527±13[b]	78±2[c]	266±8[c]
p-values	Homoscedasticity[b]	0.039	0.055	0.007	<0.001	<0.001	0.048	0.006	0.032	0.114	0.001
	Normal distribution[c]	0.002	0.316	0.002	<0.001	0.009	0.002	0.001	0.018	0.002	<0.001
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MeOH- Methanol; GAE- Gallic acid equivalents; CE- Catechin equivalents. [1]The results are presented as the mean±SD. [2]Homoscedasticity among GR doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one GR dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p<0.05$).

3.1.2.3 Principal component analysis (PCA)

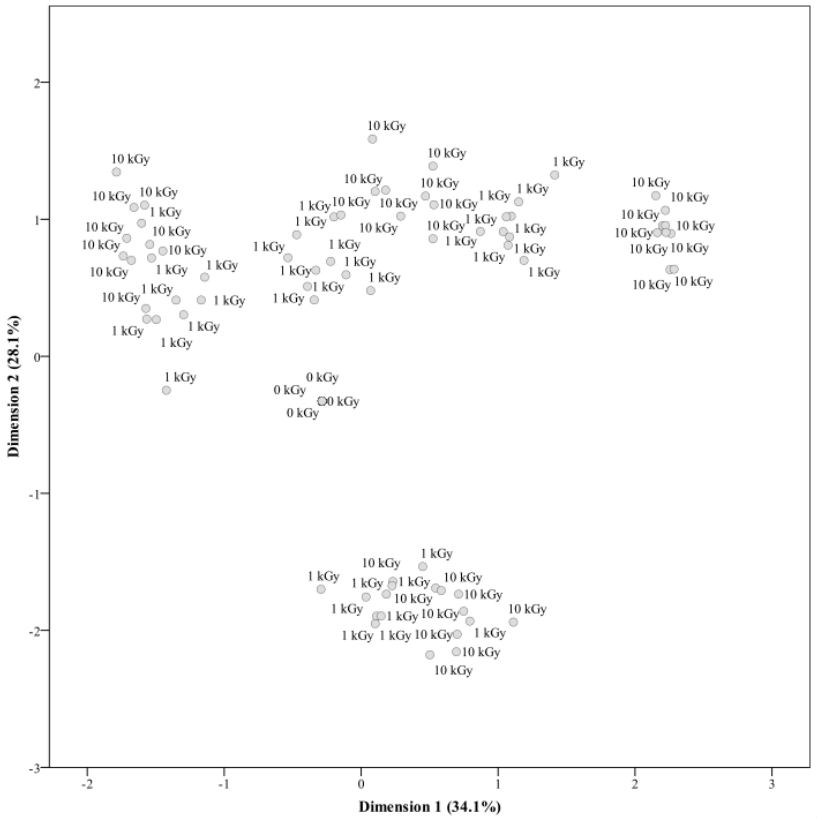
In the former section, the differences resulting from gamma radiation were compared considering the individual effect inside each species. Despite the observation of statistically significant changes in the different variables among samples, it was not possible to identify overall trends that might characterize the effects of gamma radiation. For that reason, it was tried to evaluate the effects of the irradiation independently of the treated plant species. Accordingly, in the present section the whole data obtained for all species and variables were considered simultaneously and submitted to principal components analysis (PCA). With that approach, instead of evaluating individual changes caused by the irradiation in a particular variable, it was intended to obtain an integrated output including all the effects at once. Due to the variable magnitude for some variables among species, the values were normalized by subtracting the value corresponding to 0 kGy to each of the values corresponding to 1 and 10 kGy, and the obtained differences were further divided by the value of the respective control. In this way, the classification procedure was applied to the differences caused by irradiation and not to the absolute values measured for each variable. For practical reasons, only data concerning the variables available for the four species were included in the study.

The plot of object scores (**Figure 33A**) for gamma radiation dose indicates that the first two dimensions (first: Cronbach's α, 0.941; eigenvalue, 13.031; second: Cronbach's α, 0.915; eigenvalue, 9.819) account for more than 60% of the variance observed for all quantified variables (34.1% and 28.1%, respectively). The considered variance should ideally be higher, but the inclusion of additional dimensions, despite being significant, would not allow a meaningful interpretation. Groups corresponding to each gamma radiation dose (0 kGy, 1 kGy and 10 kGy) were not delimited, as it could have been expected from **Tables 16-21**. In fact, and as it can be concluded by comparing the plots of object scores (**Figure 35A**) and component loadings (**Figure 35B**), the four defined groups included non-irradiated samples, but also samples irradiated at 1 and 10 kGy, making impossible to point out which variable changes better characterize each of the studied groups (0, 1 and 10 kGy). This result clearly indicates that, when considered from a global point of view, the changes resulting from the irradiation treatment are not enough to separate each of the corresponding groups.

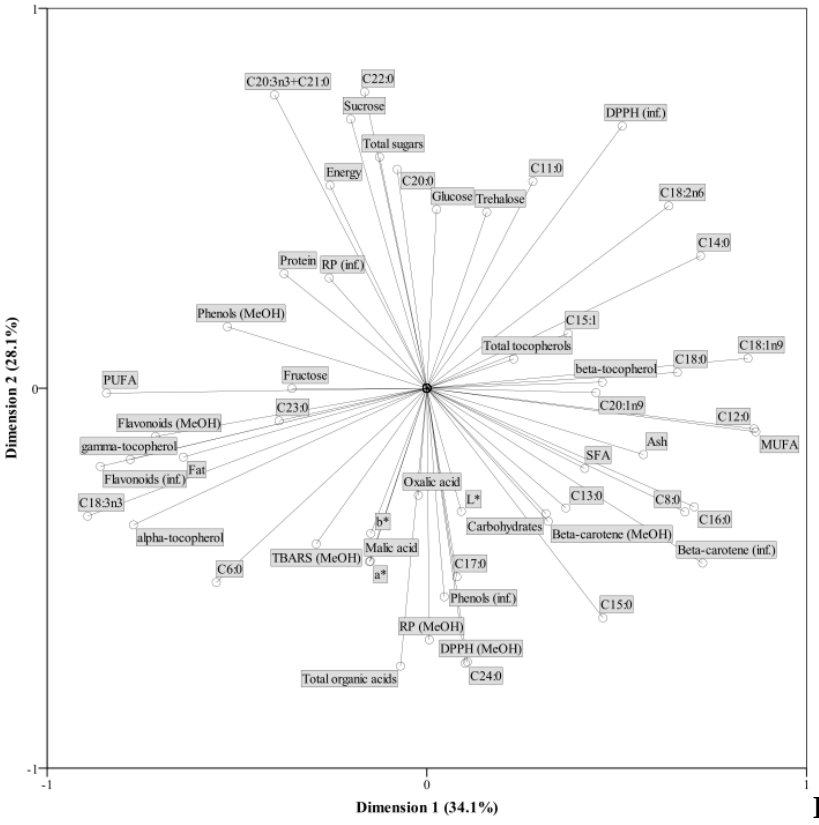
Nevertheless, gamma radiation seemed to have caused changes in a species-dependent manner. In fact, the object score corresponding to each plant species were clearly separated (**Figure 35C**), especially for *A. citrodora*. The defined dimensions had, of course, the same Cronbach's α and eigenvalues, including also the same percentage of variance. By comparing **Figures 35B** and **35C**, it is evident that the major differences in lemon verbena were caused

on carbohydrates, physical variables, malic acid, oxalic acid, total organic acids, C17:0, TBARS formation inhibition, reducing power and DPPH scavenging activity (all in the methanolic extracts), and total phenolics content in infusions; on the other hand, energy, reducing sugars, C11:0, C22:0 and C20:3n3 + C21:0 suffer minor changes. The main differences in lemon balm were observed for protein, total phenolics (methanolic extracts) and reducing power (infusions), while ash, carbohydrates, C8:0, C13:0, C15:0, C16:0, SFA, and β-carotene bleaching inhibition remain almost unchanged. Since the object scores of peppermint are in symmetric position in relation to lemon balm, the main characteristic changes for peppermint are exactly the inverse to those verified in lemon balm. Lastly, the most sensitive variables in bastard balm samples were C11:0, C14:0, C18:2n6 and DPPH scavenging activity (infusion), whereas fat, α-tocopherol, γ-tocopherol, C6:0, C18:3n3 and flavonoids suffered less alterations in this species.

Thus, taking into account the results obtained in the study of *G. biloba*, irradiation could represent a possibility for the processing of this species, because the changes that have occurred in some variables are not enough to significantly alter its global chemical profile.



A



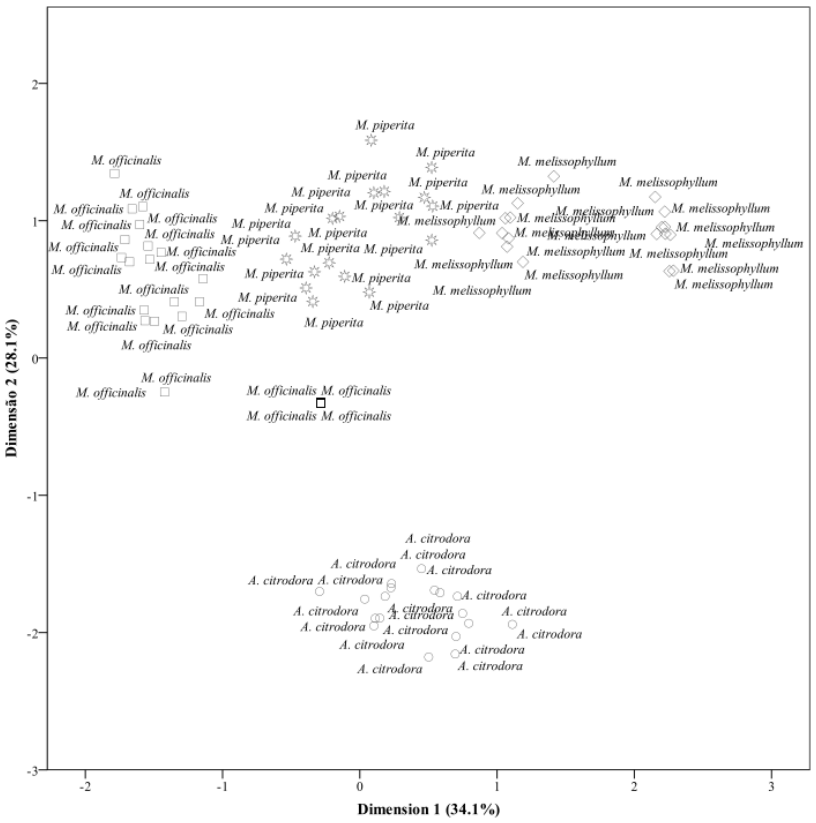


Figure 35. Plots of object scores and component loadings. A: using gamma radiation doses as objects; B: using the differences in the evaluated variables as component loadings. C: using the studied *Lamiaceae* and *Verbenaceae* species as objects.

3.1.3 *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x piperita* L.: Changes in composition and antioxidant properties of irradiated and non-irradiated plants during storage.

With the purpose of evaluating the effects of gamma radiation over nutritional profiles, individual compounds and antioxidant variables, the plants used in this study were previously characterized comparing non-irradiated and gamma-irradiated samples after the irradiation treatment (see section 3.1.2). The results obtained in that study were used as reference values to assess variations arising from the storage time (ST) in both irradiated and non-irradiated plants. The main purpose was to check whether gamma-radiation could prevent or minimize the potentially degrading changes suffered by the plants throughout storage at room temperature in a dry place for 12 and 18 months. Differences were considered irrelevant when variations were below ±5%. To validate the influence of the irradiation treatment independently of plant species (PS), the results from all assayed plants were combined, both for non-irradiated and irradiated samples. Thereby, the values presented in the tables for 0 kGy and 10 kGy levels were calculated considering the contributions of all species simultaneously. On the other hand, the values presented for each plant species reflect the average of the results measured for 0 and 10 kGy assays. Accordingly, the standard deviation values should not be regarded as a measure of the certainty of a set of results, but instead as an indication of their amplitude when the variables were assayed under different conditions. To allow a more reliable comparison, only variables for which results were available for all plant species and time points were considered, since, for instance, some individual compounds (e.g. β-tocopherol) could only be detected in the control samples.

3.1.3.1 Effects on chemical variables

The results obtained regarding the proximate composition of the plants at 12 and 18 months are shown in **Table 22**. The observed changes were characterized by a significant interaction among gamma radiation (GR) and plant species (PS), either for samples stored during 12 or 18 months. This is to say that the changes induced by GR depended on the PS and vice-versa. Regarding the influence of each factor, it was interesting to verify that longer storage periods increased the differences in nutritional variables variations, while the significant differences observed among irradiated and non-irradiated samples at 12 months of storage tended to

diminish at 18 months. In fact, at this time, no apparent difference exists between the nutritional variables in irradiated and non-irradiated samples. Even so, it might be concluded that fat and protein levels tended to decrease along ST, and that this decrease was more noticeable in irradiated samples.

On the other hand, the effects of ST and PS seem to act independently over the color variables, since their interaction was generally not significant ($p > 0.05$). The variables a* (redness) and b* (blueness) revealed the highest percent variations, especially after 18 months of storage, whereas lightness variations were always negligible. Independently of PS and GR, a* and b* tended to decrease, indicating slightly greener samples, but also a minor increase of the yellow color. The decrease in both variables is in agreement with the observed in gamma irradiated green tea extracts (Jo et al., 2003b).

Table 22. Proximate composition and color variables variations (differential percentage in comparison to the control values: section 3.1.2) as influenced by gamma radiation at 10 kGy and storage time. The results are presented as the mean±SD.

		Fat	Protein	Ash	Carbohydrates	Energy	*L**	*a**	*b**
12 months									
Gamma-radiation (GR)	0 kGy	-3±24	-6±8	3±5	1±1	-1±1	1±4	-12±10	-8±8
	10 kGy	-10±13	-23±9	1±3	2±1	-1±1	4±5	-9±11	-3±7
	p-value (n = 36)[2]	0.134	<0.001	0.013	<0.001	0.259	0.034	0.252	0.005
Plant species (PS)	*A. citrodora*	-2±6	-13±8	1±3	1±1	-1±1	4±2	-9±8[ab]	-8±2[b]
	M. officinalis	9±26	-16±19	2±4	1±2	-1±1	-1±3	-14±12[b]	-2±4[a]
	M. melissophyllum	-26±7	-15±11	3±6	1±1	-1±1	4±7	-4±7[a]	-1±10[a]
	M. piperita	-6±12	-12±9	1±4	2±1	-1±1	3±4	-16±9[b]	-11±4[b]
	p-value (n = 18)[2]	<0.001	0.772	0.256	0.077	<0.001	0.012	0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	0.034	<0.001	<0.001	<0.001	0.034	0.628	0.357
18 months									
Gamma-radiation (GR)	0 kGy	-15±34	-19±13	-2±4	2±1	-1±1	3±4	-17±10	-9±8
	10 kGy	-27±9	-25±37	-2±2	3±4	-1±1	4±4	-11±11	-5±8
	p-value (n = 36)[2]	0.061	0.393	0.406	0.059	0.567	0.145	0.017	0.018
Plant species (PS)	*A. citrodora*	-24±6	-30±8	-2±3	2±1	-1±1	4±2[ab]	-16±7	-14±3[c]
	M. officinalis	10±29	-20±22	-5±4	2±2	1±1	2±3[b]	-16±15	-3±3[b]
	M. melissophyllum	-43±11	1±34	-1±3	1±2	-1±1	6±5[a]	-8±11	3±6[a]
	M. piperita	-27±9	-40±22	-2±2	6±3	-1±1	4±3[ab]	-16±8	-14±4[c]
	p-value (n = 18)[2]	<0.001	<0.001	0.001	<0.001	<0.001	0.003	0.081	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	<0.001	0.005	<0.001	<0.001	0.412	0.830	0.881

[1] Values within a column with different letters differ significantly ($p<0.05$); [2] $p<0.05$ indicates that the mean value of at least one ratio differs from the others; [3] $p<0.05$ indicates a significant interaction (in this case multiple comparison tests results could not be indicated).

In general, GR and PS showed a strong interaction regarding their effects on free sugars and organic acids of samples stored 12 months (**Table 23**). Curiously, the interdependence of these factors was nearly absent from the results obtained at 18 months. Furthermore, the variations measured for irradiated and non-irradiated samples were similar in all cases (except for sucrose and trehalose at 12 months of storage), clearly indicating that gamma radiation did not have the ability to prevent the free sugars and organic acids losses throughout storage. Concerning the variations according to the plant species, several significant differences were found, being bserved that *M. melissophyllum* was less prone to suffer a decrease in free sugars content (except trehalose), and that *A. citrodora* kept better organic acids contents. As previously discussed (section 3.1.2) and also reported by other authors (Byun et al., 1997), the contents in free sugars tend to increase in response to gamma radiation, probably due to the shortening or depolymerization of polysaccharide molecules, but those studies were conducted in recently processed samples or samples stored for a maximum period of 6 months.

On the other hand, while the decrease in organic acids was similar for both storage times, the losses in free sugars were clearly aggravated after the 18 months period, an undesirable effect that could not be prevented by applying gamma radiation at 10 kGy, contrarily to the outcomes further achieved with e-beam radiation, which showed ability to attenuate the decrease of both groups of compounds (section 3.2.2).

Table 23. Free sugars and organic acids content variations (differential percentage in comparison to the control values: section 3.1.2) as influenced by gamma radiation at 10 kGy and storage time. The results are presented as the mean±SD.

		Fructose	Glucose	Sucrose	Trehalose	Total sugars	Oxalic acid	Malic acid	Organic acids
12 months									
Gamma-radiation (GR)	0 kGy	1±15	7±32	1±19	-5±26	-4±10	-11±17	-11±18	-8±8
	10 kGy	-1±14	-2±20	-9±15	-22±21	-9±12	-10±19	-2±25	-6±9
	p-value (n = 36)[2]	0.710	0.147	0.029	0.002	0.126	0.864	0.087	0.264
Plant species (PS)	*A. citrodora*	-2±17	-16±15	-11±8	-14±12	-11±7	10±13[a]	-24±19[c]	-2±7
	M. officinalis	2±15	-10±14	-19±9	-9±39	-14±12	-14±18[b]	-6±21[b]	-8±12
	M. melissophyllum	4±9	9±15	9±11	-17±25	3±7	-25±9[c]	-11±9[bc]	-10±8
	M. piperita	-6±15	28±32	5±22	-15±19	-3±11	-12±10[b]	14±19[a]	-7±5
	p-value (n = 18)[2]	0.218	<0.001	<0.001	0.805	<0.001	<0.001	<0.001	0.020
(GR×PS)	*p*-value (n = 72)[3]	0.011	0.006	0.003	<0.001	<0.001	0.091	0.050	0.022
18 months									
Gamma-radiation (GR)	0 kGy	-12±17	5±48	-13±17	-63±38	-16±3	-11±15	2±25	-11±10
	10 kGy	-13±14	-6±32	-14±20	-64±37	-21±12	-13±14	1±18	-8±10
	p-value (n = 36)[2]	0.817	0.253	0.796	0.882	0.065	0.527	0.892	0.209
Plant species (PS)	*A. citrodora*	-22±9[b]	-37±10	-19±7[bc]	-30±10[b]	-23±6	-11±10	1±29[ab]	-6±7[a]
	M. officinalis	-19±12[b]	-22±11	-29±9[c]	-100*[c]	-32±7	-5±18	5±20[a]	-5±13[a]
	M. melissophyllum	4±13[a]	2±12	8±14[a]	-100*[c]	-10±10	-13±11	-14±10[b]	-11±9[ab]
	M. piperita	-14±14[b]	53±41	-13±16[b]	-23±9[a]	-9±8	-18±14	16±13[a]	-16±7[b]
	p-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	0.049	<0.001	0.002
(GR×PS)	*p*-value (n = 72)[3]	0.065	0.015	0.279	0.136	0.014	0.004	0.291	0.181

*This variable was not detected in the samples treated under these conditions. [1] Values within a column with different letters differ significantly ($p<0.05$); [2]$p<0.05$ indicates that the mean value of at least one ratio differs from the others; [3]$p<0.05$ indicates a significant interaction (in this case multiple comparison tests results could not be indicated).

The interaction among factors was significant for tocopherols variations in all cases, in line with the individual effect observed for PS (**Table 24**). In general, between 2/3 and 3/4 of the tocopherols contents were lost, without relevant differences among 12 and 18 months of storage, as well as with no significant differences among irradiated and non-irradiated samples in any of the cases. Thus, the application of gamma radiation was not able to reduce the high tocopherol losses, contrarily to the observed in studies using e-beam radiation (Section 3.2.2).

Table 24. Tocopherols content variation (differential percentage in comparison to the control values: section 3.1.2) as influenced by gamma radiation at 10 kGy and storage time. The results are presented as the mean±SD.

		α-Tocopherol	β-Tocopherol	γ-Tocopherol	Total tocopherols
		12 months			
Gamma-radiation (GR)	0 kGy	-76±13	-87±23	-53±41	-65±12
	10 kGy	-74±7	-88±21	-61±28	-65±8
	p-value (n = 36)[2]	0.378	0.868	0.316	0.810
Plant species (PS)	*A. citrodora*	-74±3	-100*	-69±6	-74±3
	M. officinalis	-74±2	-100*	-50±7	-74±2
	M. melissophyllum	-89±5	-50±3	-100*	-52±2
	M. piperita	-61±5	-100*	-8±20	-59±6
	p-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	<0.001	<0.001	<0.001
		18 months			
Gamma-radiation (GR)	0 kGy	-76±11	-88±21	-59±35	-66±10
	10 kGy	-73±7	-90±18	-64±25	-67±6
	p-value (n = 36)[2]	0.210	0.746	0.471	0.919
Plant species (PS)	*A. citrodora*	-74±3	-100*	-71±5	-74±3
	M. officinalis	-73±2	-100*	-54±5	-73±3
	M. melissophyllum	-87±5	-56±4	-100*	-58±3
	M. piperita	-62±4	-100*	-20±15	-61±4
	p-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	<0.001	<0.001	<0.001

*This variable was not detected in the samples treated under these conditions. [1] Values within a column with different letters differ significantly ($p<0.05$); [2]$p<0.05$ indicates that the mean value of at least one ratio differs from the others; [3]$p<0.05$ indicates a significant interaction (in this case multiple comparison tests results could not be indicated).

The changes in fatty acids percentages are presented in **Table 25**. Only those fatty acids cumulatively found in all species and presenting relative percentages higher than 0.5% in the respective controls were included. Besides those ones, C6:0, C8:0, C10:0, C11:0, C13:0, C14:1, C15:1, C16:1, C18:3n6, C20:1, C20:2, C20:5n3, C22:1, C22:6n3 and C23:0 were also detected in most samples. Nevertheless, all the determined fatty acids will be included in the PCA presented in section 3.3.

As for the previous variables, the interaction among factors was significant in most cases, except for the variation in C15:0 and C17:0 in the samples stored for 12 months. In line with the observed interaction, the fatty acids variations were significantly different for each PS. On the other hand, and for a few cases, there were no significant changes regarding changes in fatty acids contents between non-irradiated and irradiated samples. Overall, polyunsaturated fatty acids (PUFA) tended to decrease along time, showing lower percentages in the samples stored during 18 months. Accordingly, the percentages of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) showed a general tendency to increase, intensified in the longer storage period. This is a usual effect of the irradiation treatments, since PUFA species are more susceptible to radiolysis (Molins, 2001).

When evaluating each fatty acid individually, the application of GR seemed, however, to prevent the loss of oleic acid, independently of the PS, particularly in the samples stored for 18 months. Likewise, irradiated samples stored for 18 months tended to suffer less decrease in the percentage of linolenic acid, the majority fatty acid in all analyzed species, although that attenuations was not statistically significant ($p = 0.170$). Nevertheless, the same effect was not obtained for the grouped PUFA, once again pointing to e-beam radiation as a better conservation technology for this type of samples (section 3.2.2).

Table 25. Variations in majority fatty acids (differential percentage in comparison to the control values: section 3.1.2) as influenced by gamma radiation at 10 kGy and storage time. The results are presented as the mean±SD.

		C12:0	C14:0	C15:0	C16:0	C17:0	C18:0	C18:1	C18:2	C18:3n3	C20:0	C22:0	C24:0	SFA	MUFA	PUFA
							12 months									
Gamma-radiation (GR)	0 kGy	13±22	-23±15	27±43	29±5	43±28	39±15	47±22	2±5	-14±10	10±54	82±23	-15±45	11±10	23±21	-9±7
	10 kGy	16±41	-30±16	31±46	24±12	37±24	34±23	39±23	-4±8	-15±11	10±53	91±36	-29±21	14±14	17±19	-11±10
	p-value (n = 36)[2]	0.697	0.054	0.720	0.018	0.318	0.288	0.132	0.002	0.910	0.988	0.222	0.085	0.282	0.202	0.469
Plant species (PS)	*A. citrodora*	-16±21	-17±10	-18±13[c]	23±9	63±21[a]	43±11	50±19	5±3	-7±2	-74±4	113±31	-58±6	7±5	45±14	-4±2
	M. officinalis	37±36	-29±8	17±21[b]	32±8	37±13[b]	59±15	51±15	-1±3	-31±3	67±11	78±15	8±50	28±5	25±10	-23±3
	M. melissophyllum	18±18	-29±25	22±13[b]	23±9	46±23[b]	24±12	14±9	-8±8	-13±6	18±19	106±12	-25±13	-1±3	11±8	-2±2
	M. piperita	21±29	-32±8	94±20[a]	28±8	12±17[c]	21±9	56±16	1±6	-7±5	28±5	51±11	-12±12	16±8	-2±6	-11±5
	p-value (n = 18)[2]	<0.001	0.016	<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	<0.001	0.475	<0.001	0.055	<0.001	<0.001	<0.001	<0.001	0.027	<0.001	<0.001	<0.001	<0.001	<0.001
							18 months									
Gamma-radiation (GR)	0 kGy	48±26	34±55	76±50	39±16	59±42	85±80	44±50	-14±22	-43±23	-17±35	39±27	-31±26	32±19	51±53	-32±20
	10 kGy	36±30	32±43	59±52	34±9	41±17	48±34	20±13	-17±21	-35±21	-22±37	41±35	19±40	31±21	24±20	-28±23
	p-value (n = 36)[2]	0.070	0.856	0.163	0.105	0.080	0.013	0.008	0.533	0.170	0.560	0.789	0.003	0.853	0.008	0.367
Plant species (PS)	*A. citrodora*	51±36	-34±7	24±25	49±14	95±36	144±76	82±49	-8±12	-27±12	-75±5	39±30	48±47	32±16	86±53	-24±12
	M. officinalis	71±17	80±12	138±28	29±8	36±8	79±11	26±2	-48±4	-69±1	-14±7	25±15	-63±8	58±8	48±4	-63±2
	M. melissophyllum	24±10	63±39	31±13	28±8	44±12	19±15	4±3	-2±5	-45±11	-12±13	74±30	47±12	15±5	16±13	-16±7
	M. piperita	22±7	24±16	78±27	39±6	25±11	24±7	16±2	-4±6	-15±9	22±4	23±12	-56±12	20±13	15±6	-17±9
	p-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Values within a column with different letters differ significantly ($p<0.05$); [2]$p<0.05$ indicates that the mean value of at least one ratio differs from the others; [3]$p<0.05$ indicates a significant interaction (in this case multiple comparison tests results could not be indicated).

3.1.3.2 Effects on antioxidant variables

As for most of the composition variables, the effects of GR on the antioxidant capacity of the samples depended on the PS, as indicated by the significant interactions observed in all cases (**Table 26**). Among the samples stored for 12 months and further submitted to infusion extraction, the most affected capacity was β-carotene bleaching inhibition, which was highly weakened in non-irradiated samples. The results on antioxidant capacity obtained at that storage time for the methanolic extracts from the same samples were still worse than those of the corresponding infusions. The irradiated samples, however, showed better antioxidant indicators. Besides the ability to maintain the β-carotene bleaching inhibition in the infusions, GR treatment had a positive effect on the DPPH scavenging activity and on the reducing power of the methanolic extracts. Concerning the samples stored for 18 months, the advantageous effects of GR over the DPPH scavenging activity and the reducing power of the methanolic extracts were maintained, but not the β-carotene bleaching inhibition activity measured in the infusions.

Independently of the storage time and irradiation treatment, the analysed species showed different responses in the different antioxidant activity assays, either when evaluating their infusions or methanolic extracts. Similar observations were made regarding total phenolics and flavonoids contents (**Table 26**), which however were not correlated to the antioxidant assays results. In general, the variations observed in the infusions and the methanolic extracts were similar for all PS, except for *M. officinalis* that showed high differences in the phenolic contents variations according to the type of extract.

In general, the interaction among factors (PS and GR) was significant for most of the evaluated variables. This implies that different changes in a particular variable can be induced in response to the application of GR depending on the PS. Thereby, it became difficult to found statistically significant changes among irradiated and non-irradiated samples, when the results from all the different PS were analysed together. Perhaps, if the analysis was performed considering each PS individually, some particular differences among irradiated and non-irradiated samples could have been found. However, the main objective of this work was validating GR treatment as an alternative to improve the shelf life in different aromatic plants, and not finding an alternative treatment specific to a single PS.

Table 26. Variations in the antioxidant capacity (EC_{50} values) determined by different assays, and total phenolics and flavonoids contents (presented as differential percentages in comparison to the control values: section 3.1.2) as influenced by gamma radiation at 10 kGy and storage time. The results are presented as the mean±SD.[1]

		DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		Total phenolics		Total flavonoids	
		Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
						12 months					
Gamma-radiation (GR)	0 kGy	-2±18	110±62	-2±11	32±39	97±69	87±36	84±30	-24±26	52±36	-38±30
	10 kGy	-7±8	13±16	-9±17	-18±16	-1±45	93±89	66±26	-1±34	81±27	-17±38
	p-value (n = 36)[2]	0.100	0.001	0.028	<0.001	<0.001	0.089	0.548	0.001	0.322	0.009
Plant species (PS)	*A. citrodora*	-2±8	110±81	6±5	64±35	75±84	98±60	5±19	4±25	2±15	-1±21
	M. officinalis	-9±5	22±12	-27±8	-30±9	89±79	83±50	93±14	-63±5	73±11	-75±4
	M. melissophyllum	5±21	10±10	6±4	2±13	-31±36	-45±26	-2±6	-1±16	-10±21	-41±13
	M. piperita	-11±14	3±7	-7±6	-8±12	58±13	43±23	3±11	8±5	2±6	6±8
	p-value (n = 18)[2]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
						18 months					
Gamma-radiation (GR)	0 kGy	3±5	33±28	32±11	-5±14	-31±33	19±38	-19±28	-54±13	-26±29	-55±9
	10 kGy	-5±6	13±18	26±32	-18±14	-47±38	15±53	-28±33	-40±18	-6±19	-44±7
	p-value (n = 36)[2]	0.001	0.001	0.310	<0.001	0.068	0.697	0.242	<0.001	0.001	<0.001
Plant species (PS)	*A. citrodora*	-3±5	12±38	28±6	9±8	-64±12	26±2	-37±11	-39±13	-34±9	-48±8
	M. officinalis	1±4	9±12	2±15	-23±9	18±3	18±2	28±1	-73±2	23±1	-54±2
	M. melissophyllum	-3±9	28±14	62±15	-17±12	-48±18	-50±18	-44±3	-40±10	-26±22	-57±9
	M. piperita	2±2	43±13	24±7	-15±3	-61±15	73±18	-41±7	-37±4	-27±11	-39±4
	p-value (n = 18)[2]	0.472	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(GR×PS)	*p*-value (n = 72)[3]	<0.001	0.046	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] Values within a column with different letters differ significantly ($p<0.05$); [2] $p<0.05$ indicates that the mean value of at least one ratio differs from the others; [3] $p<0.05$ indicates a significant interaction (in this case multiple comparison tests results could not be indicated).

3.1.3.3 Principal component analysis (PCA)

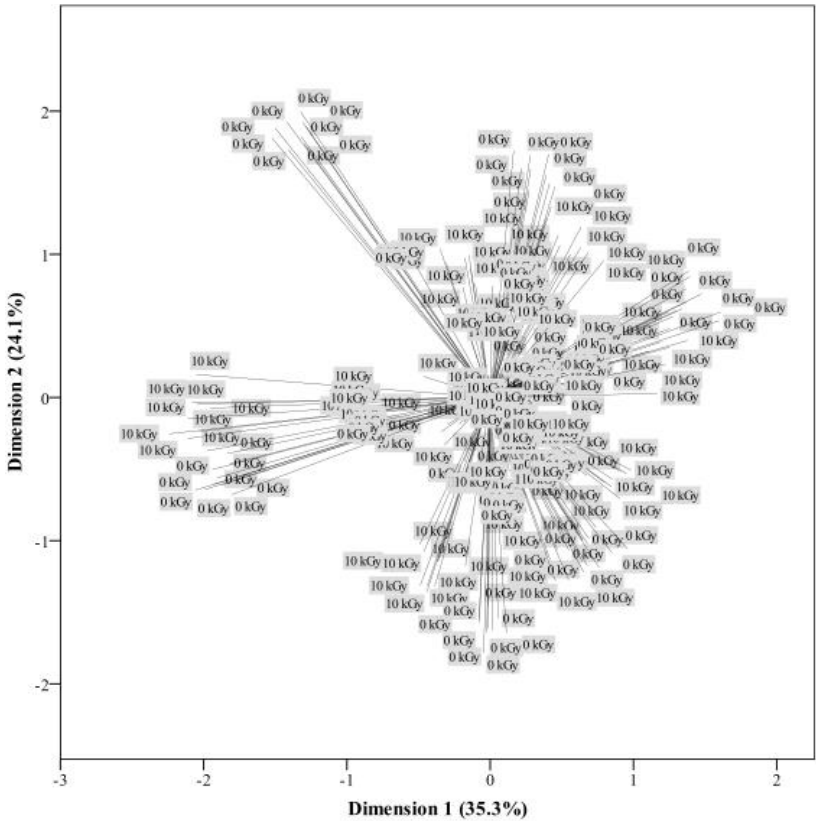
In the former sections, the variations induced by GR throughout ST were assessed in several individual variables. Despite the observed differences, the dissimilar behaviour among species did not allow obtaining overall conclus

ions regarding the effect of GR in each of the evaluated variables. In this section, PCA was applied by considering the variations in all obtained variables simultaneously. In the performed analysis, five significant dimensions were obtained, from which the first two (1st: Cronbach's $\alpha = 0.942$; eigenvalue = 13.176; 2nd: Cronbach's $\alpha = 0.880$; eigenvalue = 7.320) were plotted, sequentially using GR and ST as labelling variables.

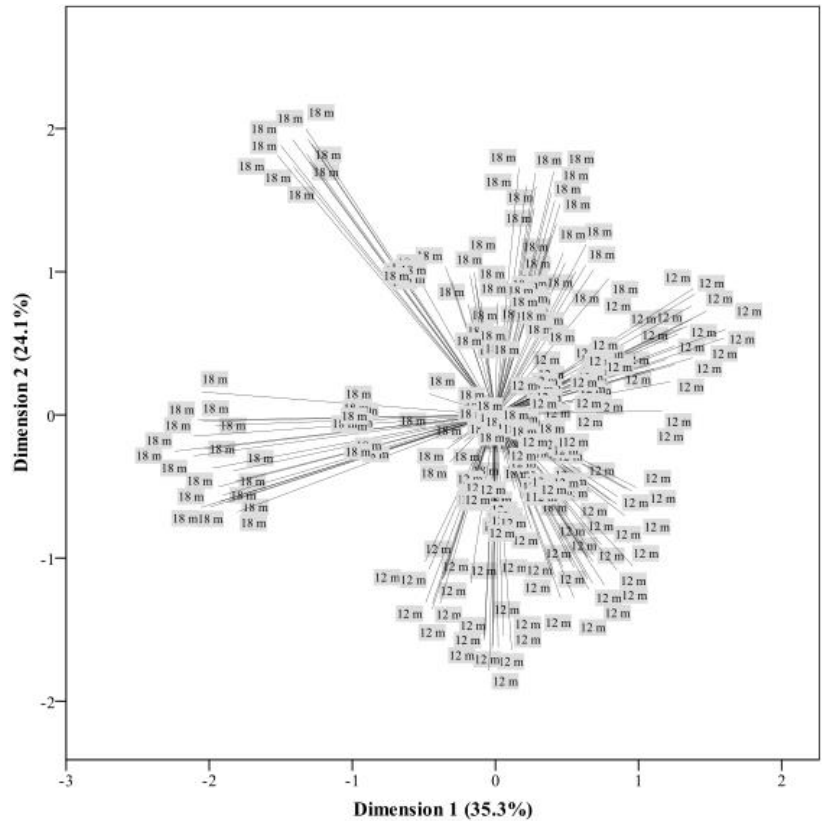
Concerning GR, the plot of object scores (**Figure 36A**) shows that the markers corresponding to non-irradiated and irradiated samples were distributed without a clear separation in distinct groups. Thus, as for the individual compounds, considering all variables together does not allow improving the separation of the samples as irradiated or non-irradiated indicating that the applied irradiation treatment does not induce differential effects on the studied variables in relation to non-treated samples.

However, groups corresponding to each storage time correlated differently with the defined principal components (**Figure 36B**). The markers corresponding to the 12 months period are mainly located in the bottom right quadrant, while those corresponding to the 18 months are concentrated in the top left quadrant. Besides highlighting their separation, the PCA also revealed which of the assayed variables correlate mostly with the markers corresponding to each storage period. As it can be visualized in **Figure 36C**, the analyzed variables might be easily divided according to the changes suffered in response to gamma radiation treatment throughout their storage. All the variables located in the inner circle were less susceptible to suffer variation, while those located between the two circles were more prone to be more affected. The 12 months group was mainly characterized by the highest increases (or lowest decreases) in fructose, glucose, trehalose, total sugars, C18:2, C18:3, C20:0, PUFA and phenolic contents (in the methanolic extracts) and also for the improved reducing power (in the aqueous extracts). On the other hand, the 12 months stored samples showed the most relevant reductions (or less pronounced increases) in C6:0, C8:0, C12:0, C17:0, C18:0, C18:1, SFA and MUFA. Surprisingly, samples stored during 18 months showed opposite distribution of their markers compared to those of the 12 months period, and they are characterized by greater increases in C6:0, C8:0, C12:0, C17:0, C18:0, C18:1, SFA and MUFA, and maximum decreases in all the variables that revealed the highest increases for the 12 months period. All

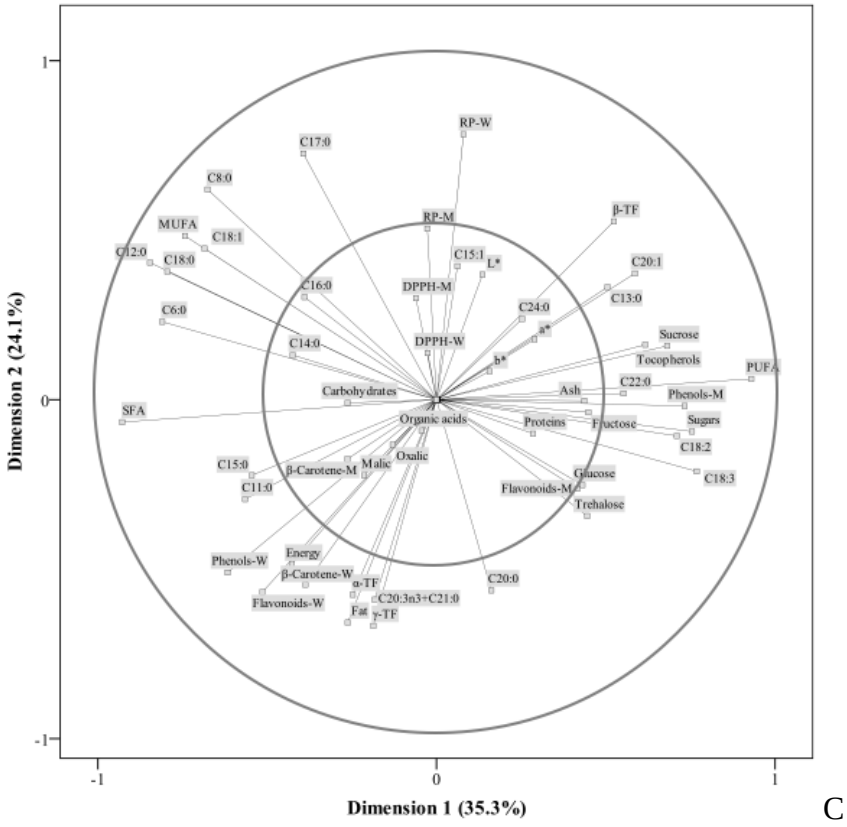
these variations are mainly dependent of the storage time, showing no significant association with the plant species.



A



B



C

Figure 36. Plots of objects scores according to irradiation doses (A), storage time (B) and component loadings (C).

3.1.4 *Thymus vulgaris* L. and *Mentha x piperita* L.: Effects of gamma radiation on cytotoxicity and phenolic composition of samples

3.1.4.1 Cytotoxicity of non-irradiated and irradiated samples

For this study, the plant samples were irradiated at different doses (0, 2, 5 and 10 kGy) and the cytotoxicy was further evaluated in their methanolic extracts on four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) and a non-tumor cell line (PLP2). The results obtained (**Table 27**) revealed that all samples showed cytotoxicity on the assayed tumor cells. Berdowska et al. (2013), in dried aqueous extracts of *Thymus vulgaris* from Poland, also found cytotoxicity on MCF-7 (breast carcinoma) tumor cell line, whereas Lv et al. (2012) described the anti-proliferative activity of a peppermint extract against the human tumor cell line HT-29.

Regarding the effect of the irradiation, the thyme samples irradiated at 2 and 5 kGy showed lower toxicity (higher GI_{50} values) than the control sample (0 kGy) on MCF-7, HeLa and HepG2 cell lines, while the one irradiated at 10 kGy showed similar activity on those cells as the non-irradiated sample. In the case of the NCI-H460 line greater toxicity was found for the 10 kGy sample than the others. As for peppermint samples, no significant differences were, in general, observed among the results obtained at the different irradiation doses, but for MCF-7 cell line, where higher cytotoxicity was observed in the control sample (0 kGy). None of the samples presented hepatotoxicity ($GI_{50} > 400$ μg/mL) on non-tumor cells (PLP2).

In general and overall, gamma radiation did not produce notable effects on the cytotoxicity of the studied plants. However, when applied at low doses, the antiproliferative capacity against tumor cells undergoes a slight increase, which can considerer a positive effect of this technology.

Table 27. Cytotoxicity of thyme and peppermint extracts prepared from non-irradiated and irradiated samples on different tumor and non-tumor cell lines.

	Doses			
	0 kGy	2 kGy	5 kGy	10 kGy
Thyme				
MCF-7 (breast carcinoma)	88 ± 7^{b}	104 ± 6^{a}	106 ± 10^{a}	83 ± 9^{b}
NCI-H460 (non-small cell lung cancer)	294 ± 12^{a}	276 ± 16^{ab}	297 ± 11^{a}	262 ± 4^{b}
HeLa (cervical carcinoma)	161 ± 6^{b}	189 ± 13^{a}	191 ± 9^{a}	160 ± 13^{b}
HepG2 (hepatocellular carcinoma)	103 ± 10^{a}	110 ± 13^{a}	106 ± 8^{a}	100 ± 10^{a}
Hepatotoxicity PLP2 (non-tumor cells)	> 400	> 400	> 400	> 400
Peppermint				
MCF-7 (breast carcinoma)	114 ± 12^{b}	175 ± 15^{a}	150 ± 4^{ab}	154 ± 7^{ab}
NCI-H460 (non-small cell lung cancer)	226 ± 11^{a}	224 ± 2^{a}	213 ± 20^{a}	229 ± 16^{a}
HeLa (cervical carcinoma)	221 ± 13^{a}	206 ± 11^{a}	211 ± 21^{a}	214 ± 12^{a}
HepG2 (hepatocellular carcinoma)	98 ± 9^{a}	115 ± 9^{a}	106 ± 11^{a}	111 ± 12^{a}
Hepatotoxicity PLP2 (non-tumor cells)	> 400	> 400	> 400	> 400

Positive control (Ellipticine) - MCF-7: 1.21 ± 0.02; NCI-H460: 1.03 ± 0.09; HeLa: 0.91 ± 0.11; HepG2: 1.10 ± 0.09; PLP2: 2.29 ± 0.18. GI_{50} values (μg/mL) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

3.1.4.2 Comparative analysis of the phenolic composition in non-irradiated and irradiated samples

Since, in general, no significant differences were found in the cytotoxic properties of the extracts prepared from samples irradiated at three different doses (2, 5 and 10 kGy), only control samples and samples irradiated at the highest dose (10 kGy) were used for phenolic compounds analysis, due to the higher efficiency of higher doses in the decontamination process.

Thirteen and fourteen phenolic compounds were identified in the methanolic extracts of thyme and peppermint, respectively. **Figure 37** shows HPLC chromatograms recorded at 280 nm with the phenolic profiles of thyme and peppermint samples, and **Tables 28** and **29** present data regarding compound identification (retention time, λ_{max} in the visible region, pseudomolecular ion, main fragment ions in MS^2, and tentative identities) and quantification for both species.

Compounds 2 (apigenin 6,8-di-*C*-glucoside), 3 (caffeic acid), 6 (rosmarinic acid hexoside), 7 (luteolin-7-*O*-glucuronide), 8 (luteolin-7-*O*-glucoside), 10 (rosmarinic acid), 12 (lithospermic acid A) and 13 (eriodictyol) in thyme were already described and identified in a previous study carried out by our research group, but using a different commercial sample (Martins et al., 2015). Moreover, most of the mentioned compounds have been previously cited in thyme samples by other authors (Dapkevicius et al., 2002; Fecka and Turek, 2008; Boros et al., 2010; Hossain et al., 2010; Costa et al., 2012; Vergara-Salinas et al., 2012; Roby et al., 2013; Vallverdú-Queralt et al., 2014).

Compounds 1, 4, 5, 9 and 11 detected in thyme were not described in our previous study (Martins et al., 2015), which may be due to the existence of different composition and/or distribution of phenolic compounds depending on the tissue, the origin of the plant and the edaphoclimatic conditions (Boros et al., 2010; Costa, et al., 2012). Based on their UV and mass spectra, compounds were tentatively identified as caffeic acid hexoside (peak 1), methyleriodictyol-*O*-pentosylhexoside (peak 4) and quercetin-*O*-glucuronide (peak 5). The presence of caffeic acid hexoside has been already reported in thyme by Hossain et al. (2010), Nagy et al. (2011), Vergara-Salinas et al. (2012) and Vallverdú-Queralt et al. (2014). Peak 9 ($[M-H]^-$ at *m/z* 537) showed similar UV and mass spectra as lithospermic acid A (peak 12), although it

eluted earlier. Although no other elements exist for assigning a definite identity to this peak, it was tentatively identified as salvianolic acid I, a compound with the same molecular previously reported in thyme by Dapkevicius et al. (2002) and Nagy et al. (2011). Compound 11 ($[M-H]^-$ at *m/z* 567) should also correspond to a caffeic acid derivative, owing to its UV spectrum and the observation of an MS^2 fragments at *m/z* 493, coherent with salvianolic acid A; furthermore, the fragment at *m/z* 197 could be attributed to dihydroxyphenyl-lactic acid (danshensu); however, no definite structure could be matched for the compound, so that it remains as an unidentified caffeic acid derivative. To the best of our knowledge, the presence of eriodictyol-*O*-methylhexoside, quercetin-*O*-glucuronide and the unknown caffeic acid derivative has not been previously reported in *T. vulgaris.*

Regarding peppermint, caffeic acid (compound 3'), chlorogenic acid (i.e., 5-*O*-caffeoylquinic acid; compound 2'), luteolin-7-*O*-rutinoside (compound 8') and rosmarinic acid (compound 13') were positively identified according to their retention, mass spectra and UV-vis characteristics in comparison with commercial standards. These compounds were also described in *M. piperita* leave of both commercial and non-commercial samples (Areias et al.,

2001), in *M. piperita* infusions (Kapp et al., 2013; Pérez et al., 2014), and in extracts from conventional and organically grown peppermint samples (Lv et al., 2012).

Compound 1' was identified as 3-*O*-caffeoylquinic acid based on its MS^2 fragmentation, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity of 72% relative to the base peak, considered characteristic of 3-acylchlorogenic acids as reported by Clifford et al. (2003). The sample also presented other caffeic acid derivatives, namely compounds 5', 11' and 14'. The pseudomolecular ion ($[M-H]^-$ at *m/z* 537) and fragmentation pattern of peak 5' were consistent with a caffeic acid trimer, although it eluted at an earlier retention time than lithospermic acid A. As above discussed for peak 9 in the thyme sample, the molecular weight of the compound would also match that of other caffeic acid trimers, such as salvianolic acid H/I, reported by Kapp et al. (2013) in peppermint teas; since the retention of the compound differs from that of peak 9 in thyme, it might be speculated to correspond to salvianolic acid H, an identity that was tentatively assigned although no further support for it was obtained, being justa assigned as a caffeic acid trimer. The molecular weight of compound 14' ($[M-H]^-$ at *m/z* 493) might correspond to salvianolic acid A, whereas that of compound 11' ($[M-H]^-$ at *m/z* 717) would match salvianolic acids B or E, whose presence was reported in peppermint teas by Kapp et al. (2013), or salvianolic acid L, cited by Krzyzanowska et al. (2011) in the aerial parts of *Mentha* species. Similar caffeoyl derivatives have been cited in mentha samples by other authors, as reviewed by Riachi and Maria (2015). All these compounds were quantified based on caffeic and rosmarinic acid calibration curves.

The remaining compounds in peppermint corresponded to flavonoids. Peaks 4' ($[M-H]^-$ at *m/z* 637) and 9' ($[M-H]^-$ at *m/z* 461) were assigned as luteolin glycosides, based on their UV spectra (λ_{max} around 350 nm) and the production of an MS^2 fragment ion at *m/z* 285. Compound 9' was positively identified as luteolin-7-*O*-glucuronide by comparison with a commercial standard, whereas compound 4' was tentatively assigned as luteolin-*O*-diglucuronide. The presence of luteolin glucuronides in *M. piperita* samples was also reported by Krzyzanowska et al. (2011), Kapp et al. (2013) and Riachi and Maria (2015). Compounds 6' ($[M-H]^-$ at *m/z* 595) and 7' ($[M-H]^-$ at *m/z* 449) were associated to eriodictyol-7-*O*-rutinoside and eriodictyol-*O*-hexoside, respectively, previously cited in *M. piperita* by Krzyzanowska et al. (2011) and Riachi and Maria (2015). Based on their UV and mass spectra, compounds 10' ($[M-H]^-$ at *m/z* 579) and 12' ($[M-H]^-$ at *m/z* 609) were identified as rutinosyl derivatives of the flavanones naringenin and hesperetin, respectively. The presence of narirutin (i.e., naringenin-7-*O*-rutinoside) in peppermint samples was also cited by Kapp et al. (2013) and Riachi and Maria (2015).

In a previous study, rosmarinic acid and luteolin-7-*O*-glucuronide were found as the most abundant compounds in thyme (Martins et al., 2015). These compounds were also relevant components in the samples analysed herein, although in this case rosmarinic acid hexoside appeared as the most abundant phenolic compound. These differences could be related with the natural variability inherent to plants grown under different environmental conditions that influence their secondary metabolism (Riachi and Maria 2015). Eriodictyol-7-*O*-rutinoside was the most abundant compound in peppermint, in agreement with a previous study performed by Areias et al. (2001).

The irradiation at 10 kGy did not affect the phenolic composition in both plant samples in relation to non-irradiated samples. The retention of molecules during irradiation mostly depends on the water content of food, temperature and the presence or absence of oxygen in the process (Sàdeká, 2007). Thus, a reduced formation of radiolytic products is expected in dried materials as the studied plant materials, leading to less composition changes. A different observation was, however, made in the study previously performed on *G. biloba* (section 3.1.1.8) where the samples irradiated at 10 kGy showed greater contents of phenolic compounds than the non-irradiated ones, which was explained by an increase in the extractability of certain phenolic compounds favoured by the irradiation treatment at high doses. The different behaviour among the distinct plant samples might be due to the water activity remaining after the drying process. *Ginkgo biloba* samples might have more water content than thyme or peppermint samples, thus leading to higher formation of radiolytic products and subsequently to greater structural and compositional changes (Tezotto-Uliana et al., 2015). Therefore, it can be concluded that gamma radiation does not conduct to a linear behaviour towards compounds preservation in plants, always depending on other concomitant factors (e.g., water content, presence of other compounds, applied dose). Nonetheless, as for the results herein obtained, a radiation dose of 10 kGy could be recommended as adequate to decontaminate these plants without affecting their phenolic composition. The same dose was also recommended by Machhour et al. (2011) with similar purposes.

In order to correlate the samples' cytotoxic effects with the phenolic composition, the correlation factors between the contents of total phenolic acids and total flavonoids, and the GI_{50} values obtained for the four tumor cell lines were calculated. High correlations were observed in both plant samples for three of the cell lines, with the exception of MCF-7, where no correlation was found between cytotoxicity and these phenolic groups. Nevertheless, the cytotoxic activity of thyme on the MCF-7 cell line was highly correlated with the contents of caffeic acid (R^2=0.7100), caffeic acid trimer (salvianolic acid H/I; R^2=0.7709),

methyleriodictyol-*O*-pentosylhexiside (R^2=0.4375), rosmarinic acid hexoside (R^2=0.4247), luteolin-7-*O*-glucoside (R^2=0.4305), and lithospermic acid A (R^2=0.4272), while in peppermint the main contributor compounds were caffeic acid (R^2=0.8586), caffeic acid trimer (salvianolic acid H/I; R^2=0.7667), luteolin-7-*O*-rutinoside (R^2=0.6649) and luteolin-7-*O*-glucuronide (R^2=0.6466).

The cytotoxic effects of thyme extracts on the other three cell lines, also presented high correlation factors, being NCI-H460 cell line highly correlated with total flavonoids (R^2=0.9991), and HeLa and HepG2 cell lines with total phenolic acids (R^2=0.7483 and 0.8139, respectively). The individual flavonoids that were highly correlated with NCI-H460 cell line were apigenin 6,8-di-C-glucoside (R^2=0.8294), methyleriodictyol-*O*-pentosylhexiside (R^2=0.7416), luteolin-7-*O*-glucoside (R^2=0.7708) and eriodictyol (R^2=0.9235), while the phenolic acids were caffeic acid hexoside (R^2=0.9226, for HeLA), caffeic acid (R^2=0.5037, for HeLA), rosmarinic acid hexoside (R^2=0.7211 and 0.5939, for HeLA and HepG2, respectively), rosmarinic acid (R^2=0.5748, for HepG2) and caffeic acid trimer (salvianolic acid H/I; R^2=0.8894 and 0.5696, for HeLA and HepG2, respectively). In the case of peppermint extracts an opposite effect was observed in relation to thyme, since the effects on NCI-H460 cell line correlated with total phenolic acids (R^2=0.5319), while on HeLa and HepG2 cell lines were correlated with total flavonoids (R^2=0.6946 and 0.6214, respectively). The individual compounds with greater influence were caffeic acid trimer (salvianolic acid H/I; R^2=0.5246, for NCI-H460), rosmarinic acid (R^2=0.5917, for NCI-H460), luteolin-*O*-diglucuronide (R^2=0.4148 and 0.6462, for HeLA and HepG2, respectively), eriodictyol-7-*O*-rutinoside (R^2=0.4075 and 0.6986, for HeLA and HepG2, respectively), luteolin-7-*O*-rutinoside (R^2=0.6409 and 0.5638, for HeLA and HepG2, respectively) and luteolin-7-*O*-glucuronide (R^2=0.4207 and 0.7956, for HeLA and HepG2, respectively). Most of the selected phenolic compounds were not majority molecules in the sample, suggesting that they could be more active and/or synergistic effects are produced among compounds enhancing their activity.

Table 28. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in thyme extracts prepared from non-irradiated and irradiated samples (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identity	Type of identification	Quantification (mg/g)		*t*-Students test *p*-value
							0 kGy	10 kGy	
1	7.3	320	341	179(100),135(88)	Caffeic acid hexoside	DAD/MS data References 1,2,3,4	1.7 ± 0.1	1.5 ± 0.1	0.988
2	10.8	338	593	473(20),383(33),353(27),297(5)	Apigenin 6,8-di-*C*-glucoside	DAD/MS data Reference 5	3.45 ± 0.04	3.31 ± 0.04	0.212
3	11.3	326	179	135(100)	Caffeic acid	Standard DAD/MS data Reference 5	2.69 ± 0.01	2.5 ± 0.4	0.258
4	17.4	284,336sh	595	301(47),286(100)	Methyleriodictyol-*O*-pentosylhexiside	DAD/MS data	3.12 ± 0.01	1.9 ± 0.4	0.005
5	18.1	350	461	301(100)	Quercetin-*O*-glucuronide	DAD/MS data	0.4 ± 0.1	0.35 ± 0.03	0.742
6	18.8	322	521	359(100),197(13),179(36),161(62),135(21)	Rosmarinic acid hexoside	DAD/MS data Reference 5	16.8 ± 0.1	14.7 ± 0.2	0.378
7	20.0	348	461	285(100)	Luteolin-7-*O*-glucuronide	DAD/MS data Reference 5	8.4 ± 0.2	7.1 ± 0.2	0.381
8	20.8	350	447	285(100)	Luteolin-7-*O*-glucoside	Standard DAD/MS data Reference 5	3.34 ± 0.01	3.2 ± 0.1	0.003
9	23.4	286,320sh	537	493(20),359(70),295(5),197(13),179(28),161(100),135(63)	Caffeic acid trimer	DAD/MS data References 2,6	9.1 ± 0.1	8.0 ± 0.1	0.027
10	24.0	330	359	197(17),179(35),161(100),135(29)	Rosmarinic acid	DAD/MS data Reference 5	12.7 ± 0.4	10.4 ± 0.2	0.038
11	25.6	282	567	535(23),493(49),387(32),285(25),197(13)	Caffeic acid derivative	DAD/MS data	2.3 ± 0.1	1.68 ± 0.05	0.001
12	27.5	290,326sh	537	493(50),359(17),295(33),179(75),135(100)	Lithospermic acid A	DAD/MS data Reference 5	2.25 ± 0.01	1.9 ± 0.1	0.999
13	30.6	288,334sh	287	151(35),135(100)	Eriodictyol	Standard DAD/MS data Reference 5	0.87 ± 0.01	0.53 ± 0.04	0.001
					Total phenolic acids		48 ± 1	47 ± 1	0.251
					Total flavonoids		19.5 ± 0.3	19.0 ± 0.1	0.010
					Total phenolic compounds		67 ± 1	66 ± 1	0.097

References: (1) Hossain et al. (2010); (2) Nagy et al. (2011); (3) Vergara-Salinas et al. (2012); (4) Vallverdú-Queralt et al. (2014); (5) Martins et al. (2015); (6) Dapkevicius et al. (2002).

Table 29. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in peppermint extracts prepared from non-irradiated and irradiated samples (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identity	Quantification (mg/g)			*t*-Students test *p*-value
						Type of identification	0 kGy	10 kGy	
1´	5.1	328	353	191(100),179(27),173(5),161(15),135(30)	3-*O*-Caffeoylquinic acid	DAD/MS data Reference 1 Standard	0.87 ± 0.02	0.76 ± 0.01	0.001
2´	7.1	328	353	191(100),179(90),173(50),161(20),135(57)	5-*O*-Caffeoylquinic acid	DAD/MS data References 2,3	1.4 ± 0.1	1.2 ± 0.1	0.025
3´	11.1	326	179	135(100)	Caffeic acid	DAD/MS data References 2,3,4,5	0.44 ± 0.01	0.5 ± 0.1	0.134
4´	14.5	348	637	285(100)	Luteolin-*O*-diglucuronide	DAD/MS data References 2,3,7	7.1 ± 0.2	6.22 ± 0.01	0.001
5´	15.7	288,330sh	537	493(45),313(18),295(36),269(55),197(36),179(64),135(100)	Caffeic acid trimer	DAD/MS data Reference 2	3.1 ± 0.2	2.9 ± 0.2	0.115
6´	16.1	284,332sh	595	287(100)	Eriodictyol-7-*O*-rutinoside	DAD/MS data References 3,6,7	100 ± 1	102.47 ± 0.01	0.005
7´	17.1	286,336sh	449	287(100)	Eriodictyol-*O*-hexoside	DAD/MS data References 3,7 Standard	2.2 ± 0.2	2.1 ± 0.1	0.157
8´	19.0	350	593	285(100)	Luteolin-7-*O*-rutinoside	DAD/MS data References 2,3,6	30.2 ± 0.1	30.3 ± 0.6	0.677
9´	19.8	348	461	285(100)	Luteolin-7-*O*-glucuronide	DAD/MS data References 2,3,7	11.2 ± 0.2	10.0 ± 0.4	0.002
10´	20.0	282,330sh	579	271(100)	Naringenin-*O*-rutinoside	DAD/MS data References 2,3,7	3.1 ± 0.1	3.0 ± 0.2	0.362
11´	21.5	278,338sh	717	537(34),519(50),493(39),339(29),321(37),313(6),295(100),197(3),179(11),161(5),135(11)	Salvianolic acid B/E/L	DAD/MS data References 2,3	13 ± 1	13.4 ± 0.3	0.276
12´	22.6	286,338sh	609	301(100)	Hesperetin-*O*-rutinoside	DAD/MS Standard	5.5 ± 0.3	5.6 ± 0.2	0.302
13´	23.6	330	359	197(13),179(20),161(100),135(21)	Rosmarinic acid	DAD/MS data References 2,3,4,5	25 ± 1	25.1 ± 0.1	0.291
14´	24.0	288,340sh	493	313(5),295(100),279(3),197(14),179(8),135(5)	Salvianolic acid A	DAD/MS data	10.3 ±0.5	9.72 ± 0.01	0.065
					Total phenolic acids		53 ± 3	54 ± 1	0.939
					Total flavonoids		159 ± 2	159.7 ± 0.1	0.248
					Total phenolic compounds		212 ± 4	213.7 ± 0.5	0.607

References: (1) Clifford et al. (2003); (2) Kapp et al. (2013); (3) Riachi and Maria (2015); (4) Pérez et al. (2014); (5) Lv et al. (2012); (6) Areias et al. (2001); (7) Krzyzanowska et al. (2011).

3.1.5 *Thymus vulgaris* L.: Effects on antioxidant properties and phenolic compounds of infusions

Table 30 presents the results obtained for the total phenolic and total flavonoid contents and antioxidant evaluation (tested by four *in vitro* assays: DPPH scavenging activity, reducing power, β-carotene bleaching and lipid peroxidation inhibition) of infusions prepared from non-irradiated and irradiated samples of *T. vulgaris*. Higher values of total phenolic and flavonoid contents were found in the samples irradiated at 10 kGy (0.168 mg GAE/mL of infusion and 0.06 mg CE/mL of infusion, respectively). The increase in total phenolic and flavonoid contents with the irradiation could be related with the release of these compounds from the matrix structures, increasing extractability of certain compounds and the degradation of larger compounds into smaller ones (Polovka and Suhaj, 2010; Taheri et al., 2014). In all the antioxidant assays, statistically significant differences were observed in the EC_{50} values among samples submitted to different radiation doses, with those treated at 10 kGy showing the highest antioxidant potential (lowest EC_{50} values). The increase in the antioxidant capacity in the samples submitted to 10 kGy is in agreement with previous findings in infusions of irradiated *Ginkgo biloba* L. (section 3.1.1.7), as well as in extracts of other plant materials like borututu (a folk medicine obtained from the African tree *Cochlospermum angolense* Welw.) (Pereira et al., 2014a) or *Hizikia fusiformis* Harvey (edible brown seaweed consumed in Korea and Japan) (Kim et al., 2009a).

Table 30. Total phenolic and total flavonoid contents, and *in vitro* antioxidant actuvity of infusions prepared from *Thymus vulgaris* submitted to gamma radiation.

Dose (kGy)	0	1	10
Total phenolics (mg GAE/mL of infusion)	0.138 ± 0.001c	0.150 ± 0.001b	0.168 ± 0.001a
Total flavonoids (mg CE/ mL of infusion)	0.048 ± 0.001c	0.053 ± 0.001b	0.060 ± 0.001a
DPPH scavenging activity (EC_{50} value, mg/mL)	0.87 ± 0.05a	0.76 ± 0.02b	0.66 ± 0.02c
Reducing power (EC_{50} value, mg/mL)	0.48 ± 0.01a	0.43 ± 0.01b	0.41 ± 0.01c
β-carotene bleaching inhibition (EC_{50} value, mg/mL)	1.63 ± 0.01a	1.66 ± 0.04a	1.25 ± 0.01b
TBARS inhibition (EC_{50} value, mg/mL)	0.22 ± 0.01a	0.22 ± 0.01a	0.13 ± 0.01b

GAE- Gallic acid equivalents; CE- Catechin equivalents. In each row different letters mean significant differences ($p < 0.05$).

In this study, the phenolic profile was only analysed in the samples irradiated with at 10 kGy (as they were the ones with higher antioxidant potential) and compared with the control sample. The HPLC-DAD-ESI/MS data used for phenolic compounds identification (retention time, λ_{max} in the visible region, pseudomolecular ion, and main fragment ions in MS^2) and tentative identities are presented in **Table 31**. The phenolic profile of the infusion obtained from a thyme sample irradiated at 10 kGy is shown in **Figure 37**; it was characterised by the presence of thirteen phenolic compounds, from which seven were phenolic acids derivatives and six were flavonoid glycosides. This phenolic profile was similar to the one previously observed in methanolic extracts obtained from irradiated samples of *T. vulgaris* (section 3.1.2.4). As above discussed, Martins et al. (2015) also reported apigenin 6,8-di-*C*-glucoside (peak 2), caffeic acid (3), rosmarinic acid hexoside (6), luteolin-7-*O*-glucuronide (7), luteolin-7-*O*-glucoside (8), rosmarinic acid (10), lithospermic acid A (12) and eriodictyol (13) in *T. vulgaris* samples. The presence of caffeic acid, luteolin-7-*O*-glucuronide, rosmarinic acid and eriodictyol was also found in aqueous and methanolic extracts of thyme by Fecka and Turek (2008), whereas Boros et al. (2010) detected apigenin 6,8-di-*C*-glucoside, caffeic acid, rosmarinic acid and eriodictyol in hydroalcoholic extracts of different *Thymus* species (*T. pannonicus*, *T. glabrescens*, *T. pulegioides*, *T. praecox*, *T. serpyllum*).

The majority phenolic compounds found in *T. vulgaris* infusions were rosmarinic acid hexoside (peak 6), luteolin-7-*O*-glucuronide (7), caffeic acid trimer (possible salvianolic acid I; 9) and rosmarinic acid (10), as previously found in thyme methanolic extracts (section 3.1.2.4). The presence of luteolin-7-*O*-glucuronide and rosmarinic acid as major compounds in thyme infusions was also reported by Martins et al. (2015).

Little changes were observed in the phenolic composition of the infusion of the sample irradiated at 10 kGy in relation to the non-irradiated one. A small but statistically significant increase was observed in the levels of methyleriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside and eriodictyol, and consequently in the content of total flavonoids, which is in agreement with previous observations in infusions of irradiated samples of *Ginkgo biloba* (see section 3.1.1.8), explained by an increase in compound extractability due to depolymerization and release from cell wall polysaccharides produced by the irradiation process. On the contrary, also small but significant decreases were observed in the levels of caffeic acid hexoside, quercetin-*O*-glucuronide, rosmarinic acid hexoside, caffeic acid trimer, rosmarinic acid, caffeic acid derivative and lithospermic acid A, after irradiation at 10 kGy, that are reflected in the contents of total phenolic acids and total phenolic compounds. It can be supposed that phenolic acid derivatives have lower stability against gamma radiation than

flavonoids and, therefore, they are degraded to some extent at higher irradiation doses. Thus, whereas radiation may contribute to increase compounds extractability, on the other hand, it may also lead to some degradation of less stable compounds.

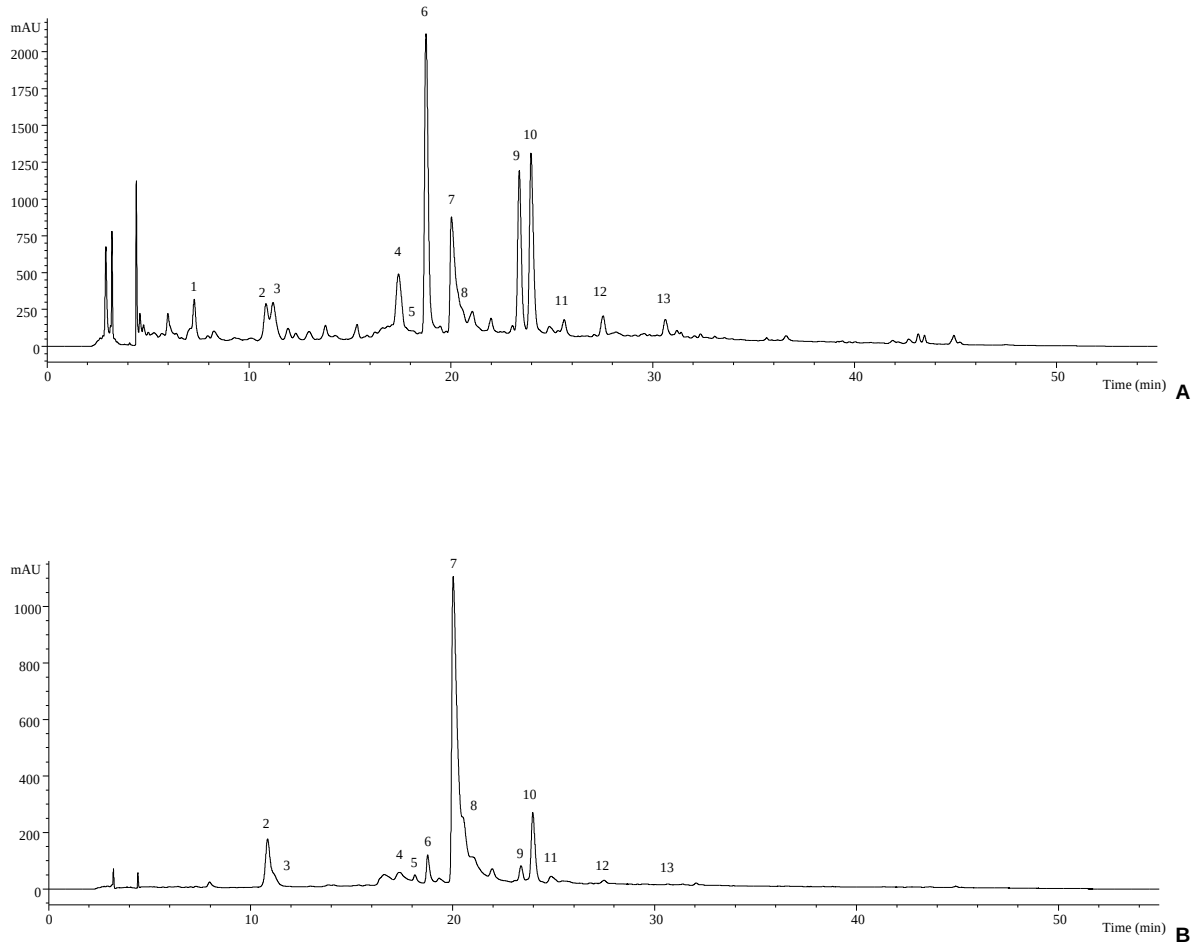


Figure 37. Phenolic compounds profile in infusions prepared from thyme samples irradiated with 10 kGy, recorded at 280 nm (A) and 370 nm (B).

Table 31. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification (μg/mL of infusion) of phenolic compounds in infusions prepared from *Thymus vulgaris* submitted to gamma radiation.

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Infusion		*t*-Students test
						0 kGy	10 kGy	*p*-value
1	7.3	320	341	179(100),135(88)	Caffeic acid hexoside	3.1 ± 0.1	2.5 ± 0.2	0.003
2	10.8	338	593	473(20),383(33),353(27),297(5)	Apigenin 6,8-di-*C*-glucoside	6.6 ± 0.4	6.2 ± 0.1	0.122
3	11.3	326	179	135(100)	Caffeic acid	4.54 ± 0.1	4.19 ± 0.4	0.091
4	17.4	284,336sh	595	301(47),286(100)	Methyleriodictyol-*O*-pentosylhexoside	5.03 ± 0.01	6.34 ± 0.04	<0.001
5	18.1	350	461	301(100)	Quercetin-*O*-glucuronide	0.21 ± 0.01	0.13 ± 0.02	0.002
6	18.8	322	521	359(100),197(13),179(36),161(62),135(21)	Rosmarinic acid hexoside	33 ± 1	32 ± 1	0.035
7	20.0	348	461	285(100)	Luteolin-7-*O*-glucuronide	24.0 ± 0.1	23.8 ± 0.1	0.062
8	20.8	350	447	285(100)	Luteolin-7-*O*-glucoside	4.7 ± 0.1	5.57 ± 0.01	<0.001
9	23.4	286,320sh	537	493(20),359(70),295(5),197(13),179(28),161(100),135(63)	Caffeic acid trimer	19.36 ± 0.02	17.8 ± 0.1	<0.001
10	24.0	330	359	197(17),179(35),161(100),135(29)	Rosmarinic acid	22.3 ± 0.4	21 ± 1	0.012
11	25.6	282	567	535(23),493(49),387(32),285(25),197(13)	Caffeic acid derivative	3.0 ± 0.1	2.6 ± 0.3	0.034
12	27.5	290,326sh	537	493(50),359(17),295(33),179(75),135(100)	Lithospermic acid A	3.53 ± 0.04	2.1 ± 0.4	0.004
13	30.6	288,334sh	287	151(35),135(100)	Eriodictyol	1.51 ± 0.02	1.6 ± 0.1	0.019
					Total phenolic acids	89 ± 1	82 ± 2	0.001
					Total flavonoids	42.0 ± 0.4	43.7 ± 0.4	0.002
					Total phenolic compounds	131 ± 1	125 ± 1	0.001

In order to try to establish possible relationships between antioxidant capacity and phenolic composition of the samples, correlation factors were obtained considering the contents of individual compounds and the sums of phenolic acid derivatives, flavonoids and total phenolic compounds. A Pearson's correlation analysis was performed, because the normality was verified through a Shapiro-Wilk test. The correlations for the four antioxidant assays (DPPH scavenging activity, reducing power, β-carotene bleaching and lipid peroxidation inhibition) with phenolic compounds and groups are presented in **Table 32**. In general, the majority of the compounds showed correlations statistically significant with the antioxidant activity assays. Methyleriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside and caffeic acid trimer (peaks 4, 8 and 9, respectively) were the compounds that presented the most statistically significant correlations with antioxidant activity assays (reducing power, β-carotene bleaching and lipid peroxidation inhibition), at a significance level $p < 0.001$. The compounds corresponding to peaks 1, 5, 6, 10 and 12 also revealed statistically significant correlations at $p < 0.05$. Otherwise, apigenin 6,8-di-*C*-glucoside (peak 2) and caffeic acid (peak 3) were the only compounds that did not statistically significantly relate to any of the antioxidant assays ($p > 0.05$). The majority individual phenolic compound (peak 6 - rosmarinic acid hexoside) also showed a high correlation with all the antioxidant activity assays, with a correlation factor between 0.815 and 0.865. Overall, total phenolic acids, total flavonoids and total phenolic compounds showed statistically significant correlations with all assays ($p \leq 0.05$).

Table 32. Correlations between phenolic compounds and *in vitro* antioxidant activity of infusions prepared from *Thymus vulgaris* submitted to gamma radiation (10 kGy).

		DPPH scavenging activity		**Reducing power**		**β-carotene bleaching inhibition**		**TBARS inhibition**	
Peaks	**Compounds**	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*
1	Caffeic acid hexoside	0.878	0.021	0.969	0.001	0.952	0.003	0.956	0.003
2	Apigenin 6,8-di-*C*-glucoside	0.530	0.280	0.742	0.091	0.689	0.137	0.698	0.123
3	Caffeic acid	0.719	0.107	0.724	0.104	0.750	0.086	0.742	0.091
4	Methyleriodictyol-*O*-pentosylhexoside	-0.954	0.003	-0.997	<0.001	-1.000	<0.001	-1.000	<0.001
5	Quercetin-*O*-glucuronide	0.945	0.004	0.962	0.002	0.965	0.002	0.967	0.002
6	Rosmarinic acid hexoside	0.865	0.026	0.815	0.048	0.851	0.032	0.843	0.035
7	Luteolin-7-*O*-glucuronide	0.679	0.138	0.817	0.047	0.778	0.068	0.788	0.063
8	Luteolin-7-*O*-glucoside	-0.926	0.008	-0.998	<0.001	-0.992	<0.001	-0.993	<0.001
9	Caffeic acid trimer	0.956	0.003	0.997	<0.001	0.999	<0.001	1.000	<0.001
10	Rosmarinic acid	0.906	0.013	0.890	0.017	0.916	0.010	0.911	0.012
11	Caffeic acid derivative	0.791	0.061	0.856	0.029	0.837	0.038	0.844	0.035
12	Lithospermic acid A	0.906	0.013	0.941	0.005	0.953	0.003	0.950	0.004
13	Eriodictyol	-0.808	0.052	-0.885	0.019	-0.887	0.018	-0.884	0.011
	Total phenolic acids	0.945	0.004	0.961	0.002	0.975	0.001	0.972	0.001
	Total flavonoids	-0.955	0.003	-0.943	0.005	-0.965	0.002	-0.961	0.002
	Total phenolic compounds	0.940	0.005	0.964	0.002	0.977	0.001	0.974	0.001

3.1.6 *Aloysia citrodora* L. and *Mentha x piperita* L.: Effects of plant irradiation on cytotoxicity and phenolic composition of infusions

3.1.6.1 Cytotoxic activity of infusions from non-irradiated and irradiated samples

Table 33 shows the results obtained for the cytotoxic evaluation on non-tumor (PLP2) and four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) of infusions prepared from non-irradiated and irradiated lemon verbena and peppermint samples. It can be seen that all samples exhibited antiproliferative activity on the four explored tumor cell lines, with GI_{50} values ranging between 74-318 μg/mL and 43-300 μg/mL for lemon verbena and peppermint, respectively. Hepatocellular carcinoma cell line (HepG2) was the most sensitive cell line against the cytotoxic effect of both plants, regardless of the dose applied, giving rise to the lowest GI_{50} values, i.e., 74 μg/mL (lemon verbena infusion irradiated at 10 kGy) and 43 μg/mL (infusion of peppermint control sample).

No significantly different effects on almost all the assayed cell lines were observed for the distinct lemon verbena samples, either irradiated or not, with the exception of the most sensitive cell line (HepG2). Otherwise, regarding peppermint samples, particular relevant effects were found for the sample irradiated at 1 kGy on the line NCI-H460 (GI_{50}= 99 μg/mL) and of all the studied samples on HepG2, especially in the case of the non-irradiated one (GI_{50}= 43 μg/mL). MCF-7 was the only cell line for which no significant difference was observed for the cytotoxic activity among the different samples. In the previous study performed with methanolic extracts of peppermint (section 3.1.4.1), similar cytotoxic activity was also observed on all the explored cell lines.

Table 33. Cytotoxicity of lemon verbena and peppermint infusions prepared from non-irradiated and irradiated samples on different tumor and non-tumor cell lines.

	Doses		
	0 kGy	1 kGy	10 kGy
Lemon verbena			
MCF-7 (breast carcinoma)	318 ± 31^{a}	302 ± 12^{a}	305 ± 1^{a}
NCI-H460 (non-small cell lung cancer)	234 ± 19^{a}	235 ± 4^{a}	234 ± 9^{a}
HeLa (cervical carcinoma)	245 ± 3^{a}	233 ± 22^{a}	249 ± 14^{a}
HepG2 (hepatocellular carcinoma)	87 ± 1^{a}	79 ± 8^{ab}	74 ± 4^{b}
Hepatotoxicity PLP2 (non-tumor cells)	> 400	> 400	> 400
Peppermint			
MCF-7 (breast carcinoma)	267 ± 17^{a}	296 ± 28^{a}	300 ± 20^{a}
NCI-H460 (non-small cell lung cancer)	140 ± 13^{b}	99 ± 8^{c}	205 ± 18^{a}
HeLa (cervical carcinoma)	242 ± 14^{b}	254 ± 4^{ab}	263 ± 10^{a}
HepG2 (hepatocellular carcinoma)	43 ± 3^{c}	55 ± 4^{b}	66 ± 7^{a}
Hepatotoxicity PLP2 (non-tumor cells)	> 400	> 400	> 400

Positive control (Ellipticine) - MCF-7: 0.91 ± 0.04; NCI-H460: 1.03 ± 0.09; HeLa: 1.91 ± 0.06; HepG2: 1.14 ± 0.21; PLP2: 3.22 ± 0.67. GI50 values (μg/mL) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

3.1.6.2 Comparative analysis of the phenolic composition in non-irradiated and irradiated samples

The identification and quantification of the phenolic compounds in non-irradiated (0 kGy) and irradiated samples (1 and 10 kGy) of lemon verbena and peppermint are shown in **Table 34** and **Table 35**, respectively.

Up to eleven phenolic compounds were detected in lemon verbena infusions, and the phenolic profile of the control sample, recorded at 280 nm, is shown in **Figure 38**. As it can be seen in **Table 34**, four flavonoids (peaks 2, 4, 6 and 9), five caffeoyl derivatives (peaks 5, 7, 8, 10 and 11), a phenylethanoid glycoside (peak 1), and a hydroxycinnamic acid (peak 3) were tentatively identified. The compounds were identified based on their mass and UV-vis spectra and retention characteristics. The majority of the detected compounds (verbasoside, luteolin-7-*O*-diglucuronide, apigenin-7-*O*-diglucuronide, verbascoside, chrysoeriol-7-*O*-diglucuronide, isoverbascoside, forsythoside, eukovoside and martinoside) have been already

reported in *A. citrodora* (Bilia et al., 2008; Quirantes-Piné et al., 2009; Quirantes-Piné et al., 2010), which has been used to support compounds identities. The identity of compounds 3 (*p*-coumaric acid) and 9 (isorhamnetin-3-*O*-glucuronide) was confirmed by comparison with authentic standards. As far as we know, these two compounds have not been previously reported in *A. citrodora*.

The most abundant compound detected was verbascoside (peak 5), with contents ranging between 83 and 134 μg/mL of infusion. This compound has been described to have a strong antioxidant activity, being related to the presence of two catechol groups in its structure (D'Imperio et al., 2014). The obtained results showed that a statistically significant degradation of chrysoeriol-7-*O*-diglucuronide (peak 6), forsythoside (peak 8), and eukovoside (peak 10) was produced at the dose of 10 kGy. In contrast, the levels of luteolin-7-*O*-diglucuronide (peak 2), *p*-coumaric acid (peak 3), verbascoside (peak 5) and isoverbascoside (peak 7) increased after irradiation at 10 kGy.

In peppermint infusions seven phenolic acids (peaks 1', 2', 3', 5', 11', 13' and 14') and seven flavonoids (peaks 4', 6', 7', 8', 9', 10' and 12') were detected. All these compounds have been already identified in methanolic extracts of *Menta x piperita* L. (section 3.1.4.2). In accordance to the previous study, eriodictyol-*O*-rutinoside (peak 6') was the majority compound present in the infusions. Eriodictyol has been reported to possess a relevant bioactive potential, expressing scavenging activity of intracellular free radicals (Imen et al., 2015). The obtained results revealed a statistically significant ($p < 0.05$) increase in the concentration of all detected phenolic compounds in the sample irradiated at 10 kGy, with the exception of caffeic acid trimer (peak 5') whose levels were higher in the sample submitted to 1 kGy. The increase in the levels of phenolic compounds following gamma irradiation at 10 kGy was also observed in other samples studied herein, such a *Ginkgo biloba* (section 3.1.1.8). The changes in the phytochemicals contents that can occur using irradiation would depend on different factors (kind of radiation, applied dose, exposure time or type sample) and, as previously commented, they might be due to either an increase in enzymatic activity that would favour the release of matrix-linked compounds or an increased accessibility and subsequent extractability from the tissues (Alothman et al., 2009).

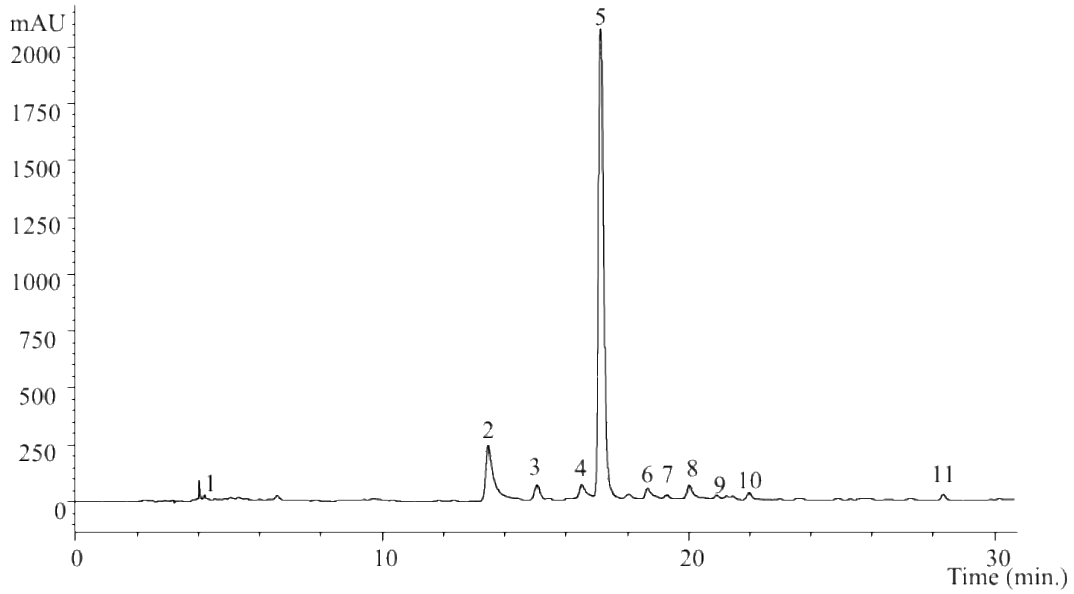


Figure 38. *Aloysia citrodora* phenolic profile recorded at 280 nm.

Peak numbering is the same as in **Table 35**.

Table 34. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification of phenolic compounds in lemon verbena infusions prepared from non-irradiated and irradiated samples (μg/mL infusion).

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Type of indentification	Quantification (μg/g)		
							0 kGy	1 kGy	10 kGy
1	4.1	280	461	315(8),135(28)	Verbasoside	DAD/MS data; References 1,2	2.6 ± 0.5[a]	2.9 ± 0.4[a]	2.4 ± 0.4[a]
2	15.0	344	637	351(100),285(89)	Luteolin-7-*O*-diglucuronide	DAD/MS data; References 1,2,3	68.7 ± 0.3[b]	64.2 ± 0.2[c]	70.0 ± 0.5[a]
3	16.7	314	163	119(100)	*p*-Coumaric acid	DAD/MS data	2.1 ± 0.1[b]	2.2 ± 0.1[b]	2.5 ± 0.1[a]
4	18.0	338	621	351(100),269(20)	Apigenin-7-*O*-diglucuronide	DAD/MS data; Reference 3	9.5 ± 0.2[a]	9.9 ± 0.1[a]	10.1 ± 0.6[a]
5	18.2	330	623	461(18), 315(5)	Verbascoside	DAD/MS data; References 1,2,3	114 ± 2[b]	83 ± 2[c]	134 ± 1[a]
6	20.2	350	651	351(100), 299(5)	Chrysoeriol-7-*O*-diglucuronide	DAD/MS data; References 1,2	10.4 ± 0.1[a]	9.3 ± 0.4[b]	9.5 ± 0.3[b]
7	20.4	330	623	461(18), 315(5)	Isoverbascoside	DAD/MS data; Reference 3	1.2 ± 0.1[b]	1.5 ± 0.3[ab]	1.7 ± 0.1[a]
8	21.2	330	623	461(15), 315(10)	Forsythoside	DAD/MS data; Reference 1	27 ± 1[a]	21.9 ± 0.6[b]	19 ± 2[c]
9	21.8	350	491	315(100),300(23)	Isorhamnetin-7-*O*-glucuronide	DAD/MS data	4.4 ± 0.1[a]	4.36 ± 0.06[a]	4.44 ± 0.07[a]
10	23.1	330	637	491(5),461(60), 315(13)	Eukovoside	DAD/MS data; References 1,2,3	1.00 ± 0.01[a]	0.9 ± 0.1[ab]	0.8 ± 0.1[b]
11	29.2	330	651	505(7),475(22)	Martinoside	DAD/MS data; References 1,2	0.52 ± 0.06[a]	0.40 ± 0.01[b]	0.56 ± 0.03[a]
					Total caffeoyl derivatives		146.0 ± 0.1[b]	111 ± 1[c]	159 ± 2[a]
					Total phenolic acids		2.1 ± 0.1[b]	2.2 ± 0.1[b]	2.4 ± 0.1[a]
					Total flavonoids		92.9 ± 0.1[a]	87.7 ± 0.4[b]	94 ± 1[a]
					Total phenolic compounds		241.04 ± 0.01[b]	201 ± 1[c]	255 ± 4[a]

References: Quirantes-Piné et al., 2009 (1) and 2010 (2); Bilia et al., 2008 (3)

Table 35. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification of phenolic compounds in peppermint infusions prepared from non-irradiated and irradiated samples (μg/mL infusion).

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (μg/g) 0 kGy	1 kGy	10 kGy
1´	5.3	328	353	191(100),179(27),173(5),161(15),135(30)	3-*O*-Caffeoylquinic acid	1.5 ± 0.1^{b}	1.0 ± 0.0^{c}	2.3 ± 0.0^{a}
2´	7.2	328	353	191(100),179(90),173(50),161(20),135(57)	5-*O*-Caffeoylquinic acid	1.7 ± 0.1^{b}	1.5 ± 0.0^{b}	4.4 ± 0.3^{a}
3´	11.3	326	179	135(100)	Caffeic acid	0.4 ± 0.2^{b}	0.3 ± 0.0 ^{b}	3.0 ± 0.3^{a}
4´	14.7	348	637	285(100)	Luteolin-*O*-dihexoside	16.4 ± 0.0^{c}	18.7 ± 0.3^{b}	19.9 ± 0.7^{a}
5´	16.1	288,330sh	537	493(45),313(18),295(36),269(55),197(36),179(64),135(100)	Caffeic acid trimer	6.0 ± 0.3^{c}	9.0 ± 0.2^{a}	7.2 ± 0.3^{b}
6´	16.3	284,332sh	595	287(100)	Eriodictyol-7-*O*-rutinoside	127 ± 2^{c}	170 ± 6^{b}	205 ± 3^{a}
7´	17.5	286,336sh	449	287(100)	Eriodictyol-*O*-hexoside	2.7 ± 0.2 ^{b}	2.3 ± 0.0^{c}	5.2 ± 0.3^{a}
8´	19.4	350	593	285(100)	Luteolin-7-*O*-rutinoside	34.4 ± 0.3^{b}	34.5 ± 0.1^{b}	41.2 ± 0.8^{a}
9´	20.2	348	461	285(100)	Luteolin-7-*O*-glucuronide	15.2 ± 0.6^{b}	17.8 ± 0.3^{b}	20.0 ± 0.9^{a}
10´	20.5	282,330sh	579	271(100)	Naringenin-*O*-rutinoside	4.5 ± 0.2^{b}	3.8 ± 0.0^{c}	6.2 ± 0.1^{a}
11´	21.9	278,338sh	717	537(34),519(50),493(39),339(29),321(37),313(6),295(100),197(3),179(11),161(5),135(11)	Salvianolic acid B/E/L	20.6 ± 0.5^{c}	21.7 ± 0.1^{b}	27.6 ± 0.2^{a}
12´	23.2	286,338sh	609	301(100)	Hesperetin-*O*-rutinoside	5.8 ± 0.9^{b}	5.7 ± 0.1^{b}	11.0 ± 0.3^{a}
13´	24.1	330	359	197(13),179(20),161(100),135(21)	Rosmarinic acid	33.7 ± 1.1^{b}	35.1 ± 0.6^{b}	51.0 ± 0.8^{a}
14´	24.5	288,340sh	493	313(5),295(100),279(3),197(14),179(8),135(5)	Salvianolic acid A	15.7 ± 0.7^{b}	17.9 ± 0.4^{b}	23 ± 2^{a}
					Total phenolic acids	80 ± 3^{c}	86.6 ± 0.5^{b}	119 ± 1^{a}
					Total flavonoids	206 ± 4^{c}	253 ± 6^{b}	308 ± 6^{a}
					Total phenolic compounds	286 ± 7^{c}	340 ± 7^{b}	427 ± 6^{a}

A correlation analysis was also performed between results obtained in the cytotoxicity assays (MCF-7, NCI-H460, HeLa and HepG2) and the phenolic composition for lemon verbena and peppermint samples (**Tables 36** and **37)**, using a Pearson's correlation analysis, in which the normality was verified through a Shapiro-Wilk test. In general, hardly statistically significant ($p < 0.05$) correlations were found for the samples of lemon verbena, but just for NCI-H460 with verbasoside (peak 1, Pearson's r=0.749), and HepG2 with chrysoeriol-7-*O*-diglucuronide (peak 6, Pearson's r=0.763) and forsythoside (peak 8, Pearson's r=0.812). The main compound (verbascoside) did not show correlation with the effects on any cellular line studied, and similarly happened with eukovoside and martinoside. Nonetheless, total flavonoid contents correlated well with the results obtained on the HeLa cell line (Pearson's r=0.705).

In the case of peppermint infusions statistically significant correlations ($p<0.05$) were found within all cell lines, especially HepG2, for which the most significant correlations were obtained for most of the compounds, and in particular with peaks 4' (Pearson's r=0.960), 6' (Pearson's r=0.953), 9' (Pearson's r=0.923), and 14' (Pearson's r=0.954). Total phenolic acids (Pearson's r=0.888), total flavonoids (Pearson's r=0.954) and total phenolic compounds (Pearson's r=0.944) also presented high correlation factors with the citotoxic effect on HepG2 cell line. The results on MCF-7 cell line presented statistically significant correlation with the content of luteolin-*O*-dihexoside (Pearson's r=0,759), and the NCI-H460 cell line correlated with various compounds, although the best correlation was with 3-*O*-caffeoylquinic acid (Pearson's r=0.985). Effects on HeLa cell line were highly correlated with eriodictyol-7-*O*-rutinoside (Pearson's r=0.818).

Salvianolic acid A (peak 14') correlated significantly with the effects observed in all cell lines (MCF-7: 0.690; NCI-H460: 0.781; HeLa: 0.679 and HepG2: 0.954), whereas caffeic acid trimer (peak 5') was the only compound for which no correlation could be established with any of the cell lines studied. The main compound, eriodictyol-7-*O*-rutinoside (peak 6'), showed significant correlation factors with all cell lines, with the exception of NCI-H460.

Table 36. Correlations between phenolic compounds and cytotoxicity activity of infusions prepared from lemon verbena submitted to gamma radiation (1 and 10 kGy).

		MCF-7		**NCI-H460**		**HeLa**		**HepG2**	
Peak	**Compounds**	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*
1	Verbasoside	0.452	0.222	0.749	0.020	0.063	0.871	0.255	0.509
2	Luteolin-7-*O*-diglucuronide	0.219	0.571	-0.103	0.793	0.653	0.056	-0.072	0.853
3	*p*-Coumaric acid	-0.014	0.972	0.300	0.432	0.524	0.148	-0.706	0.034
4	Apigenin-7-*O*-diglucuronide	-0.213	0.582	0.450	0.224	0.162	0.677	-0.861	0.003
5	Verbascoside	0.145	0.710	-0.119	0.761	0.578	0.103	-0.226	0.559
6	Chrysoeriol-7-*O*-diglucuronide	0.627	0.071	0.156	0.689	0.537	0.136	0.763	0.017
7	Isoverbascoside	-0.625	0.068	-0.271	0.480	-0.237	0.539	-0.917	<0.001
8	Forsythoside	0.557	0.119	0.222	0.565	0.072	0.855	0.812	<0.001
9	Isorhamnetin-7-*O*-glucuronide	-0.625	0.072	-0.422	0.258	0.288	0.453	-0.548	0.008
10	Eukovoside	0.200	0.605	0.178	0.648	-0.273	0.477	0.443	0.233
11	Martinoside	0.424	0.255	0.207	0.592	0.569	0.110	-0.167	0.668
	TCP	0.238	0.537	-0.077	0.844	0.606	0.084	-0.110	0.778
	TPA	-0.014	0.972	0.300	0.432	0.524	0.148	-0.706	0.034
	TF	0.270	0.482	-0.021	0.956	0.705	0.034	-0.039	0.920
	TPC	0.242	0.531	-0.068	0.862	0.620	0.075	-0.107	0.785

TPC-total caffeoyl phenylethanoid derivatives (including verbasodide); TPA- total hydroxycinnamic acids; TF- total flavonoids; TPC – total phenolic compounds

Table 37. Correlations between phenolic compounds and cytotoxicity activity of infusions prepared from peppermint submitted to gamma radiation (1 and 10 kGy).

		MCF-7		**NCI-H460**		**HeLa**		**HepG2**	
Peak	**Compounds**	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*
1´	3-*O*-Caffeoylquinic acid	0.204	0.599	0.985	<0.001	0.421	0.259	0.552	0.123
2´	5-*O*-Caffeoylquinic acid	0.452	0.222	0.936	<0.001	0.617	0.077	0.797	0.010
3´	Caffeic acid	0.429	0.250	0.906	0.001	0.674	0.047	0.781	0.013
4´	Luteolin-*O*-dihexoside	0.759	0.018	0.457	0.216	0.766	0.016	0.960	<0.001
5´	Caffeic acid trimer	0.568	0.111	-0.450	0.225	0.357	0.346	0.437	0.240
6´	Eriodictyol-7-*O*-rutinoside	0.697	0.037	0.558	0.118	0.818	0.007	0.953	<0.001
7´	Eriodictyol-*O*-hexoside	0.370	0.327	0.953	<0.001	0.605	0.061	0.735	0.024
8´	Luteolin-7-*O*-rutinoside	0.505	0.166	0.912	0.001	0.644	0.061	0.836	0.005
9´	Luteolin-*O*-glucuronide	0.702	0.035	0.537	0.136	0.814	0.008	0.923	<0.001
10´	Naringenin-*O*-rutinoside	0.265	0.491	0.977	<0.001	0.500	0.170	0.625	0.072
11´	Salvianolic acid B	0.541	0.133	0.842	0.004	0.731	0.025	0.871	0.002
12´	Hesperetin-*O*-rutinoside	0.495	0.176	0.924	<0.001	0.599	0.088	0.817	0.007
13´	Rosmarinic acid	0.486	0.185	0.878	0.002	0.706	0.034	0.833	0.005
14´	Salvianolic acid A	0.690	0.040	0.781	0.013	0.679	0.044	0.954	<0.001
	Total phenolic acids	0.566	0.112	0.840	0.005	0.721	0.029	0.888	0.001
	Total flavonoids	0.676	0.046	0.645	0.061	0.804	0.009	0.954	<0.001
	Total phenolic compounds	0.650	0.058	0.709	0.033	0.788	0.012	0.944	<0.001

3.1.7 *Aloysia citrodora* Paláu: Effects of irradiation on mycotoxins

The calibration variables of instrumentation (linear range, coefficient of correlation (R^2), equations of linear regression, limits of detection (LOD) and limits of quantification (LOQ)) for aflatoxin B_1 (AFB_1) and ochratoxin A (OTA) are shown in **Table 38**. The analytical methods for quantification of the two mycotoxins in samples of dried aromatic plants were further validated. **Table 39** displays the accuracy and precision of the optimised OTA and AFB_1 analysis methods. Recovery, as well as RSD_r and RDS_R, are within recommended ranges (EU, 2006).

Table 38. Calibration variables of instrumentation for aflatoxin B_1 (AFB_1) and ochratoxin A (OTA).

Standard		AFB_1	OTA
R_t (retention time)	Min	6.79	2.20
	CV, % (n=11)	0.76	2.45
Calibration curve		y = 312.36x - 27.24	y = 362.40x - 31.13
Correlation coefficient (r^2)		0.999	0.999
Linearity range (ng/mL)		20-0.05	20-0.05
Limits	LOD[a] (ng/mL)	0.6	0.5
	LOQ[b] (ng/mL)	1.9	1.7

r^2: Correlation coefficient.
[a]LOD: limit of detection of the chromatographic method.
[b]LOQ: limit of quantification of the chromatographic method.

Table 39. Accuracy and precision of the analytical methods for AFB_1 and OTA using spiking levels of 10 ng/g and 30 ng/g.

	AFB_1		**OTA**	
	10 ng/g	30 ng/g	10 ng/g	30 ng/g
Mean Recovery (%)	88.3	88.9	76.4	92.0
RSD_r (%)[a]	8.3-14.4	0.1	2.5-9.3	5.1
RSD_R (%)[b]	3.3	-	5.6	-
Recommended Range (European Regulation No 401/2006)				
Recovery (%)		70-110		
RSD_r (%)	<21	<22	<21	<22
RSD_R(%)	<32	<34	<32	<34

[a]RSDr: Repeatability relative standard deviation
[b]RSDR: Reproducibility relative standard deviation

Data presented in **Table 40** show the effect of gamma radiation doses (1, 5 and 10 kGy) on the reduction of AFB_1 and OTA in dried leaves of *A. citrodora*. Assays were carried out in powdered samples spiked with 30 ng/g of AFB_1 and OTA. This concentration was selected because it is an average value commonly used in this type of studies. When compared with non-irradiated samples (0 kGy), the rates of mycotoxin reduction at the different irradiation doses (1, 5 and 10 kGy) ranged between 4.9 and 5.2% for OTA, and 5.3 to 9.6% for AFB_1, with no statistically significant differences ($p > 0.05$) between irradiated and non-irradiated samples, independently of the applied dose, as well as no apparent dose-dependent effects on the rate of mycotoxins decrease, either. These results suggest that irradiation at the tested doses is not an effective treatment for AFB_1 and OTA decontamination of the studied dried plants.

The effect of gamma radiation on mycotoxin decontamination has been investigated in several food products (spices, feedstuff, coffee beans, fruits, seeds, vegetables, cured meat and others), but divergent results have been reported. Some studies report the effectiveness of gamma radiation to reduce mycotoxin levels in various low moisture foods (Aziz and Moussa, 2004; Iqbal et al., 2013; Prado et al., 2003), although in some cases this effect was only observed at irradiation doses of 30 to 60 kGy (Jalili, et al., 2010, 2012), higher than the maximum dose allowed by EU regulations, i.e., 10 kGy, assayed herein. However, most reports conclude that at the admissible dose levels no significant positive effects on mycotoxin decontamination are obtained for low moisture content foods or feeds (Akueche et al., 2012; Herzallah et al., 2008; Hooshmand and Klopenstein, 1995; Jalili, et al., 2010, 2012; Kumar et al., 2012; Vita et al, 2014). In the study performed by Jalili et al. (2012), gamma radiation was applied to black and white pepper, finding significant AF and OTA reductions only at irradiation doses of 30 kGy or higher and, even at 60 kGy, gamma rays were not completely effective in destroying those mycotoxins. Moreover, at 10 kGy, mycotoxin reduction varied between 1.4% in OTA to 7.2% in AFB_1 for samples with 12% of moisture content.

The limited effect of this technique in low moisture matrices seems to be a direct result of the reduced water content. The presence of water is an important factor in the destruction of AF's and OTA by gamma radiation, since water radiolysis leads to formation of highly reactive free radicals that degrade the mycotoxins (Calado et al., 2014; Rustom, 1997). This is supported in a study by Kumar et al. (2012), where the elimination of OTA in coffee grains with different moisture contents (9, 10, 12 and 23%) was tested. OTA degradation in the lowest moisture content grains was 5% at 10 kGy, similar to the one obtained in our study, while its reduction

reached 90% in the highest moisture content samples. The inefficient degradation of these mycotoxins may also be related to their concentration, as suggested by Jalili et al. (2010) that observed greater percentages of reduction as mycotoxins concentration increased. Mycotoxin degradation by gamma radiation has mostly been tested in dried herbs and spices. To our knowledge, no similar studies have been performed in fresh plants, although there are several studies on the effects of gamma radiation on the nutritional value of different parts of edible fresh plants that, in general, concluded that this treatment does not cause significant changes in the nutritional quality (e.g. Fan and Sokorai, 2008; Koike et al., 2015; Rezende et al., 2014).

Table 40. Reduction (%) (mean ± SD; n= 6) of aflatoxin B_1 and ochratoxin A in spiked dried samples (30 ng/g of each mycotoxin) of *Aloysia citrodora* Paláu submitted to irradiation treatments at 1, 5 and 10 kGy, in comparison with non-irradiated samples (0 kGy).

Irradiation dose	Mycotoxin decrease (%)	
	AFB_1	**OTA**
1 kGy	5.3 ± 1.6[a]	5.0 ± 0.3[a]
5 kGy	9.6 ± 5.6[a]	4.9 ± 0.7[a]
10 kGy	6.9 ± 12.6[a]	5.2 ± 1.4[a]

In each column different letters mean significant differences ($p < 0.05$).

3.2 Studies with electron beam radiation

3.2.1 *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha x piperita* L.: Effects on nutrients and antioxidant properties of methanolic extract and infusions.

In the following sections, the effects of electron beam radiation on different composition and activity variables of these four plants are evaluated as previously studied for gamma radiation. The results obtained in non-irradiated samples are recalled for each plant species and assayed variable. Furthermore, in order to allow a more immediate comparison of the effects of gamma and electron-beam radiations the percentages of variances (calculated as explained in the Materials and methods section) will be indicated for both types of irradiation. Such percentages corresponded to the previous results obtained for gamma radiation (GR) (section 3.1.2) and to the newly assessed values resulting from applying electron-beam radiation (EB) in the same doses as those used for GR. In every case where the variation laid below 5% (either representing an increase or a decrease), it was assumed that the irradiation had no identifiable effect.

3.2.1.1 Effects on nutrients and color variables

Regarding the proximate composition and color variables (**Table 41**), it became obvious that fat and protein levels are the ones suffering higher changes with the irradiation treatments. Nevertheless, the observed effect was highly dependent on the plant species. Fat content, for instance, tended to increase in *A. citrodora* (lemon verbena) and *M. officinalis* (lemon balm), but an opposite effect was produced in *M. melissophyllum* (bastard balm) and *M. piperita* (peppermint). Likewise, no general trend could be identified for the effect on protein content, despite the similar variation in lemon verbena, bastard balm and peppermint obtained at 1 kGy of GR. Furthermore, the effects on the remaining variables, despite lower in magnitude, were significantly different (p<0.05) for each of the applied conditions in most occasions (21 out of 32 cases). In general, the 10 kGy dose tended to have a more pronounced effect than the 1 kGy dose, independently of the irradiation technology (except for a^* in all plants and fat content in bastard balm).

In this first approach, it is important to highlight the slight effects caused on L^* and b^*, which is important since colour variables are usually used in the quality control of post-harvest

preservation processes (Hsu et al., 2010). In the case of *a**, the results are still more promising, since a general decrease was observed in response to the irradiation treatment, which should be interpreted as an increase of samples greenness that result more appealing to the consumers. The variation in colour variables is in general agreement with those available from similar reports (Jo et al., 2003; Hsu et al., 2010).

Table 41. Proximate composition (g/100 g dw), energy (kcal/100 g dw) and color variables (L*: lightness, a*: redness, b*: yellowness) in non-irradiated samples (controls) of aromatic species. Values for irradiated samples are presented as percentage of variation in relation to the control.[1]

		Fat	Protein	Ash	Carbohydrates	Energy	*L**	*a**	*b**
Dose	Irradiation type								
					Aloysia citrodora (Lemon verbena)				
0 kGy	Control	1.6±0.1	3.0±0.1	8.2±0.1	87.1±0.1	375±1	49±1	-8.4±0.2	27.2±0.3
1 kGy	Electron beam	20±4^{b}	1±2^{b}	-1±2^{b}	-1±1^{b}	1±1	1±2	-8±8	7±7
	Gamma rays	32±5^{a}	-42±5^{c}	3±2^{a}	1±1^{a}	1±1	3±2	5±5	3±1
10 kGy	Electron beam	19±8^{b}	45±10^{a}	-1±1^{b}	-2±1^{c}	1±1	2±3	-10±10	3±2
	Gamma rays	6±3^{c}	-2±3^{b}	4±2^{a}	-1±1^{b}	-1±1	-2±2	-1±3	-3±1
p-values	Homoscedasticity[2]	0.012	0.172	0.073	0.016	0.008	0.310	0.003	0.030
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	0.081	0.104	0.087	0.117
					Melissa officinalis (Lemon balm)				
0 kGy	Control	1.2±0.1	2.5±0.3	8.4±0.4	88±1	372±2	48±1	-5.1±0.5	20.9±0.4
1 kGy	Electron beam	-7±4^{c}	4±2^{b}	-3±2	1±1^{a}	1±1	1±1^{b}	-10±4^{b}	-2±1^{c}
	Gamma rays	65±5^{a}	167±11^{a}	-3±2	-5±1^{b}	1±1	-1±1^{bc}	-1±2^{a}	-1±1^{b}
10 kGy	Electron beam	11±3^{b}	5±2^{b}	-1±2	-1±1^{a}	1±1	4±1^{a}	-13±2^{b}	6±1^{a}
	Gamma rays	60±2^{a}	156±20^{a}	1±1	-5±1^{b}	1±1	-2±1^{c}	-2±4^{a}	-3±1^{d}
p-values	Homoscedasticity[2]	0.731	0.002	0.045	0.009	0.003	0.850	0.180	0.261
	1-way ANOVA[3]	<0.001	<0.001	0.082	<0.001	0.071	<0.001	<0.001	<0.001
					Melittis melissophyllum (Bastard balm)				
0 kGy	Control	1.8±0.1	4.6±0.2	7.6±0.1	86.0±0.4	378±1	42±2	-8.4±0.5	18±3
1 kGy	Electron beam	-7±7	-7±5^{b}	-2±4^{bc}	1±1^{a}	1±1	-1±3	36±11^{a}	1±2
	Gamma rays	-8±5	-45±4^{c}	7±2^{ab}	2±1^{a}	1±1	3±3	-3±4^{b}	-1±2
10 kGy	Electron beam	-13±8	2±4^{b}	-4±5^{c}	1±1^{a}	-1±1	-2±4	28±13^{a}	-4±4
	Gamma rays	-13±5	22±5^{a}	13±3^{a}	-2±1^{b}	-1±1	-3±4	-4±4^{b}	-5±5
p-values	Homoscedasticity[2]	0.064	<0.001	0.059	0.053	0.012	0.111	0.188	0.962
	1-way ANOVA[3]	0.400	<0.001	<0.001	<0.001	0.082	0.743	<0.001	0.698
					Mentha x piperita (Peppermint)				
0 kGy	Control	2.4±0.1	5.1±0.3	9.2±0.2	83.3±0.5	375±1	40±1	-5.9±0.1	23.9±0.3
1 kGy	Electron beam	-5±2^{b}	19±8^{b}	-4±3^{a}	1±1^{b}	1±1	-1±2^{a}	17±8	3±2^{a}
	Gamma rays	13±5^{a}	-45±10^{d}	-10±2^{b}	3±1^{a}	1±1	-3±3^{a}	-5±2	-3±2^{b}
10 kGy	Electron beam	-4±5^{b}	3±4^{c}	-3±2^{a}	1±1^{b}	1±1	1±2^{a}	-26±15	2±2^{ab}
	Gamma rays	-21±6^{c}	91±3^{a}	-6±3^{ab}	-6±1^{c}	1±1	-7±3^{b}	-25±14	-16±5^{c}
p-values	Homoscedasticity[2]	0.056	0.045	0.306	0.544	0.053	0.376	0.064	0.580
	1-way ANOVA[3]	<0.001	<0.001	0.002	<0.001	0.082	<0.001	0.077	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]$p<0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

Concerning free sugars composition (**Table 42**), the induced variations were more pronounced, although with variable effect depending on the plant species. Sucrose and trehalose seemed to be the most susceptible sugars to irradiation, as they suffered significant changes almost in all cases, which might be an indicator of the vulnerability of the glycosidic bond, since the monosaccharides presented higher resistance. As for total sugars, only minor variations were detected, which could be anticipated from the changes in individual sugars, since the decrease in sucrose and trehalose was accompanied of an increase in fructose and, especially, glucose. Other less coherent variations might be explained by changes in the optical rotation, which is common under irradiation treatments (Molins, 2001).

Significant variations were also detected in the organic acids (**Table 42**), with quinic and citric acids as the compounds more prone to suffer quantitative changes. It could also be observed that the species with the highest contents in organic acids (bastard balm and peppermint) were the ones with more cases of significant variations. Another interesting observation was the higher propensity of lemon verbena and peppermint to increase the levels of organic acids when GR was applied, while lemon balm and bastard balm showed a general trend to lower amounts of organic acids when irradiated with EB.

Table 42. Free sugars and organic acids composition (g/100 g dw) in non-irradiated samples (controls) of aromatic species. Values for irradiated samples are presented as percentage of variation in relation to the control.[1]

		Fructose	Glucose	Sucrose	Trehalose	Total sugars	Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Citric acid	Organic acids
Dose	Irradiation type											
						Aloysia citrodora (Lemon verbena)						
0 kGy	Control	1.0±0.1	1.3±0.1	7.1±0.3	1.2±0.1	10.7±0.4	1.1±0.1	nd	0.14±0.03	1.4±0.1	1.4±0.1	4.1±0.1
1 kGy	Electron beam	1±5	-7±6	16±6^{b}	12±8^{ab}	3±5^{b}	-3±3^{ab}	-	-5±7^{ab}	-6±5^{c}	-4±3^{c}	-4±2^{c}
	Gamma rays	-2±4	-9±7	-10±7^{c}	-1±2^{b}	-8±2^{c}	-2±2^{ab}	-	29±16^{a}	29±5^{a}	40±9^{a}	24±9^{a}
10 kGy	Electron beam	18±13	-2±6	28±9^{a}	18±7^{a}	13±6^{a}	-9±6^{b}	-	-10±6^{b}	-11±4^{c}	-3±4^{c}	-8±4^{c}
	Gamma rays	-1±3	-5±5	-8±4^{c}	-2±5^{b}	-6±3^{c}	5±7^{a}	-	3±7^{ab}	12±3^{b}	20±7^{b}	12±5^{b}
p-values	Homoscedasticity[2]	0.023	0.029	0.003	0.012	0.002	0.354	-	0.056	0.390	0.059	0.459
	1-way ANOVA[3]	0.131	0.726	<0.001	0.007	<0.001	0.035	-	0.044	<0.001	<0.001	<0.001
						Mellissa officinalis (Lemon balm)						
0 kGy	Control	1.2±0.1	1.0±0.1	4.8±0.2	0.49±0.05	7.5±0.2	0.5±0.1	0.26±0.04	0.4±0.1	4.1±0.2	nd	5.3±0.3
1 kGy	Electron beam	9±5^{a}	21±9^{a}	-11±7^{b}	5±3^{c}	11±4^{ab}	-48±3^{c}	-24±5^{d}	-27±3^{c}	-30±3^{c}	-	-36±2^{c}
	Gamma rays	9±3^{a}	1±1^{b}	12±5^{a}	37±17^{b}	11±4^{ab}	-2±3^{a}	-12±5^{c}	-8±8^{b}	1±2^{b}	-	-1±2^{b}
10 kGy	Electron beam	1±2^{b}	8±6^{b}	-59±16^{c}	16±4^{c}	4±4^{b}	-10±3^{b}	25±5^{a}	8±4^{a}	16±4^{a}	-	6±3^{a}
	Gamma rays	5±3^{ab}	1±2^{b}	17±1^{a}	72±8^{a}	17±1^{a}	-3±5^{a}	-4±4^{b}	-1±4^{b}	-1±2^{b}	-	-1±2^{b}
p-values	Homoscedasticity[2]	0.030	0.026	<0.001	0.004	0.095	0.188	0.934	0.009	0.306	-	0.160
	1-way ANOVA[3]	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-	<0.001
						Melittis melissophyllum (Bastard balm)						
0 kGy	Control	1.0±0.1	0.8±0.1	0.9±0.1	0.28±0.03	5.5±0.3	1.4±0.1	0.17±0.01	6.0±0.3	0.97±0.05	0.022±0.001	8.6±0.4
1 kGy	Electron beam	-11±5^{b}	5±5^{ab}	4±3^{a}	-11±8^{b}	-1±2^{b}	-11±4^{b}	-31±8^{c}	8±5^{a}	-24±9^{b}	41±17^{a}	-3±2^{a}
	Gamma rays	-8±6^{ab}	-3±5^{b}	4±4^{a}	84±20^{a}	6±4^{b}	-16±5^{b}	-10±5^{b}	-26±4^{c}	-12±6^{ab}	-12±6^{c}	-22±2^{b}
10 kGy	Electron beam	-24±4^{c}	-26±3^{c}	-17±4^{b}	-21±10^{b}	-21±3^{c}	-12±2^{b}	-45±9^{d}	12±4^{a}	-13±7^{ab}	18±7^{b}	1±2^{a}
	Gamma rays	1±2^{a}	9±6^{a}	8±6^{a}	119±32^{a}	17±4^{a}	1±2^{a}	10±4^{a}	-1±1^{b}	-3±4^{a}	16±6^{b}	-1±1^{a}
p-values	Homoscedasticity[2]	0.040	0.030	0.017	0.511	0.338	0.575	0.055	0.064	0.364	0.369	0.032
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.045	<0.001	<0.001
						Mentha x piperita (Peppermint)						
0 kGy	Control	0.47±0.05	0.30±0.05	0.7±0.1	1.0±0.1	2.4±0.2	1.1±0.1	0.040±0.003	0.9±0.1	nd	8.5±0.2	10.6±0.3
1 kGy	Electron beam	-3±5	5±5^{a}	10±6^{a}	-6±6^{ab}	3±3^{a}	-11±8	-4±5	9±5^{a}	-	7±7^{a}	6±6^{a}
	Gamma rays	-12±8	-1±2^{ab}	12±8^{a}	3±4^{a}	3±4^{a}	6±5	-10±8	-2±4^{a}	-	-30±4^{c}	-20±4^{b}
10 kGy	Electron beam	-1±4	-11±5^{b}	-26±10^{b}	-29±10^{c}	-11±5^{b}	4±5	-21±10	8±8^{a}	-	11±7^{a}	9±8^{a}
	Gamma rays	-1±2	5±5^{a}	5±5^{a}	-24±8^{bc}	-6±4^{b}	-11±5	-17±8	-32±12^{b}	-	-10±3^{b}	-10±2^{b}
p-values	Homoscedasticity[2]	0.742	0.199	0.065	0.011	0.660	0.311	0.720	0.255	-	0.033	0.164
	1-way ANOVA[3]	0.157	0.004	<0.001	<0.001	<0.001	0.052	0.118	<0.001	-	<0.001	0.062

[1]The results are presented as the mean±SD. [2]Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]$p<0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

Among tocopherols (**Table 43**), α and β isoforms were the ones presenting higher number of significant variations, but the produced effect was once again highly dependent on the assayed plant species. α-and β-Tocopherols are known for being less stable to irradiation than γ-tocopherol (Warner et al., 2008). Regarding total tocopherols, this dissimilarity among effects was also observed. For instance, lemon verbena presented higher levels of tocopherols in samples irradiated at 1 kGy, while the 10 kGy dose induced a very important increase in bastard balm (independently of irradiation technology in both cases), and peppermint' tocopherols were increased when EB was applied. The occurrence of significant changes in tocopherols profile in response to irradiation treatment has already been reported in different species (Taipina et al., 2009).

Table 43. Tocopherols composition (mg/100 g dw) in non-irradiated samples (controls) of aromatic species. Values for irradiated samples are presented as percentage of variation in relation to the control.[1]

		α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
Dose	Irradiation type					
		Aloysia citrodora (Lemon verbena)				
0 kGy	Control	15.3±0.4	0.41±0.04	1.8±0.1	nd	17.5±0.4
1 kGy	Electron beam	22±5^{a}	2±4^{a}	5±5	-	17±3^{a}
	Gamma rays	14±4^{a}	7±9^{a}	4±5	-	13±4^{a}
10 kGy	Electron beam	5±5^{b}	-12±10^{ab}	-5±6	-	2±2^{b}
	Gamma rays	-12±4^{c}	-29±10^{b}	-5±5	-	-12±3^{c}
p-values	Homoscedasticity[2]	0.053	0.279	0.168	-	0.426
	1-way ANOVA[3]	<0.001	0.004	0.050	-	<0.001
		Melissa officinalis (Lemon balm)				
0 kGy	Control	29±1	1.3±0.1	1.5±0.1	0.37±0.05	32±1
1 kGy	Electron beam	-10±2^{d}	-22±5^{b}	-15±3^{d}	1±1^{b}	-10±2^{d}
	Gamma rays	16±1^{a}	-15±4^{a}	18±5^{a}	2±4^{b}	14±1^{a}
10 kGy	Electron beam	-2±2^{c}	-30±3^{c}	-7±3^{c}	2±2^{b}	-3±1^{c}
	Gamma rays	2±1^{b}	-25±3^{bc}	12±6^{b}	31±9^{a}	1±1^{b}
p-values	Homoscedasticity[2]	<0.001	0.148	0.802	<0.001	0.304
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001
		Melittis melissophyllum (Bastard balm)				
0 kGy	Control	0.88±0.05	13.4±0.3	0.18±0.02	0.14±0.02	14.6±0.4
1 kGy	Electron beam	1±3^{b}	-22±5^{d}	-25±10^{bc}	-34±10^{b}	-21±4^{d}
	Gamma rays	-8±5^{b}	-1±1^{c}	-8±5^{b}	3±3^{a}	-2±1^{c}
10 kGy	Electron beam	60±24^{a}	21±5^{b}	14±8^{a}	-39±7^{b}	21±5^{b}
	Gamma rays	-48±6^{c}	115±6^{a}	-40±9^{c}	-44±6^{b}	102±6^{a}
p-values	Homoscedasticity[2]	0.002	0.559	0.749	0.098	0.363
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001
		Mentha x piperita (Peppermint)				
0 kGy	Control	16.5±0.4	1.1±0.1	1.8±0.1	0.23±0.03	19.7±0.5
1 kGy	Electron beam	18±6^{a}	27±10^{a}	8±10	-4±2^{b}	18±6^{a}
	Gamma rays	-5±3^{c}	-42±12^{b}	-3±5	15±6^{a}	-6±3^{c}
10 kGy	Electron beam	7±4^{b}	15±10^{a}	-2±5	5±7^{b}	6±4^{b}
	Gamma rays	-25±4^{d}	-29±10^{b}	-1±4	22±7^{a}	-21±4^{d}
p-values	Homoscedasticity[2]	0.648	0.229	0.097	<0.001	0.906
	1-way ANOVA[3]	<0.001	<0.001	0.278	<0.001	<0.001

Due to the number of individual fatty acids (FA), they were divided in minority (those quantified below 1% in all species, **Table 44**) and majority compounds (above 1% at least in one species, **Table 45**). As it was verified for the previous variables, the variations in FA were highly dependent on the analyzed plant species. Nevertheless, it is easily observable that irradiated samples (except for bastard balm) presented higher percentages of monounsaturated fatty acids (MUFA), which represents an interesting result. A similar observation was also made for some particular polyunsaturated fatty acids (PUFA), such as C18:2n6, C18:3n6 (bastard balm), C18:3n3 (lemon balm) and C20:5n3 (peppermint). Besides C18:2n6, the variations for the remaining predominant FAs (C16:0 and C18:3n3) were not particularly noticeable (but for the decrease of C18:3n3 in bastard balm samples irradiated at 10 kGy). Among the studied plants, lemon balm was the one showing less variation in the FA profiles, especially in the samples irradiated with EB. The higher effect in the remaining species might be related with their higher fat contents (**Table 41**).

Table 44. Minority (< 1% in all species) fatty acids in the studied aromatic species. In controls (non-irradiated samples), the results are presented as relative distribution percentages. Values for irradiated samples are presented as percentage of variation in relation to the control.[1]

		C6:0	C8:0	C11:0	C12:0	C13:0	C15:0	C15:1	C17:0	C20:1n9	C20:2n6	C20:3n3+C21:0	C22:1n9
Dose	Irradiation type												
						Aloysia citrodora (Lemon verbena)							
0 kGy	Control	0.30±0.01	0.11±0.01	0.26±0.02	0.26±0.02	0.32±0.01	0.58±0.02	0.10±0.01	0.22±0.01	0.25±0.03	0.21±0.01	0.30±0.01	0.27±0.02
1 kGy	Electron beam	-37±6^{c}	-49±12^{c}	-23±8^{c}	10±6^{b}	-50±3^{d}	-19±5^{c}	-26±6^{c}	2±4^{bc}	-16±7^{b}	-14±6^{b}	28±9^{a}	83±18^{b}
	Gamma rays	-7±7^{a}	-4±5^{b}	-19±5^{bc}	9±5^{b}	41±9^{b}	5±5^{b}	-14±3^{b}	10±5^{b}	62±20^{a}	-19±3^{b}	-12±1^{bc}	36±11^{c}
10 kGy	Electron beam	-22±8^{b}	-42±9^{c}	15±6^{a}	5±4^{b}	83±6^{a}	-13±8^{c}	-13±3^{b}	-6±6^{c}	-18±9^{b}	-46±6^{c}	-17±4^{c}	181±43^{a}
	Gamma rays	-24±8^{b}	17±6^{a}	-6±8^{b}	40±11^{a}	9±5^{c}	23±7^{a}	2±4^{a}	27±4^{a}	-11±7^{b}	27±5^{a}	-8±4^{bc}	-32±5^{d}
p-values	Homoscedasticity[2]	0.104	0.836	0.374	0.055	0.021	0.272	0.007	0.097	0.147	0.078	<0.001	<0.001
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
						Melissa officinalis (Lemon balm)							
0 kGy	Control	0.22±0.01	0.40±0.02	0.13±0.01	0.46±0.01	0.14±0.01	0.44±0.03	0.55±0.01	0.81±0.01	0.18±0.02	nd	0.28±0.01	nd
1 kGy	Electron beam	1±2^{a}	-3±2^{a}	1±2^{b}	1±2^{a}	-1±5^{a}	-3±4^{a}	-2±2^{a}	2±4	-1±3^{a}	-	-1±2^{b}	-
	Gamma rays	-30±4^{b}	-25±12^{b}	-2±1^{bc}	-27±1^{b}	15±2^{a}	-4±4^{a}	-12±1^{b}	7±1	-18±2^{b}	-	25±1^{a}	-
10 kGy	Electron beam	-3±4^{a}	1±2^{a}	-10±5^{c}	-45±6^{d}	-40±10^{b}	-8±6^{ab}	-30±4^{c}	8±10	-20±12^{bc}	-	-8±5^{b}	-
	Gamma rays	-36±2^{b}	-27±1^{b}	27±2^{a}	-36±2^{c}	1±2^{a}	-19±6^{b}	-7±2^{a}	-1±1	-33±12^{c}	-	28±1^{a}	-
p-values	Homoscedasticity[2]	<0.001	0.008	0.001	0.008	0.002	0.006	0.025	0.006	0.026	-	0.001	-
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	0.015	<0.001	0.578	<0.001	-	<0.001	-
						Melittis melissophyllum (Bastard balm)							
0 kGy	Control	0.18±0.01	0.07±0.01	0.04±0.01	0.18±0.01	0.05±0.01	0.90±0.02	0.09±0.01	0.24±0.02	0.16±0.01	0.09±0.02	0.24±0.01	nd
1 kGy	Electron beam	78±24^{a}	79±34^{b}	1±2^{b}	5±5^{b}	26±5^{b}	-10±4^{b}	14±4^{a}	-20±6^{b}	-18±7^{c}	-20±3^{b}	-10±7^{b}	-
	Gamma rays	-64±2^{c}	4±5^{c}	10±7^{b}	32±6^{a}	35±10^{b}	-7±2^{b}	-9±5^{b}	-2±2^{a}	26±7^{a}	68±16^{a}	10±4^{a}	-
10 kGy	Electron beam	29±13^{b}	118±13^{a}	3±5^{b}	-23±5^{c}	-11±2^{c}	-29±7^{c}	-3±3^{b}	-18±7^{b}	-12±6^{c}	-24±3^{b}	-36±9^{b}	-
	Gamma rays	-58±4^{c}	33±8^{c}	127±12^{a}	37±2^{a}	48±7^{a}	7±3^{a}	17±5^{a}	1±2^{a}	10±2^{b}	93±21^{a}	1±1^{a}	-
p-values	Homoscedasticity[2]	0.001	0.002	0.130	0.005	0.078	0.038	<0.001	0.143	0.023	<0.001	0.022	-
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	-
						Mentha x piperita (Peppermint)							
0 kGy	Control	0.15±0.02	1.0±0.1	0.12±0.01	0.14±0.01	0.15±0.01	0.59±0.05	0.04±0.01	0.44±0.01	0.25±0.01	0.19±0.01	0.45±0.04	0.11±0.01
1 kGy	Electron beam	-23±4^{b}	-22±6^{b}	-14±2^{b}	-8±2^{d}	-72±5^{c}	-15±4^{b}	-43±2^{c}	-6±5^{b}	7±7^{b}	32±9^{a}	-18±6^{c}	35±5^{d}
	Gamma rays	2±4^{ab}	-8±5^{b}	27±8^{a}	7±5^{c}	-19±6^{b}	-23±6^{b}	6±6^{a}	7±2^{ab}	7±7^{b}	-6±6^{b}	6±5^{b}	48±10^{c}
10 kGy	Electron beam	21±2^{a}	37±9^{a}	-13±2^{b}	53±9^{a}	28±7^{a}	16±4^{a}	15±1^{a}	8±8^{a}	26±13^{b}	-8±8^{b}	16±5^{a}	79±7^{a}
	Gamma rays	-60±16^{c}	-19±6^{b}	-9±4^{b}	29±5^{b}	-71±12^{c}	-12±5^{b}	-9±7^{b}	2±2^{ab}	52±2^{a}	-20±7^{b}	18±5^{a}	61±3^{b}
p-values	Homoscedasticity[2]	<0.001	0.229	0.136	0.011	<0.001	0.017	<0.001	<0.001	<0.001	<0.001	0.316	0.018
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.030	<0.001	<0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]$p<0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly.

Table 45. Majority (> 1% at least in one species) fatty acids in the studied aromatic species. In controls (non-irradiated samples), the results are presented as relative distribution percentages. Values for irradiated samples are presented as percentage of variation in relation to the control.[1]

		C10:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n6	C18:3n3	C20:0	C20:5n3	C22:0	C23:0	C22:6n3	C24:0	SFA	MUFA	PUFA
Dose	Irradiation type																			
									Aloysia citrodora (Lemon verbena)											
0 kGy	Control	nd	1.1±0.1	nd	15.7±0.2	0.50±0.02	1.17±0.01	0.95±0.02	12.6±0.1	nd	56.2±0.3	0.87±0.02	nd	1.00±0.02	5.4±0.1	nd	1.4±0.1	28.6±0.2	2.07±0.03	69.3±0.3
1 kGy	Electron beam	-	-20±5^{c}	-	5±4^{a}	-18±5^{b}	12±4^{a}	8±6^{b}	5±2^{a}	-	-3±1^{c}	10±5^{a}	-	29±9^{a}	15±5^{a}	-	9±7^{bc}	4±2^{b}	9±5^{b}	-2±1^{b}
	Gamma rays	-	26±7^{a}	-	1±2^{ab}	25±5^{a}	-5±1^{b}	1±2^{bc}	-1±1^{b}	-	1±1^{b}	13±2^{a}	-	-18±2^{c}	-22±1^{c}	-	22±2^{ab}	-2±2^{c}	17±3^{a}	1±1^{a}
10 kGy	Electron beam	-	10±8^{b}	-	-3±2^{b}	35±12^{a}	-8±4^{b}	-3±4^{c}	-8±4^{c}	-	3±2^{a}	-11±5^{b}	-	11±8^{b}	-4±2^{b}	-	-7±7^{c}	-4±2^{c}	18±6^{a}	1±1^{a}
	Gamma rays	-	-15±6^{c}	-	5±4^{a}	27±5^{a}	13±1^{a}	19±3^{a}	-1±1^{b}	-	-3±1^{c}	-33±6^{c}	-	-7±5^{c}	10±4^{a}	-	32±6^{a}	6±2^{a}	10±2^{b}	-3±1^{b}
p-values	Homoscedasticity[2]	-	0.051	-	0.620	0.012	0.001	0.002	0.001	-	0.129	0.038	-	0.001	<0.001	-	<0.001	0.600	0.002	0.470
	1-way ANOVA[3]	-	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	-	<0.001	<0.001	-	<0.001	<0.001	0.002	<0.001
									Melissa officinalis (Lemon balm)											
0 kGy	Control	0.29±0.02	2.9±0.1	0.53±0.01	22.7±0.3	nd	3.6±0.1	4.9±0.2	15.3±0.4	nd	33.2±0.5	3.4±0.1	3.9±0.1	1.3±0.1	3.3±0.2	nd	1.2±0.2	41.2±0.5	6.2±0.2	52.6±0.5
1 kGy	Electron beam	1±2^{a}	-3±4^{a}	-2±4^{c}	1±1^{a}	-	1±2^{a}	-1±2^{a}	-1±1^{bc}	-	-1±1^{c}	1±2^{bc}	-1±2^{b}	1±2^{b}	1±2^{b}	-	-10±5^{b}	1±1^{a}	-1±2^{a}	-1±1^{c}
	Gamma rays	-13±3^{b}	-9±2^{ab}	-1±2^{c}	-8±1^{c}	-	1±1^{a}	-2±2^{a}	-1±1^{c}	-	4±1^{b}	17±2^{a}	16±1^{a}	12±5^{a}	-1±2^{b}	-	12±5^{a}	-4±1^{b}	-3±1^{a}	3±1^{b}
10 kGy	Electron beam	-18±5^{b}	-48±6^{c}	39±10^{a}	-15±2^{d}	-	-2±2^{a}	-4±4^{a}	5±2^{a}	-	8±2^{a}	-1±2^{c}	-16±7^{c}	-5±5^{b}	29±8^{a}	-	-8±4^{b}	-9±2^{d}	1±2^{a}	7±1^{a}
	Gamma rays	-26±3^{c}	-15±1^{b}	18±2^{b}	-5±1^{b}	-	-11±2^{b}	-12±3^{b}	2±2^{b}	-	9±1^{a}	4±2^{b}	-9±1^{c}	11±5^{a}	-5±5^{b}	-	-8±6^{b}	-6±1^{c}	-10±2^{b}	6±1^{a}
p-values	Homoscedasticity[2]	0.106	<0.001	<0.001	0.196	-	0.045	0.005	0.621	-	0.080	0.177	<0.001	0.093	0.274	-	0.072	0.581	0.010	0.659
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	<0.001
									Melittis melissophyllum (Bastard balm)											
0 kGy	Control	nd	0.58±0.03	nd	14.3±0.2	1.29±0.05	2.41±0.05	11.5±0.3	14.8±0.4	5.8±0.1	36±1	0.88±0.02	nd	1.3±0.1	6.2±0.2	nd	3.0±0.1	30.4±0.2	13.1±0.2	56.5±0.2
1 kGy	Electron beam	-	36±7^{b}	-	-7±3^{c}	12±6^{a}	5±4^{ab}	16±4^{c}	10±5^{c}	33±10^{a}	-7±3^{a}	5±5^{a}	-	-13±4^{b}	-10±4^{a}	-	1±2	-5±2^{b}	15±3^{c}	-2±1^{a}
	Gamma rays	-	39±5^{b}	-	-1±1^{b}	-11±4^{bc}	1±1^{b}	13±2^{c}	9±3^{c}	1±1^{b}	-7±1^{a}	8±2^{a}	-	3±3^{a}	-4±2^{a}	-	-5±4	-1±1^{a}	10±2^{c}	-2±1^{a}
10 kGy	Electron beam	-	-2±4^{c}	-	-7±4^{c}	-25±8^{c}	1±2^{b}	51±5^{a}	31±6^{a}	18±6^{ab}	-21±3^{b}	-26±8^{b}	-	-35±7^{c}	-11±8^{a}	-	-5±4	-10±3^{c}	46±4^{a}	-7±1^{b}
	Gamma rays	-	59±12^{a}	-	6±1^{a}	-3±3^{ab}	14±2^{a}	31±6^{b}	23±1^{b}	9±4^{b}	-21±2^{b}	10±4^{a}	-	11±4^{a}	-33±2^{b}	-	1±2	-1±1^{a}	27±5^{b}	-6±1^{b}
p-values	Homoscedasticity[2]	-	0.463	-	0.014	0.012	0.008	0.024	0.003	<0.001	0.003	0.001	-	0.802	<0.001	-	0.993	0.045	0.007	0.053
	1-way ANOVA[3]	-	<0.001	-	<0.001	<0.001	0.001	<0.001	<0.001	0.005	<0.001	<0.001	-	<0.001	<0.001	-	0.216	<0.001	<0.001	<0.001
									Mentha x piperita (Peppermint)											
0 kGy	Control	0.07±0.01	1.4±0.1	1.2±0.1	10.4±0.3	0.88±0.05	2.47±0.03	1.62±0.05	7.3±0.1	nd	46±1	15.8±0.5	2.8±0.2	2.6±0.1	0.24±0.0	1.4±0.1	2.1±0.1	38±1	4.1±0.1	58±1
1 kGy	Electron beam	-34±2^{a}	-2±4^{c}	6±6^{a}	-5±3^{b}	-12±5^{b}	8±4^{b}	33±6^{b}	3±3^{b}	-	1±1^{a}	2±4^{b}	-17±4^{b}	1±2^{b}	2±4^{ab}	-6±4^{b}	-5±2^{ab}	-2±2^{c}	8±4^{b}	1±1^{a}
	Gamma rays	-76±9^{a}	11±5^{bc}	-1±2^{a}	-1±1^{b}	9±5^{a}	3±1^{b}	-1±2^{d}	2±1^{b}	-	-4±2^{b}	5±3^{b}	8±4^{a}	7±2^{ab}	-20±8^{b}	9±3^{a}	-12±5^{b}	2±2^{b}	4±2^{b}	-2±1^{b}
10 kGy	Electron beam	-20±3^{a}	30±9^{a}	4±4^{a}	13±4^{a}	8±8^{a}	21±6^{a}	42±4^{a}	8±3^{a}	-	-6±2^{bc}	4±2^{b}	8±6^{a}	10±5^{a}	-5±8^{ab}	-17±3^{c}	4±4^{a}	10±2^{a}	20±3^{a}	-5±1^{c}
	Gamma rays	-376±53^{b}	16±4^{ab}	-20±6^{b}	-3±3^{b}	-9±4^{b}	5±3^{b}	15±3^{c}	-2±1^{c}	-	-7±2^{c}	12±2^{a}	15±4^{a}	9±4^{a}	9±2^{a}	10±3^{a}	-12±3^{b}	5±2^{b}	10±2^{b}	-4±1^{c}
p-values	Homoscedasticity[2]	<0.001	0.001	0.265	0.104	0.179	0.014	0.014	0.001	-	0.143	0.007	0.013	0.093	<0.001	0.090	0.124	0.787	0.007	0.092
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	-	<0.001	<0.001	<0.001	0.002	0.042	<0.001	<0.001	<0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]$p<0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly.

3.2.1.2 Effects of irradiation on antioxidant variables

The effects of the irradiation treatments on the antioxidant activity, namely the scavenging effects on DPPH radicals, reducing power and inhibition of β-carotene bleaching, as well as the amounts of total phenolic compounds and flavonoids were also compared and they are shown in **Table 46**. In general, EB produced an increase in the ability to scavenge DPPH radicals and in the reducing power (especially the 10 kGy dose), while GR caused the opposite effect. On the other hand, the effect of irradiation on β-carotene bleaching inhibition did not seem to be ruled by any overall trend, being highly dependent on the extract type (aqueous or methanolic) and the plant species. Regarding phenolic compounds, the irradiation tended to increase the levels of total phenolics in infusions, while the opposite effect was observed for methanolic extracts. Flavonoids tended to diminish with irradiation, independently of the plant species, extract type or irradiation technology.

Table 46. Antioxidant properties of extracts from the studied aromatic species. EC_{50} values (µg/mL) for the controls (non-irradiated samples) are presented for all assays, except phenolic compounds and flavonoids, which are expressed as mg GAE/g extract and mg CE/g extract, respectively. Values for irradiated samples are presented as percentage of variation in relation to the control.[1]

		DPPH scavenging Activity		Reducing Power		β-carotene bleaching inhibition		Phenolic compounds		Flavonoids	
Dose	Irradiation type	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
						Aloysia citrodora (Lemon verbena)					
0 kGy	Control	232±8	39±4	169±1	22.8±0.3	580±31	208±9	134±8	665±13	92±1	369±5
1 kGy	Electron beam	-1±2^{a}	13±8^{c}	-13±2^{d}	-9±1^{c}	-10±5^{c}	254±63^{a}	4±4^{d}	5±2^{a}	3±6^{a}	7±2^{a}
	Gamma rays	2±1^{a}	130±16^{b}	9±1^{a}	115±1^{b}	73±7^{a}	14±7^{c}	41±11^{b}	-20±6^{b}	-35±2^{c}	-3±3^{b}
10 kGy	Electron beam	-8±5^{b}	-13±5^{d}	-11±1^{c}	-10±1^{c}	67±25^{a}	60±28^{b}	30±5^{c}	6±1^{a}	12±6^{a}	7±2^{a}
	Gamma rays	-12±6^{b}	177±15^{a}	1±1^{b}	172±2^{a}	43±6^{b}	-5±3^{c}	54±8^{a}	-31±3^{c}	-18±4^{b}	-25±1^{c}
p-values	Homoscedasticity[2]	<0.001	<0.001	<0.001	0.014	<0.001	<0.001	0.038	<0.001	<0.001	0.001
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
						Melissa officinalis (Lemon balm)					
0 kGy	Control	101±3	67±1	80±1	44±1	165±4	125±3	100±1	829±6	63±1	448±4
1 kGy	Electron beam	-7±5^{c}	17±8^{a}	1±1^{c}	20±1^{b}	86±8^{b}	-14±3^{b}	-5±2^{c}	-12±1^{c}	8±8	-12±1^{d}
	Gamma rays	1±1^{b}	9±3^{b}	-6±1^{d}	8±1^{c}	-21±2^{c}	-10±1^{a}	8±1^{a}	-5±2^{b}	9±1	11±1^{a}
10 kGy	Electron beam	-14±5^{d}	-9±3^{c}	9±1^{b}	1±1^{d}	118±15^{a}	-14±4^{b}	-6±2^{c}	1±1^{a}	5±5	4±1^{b}
	Gamma rays	7±2^{a}	8±2^{b}	28±1^{a}	25±1^{a}	-18±1^{c}	-13±1^{ab}	4±1^{b}	-10±1^{c}	4±1	-7±1^{c}
p-values	Homoscedasticity[2]	<0.001	<0.001	0.075	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	0.499	<0.001
						Melittis melissophyllum (Bastard balm)					
0 kGy	Control	583±24	354±39	512±16	249±2	1648±154	447±66	70±4	160±3	29±2	108±4
1 kGy	Electron beam	-12±4^{c}	36±7^{a}	-7±2^{c}	35±1^{a}	-13±2^{c}	-22±4^{b}	10±1^{a}	6±2^{a}	12±1^{a}	1±1^{b}
	Gamma rays	19±7^{b}	2±4^{b}	18±4^{a}	-20±2^{c}	28±5^{b}	21±5^{a}	3±4^{ab}	-37±2^{d}	-45±5^{c}	-32±3^{d}
10 kGy	Electron beam	-1±2^{c}	-24±2^{c}	6±1^{b}	-38±2^{d}	-14±4^{c}	-15±4^{b}	-1±1^{c}	-5±1^{b}	-3±2^{b}	15±4^{a}
	Gamma rays	45±8^{a}	1±2^{b}	-11±2^{c}	16±2^{b}	40±10^{a}	35±7^{a}	-1±2^{c}	-16±2^{c}	-49±3^{c}	-23±2^{c}
p-values	Homoscedasticity[2]	<0.001	<0.001	<0.001	0.487	<0.001	0.081	<0.001	<0.001	0.002	0.001
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011	<0.001	<0.001	<0.001
						Mentha x piperita (Peppermint)					
0 kGy	Control	184±5	83±7	119±2	52±2	597±44	184±5	218±2	591±19	117±2	319±6
1 kGy	Electron beam	-12±4^{c}	18±3^{a}	16±1^{c}	1±1^{a}	-27±4^{c}	92±19^{b}	-1±1^{a}	-6±1^{a}	-1±1^{a}	-8±1^{b}
	Gamma rays	4±2^{b}	15±3^{b}	13±1^{a}	-22±3^{c}	-28±8^{b}	-35±4^{a}	21±1^{ab}	-4±1^{d}	-23±3^{c}	10±1^{d}
10 kGy	Electron beam	-14±3^{c}	-7±2^{c}	35±1^{b}	15±1^{d}	63±15^{c}	32±6^{b}	1±1^{c}	-6±1^{b}	-1±2^{b}	-11±1^{a}
	Gamma rays	18±3^{a}	4±2^{b}	18±3^{c}	1±2^{b}	15±5^{a}	-64±10^{a}	10±1^{c}	-12±2^{c}	-51±4^{c}	-20±2^{c}
p-values	Homoscedasticity[2]	0.140	0.086	0.002	0.066	0.003	<0.001	0.006	<0.001	0.499	0.001
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MeOH- Methanol extracts; GAE- Gallic acid equivalents; CE- Catechin equivalents. [1]All results are presented as the mean±SD. [2]Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]$p<0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

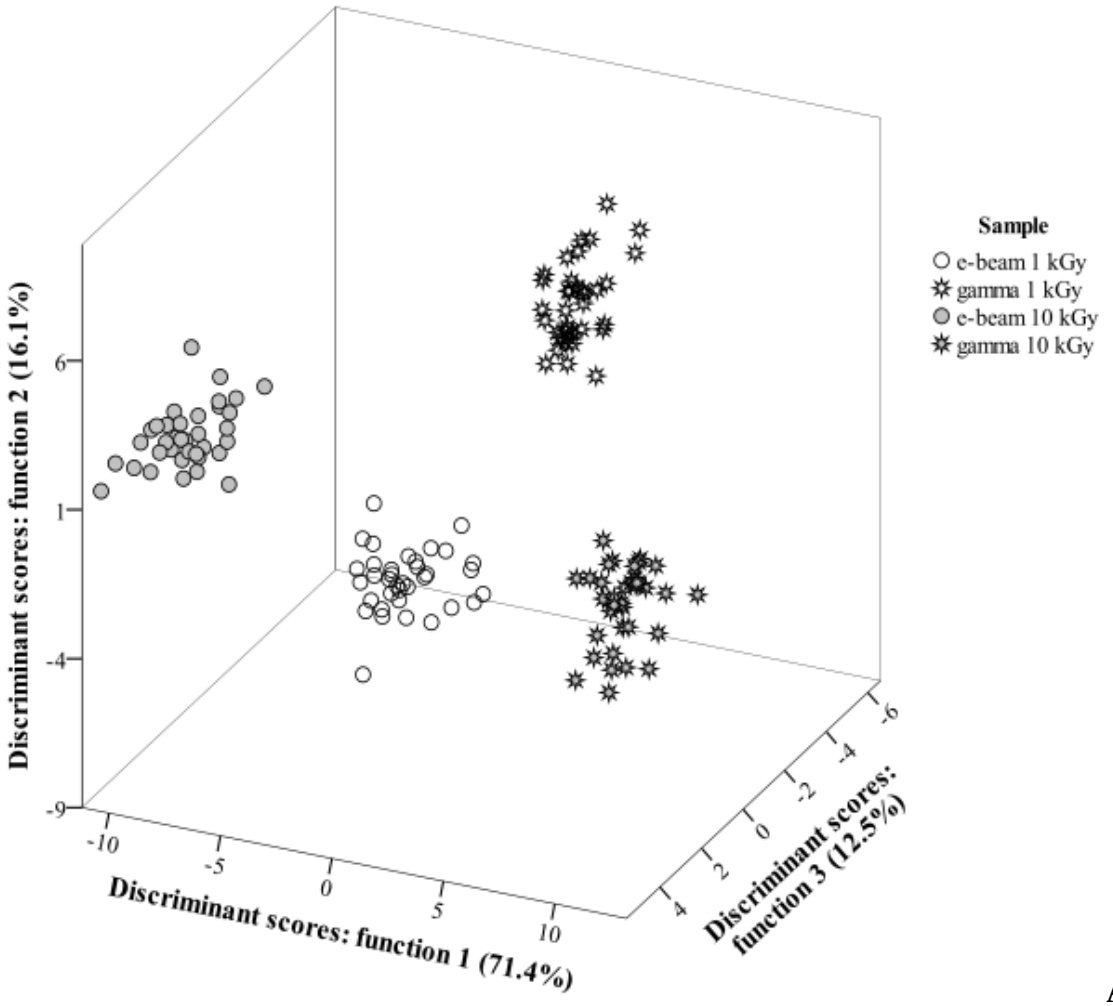
3.2.1.3 Linear discriminant analysis (LDA)

In the former sections, the differences resulting from the irradiation treatments at different doses were compared for each individual variable within each species. Despite the significant variations verified in several cases, it was not possible to identify unequivocal tendencies. Accordingly, the results were evaluated considering data for all irradiation conditions and evaluated variables simultaneously. In the performed LDA, irradiation conditions and plant species were sequentially used as grouping factors. Only variables for which data existed for the four species were used in the analysis. The significant independent evaluated variables were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only those with a statistical significant classification performance ($p < 0.050$) were kept in the analysis.

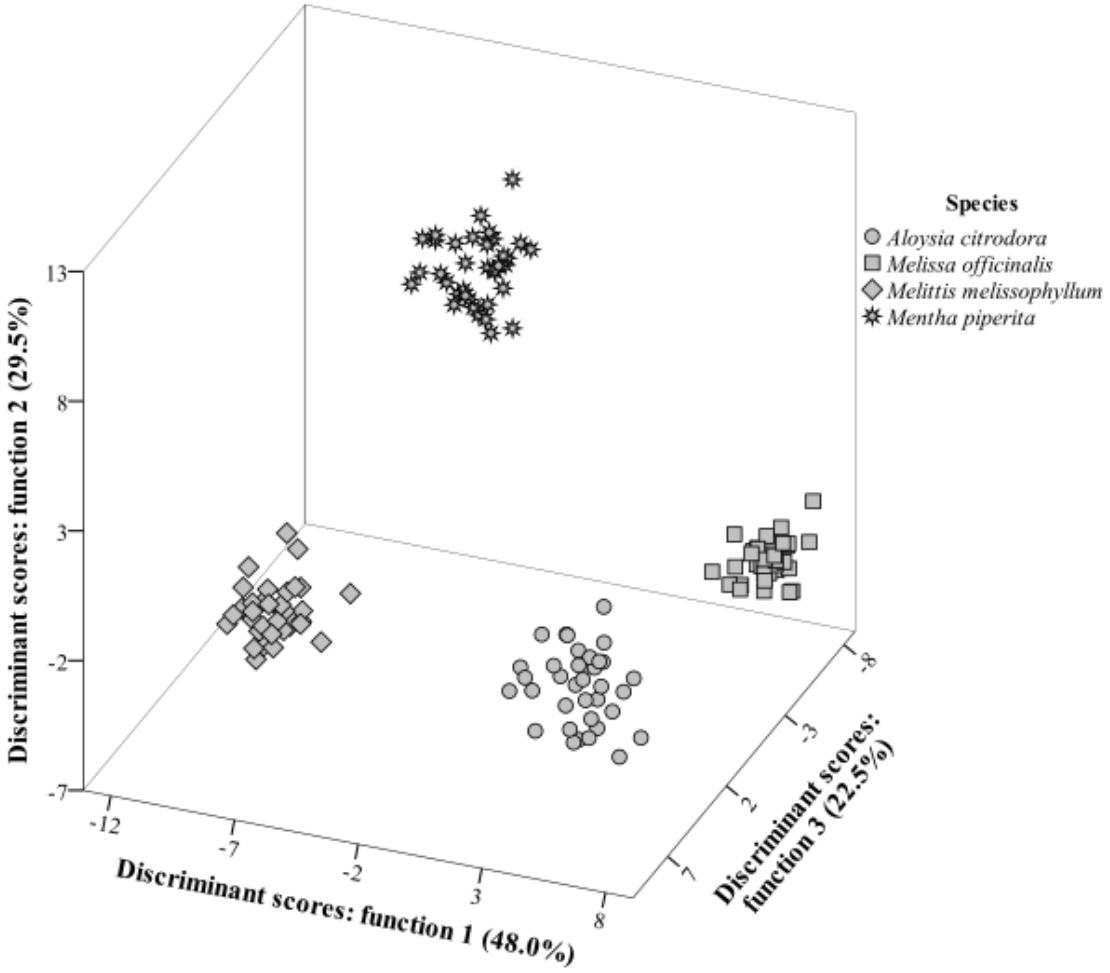
In the discriminant model obtained to verify if the different irradiation treatments (EB, 1 kGy; EB 10 kGy; GR, 1 kGy; GR 10 kGy) exerted variations in a specific way, the three defined functions (plotted in **Figure 39A**) integrated 100% of the observed variance (first: 71.4%; second: 16.1%; third: 12.5%). Among the tested variables 26 were selected as having discriminant ability: fat, carbohydrates, energy, sucrose, organic acids, C6:0, C11:0, C13:0, C14:0, C15:0, C18:0, C18:2n6, C20:0, C20:1, C20:3n3+C21:0, MUFA and all antioxidant activity variables (**Table 46)** , which indicates that the fatty acids profile and the antioxidant activity were the most affected variables considering the overall results of the different irradiation treatments. The groups corresponding to each condition were completely individualized, thereby indicating that the irradiation effects are highly specific. Function 1 (more correlated with DPPH scavenging activity in infusions, and total phenolics and flavonoids in methanolic extracts) separated mainly the groups corresponding to the 10 kGy dose of both types of irradiation. Function 2 (more correlated with C13:0, β-carotene bleaching inhibition in methanolic extracts, and flavonoids in infusions) separated mainly EB at 1 kGy dose; while function 3 (more correlated with C20:0, carbohydrates, β-carotene bleaching inhibition in infusions, and MUFA) was more effective in separating the doses of 1 kGy and 10 kGy for both irradiation sources.

In the assessment of the interaction with the plant species the three defined functions also included 100% of the observed variance (first: 48.0%; second: 29.5%; third: 22.5%), selecting 30 variables (fat, protein, ash, fructose, sucrose, trehalose, oxalic acid, organic acids, α-tocopherol, tocopherols, C6:0, C8:0, C13:0, C14:0, C16:0, C18:1n9, C18:3n3, C20:0, C20:1, C23:0, C24:0, SFA, MUFA and all the variables in **Table 46** , except DPPH scavenging activity and flavonoids content in infusions). Likewise, the defined functions separate the

markers corresponding to each of the assayed species (**Figure 39B**). Function 1 (highly correlated to C18:3n3, C8:0, C18:1n9, C14:0 and fat) separated mainly bastard balm (*M. melissophyllum*); function 2 (more correlated to reducing power in infusions, and trehalose and C13) contributed mainly to discriminate peppermint (*M. piperita*); finally, function 3 (closely correlated to phenolic compounds in infusions, and MUFA, protein and β-carotene bleaching inhibition in methanolic extracts) allowed to separate lemon verbena (*M. officinalis*).



A



B

Figure 39. Mean scores for different irradiation conditions (A) and different plant species (B), projected for the three discriminant functions considering variations measured in all evaluated variables.

3.2.2 *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha piperita* L.: Changes in nutrients and antioxidant properties during storage of plants submitted to electron beam radiation

Previously, the effects of EB radiation on the plant species were evaluated by measuring changes in the herein considered variables in samples analyzed soon after the irradiation treatment (section 3.1.2). Now, the main purpose of the study was to perform a comprehensive characterization of the influence of EB radiation in maintaining plant properties throughout storage. In order to evaluate the effects of EB independently of storage time (ST) and *vice versa*, the results of each factor were consecutively aggregated. Therefore, the variation in the values obtained for each result should not be regarded as a strict standard deviation, but more as the amplitude of values obtained after performing assays in samples corresponding to different conditions. To overcome the variability induced by each plant species, among which the magnitude of the evaluated variables was often highly different, the results were processed considering percentage variations in relation to the respective control. With this aim, the values previously obtained for the different variables in non-irradiated samples of each plant species (section 3.2.1) were used for comparison. In all cases, it was considered that the irradiation had no significant effect when the variations were below 5% (either representing an increase or a decrease).

3.2.2.1 Effects on nutrients and color variables

Concerning the proximate composition and color variables (**Table 47**), the interaction EB×ST was significant ($p < 0.050$) only in a few occasions. Similarly, the differences induced by each factor separately were not significant in many times, either. This indicates that the influence of both the irradiation process and the storage time up to 18 months is nearly irrelevant. In fact, the only significant differences were the lower fat, ash and protein contents in the irradiated samples of *A. citrodora*, the lower ash contents in the irradiated samples of *M. mellisophyllum*, and the higher fat contents in the irradiated samples of *M. piperita*. On the other hand, the differences observed as a function of the ST were only significant for fat and ash contents in *A. citrodora*, and fat contents in *M. melissophyllum*. However, the most striking result was the absence of significant effects in the assayed variables when considering both factors (**Table 47**). In fact, in 62.5% of the cases (40 out of 64) the differences calculated from the respective control laid below 5%, indicating an apparent stability of the nutritional

and color variables (except for *a**, which suffers relevant changes independently of the irradiation treatment and storage time). The maintenance of *L** and *b**, in association with the decrease in *a** (which indicates "greener" samples) might be looked as a positive result, considering that color is a good indicator of an adequate post-harvest preservation processes (Jo et al., 2003b; Hsu et al., 2010).

Table 47. Changes in proximate composition and color variables (differential percentage in relation to the control values) according to electron-beam radiation (10 kGy) and storage time. The results are presented as the mean±SD.

		Fat	Protein	Ash	Carbohydrates	Energy	*L**	*a**	*b**
					Aloysia citrodora (lemon verbena)				
Electron-beam (EB)	0 kGy	-35±9	46±18	-21±3	3±1	2±1	1±3	-35±9	-6±2
	10 kGy	-43±10	-1±11	-27±5	6±1	2±1	2±6	-27±18	-5±3
	p-value (n = 18)	0.017	<0.001	<0.001	<0.001	0.248	0.427	0.083	0.779
Storage time (ST)	12 months	-34±9	26±29	-21±3	4±1	2±1	1±5	-29±16	-5±3
	18 months	-44±8	19±26	-27±5	5±2	2±1	2±4	-32±12	-6±3
	p-value (n = 18)	0.001	0.495	<0.001	0.008	0.021	0.258	0.580	0.199
(EB×ST)	*p*-value (n = 36)	0.758	0.336	0.066	0.411	0.226	0.633	0.599	0.239
					Melissa officinalis (lemon balm)				
Electron-beam (EB)	0 kGy	-12±11	2±8	-32±4	5±1	4±1	2±2	-13±9	-1±3
	10 kGy	-8±8	4±6	-28±5	5±2	4±1	-1±3	-18±12	-2±3
	p-value (n = 18)	0.300	0.349	0.670	0.773	0.534	0.007	0.195	0.452
Storage time (ST)	12 months	-8±12	6±6	-29±5	5±1	4±1	1±4	-15±10	-1±3
	18 months	-12±6	1±7	-30±4	5±1	4±1	1±3	-17±12	-2±3
	p-value (n = 18)	0.203	0.018	0.212	0.953	0.122	0.580	0.619	0.433
(EB×ST)	*p*-value (n = 36)	0.118	0.011	0.001	0.850	0.003	0.610	0.425	0.064
					Melittis melissophyllum (bastard balm)				
Electron-beam (EB)	0 kGy	3±16	1±10	-31±6	4±1	4±1	-7±15	-18±22	-8±8
	10 kGy	-6±15	-2±8	-27±3	4±1	3±1	5±14	-23±12	-3±6
	p-value (n = 18)	0.121	0.532	0.046	0.135	0.058	0.019	0.396	0.043
Storage time (ST)	12 months	-2±15	1±8	-28±4	4±1	4±1	-2±16	-20±17	-7±8
	18 months	-2±17	-2±10	-30±6	4±1	4±1	-1±15	-22±19	-4±7
	p-value (n = 18)	0.998	0.338	0.385	0.295	0.569	0.764	0.718	0.383
(EB×ST)	*p*-value (n = 36)	0.809	0.623	0.025	0.070	0.134	0.960	0.458	0.530
					Mentha x piperita (peppermint)				
Electron-beam (EB)	0 kGy	-17±12	-3±14	-29±4	5±1	3±1	-1±4	-5±38	-4±3
	10 kGy	-3±10	-7±4	-28±4	4±1	3±1	1±4	-362±907	-4±5
	p-value (n = 18)	0.001	0.593	0.405	0.001	0.253	0.081	0.114	0.701
Storage time (ST)	12 months	-2±9	-4±12	-29±3	4±1	4±1	1±5	-208±730	-3±4
	18 months	-18±11	-4±1	-28±4	5±1	3±1	-1±3	-159±597	-4±4
	p-value (n = 18)	<0.001	0.893	0.763	0.043	0.008	0.955	0.828	0.530
(EB×ST)	*p*-value (n = 36)	0.476	0.269	0.003	0.005	0.030	0.379	0.781	0.658

The lack of significant interactions among factors was also observed in almost all cases regarding the effects over free sugars and organic acids (**Table 48**). Nevertheless, the individual effect of each factor on these components was more pronounced than the observed for nutritional and color variables. In this particular subject, the effect induced by ST was more pronounced (except for *A. citrodora*) than the one resulting from samples irradiation. The highest difference among the results collected in **Table 47** and **Table 48** was the magnitude of the induced changes. Free sugars and organic acids contents in all plants decreased significantly (except trehalose, which increased in a great extent) throughout the ST. This decrease could not be totally prevented by the EB treatment, although the loss of free sugar or organic acids was significantly attenuated in irradiated samples in most cases. The susceptibility of each individual component was highly dependent on the plant species, which hinders the unequivocal identification of the free sugars and organic acids more prone to suffer quantitative changes in response to ST and EB. Even so, glucose, sucrose, shikimic and citric acids tended to present the more important changes.

Table 48. Changes in the contents of free sugars and organic acids (differential percentage in relation to the control values) according to electron-beam radiation (10 kGy) and storage time. The results are presented as the mean±SD.

		Fructose	Glucose	Sucrose	Trehalose	Total sugars	Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Citric acid	Organic acids
Aloysia citrodora (lemon verbena)												
Electron-beam (EB)	0 kGy	-5±19	-37±16	-12±12	8±24	-14±13	-18±11	-	-25±12	-54±10	-62±39	-39±13
	10 kGy	-13±14	-29±14	-41±18	-5±18	-22±12	-13±13	-	-9±15	-51±9	-59±43	-35±14
	p-value (n = 18)	0.199	0.119	<0.001	0.072	0.062	0.204	-	0.002	0.314	0.830	0.395
Storage time (ST)	12 months	-5±15	-26±14	-21±18	-1±19	-14±11	-17±11	-	-20±18	-53±10	-21±8	-25±7
	18 months	-13±18	-40±14	-32±23	5±25	-22±14	-14±13	-	-14±12	-52±9	-100*	-49±6
	p-value (n = 18)	0.164	0.005	0.143	0.463	0.072	0.449	-	0.314	0.907	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.454	0.539	0.021	0.533	0.400	0.389	-	0.030	0.913	0.121	0.882
Melissa officinalis (lemon balm)												
Electron-beam (EB)	0 kGy	-9±13	-49±9	-100*	-53±8	-33±6	-25±14	16±26	14±25	-32±12	-	-21±11
	10 kGy	-9±16	-17±17	-100*	7±20	-9±14	-5±22	-5±20	-5±19	-36±10	-	-21±10
	p-value (n = 18)	0.986	<0.001	-	<0.001	<0.001	0.002	0.010	0.015	0.297	-	0.934
Storage time (ST)	12 months	-11±16	-32±20	-100*	-27±29	-22±14	-17±17	-5±14	10±23	-33±9	-	-21±10
	18 months	-7±14	-34±23	-100*	-20±39	-19±18	-13±24	15±30	-1±24	-35±13	-	-21±11
	p-value (n = 18)	0.429	0.768	-	0.543	0.588	0.550	0.018	0.150	0.572	-	0.813
(EB×ST)	*p*-value (n = 36)	0.487	0.423	-	0.046	0.179	0.271	0.600	0.732	0.385	-	0.807
Melittis melissophyllum (bastard balm)												
Electron-beam (EB)	0 kGy	-20±8	-25±7	-6±9	504±218	11±10	-15±8	-20±12	-63±2	-74±27	-27±13	-53±6
	10 kGy	-4±12	-5±7	-9±6	783±327	26±8	-15±8	1±19	-72±5	-72±30	-24±15	-59±8
	p-value (n = 18)	<0.001	<0.001	0.326	0.005	<0.001	0.788	<0.001	<0.001	0.809	0.569	0.010
Storage time (ST)	12 months	-11±12	-13±12	-5±7	748±339	25±9	-14±6	1±17	-65±3	-45±9	-29±14	-50±3
	18 months	-13±14	-17±13	-9±7	540±241	12±11	-15±10	-20±14	-70±7	-100*	-22±15	-63±5
	p-value (n = 18)	0.684	0.284	0.089	0.041	0.001	0.746	<0.001	0.006	<0.001	0.137	<0.001
(EB×ST)	*p*-value (n = 36)	0.671	0.446	0.065	0.727	0.688	0.183	0.078	<0.001	0.296	0.321	0.075
Mentha x piperita (peppermint)												
Electron-beam (EB)	0 kGy	-13±14	-91±2	-45±28	-33±20	-75±4	-26±19	-19±25	-25±8	-	-18±12	-21±8
	10 kGy	3±20	-88±3	4±62	15±50	-67±5	-16±13	-17±13	-39±8	-	-34±5	-34±3
	p-value (n = 18)	0.012	<0.001	0.005	0.001	<0.001	0.070	0.723	<0.001	-	<0.001	<0.001
Storage time (ST)	12 months	-2±17	-87±2	-5±60	-1±49	-68±5	-22±17	-29±9	-29±10	-	-28±13	-28±9
	18 months	-7±21	-92±2	-36±42	-17±40	-74±6	-20±17	-6±20	-35±11	-	-24±11	-27±9
	p-value (n = 18)	0.416	<0.001	0.079	0.299	0.002	0.740	<0.001	0.133	-	0.423	0.677
(EB×ST)	*p*-value (n = 36)	0.640	0.070	0.841	0.618	0.953	0.788	0.032	0.234	-	0.132	0.258

*This variable was not detected in the samples treated under these conditions.

Tocopherols also underwent very marked decreases for all of their isoforms (**Table 49**). In general, as it seems logical, the losses verified for the 18 months storage period were greater than those verified for the 12 months period. Nevertheless, wherever the variation was significant regarding the EB effect, non-irradiated samples showed, in general, more pronounced reductions in their tocopherols. Hence, and as it was also observed for free sugars and organic acids, the EB treatment seemed to attenuate (in low extent) the tocopherol losses.

Table 49. Changes in the contents of tocopherols (differential percentage in relation to the control values) according to electron-beam radiation (10 kGy) and storage time. The results are presented as the mean±SD.

		α-Tocopherol	β-Tocopherol	γ-Tocopherol	Total tocopherols
		Aloysia citrodora (lemon verbena)			
Electron-beam (EB)	0 kGy	-77±5	-31±14	-81±5	-77±5
	10 kGy	-74±7	-11±21	-78±7	-74±6
	p-value (n = 18)	0.148	0.003	0.236	0.113
Storage time (ST)	12 months	-70±4	-21±16	-75±4	-70±3
	18 months	-81±2	-21±24	-84±3	-81±2
	p-value (n = 18)	<0.001	0.990	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.033	0.363	0.138	0.010
		Melissa officinalis (lemon balm)			
Electron-beam (EB)	0 kGy	-75±2	-63±10	-43±14	-73±2
	10 kGy	-69±4	-52±22	-31±18	-66±5
	p-value (n = 18)	<0.001	0.054	0.037	<0.001
Storage time (ST)	12 months	-70±5	-45±16	-25±13	-67±5
	18 months	-74±2	-70±7	-49±11	-73±3
	p-value (n = 18)	0.002	<0.001	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	<0.001	0.002	0.437	<0.001
		Melittis melissophyllum (bastard balm)			
Electron-beam (EB)	0 kGy	-71±8	-100*	-9±11	-16±10
	10 kGy	-80±4	-100*	-20±5	-20±9
	p-value (n = 18)	<0.001	-	0.220	0.223
Storage time (ST)	12 months	-72±8	-100*	-5±9	-12±8
	18 months	-78±6	-100*	-17±7	-24±7
	p-value (n = 18)	0.018	-	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.131	-	0.234	0.653
		Mentha x piperita (peppermint)			
Electron-beam (EB)	0 kGy	-64±8	-100*	-63±11	-65±8
	10 kGy	-64±10	-100*	-52±13	-65±10
	p-value (n = 18)	0.915	-	0.011	0.828
Storage time (ST)	12 months	-56±3	-100*	-48±9	-57±3
	18 months	-73±2	-100*	-68±7	-73±2
	p-value (n = 18)	<0.001	-	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.004	-	0.973	0.005

Many fatty acids: C6:0, C8:0, C10:0, C11:0 (except in *M. melissophyllum*), C12:0, C13:0, C14:1 (except in *A. citrodora* and *M. melissophyllum*), C15:1, C18:3n6 (only in *M. melissophyllum*), C20:1, C20:2, C20:5n3 (except in *A. citrodora* and *M. melissophyllum*), C22:1 (except in *M. officinalis* and *M. melissophyllum*), C22:6n3 (except in *M. officinalis* and *A. citrodora*) and C23:0 were detected in relative percentages lower than 1%. For that reason, only majority fatty acids (>1% in all samples) are included in **Table 50**, despite the variations in all FA will be evaluated in the PCA analysis (see section 3.3.).

Contrarily to the previous variables, the interaction among factors was significant in most cases. By restricting the analysis to those results were the interaction was not significant and at least one of the factors was significant *per se*, it became obvious that the effects of ST and EB are highly dependent on the plant species in some particular cases. In *A. citrodora* and *M. piperita g*reater increase in the percentage of saturated fatty acids (SFA) was observed in non-irradiated samples than in non-irradiated ones, while the opposite observation was made for *M. officinalis* and *M. melissophyllum* samples. Monounsaturated fatty acids (MUFA) suffer, in general, higher increases in irradiated samples, while the reduction in polyunsaturated fatty acids (PUFA), such as C18:2n6, C18:3n3, was similar in all cases, independently of irradiation treatment, period of storage or plant species. Nevertheless, the EB treatment slightly attenuated PUFA losses.

In general, all plant species presented similar percentages of variation, but it is evident that the highest changes were verified among the less abundant fatty acids, which would probably be expectable. The PUFA species were previously indicated as being more prone to lipid radiolysis, (Molins, 2001). Thus, since fatty acids have not been strictly quantified but determined as relative percentages of distribution, the observed increases in more stable MUFA and SFA species could be a consequence of this process.

Table 50. Changes in majority fatty acids (differential percentage in relation to the control values) according to electron-beam at 10 kGy and storage time. The results are presented as the mean±SD.

		C14:0	C15:0	C16:0	C16:1	C17:0	C18:0	C18:1	C18:2	C18:3n3	C20:0	C20:3n3 + C21:0	C22:0	C24:0	SFA	MUFA	PUFA
Aloysia citrodora (lemon verbena)																	
Electron-beam (EB)	0 kGy	290±112	-42±46	42±9	15±76	145±39	227±53	205±60	-13±6	-32±8	-28±37	60±33	20±38	117±60	67±19	143±42	-28±8
	10 kGy	56±30	-33±53	42±5	6±80	114±24	88±13	104±63	10±4	-22±7	-1±51	117±31	45±28	138±106	47±9	43±42	-16±5
	p-value (n = 18)	<0.001	0.591	0.869	0.738	0.007	<0.001	<0.001	<0.001	0.001	0.080	<0.001	0.029	0.461	0.001	<0.001	<0.001
Storage time (ST)	12 months	131±66	-84±2	38±5	-64±7	105±20	133±52	98±57	-1±8	-20±4	-56±9	92±33	6±21	60±34	44±6	54±54	-16±5
	18 months	215±185	10±18	46±6	86±19	153±31	182±95	211±54	-2±16	-34±6	28±24	86±51	59±24	195±66	70±16	132±53	-28±7
	p-value (n = 18)	0.084	<0.001	<0.001	<0.001	<0.001	0.063	<0.001	0.627	<0.001	<0.001	0.670	<0.001	<0.001	<0.001	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	<0.001	0.155	<0.001	0.484	0.025	<0.001	0.412	<0.001	0.027	0.001	0.004	0.394	<0.001	<0.001	0.568	<0.001
Melissa officinalis (lemon balm)																	
Electron-beam (EB)	0 kGy	90±56	140±47	34±4	14±68	72±36	84±22	103±17	12±33	-48±7	15±7	92±66	10±13	78±33	36±2	74±9	-35±2
	10 kGy	409±249	197±59	50±12	6±53	68±40	133±77	166±84	-10±8	-51±14	-2±11	132±66	16±9	101±34	47±6	114±60	-41±9
	p-value (n = 18)	<0.001	0.003	<0.001	0.685	0.760	0.017	0.006	0.013	0.394	<0.001	0.081	0.179	0.049	<0.001	0.013	0.009
Storage time (ST)	12 months	110±68	196±61	47±13	-48±7	55±31	63±13	100±19	-18±4	-39±4	15±7	171±38	16±11	70±28	40±3	68±14	-34±3
	18 months	390±270	142±46	37±10	68±21	86±38	153±57	170±81	21±24	-60±6	-2±11	54±29	10±11	108±30	44±9	120±54	-41±8
	p-value (n = 18)	<0.001	0.005	0.018	<0.001	0.011	<0.001	0.002	<0.001	<0.001	<0.001	<0.001	0.128	<0.001	0.113	0.001	0.003
(EB×ST)	*p*-value (n = 36)	<0.001	0.210	0.003	0.006	0.132	<0.001	<0.001	<0.001	<0.001	0.002	0.828	0.001	0.444	<0.001	<0.001	<0.001
Melittis melissophyllum (bastard balm)																	
Electron-beam (EB)	0 kGy	462±152	185±100	69±4	24±81	95±39	50±29	14±4	-7±3	-40±7	27±33	-10±60	4±24	104±29	69±4	13±7	-25±2
	10 kGy	352±136	688±550	66±9	47±110	124±26	53±19	-8±4	-17±3	-40±5	64±41	17±95	46±43	126±34	80±11	-6±3	-22±3
	p-value (n = 18)	0.029	0.001	0.156	0.482	0.016	0.709	<0.001	<0.001	0.993	0.005	0.315	0.001	0.045	0.001	<0.001	0.016
Storage time (ST)	12 months	535±86	749±488	71±6	-55±7	101±39	37±15	4±8	-14±5	-35±3	15±21	-68±6	-2±19	140±25	78±13	1±7	-24±2
	18 months	278±72	124±42	65±6	126±39	118±31	67±22	3±14	-11±7	-44±4	76±33	75±46	53±37	91±20	72±5	6±14	-23±4
	p-value (n = 18)	<0.001	<0.001	0.005	<0.001	0.178	<0.001	0.849	0.178	<0.001	<0.001	<0.001	<0.001	<0.001	0.053	0.157	0.414
(EB×ST)	*p*-value (n = 36)	0.595	<0.001	0.009	0.001	0.251	0.002	<0.001	0.408	<0.001	0.635	<0.001	0.001	0.259	<0.001	<0.001	<0.001
Mentha x piperita (peppermint)																	
Electron-beam (EB)	0 kGy	350±223	532±383	48±12	145±64	38±17	63±15	107±59	8±18	-35±9	23±8	-28±47	19±9	19±70	51±13	82±57	-25±8
	10 kGy	177±136	197±85	38±20	99±53	33±24	24±12	45±42	4±14	-32±11	23±9	-34±46	28±25	-2±70	37±15	37±35	-19±9
	p-value (n = 18)	0.009	0.002	0.062	0.026	0.504	<0.001	0.001	0.521	0.291	0.845	0.686	0.184	0.381	0.004	0.008	0.066
Storage time (ST)	12 months	430±149	585±329	58±6	170±51	42±24	44±28	122±44	-9±5	-24±3	27±8	-76±3	35±19	-58±12	57±7	103±36	-31±3
	18 months	96±55	143±41	28±11	74±24	30±14	43±21	30±28	20±8	-43±3	18±5	13±9	13±11	75±20	31±9	16±15	-14±4
	p-value (n = 18)	<0.001	<0.001	<0.001	<0.001	0.078	0.819	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	<0.001	<0.001	<0.001	0.117	0.555	0.160	0.005	0.025	0.013	0.007	0.683	<0.001	0.920	0.073	<0.001	0.099

3.2.2.2 Effects of electron beam radiation on antioxidant variables

In line with the observations made for the fatty acids variation, the interaction among factors in the case of antioxidant variables was significant in most cases (**Table 51**). In general, the reducing power was the less susceptible to be affected by ST and EB effects. The ability to scavenge DPPH radicals was only maintained for *A. citrodora* and *M. officinalis*, while it was greatly reduced in the cases of *M. melissophyllum* and *M. piperita*. Likewise, TBARS formation inhibition was also decreased throughout ST (except for *M. melissophyllum*), contrarily to the capacity to inhibit β-carotene bleaching, which was greatly improved in response to ST and EB. Concerning the contents in total phenolics and flavonoids, the effect of the plant species was highly significant, hindering the observation of overall trends. On the other hand, some particular differences were also verified among infusions and methanolic extracts. The differences induced by EB in the contents of phenolic compounds and flavonoids were in general not significant. On the contrary, the effect of ST was significant in nearly all cases, with the greatest increases observed in samples at 12 months of storage (except in *M. melissophyllum*).

Table 51. Variations in the antioxidant capacity (EC_{50} values) evaluated by different assays, and total phenolics and flavonoids contents (presented as differential percentages in relation to the control values) according to electron-beam at 10 kGy and storage time. The results are presented as the mean±SD.

		DPPH scavenging Activity		Reducing power		β-carotene bleaching inhibition		TBARS formation inhibition		Phenols		Flavonoids	
		Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
						Aloysia citrodora (lemon verbena)							
Electron-beam (EB)	0 kGy	-9±12	-4±14	-6±14	-10±2	-74±12	-85±1	181±78	4±55	12±8	10±7	-23±32	16±4
	10 kGy	5±16	5±12	9±10	-1±1	-88±3	-91±2	422±19	70±49	-2±33	2±8	-24±40	5±3
	p-value (n = 18)	0.008	0.035	0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.098	0.003	0.955	<0.001
Storage time (ST)	12 months	-4±7	-11±7	3±4	-6±6	-74±13	-89±4	339±93	-7±35	20±13	13±5	11±9	13±7
	18 months	-1±21	12±6	-1±20	-4±3	-87±3	-87±3	264±160	81±50	-10±24	-1±5	-58±5	7±5
	p-value (n = 18)	0.468	<0.001	0.424	0.383	<0.001	0.111	0.093	<0.001	<0.001	<0.001	<0.001	0.003
(EB×ST)	*p*-value (n = 36)	<0.001	0.198	<0.001	<0.001	<0.001	0.002	<0.001	0.974	<0.001	0.268	<0.001	0.008
						Melissa officinalis (lemon balm)							
Electron-beam (EB)	0 kGy	12±10	-16±14	15±14	-22±1	4±40	-47±22	20±25	-1±5	-31±29	1±28	-29±14	38±4
	10 kGy	28±8	-13±13	13±5	-29±3	-61±8	-44±22	43±18	43±31	-26±33	9±33	-33±12	44±7
	p-value (n = 18)	<0.001	0.588	0.697	<0.001	<0.001	0.713	0.003	<0.001	0.668	0.430	0.326	0.005
Storage time (ST)	12 months	14±10	-25±7	23±6	-27±5	-43±35	-25±5	49±11	31±40	1±6	34±7	-20±8	45±6
	18 months	25±12	-4±10	5±4	-24±2	-15±47	-66±7	15±22	12±14	-59±1	-26±2	-42±6	37±2
	p-value (n = 18)	0.006	<0.001	<0.001	0.076	0.048	<0.001	<0.001	0.081	<0.001	<0.001	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.309	0.812	<0.001	<0.001	0.019	0.741	0.002	<0.001	0.002	<0.001	0.793	<0.001
						Melittis melissophyllum (bastard balm)							
Electron-beam (EB)	0 kGy	-51±32	-60±17	-35±42	-42±34	-95±2	-82±7	-18±50	-77±11	56±44	110±88	82±59	159±143
	10 kGy	-54±29	-38±29	-38±40	11±70	-93±3	-74±14	-32±32	-68±12	66±30	104±96	98±55	107±122
	p-value (n = 18)	0.743	0.013	0.824	0.007	0.022	0.035	0.330	0.036	0.413	0.858	0.398	0.243
Storage time (ST)	12 months	-23±7	-27±17	4±2	35±45	-91±2	-68±7	15±18	-62±5	26±17	18±7	35±11	4±17
	18 months	-82±1	-72±5	-76±1	-66±10	-96±1	-88±2	-64±3	-83±4	97±3	196±10	146±7	261±38
	p-value (n = 18)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.050	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.482	<0.001	0.001	<0.001	<0.001
						Mentha x piperita (peppermint)							
Electron-beam (EB)	0 kGy	143±11	127±31	18±2	1±1	-73±10	-13±33	79±10	125±37	-29±24	7±5	-23±33	6±7
	10 kGy	183±17	158±35	-12±2	-7±6	-79±10	-54±4	50±30	-29±8	-32±23	20±15	-20±36	21±15
	p-value (n = 18)	<0.001	0.009	<0.001	<0.001	0.093	<0.001	0.001	<0.001	0.728	0.003	0.770	0.001
Storage time (ST)	12 months	160±24	111±16	5±15	-6±7	-66±4	-16±37	53±33	55±87	-8±2	23±12	12±3	24±12
	18 months	165±26	173±19	1±16	-1±1	-85±4	-51±7	76±10	41±79	-53±1	4±2	-55±1	3±3
	p-value (n = 18)	0.531	<0.001	0.514	0.006	<0.001	0.001	0.014	0.634	<0.001	<0.001	<0.001	<0.001
(EB×ST)	*p*-value (n = 36)	0.588	0.190	0.047	<0.001	0.872	<0.001	<0.001	0.297	<0.001	<0.001	<0.001	<0.001

MeOH- Methanol extracts; GAE- Gallic acid equivalents; CE- Catechin equivalents. [1]The results are presented as the mean±SD. [2]Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]$p<0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

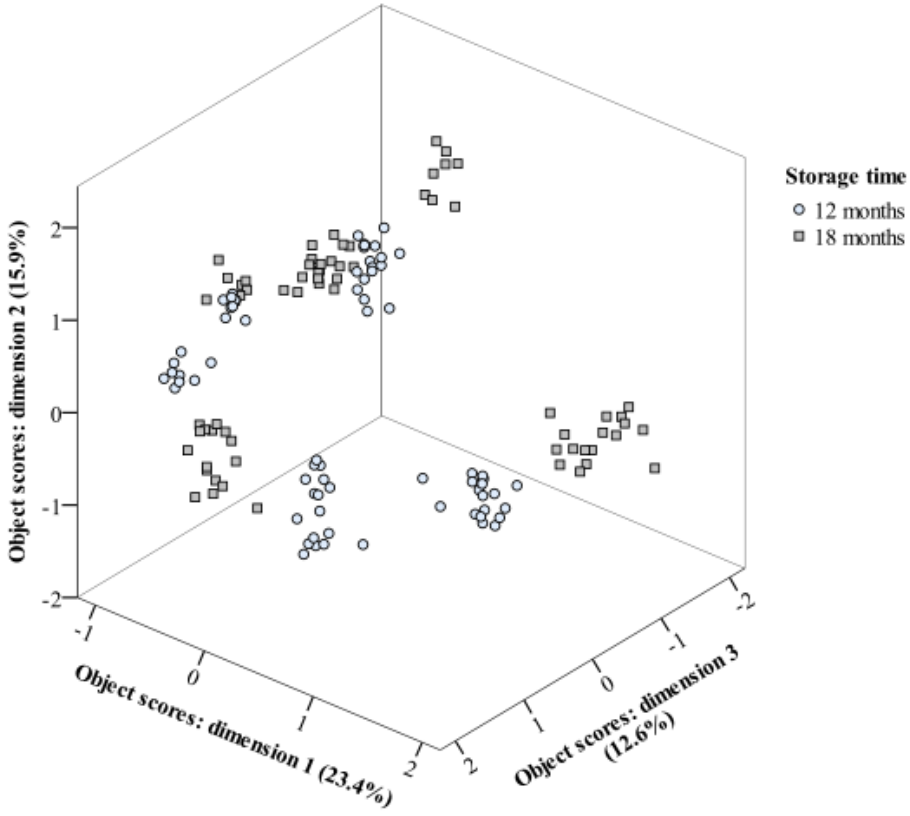
3.2.2.3 Principal component analysis (PCA)

In sections 3.2, the effects of ST and EB were studied in different chemical and antioxidant variables. As it was discussed, some significant changes were observed, but no general trends could be concluded. Therefore, it seemed interesting to ascertain which variables could be more related to each level of both studied factors, when considering the contribution of all assayed variables variations simultaneously. Accordingly, the results were evaluated considering data for all studied EB doses, ST and plant species through a categorical principal components analysis (CATPCA).

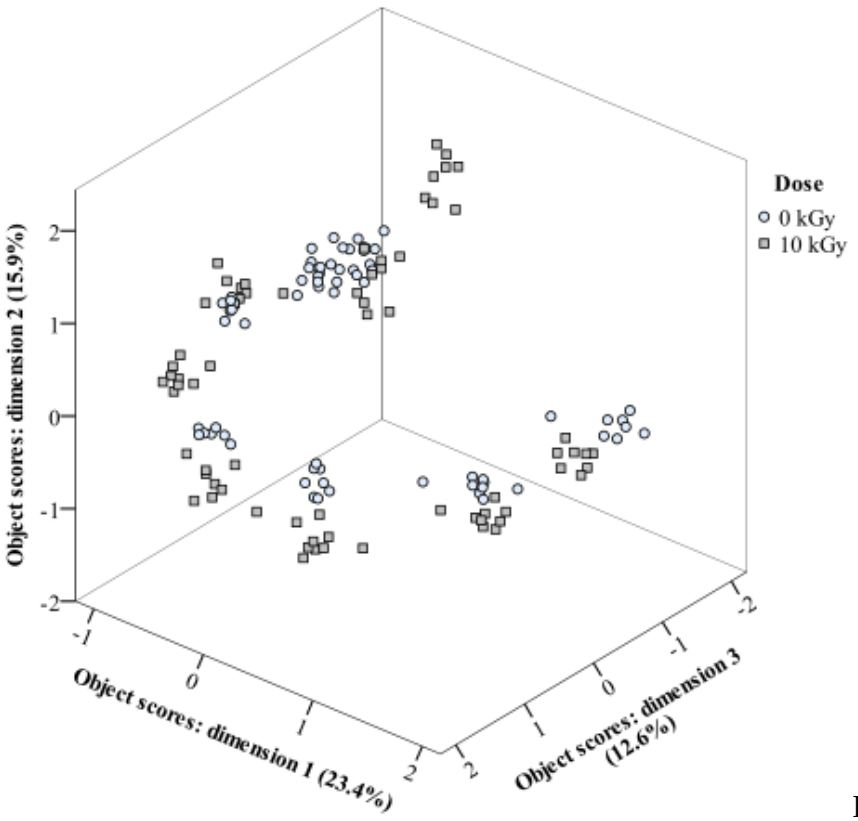
The plot of object scores for different ST and EB (**Figures 40A** and **40B**) in which the first three dimensions are represented (first: Cronbach's α = 0.942; eigenvalue = 13.346; second: Cronbach's α = 0.906; eigenvalue = 9.080; third: Cronbach's α = 0.877; eigenvalue = 7.202) account for most of the variance for all quantified variables (23.4%, 15.9% and 12.6%, respectively). The percentage of variance should ideally be higher, but it would not be possible to plot additional dimensions. As it can be seen, markers corresponding to each factor level (12 months and 18 months, or 0 kGy and 10 kGy) did not cluster in individualized groups. Therefore, there seemed not logical to proceed with the analysis of associations among principal components and variables.

However, when comparing the effects of EB and ST as a function of the plant species, the differences were obvious. Three main groups were formed: one corresponding to *A. citrodora* and *M. officinalis*, and two other groups corresponding to each of the remaining species. According to the correlations among variables and principal components (data not shown to avoid the overlapping of markers), *A. citrodora* and *M. officinalis* were mainly characterized by increases in protein content, malic acid, C18:0, C18:1n9, C23:0, MUFA, β-tocopherol and also in the TBARS formation inhibition (for either aqueous and methanolic extracts). Conversely, these same species suffer important decreases in their contents of fat, sucrose, trehalose, C20:0 and total tocopherols. As observable in **Figure 40C**, markers corresponding to *M. melissophyllum* were localized in a diametrically opposite position; thereby, it could be concluded that this species characterized by the exact inverse variations of those indicated for *A. citrodora* and *M. officinalis*. Finally, *M. piperita* was mainly characterized for presenting high increases in α-tocopherol, C8:0, C15:1 and in the values of DPPH scavenging activity (independently of the type of extract), reducing power (in the methanolic extracts) and β-carotene bleaching inhibition (in methanolic extracts, too); on the other hand, these samples showed significant decreases in glucose, total sugars, C6:0, C13:0 and C17:0.

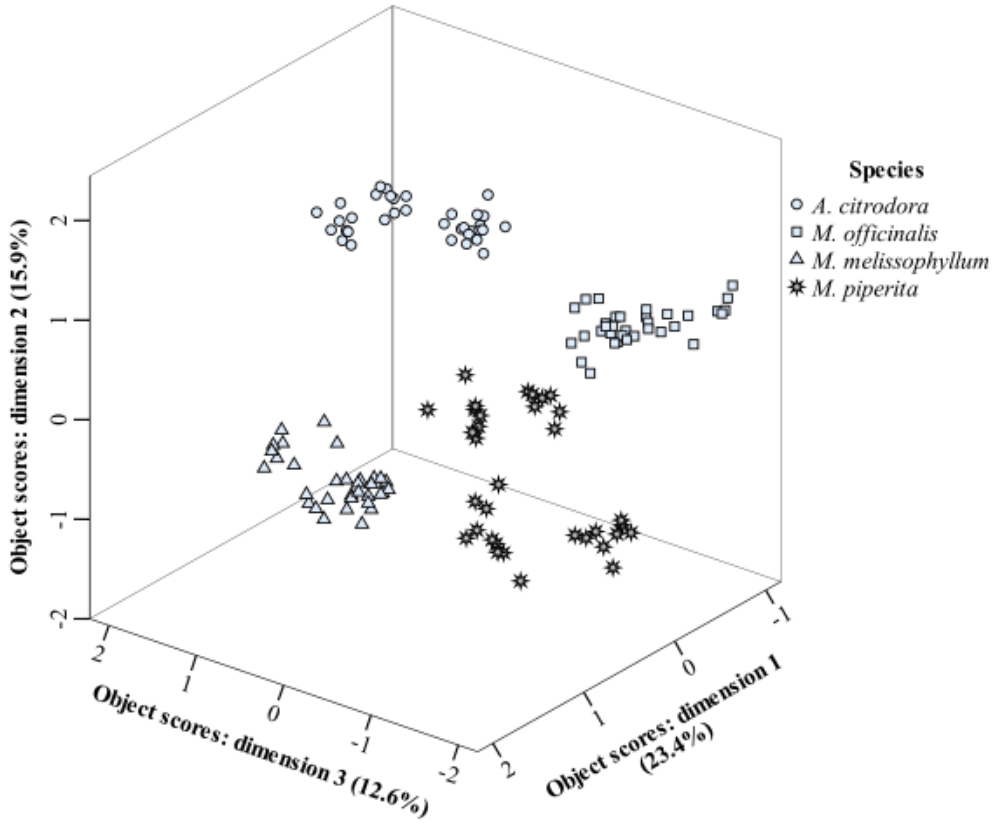
The observed plant species-specific variation may be well explains that general trends regarding the effects of ST or EB could not be concluded. Accordingly, the application of EB in a broader sense still needs further characterization studies, before it can be considered as a practical alternative for herbal plant species.



A



B



C

Figure 40. Canonical discriminant funtions coeficients obtained according to evaluated variables plotted to elucidate differences among storage time (A), electron beam radiation doses (B) and plant species (C).

3.3. Comparative studies with gamma and electron beam radiation

3.3.1 *Arenaria montana* L.: Bioactivity and phytochemical characterization of infusion and methanolic extracts.

3.3.1.1 Evaluation of antioxidant activity

The results obtained in the antioxidant activity evaluation of the infusion and methanolic extracts of *A. montana* are given in **Table 52**. The aqueous extract showed higher antioxidant activity than the methanolic extracts in all the assays, with the exception of the DPPH scavenging activity assay in which both types showed similar results.

The effects of *A. montana* extracts on the growth of five human tumor cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are also collected in **Table 52**. The aqueous extract showed anti-proliferative activity on all the assayed cell lines, while the methanolic extract only presented some activity for HeLa and HepG2 cell lines, which also were the most susceptible (lowest GI_{50} values) to the aqueous extract. Thus, it could be concluded that the bioactive compounds involved in growth inhibition are preferentially extracted in the aqueous preparation. Both the aqueous and methanolic extracts showed some hepatotoxicity on PLP2 non-tumor cells, although at high doses (332.18 mg mL^{-1} and 350.25 mg mL^{-1}, respectively). It should be highlighted that at the doses active against tumor cell lines, the aqueous extract did not show hepatotoxicity.

Trolox and ellipticine were used as positive controls in antioxidant and cytotoxic activities evaluation assays, respectively, but the comparison with the samples should be avoided because they are individual compounds and not mixtures as the studied extracts. To the best of our knowledge, no reports are available on the bioactivity of the aqueous or methanolic extracts of the aforementioned plant.

Table 52. Antioxidant and cytotoxic activity of *Arenaria montana* methanolic and aqueous extracts.

	Methanolic extract	Infusion	Positive control*
Antioxidant activity			
DPPH scavenging activity (EC_{50}, mg/mL)	0.90 ± 0.01[a]	0.93 ± 0.02[a]	0.04 ± 0.00
Reducing power (EC_{50}, mg/mL)	0.82 ± 0.01[a]	0.77 ± 0.02[b]	0.03 ± 0.00
β-carotene bleaching inhibition (EC_{50}, mg/mL)	6.25 ± 0.31[a]	1.71 ± 0.02[b]	0.003 ± 0.00
TBARS inhibition (EC_{50}, mg/mL)	0.90 ± 0.08[a]	0.20 ± 0.02[b]	0.004 ± 0.00
Cytotoxic activity			
MCF-7 (breast carcinoma) (GI_{50}, µg/mL)	>400[a]	130.05±8.05[b]	0.91±0.04
NCI-H460 (non-small cell lung cancer) (GI_{50}, µg/mL)	>400[a]	231.08±5.86[b]	1.42±0.00
HCT-15 (colon carcinoma) (GI_{50}, µg/mL)	>400[a]	183.51±15.54[b]	1.91±0.06
HeLa (cervical carcinoma) (GI_{50}, µg/mL)	329.46±12.46[a]	80.21±6.29[b]	1.14±0.21
HepG2 (hepatocellular carcinoma) (GI_{50}, µg/mL)	308.68±13.25[a]	58.57±6.59[b]	3.22±0.67
Hepatotoxicity PLP2 (GI_{50}, µg/mL)	350.25±5.70[a]	332.18±3.61[b]	2.06±0.03

*Trolox and ellipticine for antioxidant and cytotoxic activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

3.3.1.2 Composition in sugars, organic acids and phenolic compounds

The composition of the samples in sugars and organic acids was analyzed of *A. montana* and the results are shown in **Table 53**. The sugars found were fructose, glucose, sucrose trehalose and raffinose, fructose being the most abundant.

Oxalic, quinic, malic, ascorbic, citric, succinic and fumaric acids were identified and quantified (**Table 53**), oxalic and malic acids being the most abundant ones. Some of these acids (e.g., ascorbic and citric acids) have been reported as having antioxidant capacity and health benefits (Hraš et al., 2000; Kim et al., 2013). Oppositely, excessive intake of oxalic acid has been related with acute oxalate nephropathy and neurotoxicity in humans and animals (Fang et al., 2007).

Table 53. Composition in sugars and organic acids of wild *Arenaria montana*.

Free sugars	g/100 g dw	Organic acids	g/100 g dw
Fructose	5.46 ± 0.53	Oxalic acid	1.93 ± 0.09
Glucose	2.05 ± 0.33	Quinic acid	0.06 ± 0.00
Sucrose	1.41 ± 0.32	Malic acid	1.48 ± 0.00
Trehalose	0.80 ± 0.01	Ascorbic acid	0.02 ± 0.00
Raffinose	0.43 ± 0.00	Citric acid	0.30 ± 0.03
Total sugars	10.15 ± 0.99	Succinic acid	0.28 ± 0.03
		Fumaric acid	0.01 ± 0.00
		Total organic acids	4.07 ± 0.08

dw- dry weight

Ten phenolic compounds were identified in the methanolic extract of *A. montana* (flowers and leafy stems) being all of them flavone derivatives. The HPLC phenolic profile recorded at 370 nm is shown in **Figure 41**, and peak characteristics, identities and quantification are presented in **Table 54**.

Luteolin-6-*C*-glucoside (peak 3) and apigenin 6-*C*-glucoside (peak 6) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards. Peaks 2, 4 and 7 presented similar UV spectra to peak 6 with a λ_{max} at 336–338 nm, suggesting that may derive from apigenin. All these peaks showed fragments ions at *m/z* 311 and 341, corresponding to the aglycone (apigenin) +41 mu and +71 mu, respectively, that are characteristic of *C*-glycosylated flavones (Ferreres et al., 2003). Peaks 2 and 4 had the same pseudomolecular ion $[M - H]^-$ at *m/z* 563 pointing to apigenin bearing pentose and hexose sugar substituents, but yielded different MS^2 fragment ions. According to Ferreres et al. (2003) the ions aglycone + 83 mu and aglycone + 113 mu would typify di-*C*-glycosylated flavones. Thus, the observation of the ions at *m/z* 383 (agl + 113) and 353 (agl + 83) in the case of peak 2 would indicate that both sugars are *C*-attached, which is supported by the losses of -120 mu (ion at *m/z* 443), -180 mu (90 + 90; *m/z* at 383), and 210 mu (120 + 90; *m/z* at 353), characteristic of *C*-glycosylated flavones (Cuyckens and Claeys, 2004). The loss of -120 mu is typical of *C*-attached hexoses, whereas that of -90 mu is observed for *C*-attached pentoses and it is also usual in the case of 6-*C*-hexoses but less common in the case of 8-*C*-hexoses (Rechner et al., 2002). These observations allowed the tentative identification of peak 2 as apigenin 6-*C*-hexoside-8-*C*-pentoside.

The fragmentation of peak 4 would be more coherent with an *O,C*-diglycoside. The loss of -120 mu leading to the ion at *m/z* 443 ($^{0,2}X_0^-$ in **Figure 42**) supported the presence of a *C*-

attached hexose, while the absence of an ion [(M - H) - 90]$^-$ pointed to a 6-*C*-attachment. The lack of an ion [(M - H) - 132]$^-$ from the loss of the pentosyl residue suggested that this sugar was not linked to the aglycone but to the other sugar; this was confirmed by the presence of an abundant [(M - H) - 150]$^-$ ion (Z_1^- in **Figure 42**) at *m/z* 413, which according to Ferreres et al. (2003) would be characteristic of an *O*-attached pentose on the *C*-glycosylating hexose. The *O*-glycosylation should not take place in the positions 6'', 4'' or 3'' of the hexose, otherwise the fragment [(M - H) - 120]$^-$ would not be produced. Finally, the ion at *m/z* 293 would result from the fragment at *m/z* 413 by further loss of a fragment of 120 mu (partial loss of the *C*-attached hexose). All in all, peak 4 could be tentatively identified as apigenin 2''-*O*-pentosyl-6-*C*-hexoside.

Peak 7 ([M - H]$^-$ at *m/z* 605) was 42 mu greater than peak 4 and showed a similar fragmentation pattern, so that it can be assigned to an acetyl derivative of peak 4. The observation of an abundant ion at m/z 413 ([(M - H) - 42 - 150]$^-$) from the loss of the pentose after release of the acetyl residue would confirm that this sugar was *O*-linked to the *C*-hexose. The observation of an ion at *m/z* 431 ([(M - H) - 42 - 132]$^-$) might indicate that the acetyl moiety is attached to the pentose. Further losses of -120 mu and -90 mu from that ion suggested that the hexose was 6-*C*-attached. Thus, the peak was tentatively assigned as apigenin 2''-*O*-acetylpentosyl-6-*C*-hexoside.

Peaks 1 and 10 would also derive from apigenin owing to the presence of the fragments at *m/z* 311 and 341. In the case of peak 1 ([M - H]$^-$ at *m/z* 593) the observation of the ions at *m/z* 473 and 353 from two consecutive losses of -120 mu would point to a di-*C*-hexosyl derivative, so that it could be tentatively associated with apigenin 6-*C*-hexoside-8-*C*-hexoside. Peak 10 ([M - H]$^-$ at *m/z* 769) had a mass 176 mu greater than apigenin di-hexoside suggesting acylation with ferulic acid, which is coherent with its delayed elution. The cleavage of the feruloyl residue yielded the ion at *m/z* 593 that would give rise to the formation of the abundant ion at *m/z* 413 by loss of a fragment of 180 mu, which according to Ferreres et al. (2003) would be characteristic of an *O,C*-dihexoside. On the other hand, the loss of -120 mu to produce the ion at *m/z* 649 confirmed the existence of a hexose *C*-attached to the aglycone, and also that the feruloyl residue was linked to the second *O*-attached hexose. In similarity with the other apigenin *O,C*-diglycosides observed in the sample, a 6-*C*-attachment might be supposed. Thus, peak 10 was tentatively assigned as apigenin 2''-*O*-feruloylhexosyl-6-*C*-hexoside.

The fragmentation pattern of peak 5 ([M - H]$^-$ at *m/z* 769) would also be coherent with a structure similar to peak 10 although having luteolin as the aglycone. Thus, the losses of -120 mu (ion at *m/z* 489) and -176 mu (ion at *m/z* 609), and further -180 mu (ion at *m/z* 429) would

point to a *O,C*-dihexoside. The fragment at m/z 489 (loss of -120 mu from the ion at *m/z* 609) would confirm the presence of the *C*-attached hexose, and the ion at *m/z* 339 (loss of -90 mu from the ion at *m/z* 429) would suggest a 6 C attachment. Thus, the peak was tentatively identified as luteolin 2''-*O*-feruloylhexosyl-6-*C*-hexoside.

The pseudomolecular ion of peak 9 ($[M - H]^-$ at *m/z* 799) was 14 mu greater than peak 5 and showed a similar fragmentation pattern, with characteristic product ions resulting from the losses of fragments of 120 mu (ion at *m/z* 679), 176 mu (ion at *m/z* 623), 176 + 180 mu (ion at *m/z* 609) and 176 + 180 + 120 mu (ion at *m/z* 323). The observation of fragments at *m/z* 371 (aglycone + 71 mu) and 323 (aglycone + 41–18 mu) would support the presence of methyl-luteolin as the aglycone (Ferreres et al., 2003). Therefore, the compound might be tentatively assigned as methyl-luteolin 2''-*O*-feruloylhexosyl-*C*-hexoside.

Finally, peak 8 presented a pseudomolecular ion $[M - H]^-$ at *m/z* 635 and fragment ions at *m/z* 593 (-42 mu) and 515 (-120 mu), indicating the presence of an acetyl residue and a *C*-attached hexose. The abundant ion at *m/z* 443 by loss of a fragment of 150 mu from the ion at *m/z* 515 would indicate the presence of an *O*-attached pentose on the *C*-glycosylating hexose (Ferreres et al., 2003). The fragment at *m/z* 461 would result from the loss of the pentosyl residue (-132 mu) from the ion at *m/z* 515, and the ions at *m/z* 371 and 323 would also support methyl-luteolin as the aglycone. Thus, the compound was tentatively identified as methyl-luteolin 2''-*O*-acetylpentosyl-*C*-hexoside.

Methyl-luteolin 2''-*O*-feruloylhexosyl-*C*-hexoside (peak 9) was the main flavone found (450.26 mg per 100 g dw) in *A. montana* (**Table 54**), the total amount of flavones being 1204.63 mg per 100 g dw. As far as we know, there are no data regarding the phenolic composition in this plant, and thus these values cannot be compared to the literature.

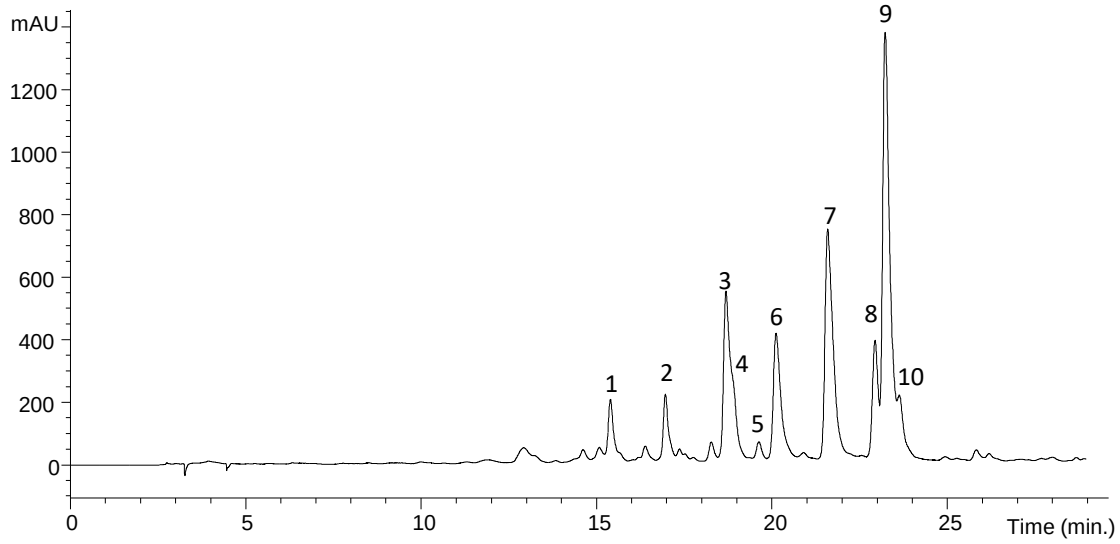


Figure 41. HPLC phenolic profile of wild *Arenaria montana*, obtained at 370 nm.

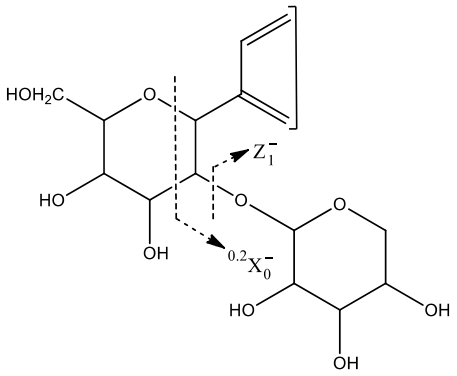


Figure 42. Fragmentation of x”-glycosyl-*C*-glycosylflavones (adapted from Ferreres et al., 2007).

Table 54. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of phenolic compounds in wild *Arenaria montana***.**

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/100 g dw)
1	14.7	330	593	473(4),353(6),341(19),311(65),283(12)	Apigenin 6-*C*-hexoside-8-*C*-hexoside	8.71 ± 0.32
2	15.4	336	563	443(29),383(33),353(34),311(4),297(6)	Apigenin 6-*C*-hexoside-8-*C*-pentoside	41.63 ± 0.27
3	16.9	350	447	429(12),357(50),327(58),285(12)	Luteolin-6-*C*-glucoside	22.57 ± 0.84
4	18.7	338	563	443(7),413(62),341(22),311(22),293(72)	Apigenin 2''-*O*-pentosyl-6-*C*-hexoside	123.74 ± 2.13
5	18.9	348	785	665(11),609(12),489(4),429(26),339(8),285(4)	Luteolin 2''-*O*-feruloylhexosyl-6-*C*-hexoside	37.95 ± 0.50
6	20.1	336	431	341(72),311(100)	Apigenin-6-*C*-glucoside	159.91 ± 1.83
7	21.6	336	605	563(4),431(6),413(44),341(13),311(13)	Apigenin 2''-*O*-acetylpentosyl-6-*C*-hexoside	179.84 ± 0.88
8	22.9	350	635	593(4),515(7),461(5),443(50),371(19),323(68)	Methyl-luteolin 2''-*O*-acetylpentosyl-*C*-hexoside	88.36 ± 1.44
9	23.2	330	799	679(7),623(21),443(64),371(6),323(33)	Methyl-luteolin 2''-*O*-feruloylhexosyl-*C*-hexoside	450.26 ± 3.50
10	23.6	332	769	649(14),593(19),443(18),413(60),341(7),311(5)	Apigenin 2''-*O*-feruloylhexosyl-6-*C*-hexoside	91.68 ± 2.54
					Total Flavones	1204.63 ± 5.76

3.3.1.3 Composition of fatty acids and tocopherols

The results obtained in the analysis of fatty acids and tocopherols of *A. montana* are shown in **Table 55**. Up to 28 fatty acids were detected, among which polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Linoleic acid (C18:2n6) was the majority fatty acid. The interest of this compound has increased over time, since it is an essential fatty acid for human development and also plays an active role in good general health (Choque et al., 2014), being related with prevention of cardiovascular diseases and some types of cancer (Whelan, 2008). PUFA, besides acting as endogenous mediators of cell signaling and being involved in regulating gene expression, are also precursors of eicosanoids, such as prostaglandins and leukotrienes, as well as docosanoids, as protectins or resolvins (Choque et al., 2014).

α-Tocopherol was the most abundant tocopherol in *A. montana*, which is also found in the isoforms λ- and δ- (**Table 55**). Tocopherols are very important natural antioxidants and can be used to delay rancidity in fatty materials in manufactured foods; they have also been suggested to help counteract undesirable effects associated to ageing and prevent oxidative stress-related diseases, such as cancer, neurodegenerative and heart diseases (Halliwell, 1999; Kagan et al., 2003).

Table 55. Composition of fatty acids and tocopherols in wild *Arenaria montana*.

Fatty acids	Relative percentage	Fatty acids	Relative percentage
C6:0	1.32 ± 0.01	C18:3n3	15.94 ± 0.14
C8:0	0.30 ± 0.01	C20:0	3.84 ± 0.67
C10:0	0.12 ± 0.03	C20:1	0.52 ± 0.29
C12:0	0.66 ± 0.21	C20:2	0.70 ± 0.09
C13:0	0.15 ± 0.00	C20:3n6	1.99 ± 0.04
C14:0	1.37 ± 0.27	C20:4n6	1.75 ± 0.06
C14:1	0.55 ± 0.04	C20:3n3+C21:0	0.50 ± 0.00
C15:0	0.93 ± 0.17	C20:5n3	0.31 ± 0.08
C15:1	0.09 ± 0.00	C22:0	3.58 ± 0.23
C16:0	22.18 ± 0.40	C22:1n9	0.08 ± 0.00
C16:1	0.36 ± 0.18	C23:0	0.20 ± 0.06
C17:0	0.68 ± 0.03	C24:0	3.45 ± 0.46
C18:0	4.38 ± 0.10	SFA	43.16 ± 0.38
C18:1n9	8.57 ± 0.28	MUFA	10.16 ± 0.43
C18:2n6	23.39 ± 0.66	PUFA	46.68 ± 0.82
C18:3n6	2.11 ± 0.02		
Tocopherols	mg/100 g dw		
α-tocopherol	1.22 ± 0.21		
γ-tocopherol	0.23 ± 0.02		
δ-tocopherol	0.84 ± 0.08		
Total tocopherols	2.29 ± 0.31		

dw-dry weight. SFA-Saturated fatty acids; MUFA-Monounsaturated fatty acids; PUFA-Polyunsaturated fatty acids.

3.3.1.4 Effects of irradiation on chemical composition

The proximate composition (in dry basis) of *A. montana* was dominated by the carbohydrates content, followed by ash, protein and fat levels (**Table 56**). Except for the fat content in gamma irradiated samples (p = 0.051), all the nutritional components showed significant changes in response to irradiation treatment, either electron beam (e-beam) or gamma radiation. In the first case (e-beam), fat and protein contents decreased in irradiated samples, while some increase was produced in ash contents; in accordance to those changes a slight increase was observed in carbohydrates levels (obtained by difference). The effect produced by gamma radiation on the nutritional variables was similar to the one observed for electron beam radiation, except for the already pointed out lack of variation in fat content and a less pronounced decrease observed in proteins.

Table 56. Proximate composition of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

		Fat (g/100 g fw)	Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)
E-beam irradiation						
Dose	0 kGy	1.4±0.1^{a}	4.9±0.3^{a}	8.4±0.3^{ab}	85.4±0.3^{b}	373±1^{a}
	1 kGy	1.2±0.1^{b}	3.9±0.3^{b}	8.2±0.2^{b}	86.7±0.4^{a}	373±1^{a}
	10 kGy	1.2±0.1^{b}	3.6±0.2^{c}	8.6±0.2^{a}	86.7±0.4^{a}	372±1^{b}
p-values	Homoscedasticity[2]	0.451	0.891	0.111	0.231	0.058
	Normal distribution[3]	0.373	0.080	0.346	0.102	0.794
	1-way ANOVA[4]	<0.001	<0.001	0.007	<0.001	0.004
Gamma irradiation						
Dose	0 kGy	1.7±0.1	4.4±0.5^{b}	9.6±0.2^{a}	84.3±0.5^{b}	370±1^{b}
	1 kGy	1.7±0.1	5.1±0.3^{a}	9.1±0.2^{b}	84.1±0.3^{b}	372±1^{a}
	10 kGy	1.6±0.1	3.8±0.2^{c}	9.5±0.2^{a}	85.1±0.2^{a}	370±1^{b}
p-values	Homoscedasticity[2]	0.824	0.011	0.851	0.004	0.760
	Normal distribution[3]	0.448	0.020	0.621	0.106	0.148
	1-way ANOVA[4]	0.051	<0.001	<0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, p>0.05; heteroscedasticity, p<0.05. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]p<0.05 indicates that the mean value of the evaluated variable of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly (p<0.05).

Regarding free sugars (**Table 57**), fructose was quantified as the main compound (4.2-4.7 g/100 g dw), followed by glucose (1.5-1.7 g/100 g dw), sucrose (0.4-1.0 g/100 g dw), trehalose (0.19-0.23 g/100 g dw) and raffinose (0.09-0.13 g/100 g dw). The observed profile and individual proportions are similar to those previously found in non-irradiated samples (see section 3.3.1.2), despite the overall quantities now detected are somewhat lower. Almost none of the characterized molecules suffer significant changes (except for sucrose with both irradiation types and total sugars when samples were treated with e-beam radiation), which can be considered a positive result since sugars are often pointed out as good indicators of an adequate conservation technology (Barreira et al., 2010).

Concerning the organic acids profile (**Table 57**), oxalic acid was the prevalent form (2.2-2.6 g/100 g dw), followed by malic acid (0.9-1.1 g/100 g dw), succinic acid (0.5-0.7 g/100 g dw), citric acid (0.29-0.38 g/100 g dw), quinic acid and fumaric acid (which were detected below the limit of quantification). This result is also closely related to the mentioned previous analysis (section 3.3.1.2). In this case, the dissimilarity observed among the effect produced by each type of irradiation is quite interesting. While e-beam radiation did not cause statistically significant changes in any case, gamma radiation induced the opposite effect, *i.e.*, all the quantified organic acids presented significant changes, with a clear tendency to increase with irradiation. This latter observation is in agreement with a previous study conducted to evaluate the effects of gamma radiation and accelerated electrons on organic acids (Semelová et al., 2008).

Table 57. Free sugars and organic acids composition (g/100 g dw) of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

		Free sugars						Oxalic acids				
		Fructose	Glucose	Sucrose	Trehalose	Raffinose	Total	Oxalic acid	Malic acid	Citric acid	Succinic acid	Total
E-beam irradiation												
Dose	0 kGy	4.5±0.3	1.6±0.1	0.6±0.1^{b}	0.22±0.03	0.11±0.02	7.1±0.3^{ab}	2.3±0.1	0.9±0.1	0.30±0.05	0.5±0.1	4.0±0.3
	1 kGy	4.2±0.3	1.5±0.1	0.9±0.1^{a}	0.19±0.02	0.12±0.01	6.9±0.4^{b}	2.2±0.2	0.9±0.1	0.29±0.04	0.5±0.1	3.8±0.2
	10 kGy	4.4±0.4	1.7±0.2	1.0±0.1^{a}	0.21±0.04	0.13±0.02	7.3±0.3^{a}	2.2±0.2	0.9±0.1	0.31±0.05	0.5±0.1	3.9±0.3
p-values	Homoscedasticity[2]	0.435	0.462	0.119	0.221	0.842	0.560	0.579	0.669	0.795	0.072	0.737
	Normal distribution[3]	0.123	0.712	0.150	0.206	0.818	0.390	0.762	0.688	0.360	0.377	0.852
	1-way ANOVA[4]	0.195	0.135	<0.001	0.217	0.082	0.034	0.182	0.369	0.743	0.988	0.278
Gamma irradiation												
Dose	0 kGy	4.7±0.3	1.7±0.1	0.6±0.1^{b}	0.23±0.05	0.09±0.01	7.3±0.4	2.3±0.1^{b}	0.9±0.1^{b}	0.30±0.04^{b}	0.5±0.1^{b}	4.0±0.3^{b}
	1 kGy	4.5±0.3	1.7±0.2	0.4±0.1^{c}	0.20±0.03	0.10±0.02	7.0±0.5	2.6±0.1^{a}	1.1±0.1^{a}	0.37±0.05^{a}	0.7±0.1^{a}	4.8±0.3^{a}
	10 kGy	4.3±0.3	1.6±0.2	0.9±0.1^{a}	0.21±0.02	0.09±0.02	7.2±0.4	2.6±0.2^{a}	1.0±0.1^{a}	0.38±0.04^{a}	0.6±0.1^{a}	4.6±0.3^{a}
p-values	Homoscedasticity[2]	0.993	0.685	0.202	0.086	0.510	0.514	0.557	0.737	0.700	0.526	0.987
	Normal distribution[3]	0.331	0.445	0.069	0.711	0.044	0.747	0.587	0.657	0.404	0.574	0.800
	1-way ANOVA[4]	0.157	0.105	<0.001	0.102	0.337	0.198	<0.001	0.002	0.001	<0.001	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

The individual fatty acids (FA) profiles are depicted in **Table 58**. Besides the presented FA, C6:0, C8:0, C10:0, C13:0, C16:1, C20:2, C20:3n3+C21:0 and C23:0 were also detected, but their relative percentages laid below 0.5%. In general, the detected profile is shows some similarity to the one reported before (section 3.3.1.2), despite the differences in linoleic acid. The main saturated fatty acid (SFA) was palmitic acid (22-26%), while oleic acid (10.1-13.4%) and α-linolenic acid (17.4-22.7%) were the predominant monounsaturated and polyunsaturated forms, respectively. Nearly half of the detected forms are polyunsaturated fatty acids (PUFA), among which ALA (α-linolenic acid) and GLA (γ-linolenic acid) deserve special attention. Despite the significant changes induced by both irradiation types in all FA, the effect of e-beam treatment was more pronounced. Considering that the results are presented in relative percentage, if some FA decrease significantly, others will, inevitably, increase. Nevertheless, when evaluated as grouped SFA, MUFA and PUFA, it became clear that e-beam treatment increased the relative percentages of SFA and MUFA, while reducing those of PUFA, indicating that this source of radiation might be more deleterious for this type of fatty acids.

Table 58. Fatty acids profile (relative percentage) of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

	E-beam irradiation			*p*-values			Gamma irradiation			*p*-values		
	0 kGy	1 kGy	10 kGy	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]	0 kGy	1 kGy	10 kGy	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
C12:0	0.9±0.1^{b}	0.7±0.1^{c}	1.0±0.1^{a}	0.050	0.435	<0.001	1.0±0.1^{b}	1.3±0.1^{a}	1.0±0.1^{b}	0.219	0.809	<0.001
C14:0	1.4±0.1^{b}	1.3±0.1^{b}	2.8±0.2^{a}	0.496	<0.001	<0.001	1.9±0.1^{a}	1.5±0.1^{b}	1.6±0.2^{b}	0.636	0.661	<0.001
C15:0	0.9±0.1^{b}	0.8±0.1^{b}	1.0±0.1^{a}	0.740	0.142	<0.001	1.0±0.1^{a}	0.7±0.1^{b}	0.8±0.1^{b}	0.680	0.101	<0.001
C16:0	26±1^{a}	24±1^{b}	23±1^{b}	0.410	0.344	<0.001	22±1^{b}	23±1^{ab}	24±1^{a}	0.576	0.670	0.001
C17:0	1.1±0.1^{b}	1.3±0.1^{a}	1.3±0.1^{a}	0.316	0.377	<0.001	1.2±0.1^{a}	1.1±0.1^{b}	1.3±0.1^{a}	0.278	0.312	0.001
C18:0	5.0±0.3^{c}	5.7±0.2^{b}	6.6±0.3^{a}	0.210	0.185	<0.001	6.0±0.2^{a}	5.5±0.2^{b}	5.6±0.3^{b}	0.654	0.624	<0.001
C18:1n9	10.1±0.4^{b}	13.4±0.3^{a}	13.2±0.5^{a}	0.563	<0.001	<0.001	12.2±0.3^{a}	11.4±0.2^{b}	10.7±0.3^{c}	0.518	0.287	<0.001
C18:2n6	17.2±0.5^{a}	17.5±0.4^{a}	14.7±0.4^{b}	0.627	0.001	<0.001	14.8±0.4^{b}	16.6±0.3^{a}	16.3±0.4^{a}	0.496	0.011	<0.001
C18:3n6	2.7±0.1^{a}	2.8±0.2^{a}	2.4±0.2^{b}	0.389	0.631	<0.001	2.6±0.2^{b}	3.1±0.2^{a}	2.8±0.3^{b}	0.765	0.593	0.002
C18:3n3	21.6±0.5^{a}	19.6±0.5^{b}	17.4±0.5^{c}	0.998	0.107	<0.001	22.0±0.4^{b}	22.4±0.4^{ab}	22.7±0.3^{a}	0.504	0.331	0.002
C20:0	2.2±0.1^{b}	2.3±0.2^{b}	2.5±0.2^{a}	0.110	0.776	0.001	2.0±0.1^{a}	1.7±0.2^{b}	1.9±0.2^{a}	0.453	0.471	0.001
C20:1	0.5±0.1	0.6±0.1	0.6±0.1	0.004	0.206	0.218	0.6±0.1^{b}	0.8±0.1^{a}	0.6±0.1^{b}	0.579	0.599	<0.001
C20:3n6	1.4±0.1^{a}	1.1±0.1^{b}	1.1±0.1^{b}	0.177	0.335	<0.001	1.5±0.1^{a}	1.4±0.2^{a}	1.2±0.2^{b}	0.466	0.170	0.002
C20:4n6	1.9±0.1^{b}	1.9±0.1^{b}	2.9±0.2^{a}	0.004	<0.001	<0.001	3.6±0.2^{a}	2.6±0.2^{b}	2.4±0.2^{b}	0.740	0.002	<0.001
C20:5n3	0.8±0.1^{b}	0.5±0.1^{c}	1.1±0.1^{a}	0.002	0.008	<0.001	0.7±0.1^{a}	0.6±0.1^{b}	0.5±0.1^{c}	0.089	0.114	<0.001
C22:0	3.2±0.2^{a}	2.9±0.1^{b}	2.9±0.2^{b}	0.038	0.210	<0.001	3.1±0.3^{a}	1.9±0.2^{c}	2.4±0.3^{b}	0.309	0.195	<0.001
C24:0	1.3±0.1^{b}	1.4±0.1^{b}	1.6±0.2^{a}	<0.001	0.020	<0.001	1.7±0.1^{a}	1.2±0.2^{c}	1.4±0.1^{b}	0.121	0.962	<0.001
SFA	42.7±0.5^{b}	41.8±0.5^{c}	45.4±0.5^{a}	0.881	0.017	<0.001	41±1^{a}	39±1^{b}	41±1^{a}	0.358	0.583	0.001
MUFA	10.9±0.3^{b}	14.2±0.2^{a}	14.0±0.5^{a}	0.193	<0.001	<0.001	13.1±0.3^{a}	13.0±0.3^{a}	12.0±0.2^{b}	0.467	0.014	<0.001
PUFA	46.4±0.5^{a}	44.0±0.5^{b}	40.6±0.5^{c}	0.709	0.030	<0.001	46±1^{b}	48±1^{a}	47±1^{ab}	0.107	0.330	0.002

[1]The results are presented as the mean±SD. [2]Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

From a qualitative point of view, the individual tocopherols proportions are similar to dose indicated in section 3.3.1.2, although the higher contents herein (**Table 59**). α-Tocopherol was also the main isoform, but in this case the determined concentrations are nearly 20-fold greater than those of the remaining vitamers. In what concerns the main subject of this study, the irradiation treatment caused statistically significant changes in the tocopherols contents, especially in samples irradiated at 10 kGy. The significant effect of irradiation over the tocopherols content was previously reported (section 3.1.2.1; Taipina et al., 2009), being probably associated to their oxidative instability (Warner et al., 2008).

Table 59. Tocopherols composition (mg/100 g dw) of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

		α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Tocopherols
		E-beam irradiation			
Dose	0 kGy	4.6±0.3^{a}	0.26±0.03^{a}	0.29±0.03^{a}	5.2±0.3^{a}
	1 kGy	3.9±0.2^{b}	0.23±0.03^{ab}	0.28±0.03^{ab}	4.4±0.3^{b}
	10 kGy	3.7±0.3^{b}	0.21±0.04^{b}	0.25±0.03^{b}	4.2±0.3^{b}
p-values	Homoscedasticity[2]	0.375	0.374	0.895	0.595
	Normal distribution[3]	0.123	0.138	0.247	0.231
	1-way ANOVA[4]	<0.001	0.040	0.029	<0.001
		Gamma irradiation			
Dose	0 kGy	4.8±0.2^{a}	0.28±0.03^{a}	0.16±0.02^{ab}	5.3±0.2^{a}
	1 kGy	4.8±0.1^{a}	0.29±0.03^{a}	0.15±0.01^{b}	5.2±0.2^{a}
	10 kGy	3.5±0.2^{b}	0.20±0.03^{b}	0.19±0.03^{a}	3.8±0.2^{b}
p-values	Homoscedasticity[2]	0.435	0.691	0.005	0.342
	Normal distribution[3]	<0.001	0.526	0.120	<0.001
	1-way ANOVA[4]	<0.001	<0.001	0.004	<0.001

[1]The results are presented as the mean±SD. [2]Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [4]$p<0.05$ indicates that the mean value of the evaluated variable of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, values within a column with different letters differ significantly ($p<0.05$).

3.3.1.5 Effects of irradiation on the antioxidant activity

The effects induced by gamma and e-beam radiation on the antioxidant activity of *A. montana* were compared both in infusions and methanolic extracts (**Table 60**). Four *in vitro* assays were applied: scavenging effects on DPPH radicals, reducing power, inhibition of β-carotene bleaching and thiobarbituric acid reactive substances (TBARS) formation inhibition. Likewise, a quantification of total phenolic compounds was also performed (**Table 60**). The methanolic extracts showed higher (approximately threefold) antioxidant activity than the corresponding infusions in all performed assays. In agreement with these results, the quantities of phenolic compounds were nearly threefold lower in the infusions. Furthermore, *A. montana* extracts were particularly active as inhibitors of TBARS formation, as indicated by the lowest EC_{50} values determined in this case.

Independently of the irradiation type, the antioxidant activity decreased in the infusions as a consequence of the irradiation treatment, an effect that was also observed in the methanolic extracts of the *A. montana* samples treated with gamma radiation. By contrast, the effect observed in the methanolic extracts submitted to e-beam radiation showed a general tendency to increased antioxidant activity (except for the β-carotene bleaching inhibition), indicating a higher suitability of electron beam radiation for this particular purpose.

Table 60. Antioxidant properties and total phenolic contents in extracts from *Arenaria montana* submitted to electron beam or gamma irradiation (GI). EC50 values (µg/mL) are presented for all assays except phenolic compounds, expressed as mg GAE/g extract. The results are presented as the mean±SD.

		DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		TBARS formation inhibition		Total phenolics	
		Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
Electron beam											
	0 kGy	3532±175^{b}	988±20^{a}	1592±15^{c}	528±3^{a}	3658±120^{c}	1772±52^{b}	310±4^{c}	97±2^{a}	40±1^{a}	102±5^{c}
	1 kGy	3998±147^{a}	813±80^{b}	1816±12^{b}	509±4^{b}	4230±227^{b}	1450±131^{c}	365±35^{b}	60±2^{c}	35±1^{b}	109±1^{b}
	10 kGy	3945±338^{a}	631±34^{c}	1954±8^{a}	441±3^{c}	7210±517^{a}	1906±70^{a}	427±29^{a}	72±2^{b}	33±1^{c}	119±1^{a}
p-values	Homoscedasticity[1]	0.011	<0.001	0.198	0.604	<0.001	0.001	0.003	0.684	0.113	<0.001
	Normal distribution[2]	0.750	0.003	0.001	<0.001	<0.001	0.054	0.003	0.001	0.001	0.008
	1-way ANOVA[3]	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Gamma irradiation											
	0 kGy	3475±228^{b}	972±55^{b}	1613±12^{b}	520±8^{a}	2895±173^{b}	665±9^{c}	487±41^{b}	164±3^{c}	37±1^{a}	116±2^{c}
	1 kGy	3619±109^{b}	958±26^{b}	1619±18^{b}	498±13^{b}	4489±325^{a}	875±18^{b}	579±22^{a}	230±2^{b}	37±1^{a}	122±1^{a}
	10 kGy	3942±86^{a}	1962±160^{a}	1709±18^{a}	507±2^{b}	4324±144^{a}	1134±21^{a}	633±107^{a}	245±3^{a}	35±1^{b}	119±1^{b}
p-values	Homoscedasticity[1]	<0.001	<0.001	0.420	0.003	0.052	0.012	0.003	0.013	<0.001	0.001
	Normal distribution[2]	0.043	<0.001	0.002	0.102	0.001	0.001	0.027	<0.001	0.008	0.014
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MeOH- Methanol extracts; GAE- Gallic acid equivalents; [1]Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p>0.05$; heteroscedasticity, $p<0.05$. [2]Normal distribution of the residuals was evaluated using Shapiro-Wilk test. [3]$p<0.05$ indicates that the mean value of the evaluated variable of at least one dose differs from the others. For each species, values within a column with different letters differ significantly ($p<0.05$).

3.3.1.6 Linear discriminant analysis

As indicated by the results presented in **Tables 56-60**, most of the characterized variables (with the exceptions of most free sugars and organic acids) showed statistically significant differences when submitted to gamma or e-beam radiation treatment. To take this comparative study a step further, the overall significance of the detected differences was also evaluated by evaluating which statistically significant differences maintain their relevance when compared globally. Accordingly, all results were analysed simultaneously by applying two sequential LDA: initially the results were grouped according to the irradiation type, while in the second case the grouping criterion was based on the irradiation dose. The significant independent variables were selected using the stepwise procedure of the LDA, according to the Wilks' λ test, which maintains only those variables with a statistically significant classification ability ($p < 0.05$).

The two discriminant functions plotted in **Figure 43**, included 100.0% of the observed variance (first: 80.0%, second: 20.0%). As an initial result, the reduction in the variables number was noteworthy. From the initial 58 considered items, only 11 (fat, carbohydrates, raffinose, C6:0, C20:4n6, C20:5n3, C24:0, reducing power in infusions, TBARS formation inhibition and β-carotene bleaching inhibition in methanolic extracts, and total phenolic content in infusions) were selected as having discriminant ability. Concerning the correlation between the selected discriminating variables and the canonical discriminant functions, function 1 was more correlated with TBARS formation inhibition in methanolic extracts (which present higher EC_{50} values in gamma irradiated samples) and fat (also higher values in gamma irradiated samples), separating mostly gamma irradiated samples from the remaining groups. Function 2, in turn, was more correlated to the reducing power (lower in e-beam irradiated samples) and phenolic content in the *A. montana* infusions (lower in e-beam irradiated samples), clearly separating the markers corresponding to e-beam irradiated samples from non-irradiated ones. The classification performance was 100% accurate, either for the originally grouped cases, as well as for the cross-validated cases.

When a similar assay was conducted to evaluate the variables undergoing the most relevant changes once submitted to different irradiation doses (independently of being generated by gamma rays or accelerated electrons), the two discriminant functions (**Figure 44**) also included 100.0% of the observed variance (first: 78.8%, second: 20.2%).

The reduction in the variables number was notable again, selecting carbohydrates, C6:0, C8:0, C20:4n6, C20:5n3, SFA, γ-tocopherol, reducing power in infusions, DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition in

methanolic extracts, and total phenolic content in the infusions as the more discriminant variables. Function 1 was highly correlated with reducing power (higher EC_{50} values at 10 kGy) and phenolic content (lower in samples irradiated at 10 kGy), clearly separating the markers corresponding to the 10 kGy dose. Function 2, on the other hand, was more correlated to C6:0 (higher in samples irradiated at 1 kGy) and SFA (lower in samples at 1 kGy), particularly contributing to separate the markers corresponding to samples irradiated with 1 kGy (independently of irradiation source). The classification performance was 100% accurate, either for the originally grouped cases, as well as for the cross-validated cases.

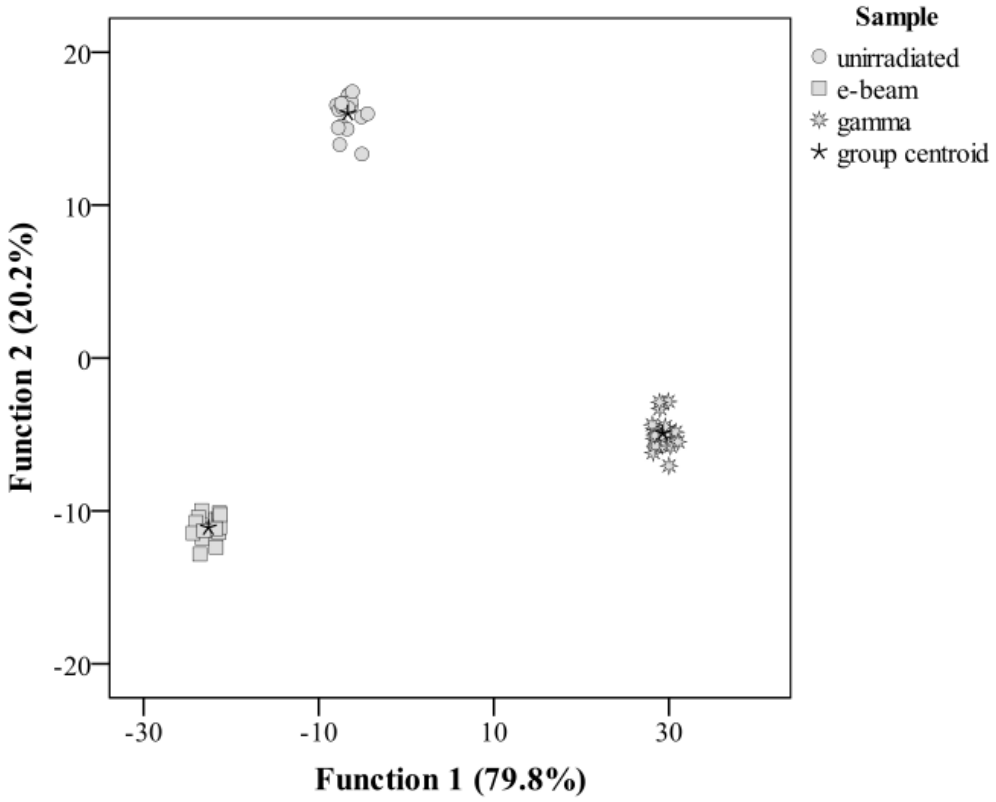


Figure 43. Canonical discriminant function coefficients defined from the evaluated variables plotted to highlight differences among irradiation technologies.

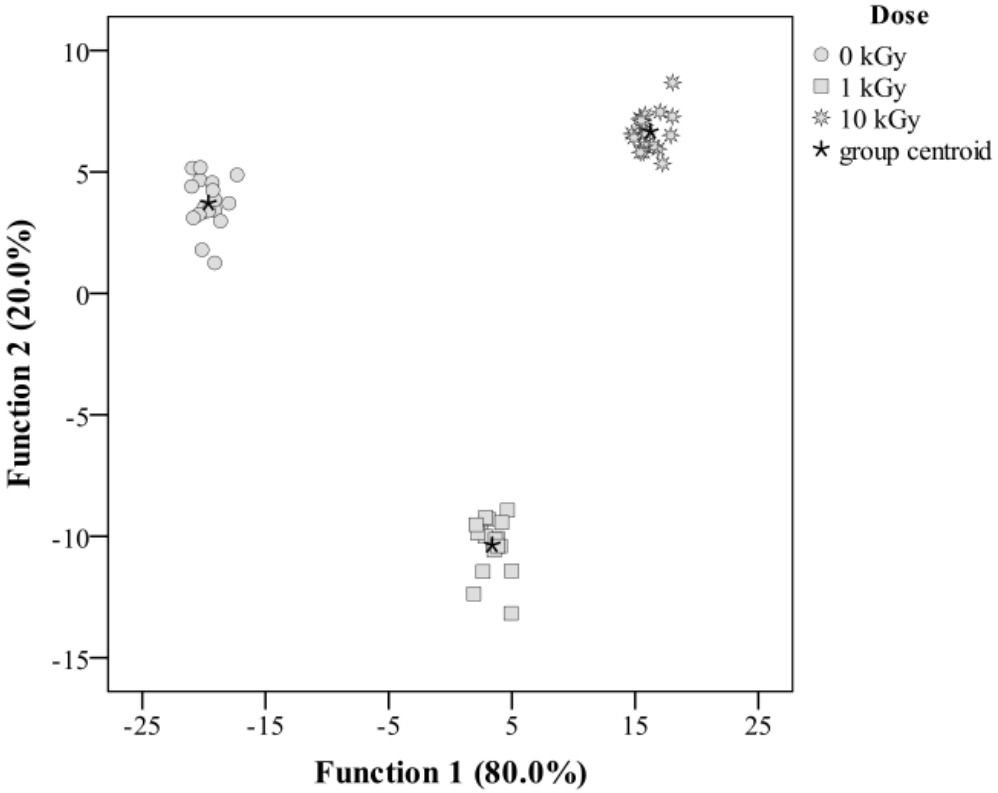


Figure 44. Canonical discriminant function coefficients defined from the evaluated variables plotted to highlight differences among irradiation doses.

3.3.2 *Melissa officinalis* L. and *Melittis melissoffillum* L. infusions: Effects of irradiation on phenolic compounds.

3.3.2.1 Individual phenolic compounds

HPLC-DAD-MS data and tentative identification of phenolic compounds detected in the infusions of bastard balm and lemon balm are presented in **Table 61** and **62**, respectively, and characteristic HPLC chromatograms are shown in **Figure 45**. In bastard balm, six compounds were detected, three phenolic acid derivatives and three coumarin derivatives (**Table 61**).

Peaks 1^{BB} and 3^{BB} were identified as 3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid (chlorogenic acid), respectively, based on their UV and mass spectra according to previous identifications made in our laboratory in different plant materials (e.g., Rodrigues et al., 2012; Martins et al., 2014; Jabeur et al., 2016) and also described in this Thesis for the samples of *M. piperita* (section 3.1.4.2). A compound with the same UV and mass characteristics of peak 4^{BB} was reported by Maggi et al (2011) as majority component in fresh leaves bastard balm and identified as *o*-coumaric acid glucoside, corresponding the signals at *m/z* 651 to a dimeric adduct ($[2M-H]^-$) and the pseudomolecular ion ($[M-H]^-$), respectively, and resulting the fragments at *m/z* 163 and 119 from the successive loss of the glucose and CO_2, respectively. Peak 2^{BB}, with similar mass characteristics, should correspond to another coumaric acid hexoside, either bearing the hydroxy group in another position (*m*- or *p*-) and/or a sugar different to glucose. The presence of *o*-coumaric and *p*-coumaric acids in bastard balm was actually reported by Skrzypczak-Pietraszeka & Pietraszek (2012), as well as that of chlorogenic acid.

No information could be obtained for peaks 5^{BB} and 6^{BB}, but according to their UV spectra, similar to peak 4^{BB}, they might correspond to other hydroxycinnamoyl derivatives or to related coumarins. Indeed, hydroxycinnamic acids are precursors of coumarins and, in particular, *o*-coumaric acid is the precursor of coumarin, whose presence in bastard balm has been reported by Maggi et al (2011). Since no elements to decide about their possible structure, they are just named as "unknown phenylpropanoids"

The phenolic profile of lemon balm infusions revealed the presence of twenty phenolic compounds, mostly corresponding to caffeic acid derivatives (**Table 63**). Similar phenolic profiles were already reported in infusions (Barros et al., 2013) and decoctions (Carocho et al., 2015) of samples of *M. officinalis* of different origins previously analyzed by our research group. Details for the identification of the herein detected compounds can be found in the

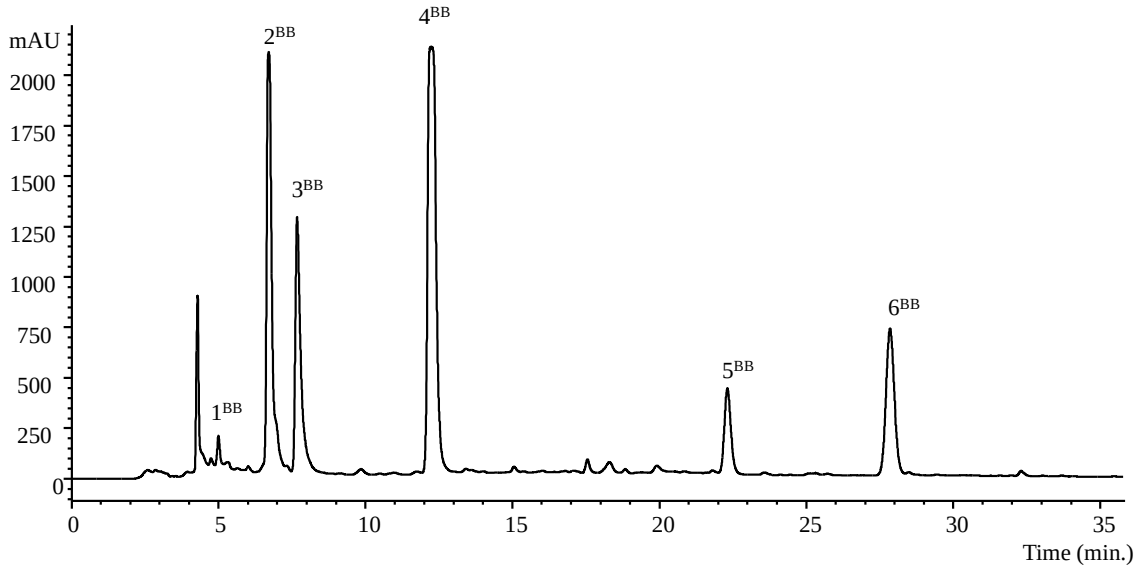
indicated articles, in relation to which only an additional salvianolic acid C derivative (peak 12^{LB}) has been newly detected in the present work.

Table 61. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of phenolic compounds in bastard balm infusions prepared from non-irradiated and irradiated samples.

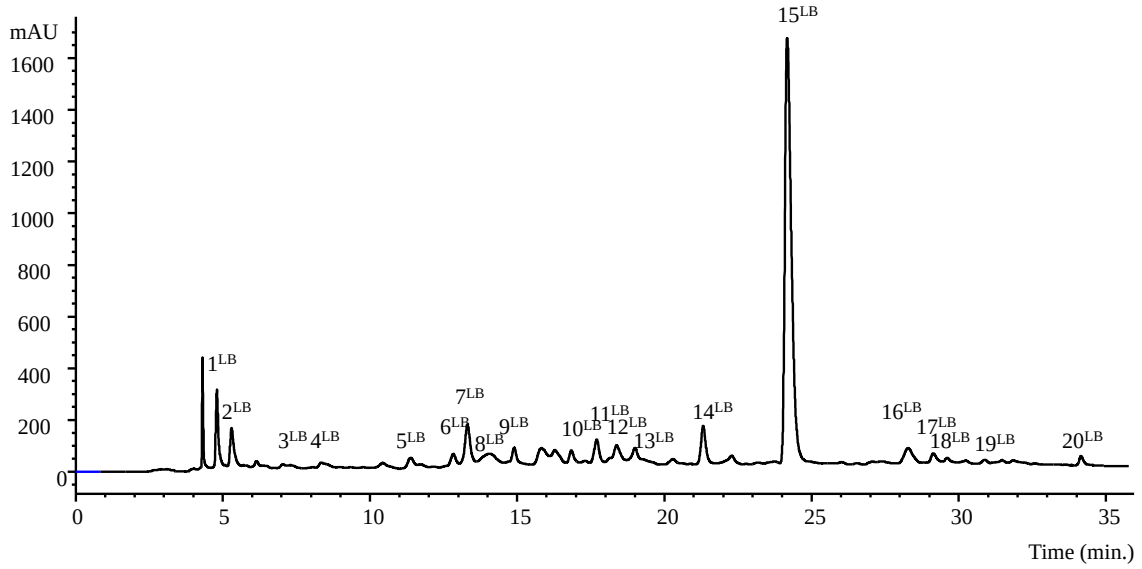
Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1^{BB}	5.2	326	353	191(100),179(80),173(5),161(5),135(20)	3-*O*-Caffeoylquinic acid
2^{BB}	7.0	264,sh300	325	163(36),191(100)	*p*-Coumaric acid hexoside
3^{BB}	8.1	326	353	191(100),179(40),173(20),161(18),135(21)	5-*O*-Caffeoylquinic acid
4^{BB}	12.7	278,sh314	651 ($[2M-H]^-$)	325(100),163(57),119(61)	*o*-Coumaric acid glucoside
5^{BB}	22.8	278,sh320	-	-	Unknown phenylpropanoid
6^{BB}	28.3	278,sh312	-	-	Unknown phenylpropanoid

Table 62. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of phenolic compounds in lemon balm infusions prepared from non-irradiated and irradiated samples.

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification
1^{LB}	4.8	280	197	179(92),135(100)	3-(3,4-dihydroxyphenyl)-lactic acid
2^{LB}	5.3	330	311	179(100),149(98),135(31)	Caftaric acid
3^{LB}	7.0	320	341	179(100),149(7),135(31)	Caffeic acid hexoside
4^{LB}	8.3	324	325	193(100),149(11),145(25),134(43)	Fertaric acid
5^{LB}	11.4	324	179	135(100)	Caffeic acid
6^{LB}	12.8	330	439	359(10),179(8),161(40),135(28)	Sulphated rosmarinic acid
7^{LB}	13.3	270	571	527(14),483(61),439(52),329(23),259(22),241(49),197(100),179(77),135(98)	Yunnaneic acid E
8^{LB}	14.0	276,324sh	537	493(57),359(13),313(27),295(100),269(27),197(19),179(78),135(45)	Lithospermic acid A isomer
9^{LB}	14.9	328	473	311(19),293(19),179(75),149(100),135(28)	Chicoric acid
10^{LB}	16.8	274,334sh	597	359(31),295(27),197(16),179(10),135(12)	Yunnaneic acid F
11^{LB}	17.7	266,336sh	553	491(9),359(3),311(5),197(3),179(21),161(12),135(100)	Salvianolic acid C derivative I
12^{LB}	18.3	266,336sh	553	491(9),359(3),311(5),197(3),179(21),161(12),135(100)	Salvianolic acid C derivative II
13^{LB}	19.0	322	521	359(100),197(16),179(32),161(72),135(16)	Rosmarinic acid hexoside
14^{LB}	21.3	284,328sh	719	539(17),521(15),359(100),197(22),179(26),161(81),135(7)	Sagerinic acid
15^{LB}	24.1	330	359	197(83),179(70),161(100),135(40)	Rosmarinic acid
16^{LB}	27.6	324	493	359(78),313(8),295(52),269(7),197(33),179(44)	Salvianolic acid A
17^{LB}	28.2	328	829	667(86),535(100),491(21),311(39),293(15),179(10)	Salvianolic acid C derivative III
18^{LB}	30.2	288,326sh	537	493(53),359(100),313(5),295(18),269(3),197(44),179(64)	Lithospermic acid A
19^{LB}	30.8	320	493	359(100),313(5),295(6),269(4),197(14),179(34)	Salvianolic acid A isomer
20^{LB}	34.6	288,320sh	715	535(100),491(38),311(69),293(4),179(5),135(20)	Salvianolic acid C derivative IV



A



B

Figure 45. HPLC chromatograms recorded at 280 nm with the phenolic profiles of bastard balm (A) and lemon balm (B) control samples. Peaks numbers correspond to those in tables 62 and 63, respectively.

3.3.2.2 Effects of gamma and electron-beam irradiation on the profiles of phenolic compounds

In order to evaluate the effect of the irradiation dose (ID), the mean values of the contents of every individual phenolic compound at each dose considering both the samples submitted to electron-beam and gamma radiation (^{60}Co) were used; with this approach it was aimed to establish the most suitable ID independently of the used irradiation source. Similarly, to assess the influence of the irradiation technology (IT), mean values of the contents of every phenolic compound obtained at each IT (electron-beam and gamma rays), regardless of the applied ID, were considered.

As it can be seen in **Tables 63** and **64**, the interaction among factors (ID×IT) was significant for all the compounds determined in bastard balm and most of the compounds quantified in lemon balm (except for 1^{LB}: 3-(3,4-dihydroxyphenyl) lactic acid; 10^{LB}: yunnaneic acid F; 13^{LB}: rosmarinic acid hexoside, and 16^{LB}: salvianolic acid A).

Regarding bastard balm (**Table 63**), the most abundant compounds were the two coumaric acid hexosides (peaks 2^{BB} and 4^{BB}) and 5-*O*-caffeoylquinic acid (3^{BB}). Despite the significant interaction among ID and IT, the estimated marginal mean (EMM) plots (data not shown) allowed to verify a marked tendency to higher individual phenolic contents in the samples of bastard balm irradiated at 10 kGy, independently of the IT. Complementarily, samples treated with gamma irradiation showed higher phenolic contents than the corresponding samples irradiated with electron-beam.

The effect produced by the ID was less pronounced over the individual phenolics characterized in lemon balm. In fact, no significant differences were found for half of the compounds (1^{LB}, 4^{LB}, 6^{LB}, 7^{LB}, 8^{LB}, 9^{LB}, 11^{LB}, 12^{LB}, 16^{LB} and 20^{LB}). For all the remaining phenolics, the irradiation treatment (independently of the source) lead to an increase in their contents. This effect was especially relevant for compounds 10^{LB} (yunnaneic acid F), 13^{LB} (rosmarinic acid hexoside) and 18^{LB} (lithospermic acid A), in samples irradiated at 1 kGy, and compounds 5^{LB} (caffeic acid), 17^{LB} (salvianolic acid C derivative III) and 19^{LB} (salvianolic acid A isomer), in samples irradiated at 10 kGy. No differences between the two irradiation doses were observed for compounds 2^{LB} (caftaric acid), 14^{LB} (sagerinic acid) and 15^{LB} (rosmarinic acid). The increasing effect in the levels of rosmarinic acid deserves particular attention, as it was by far the most abundant individual phenolic compound in lemon balm infusions from non-irradiated samples (≈90 μg/mL) and its content increased more than 20% with the irradiation treatment (≈110 μg/mL).

Regarding the potential effect induced by the IT, no significant differences were observed for compounds 3^{LB}, 10^{LB}, 15^{LB} and 19^{LB}, whereas the e-beam favored the increase in the contents of compounds 1^{LB}, 4^{LB} to 7^{LB}, 9^{LB}, 12^{LB} and 14^{LB}, and gamma irradiation tended to increase those of 2^{LB}, 8^{LB}, 11^{LB}, 13^{LB}, 16^{LB}, 17^{LB}, 18^{LB} and 20^{LB}. The increasing effect of gamma irradiation in lithospermic acid related compounds should be highlighted, since these compounds represent an important percentage of the individual phenolics in lemon balm. However, no significant differences were found in the levels of the majority compound, rosmarinic acid.

Table 63. Contents of phenolic compounds in bastard balm infusions according to the irradiation dose (ID) and irradiation technology (IT). The values indicated correspond to mean ± SD of all samples treated under the same conditions.

Compound	Tentative identification	Quantification (µg/mL of infusion)							
		Irradiation dose (ID)			*p*-value	Irradiation technology (IT)		*p*-value	ID×IT
		0 kGy	1 kGy	10 kGy	(n = 18)	Electron-beam	^{60}Cobalt	(n = 27)	*p*-value (n = 54)
1^{BB}	3-*O*-Caffeoylquinic acid[1]	1.9±0.2	2.2±0.2	1.9±0.3	0.009	1.9±0.3	2.1±0.1	0.001	<0.001
2^{BB}	*p*-Coumaric acid hexoside[2]	15±5	17±2	18±3	0.077	14±2	20±1	<0.001	<0.001
3^{BB}	5-*O*-Caffeoylquinic acid[1]	15±2	17±1	19±1	<0.001	16±2	18±1	<0.001	0.015
4^{BB}	*o*-Coumaric acid glucoside	28±4	29±1	31±3	0.003	27±2	32±2	<0.001	<0.001
5^{BB}	Unknown phenylpropanoid I*,[2]	6±2	6±2	2±2	<0.001	5±3	4±1	0.176	<0.001
6^{BB}	Unknown phenylpropanoid II*,[2]	17±3	8±8	11±11	0.011	5±5	20±3	0.001	<0.001

*These compounds were not detected in samples irradiated with e-beam at 10 kGy. Phenolic standards used for the quantification: 1- chlorogenic acid, 2- *o*-coumaric acid.

Table 64. Contents of phenolic compounds in lemon balm infusions according to the irradiation dose (ID) and irradiation technology (IT). The values indicated correspond to mean ± SD of all samples treated under the same conditions.

Compound	Tentative identification	Quantification (µg/mL of infusion)							
		Irradiation dose (ID)			*p*-value (n = 18)	Irradiation technology (IT)		*p*-value (n = 27)	ID×IT
		0 kGy	1 kGy	10 kGy		Electron-beam	^{60}Cobalt		*p*-value (n = 54)
1^{LB}	3-(3,4-dihydroxyphenyl)-lactic acid[1]	6±1	6±1	6±1	0.121	6.9±0.5	5.1±0.5	<0.001	0.167
2^{LB}	Caftaric acid[1]	5±1	6±1	6±1	0.013	4.4±0.5	7±1	<0.001	<0.001
3^{LB}	Caffeic acid hexoside[1]	0.4±0.2	0.1±0.1	0.01±0.01	<0.001	0.2±0.2	0.1±0.1	0.562	<0.001
4^{LB}	Fertaric acid[2]	0.5±0.4	0.5±0.4	0.3±0.1	0.050	0.7±0.3	0.13±0.03	<0.001	<0.001
5^{LB}	Caffeic acid[1]	1.3±0.2	1.5±0.4	1.8±0.5	0.018	1.9±0.4	1.2±0.1	<0.001	<0.001
6^{LB}	Sulphated rosmarinic acid[3]	2±1	2±1	3±1	0.098	3.3±0.5	1.6±0.3	0.001	<0.001
7^{LB}	Yunnaneic acid E isomer[3]	6±2	6±2	7±3	0.253	9±2	3.9±0.3	<0.001	<0.001
8^{LB}	Lithospermic acid A isomer[3]	26±20	39±21	32±23	0.182	12±5	53±7	<0.001	<0.001
9^{LB}	Chicoric acid[2]	2.5±0.5	2.5±0.3	2.8±0.5	0.129	2.8±0.5	2.3±0.4	0.001	<0.001
10^{LB}	Yunnaneic acid F[3]	$3.4±0.3^{b}$	$3.8±0.4^{a}$	$3.5±0.2^{b}$	0.001	3.7±0.4	3.5±0.3	0.053	0.364
11^{LB}	Salvianolic acid C derivative I[3]	5.5±0.5	7±3	6±1	0.050	5±1	7±2	<0.001	<0.001
12^{LB}	Salvianolic acid C derivative II[3]	4±1	4±1	4±1	0.350	5±1	2.9±0.5	<0.001	<0.001
13^{LB}	Rosmarinic acid hexoside[3]	$6±1^{b}$	$7±1^{a}$	$6±1^{b}$	0.001	5±1	8±1	<0.001	0.564
14^{LB}	Sagerinic acid[3]	7±1	9±1	9±1	<0.001	9±1	8±1	0.001	0.007
15^{LB}	Rosmarinic acid[3]	89±7	110±8	112±9	<0.001	105±16	102±10	0.525	<0.001
16^{LB}	Salvianolic acid A[3]	$10±3^{c}$	$12±3^{a}$	$11±3^{b}$	0.131	8±1	14±1	<0.001	0.489
17^{LB}	Salvianolic acid C derivative III[3]	7±3	8±1	11±1	<0.001	7±3	10±1	<0.001	<0.001
18^{LB}	Lithospermic acid A[3]	16±14	33±4	29±7	<0.001	18±12	34±3	<0.001	<0.001
19^{LB}	Salvianolic acid A isomer[3]	2.4±0.3	3.3±0.5	3.7±0.4	<0.001	3±1	3±1	0.073	0.032
20^{LB}	Salvianolic acid C derivative IV[3]	2.4±0.5	2.7±0.4	1.8±0.5	0.087	1.3±0.5	3.3±0.4	<0.001	<0.001

In each line, different letters mean significant differences among irradiation doses ($p<0.05$). Phenolic standards used for the quantification: 1- caffeic acid, 2- ferulic acid, 3- rosmarinic acid.

3.3.2.3 Linear discriminant analysis

In the previous section, the differences induced by each assayed factor (ID and IT) were classified considering each plant species separately. Additionally, it was considered interesting to establish whether phenolic compounds were affected independently of the plant species. With that purpose in mind, a linear discriminant analysis (LDA) was performed to evaluate the overall effects on the phenolic compounds profile of both plants. To overcome the qualitative differences among the two species, the results were previously normalized by calculating the percentage of variation in comparison to each corresponding control (non-irradiated samples), thereby being possible to analyze all the phenolic compounds simultaneously.

Regarding the effects of ID, the defined significant functions (**Figure 46**) included 100.0% of the observed variance (function 1: 59.4%%; function 2: 40.6%). The individual clustering of the markers corresponding to each of the factor levels (0 kGy, 1 kGy and 10 kGy) is obvious. From the included 26 variables (20 from lemon balm and 6 form bastard balm), only 11 were selected as having discriminant ability: 1) sagerinic acid, 2) rosmarinic acid hexoside, 3) caffeic acid hexoside, 4) lithospermic acid A, 5) salvianolic acid C derivative I, 6) yunnaneic acid F; 7) salvianolic acid A isomer, 8) caffeic acid, 9) phenylpropanoid II, 10) chicoric acid, and 11) yunnaneic acid E. As it can be observed, the differences among the non-irradiated samples and those treated with 1 kGy (independently of IT) were associated with function 1, which was more highly correlated to rosmarinic acid hexoside and lithospermic acid A, both showing higher increases in samples irradiated at 1 kGy. In addition, function 2 highlighted the differences among non-irradiatedsamples and those irradiated at 10 kGy, mainly based in its correlation with salvianolic acid A isomer and sagerinic acid, both reaching higher increases in 10 kGy-irradiated samples.

In the case of IT, the variables with highest differences among e-beam and gamma irradiation (*i.e.*, those selected as having discriminant ability) were: 1) phenylpropanoid II, 2) caffeic acid hexoside, 3) salvianolic acid C derivative I, 4) salvianolic acid A isomer, 5) phenylpropanoid I, 6) sulphated rosmarinic acid, 7) 5-*O*-caffeoylquinic acid, 8) caffeic acid, 9) caftaric acid, 10) lithospermic acid A, 11) coumarin, 12) rosmarinic acid hexoside, and 13) sagerinic acid.

The markers corresponding to each factor level were clustered individually according to the distribution by the defined significant functions (**Figure 47**). It is obvious that changes induced by electron-beam irradiation were more significant, especially in what concerns the compounds more highly correlated with function 1: phenylpropanoid derivatives I and II

(both with higher decreases in e-beam irradiated samples), lithospermic acid A and caffeic acid (both with higher increases in e-beam irradiated samples). The effects induced by gamma irradiation were mainly observable in salvianolic acid A isomer and sagerinic acid (which did not increase as much as with e-beam irradiation), caftaric acid and salvianolic acid C derivative I (both with higher increases in gamma-irradiated samples), which were the variables more correlated to function 2.

Regarding the classification performance, in both cases all samples were correctly classified, either for the original grouping, as well as for cross-validated grouped cases.

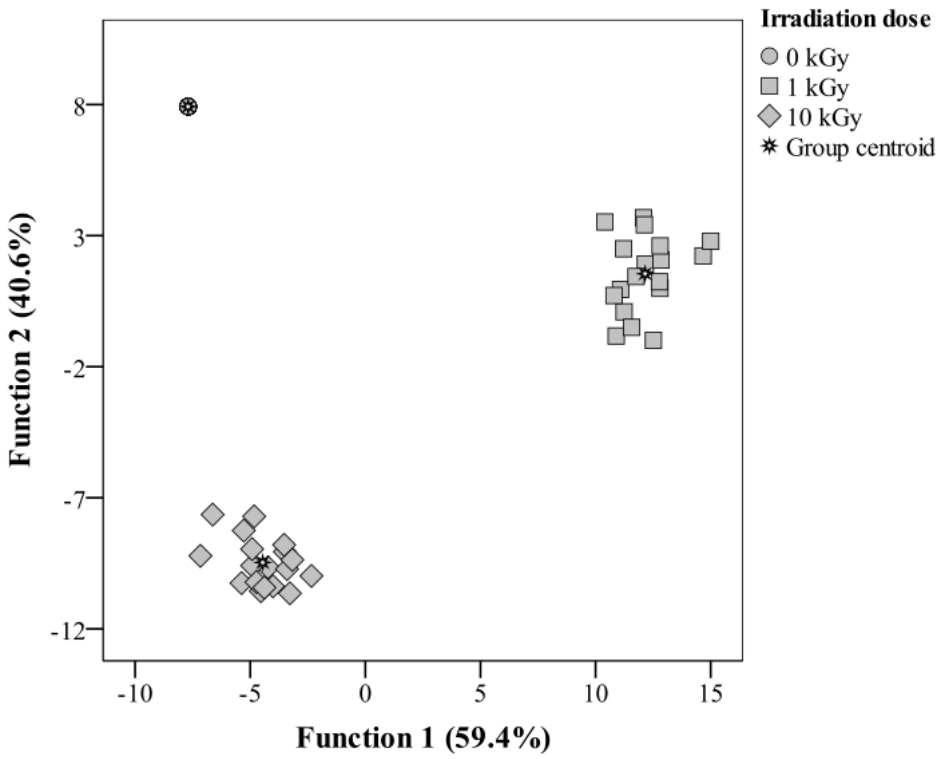


Figure 46. Mean scores of different irradiation doses distributed according to the discriminant functions defined from the variations induced by e-beam and gamma irradiation on the phenolic compounds profiles of bastard balm and lemon balm.

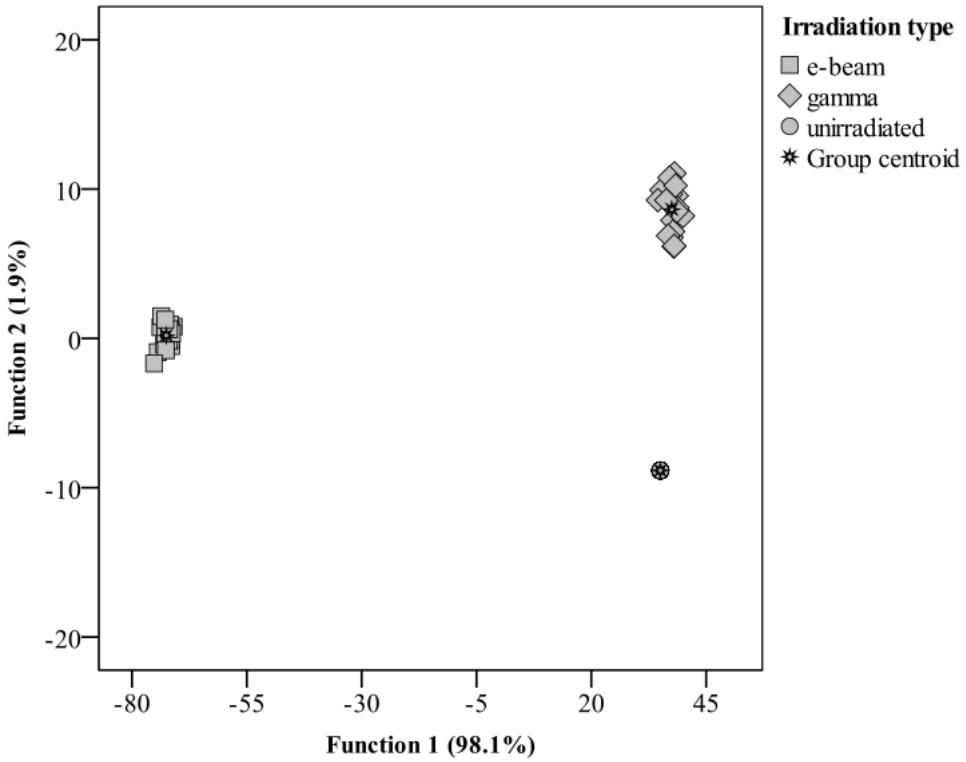


Figure 47. Mean scores of irradiation types distributed according to the discriminant functions defined from the variations induced by both irradiation doses on the phenolic compounds profiles of bastard balm and lemon balm.

CONCLUDING REMARKS

Evaluation of composition and antioxidant properties of Ginkgo biloba *and dietary supplements.*

Dietary supplements containing plant extracts are complex mixtures whose therapeutic effect is often attributed to the cumulative effects of several components with bioactive properties. Thus, it is important to have an overview of all the elements present to evaluate product quality at nutraceutical and nutritional level. Particularly in *G. biloba* plant, several bioactive compounds were identified and quantified, such as tocopherols, mainly α-tocopherol, and phenolic compounds (potent antioxidants that have been related with a reduced risk of atherosclerosis and mitigation of neurological damage in patients with Alzheimer's disease). *Ginkgo biloba* pills (and especially the sample referred to as P3, the dietary supplement with the highest concentration of plant extract) allow the intake of the highest antioxidants concentration.

Effects of gamma irradiation on Ginkgo biloba composition and properties.

The analytical results proved that gamma irradiation could be a good alternative for *G. biloba* preservation since it satisfactorily maintained macronutrients, fatty acids, γ- and δ-tocopherols, fructose, trehalose, quinic and shikimic acids. Furthermore, at the dose of 1 kGy α-tocopherol, oxalic and malic acids were also preserved. In contrast, irradiation at 10 kGy decreased α-tocopherol, glucose, sucrose, oxalic and malic acids levels. Based on the obtained results, 1 kGy would be thus the recommended dose; in addition to maintain the nutritional profile of *G. biloba* and protect specific molecules, it also led to increased antioxidant activity in the infusions and methanolic extracts prepared from its leaves.

This is also the first report that describes the phenolic composition in irradiated samples of *G. biloba* using two different doses. In this regard, the dose of 10 kGy, that is enough to guarantee product disinfestation and microbial decontamination (Molins, 2001), also favors an increase in the phenolic compounds extractability, so that greater levels of these bioactives are recovered in methanol/water and infusion preparations. The use of irradiation to improve bioactive properties as a result of an increase in the levels of phenolic compounds in the extracts obtained from different plant-derived products was also suggested by other authors (Zhu et al., 2010; Aouidi et al., 2011; Lee et al., 2013; Wanyo et al., 2014). Nevertheless, further research is needed to understand all the mechanisms involved in the effects of irradiation processing in plant constituents.

Effects of gamma irradiation on Aloysia citrodora, Melissa officinalis, Melittis melissophyllum and Mentha x piperita.

When the effects of gamma-radiation (up to 10 kGy) in the chemical/nutritional and antioxidant properties were considered individually for each plant material (i.e., lemon verbena, lemon balm, bastard balm or peppermint samples) statistical significant differences were found in particular cases. Nonetheless, when analyzed under an integrated approach, non-irradiated and irradiated samples were grouped indiscriminately (as deduced from PCA analysis), suggesting that the irradiation treatment is not sufficient to define a specific chemical/antioxidant profile in the type of changes produced. Interestingly, PCA plot of object scores indicated that irradiation affected each plant species in specific way.

Overall, it might be considered that gamma radiation (up to 10 kGy) is a feasible conservation technology for the assayed *Lamiaceae* and *Verbenaceae* species. This observation is interesting because a dose of 10 kGy would allow sample disinfestation and decontamination.

Influence of gamma irradiation in the preservation of Aloysia citrodora, Melissa officinalis, Melittis melissophyllum and Mentha x piperita properties during storage (12 and 18 months).

The nutritional variables presented similar profiles after 18 months, apparently without a significant effect of gamma-radiation, whereas a relevant protective effect was observed on oleic acid and β-carotene bleaching inhibition (when evaluated in the infusions), and DPPH scavenging activity and reducing power (in the methanolic extracts). On the other hand, gamma radiation could not prevent the losses in free sugars, organic acids and tocopherols, especially considering the 18 months period. Regarding color variables a^* and b^*, a change toward a greener color during storage of the irradiated samples was indicated, which could be considered a positive effect, as those hues tend to be more appreciated by consumers.

Despite the lack of similarity among the effects suffered by each plant species, the applied PCA allowed identifying the most affected variables (all those outside the inner circle, **Figure 36**, section 3.1.3) during storage as influenced by its duration (12 or 18 months), independently of the plant species.

Effects of gamma irradiation on cytotoxicity and phenolic compounds of Thymus vulgaris and Mentha x piperita. Evaluation in methanolic extracts.

Thyme and peppermint are rich sources of antioxidant compounds of phenolic nature: flavonols, flavones, flavanones and phenolic acid derivatives, which might contribute to the prevention and control of diseases through their incorporation into a normal diet or as supplements. The methanolic extracts of these plants showed anti-proliferative activity in different human tumor cell lines, but no toxicity on non-tumor cells. The assayed irradiation doses (1 to 10 kGy) did not affect those properties or the phenolic composition of the peppermint samples. However, thyme samples irradiated at 10 kGy increased their cytotoxicity for tumor cell lines in relation to lower doses and non-irradiated plants. As for the studied plants, it can be concluded that 10 kGy could be an appropriate irradiation dose because it does not seems to affect the evaluated bioactive properties. From this point of view, irradiation can be considered as a suitable decontamination and preservation process (as also indicated by other authors), because still when applied up to the maximum dose allowed in legislation (10 kGy) it is able to retain the factors that provide the bioactive potential. Thus, this technology represents an added-value solution to meet the requirements of the food and pharmaceutical industries in their quest of high quality raw materials.

Effects of gamma irradiation on bioactive properties of Thymus vulgaris. Evaluation in infusions.

Infusions are a form of consumption of thyme leaves and evaluation of their bioactive potential depending on the type of plant processing is extremely important. According to the obtained results it became evident that gamma radiation had an influence on the antioxidant activity of the samples, with treatment at 10 kGy increasing their antioxidant potential (lower EC_{50} values in all the assays). This irradiation dose (10 kGy) also led to infusions with higher total phenolic and flavonoid contents. As for individual compounds, only three (apigenin 6,8-di-*C*-glucoside, caffeic acid and luteolin-7-*O*-glucuronide) out of the thirteen detected phenolics did not suffer statistically significant changes in their concentrations, whereas an increase was observed in the contents of the remaining ones, especially relevant in the cases of methyl-eriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside, eriodictyol and total flavonoids.

The observations made suggest that, for the legally permitted maximum radiation dose in the EU (10 kGy), gamma radiation may even improve the antioxidant potential and phenolic contents in *T. vulgaris* infusions without significantly affecting other aspects in their chemical profile.

Effects of gamma irradiation on cytotoxicity and phenolic compounds of Aloysia citrodora and Mentha x piperita. Evaluation in infusions.

All the lemon verbena and peppermint samples showed anti-proliferative activity on the different assayed tumor cell lines, without the fact of being irradiated or not induced, in general, significant differences among them. None of the samples revealed cytotoxic effects on non-tumor cell lines (PLP2 line from liver).

Regarding phenolic compounds, a statistically significant increase was produced in their contents in the case of irradiated samples, whereas no relevant differences were observed among different lemon verbena samples. The phenolic composition presented better correlation factors with the anti-proliferative activity for peppermint infusions than for lemon verbena ones.

Effects of gamma radiation on mycotoxins in Aloysia citrodora.

The treatment with gamma radiation at doses of 1, 5 and 10 kGy did not result in a significant decrease in the levels of OTA and AFB_1 in dried leaves of *A. citrodora.* As for these results, even at the maximum legislated dose of 10 kGy, irradiation seems to be ineffective for mycotoxin decontamination, even though it may be effective for other food safety purposes. Regarding phenolic composition, no relevant changes were produced in compounds profiles and contents as a result of the irradiation.

The obtained results might point to the need of approaching the EU irradiation regulations with other countries that allow higher doses in dried herbs, as long as they do not compromise other food safety variables. Also, in the case where mycotoxin decontamination of herbs is the primary goal, and considering water content as an important variable for their destruction by gamma rays, future research should contemplate testing the effectiveness of herbs irradiation prior drying. For this matter, other features such as physical, chemical and organoleptic characteristics should also be studied.

Comparison of the effects of electron-beam and gamma radiation on Aloysia citrodora, Melissa officinalis, Melittis melissophyllum and Mentha x piperita.

The effects of electron-beam (EB) and gamma radiation (GR) on chemical variables and bioactivity indicators in the assayed aromatic plants were highly dependent on the plant species. Nevertheless, when all the results were considered together for statistical analysis, it became evident that the highest changes were induced in the fatty acids profiles and antioxidant activity, either when evaluating the effect of irradiation type, as well as in the assessment of the plant species influence.

The obtained results pointed to EB as a more suitable source for irradiation aromatic plants, as EB irradiated samples showed higher MUFA (and some PUFA) percentages and increased antioxidant activity when compared with GR ones. Nevertheless, the effects were also strongly dependent on the plant species and the irradiation dose, advising for specific studies on the particular plant species considered for irradiation.

Influence of electron-beam radiation in the preservation of Aloysia citrodora, Melissa officinalis, Melittis melissophyllum and Mentha x piperita properties during storage (12 and 18 months).

In general, hardly significant changes were observed in proximate composition and color variables during the storage time (ST) in A. *montana* samples, either irradiated or not. In contrast, important losses were produced in the levels of some individual compounds, such as free sugars, organic acids, tocopherols and PUFA, throughout ST. However, the EB treatment, despite not preventing totally those losses, had a significant attenuating action. Anyway, dissimilar effects were observed for different plant species, either in the chemical variables, antioxidant activity indicators or contents of phenolic compounds.

Bioactivity and phytochemical characterization of Arenaria montana

Phenolic compounds (namely flavones) and tocopherols were identified and quantified in *A. montana*, as also omega-3 and omega-6 fatty acids, all of them constituting important classes of phytochemicals that have been related with beneficial health effects. The infusions of this plant revealed higher antioxidant and anti-proliferative activities than its methanolic extracts. The results obtained support the documented medicinal effect of this species and open the possibilities for food and pharmaceutical applications, especially its infusions that may be

used as a nutraceutical or functional ingredient, owing to its important antioxidant activity and content of bioactive phytochemicals.

Effects of irradiation on Arenaria montana composition and properties.

Most of the evaluated nutritional and chemical variables (except for free sugars and organic acids), as well as antioxidant activity showed statistically significant variations in response to irradiation treatment. The application of linear discriminant analysis to the whole of the data allowed establishing the most affected variables by gamma or e-beam radiation, as well as according to the irradiation dose (1 or 10 kGy). Thus, the antioxidant variables proved to be the ones suffering the most significant changes, especially at the dose of 10 kGy dose and when using e-beam radiation. The obtained results might constitute a guidance to choose the irradiation type or dose in order to preserve a specific chemical or bioactive profile.

Effects of irradiation on phenolic compounds in Melissa officinalis and Melittis melissophyllum.

An increase in the levels of phenolic compounds analyzed individually was observed among irradiated samples. In previous studies on these plants carried out by our research group (Koike et al., 2015b), the differences induced by gamma or electron-beam irradiation on the proximate composition, color variables, free sugars, organic acids, tocopherols, fatty acids and antioxidant activity were found to be negligible. The improving effects of the same irradiation technologies over the individual phenolics could be considered an additional argument to propose gamma or electron-beam radiation as feasible conservation technologies for these dried plants. Furthermore, it was observed that different compounds were distinctly favored at 1 kGy or 10 kGy irradiation doses, as well as e-beam or gamma irradiation, which might be considered as a way to specifically maximize the production of target phenolic compounds.

GLOBAL REMARK

This study aimed not only to analyse several plant species used in traditional medicine, in terms of chemical and nutritional composition and bioactive potential, but also to test the decontaminant and preservative potential of two ionizing irradiation techniques (gamma rays and electron beams). According with the obtained results, this type of processing displays advantages that stand out, as it allows preserving and even increasing the levels of some nutritional and phytochemical components and sometimes improving bioactivity indicators, when applied at doses up to 10 kGy. Nevertheless, depending on the irradiated plant material and applied process, significant decreases could also occur in some molecules.

In general, compositional and bioactive properties of the irradiated samples were not more affected during further storage (up to 18 months) than the corresponding non-irradiated ones, although greater losses of some compounds were sometimes noticed in the samples treated at 10 kGy than at 1 kGy. This decreasing effect was observed for both types of irradiation, although it was less accentuated with electron beam than with gamma radiation. In general, in the cases where the two types of ionizing radiation were compared in the same plant species, it became evident that electron beam was a more suitable technology, both for preserving plants characteristics during processing as also during their further storage.

On the other hand, irradiation did not show efficient for mycotoxin decontamination, as it did not allow reducing the levels of ochratoxin A and aflatoxin B_1 in the assayed dried plant materials, within the applied doses (0, 5 and 10 kGy).

All in all, as for the results obtained in the present study, it was evident that the behaviour of molecules is heterogeneous, depending on the plant species, source of radiation and applied dose. Thus, although the irradiation may be considered a suitable and promising technology for preservation of aromatic and medicinal plants, a previous evaluation should be required before deciding the adequate type of processing according to the particular material.

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ANNEXES

Annexes 1

Chemical characterization of *Ginkgo biloba* L. and antioxidant properties of its extracts and dietary supplements

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Chemical characterization of *Ginkgo biloba* L. and antioxidant properties of its extracts and dietary supplements

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ABSTRACT

Ginkgo biloba L. is the most commercialized medicinal plant worldwide, being its consumption related to prevention, and even decrease of the progression of degenerative neurological diseases. Considering the correlation between oxidative stress and the mentioned diseases, the antioxidant activity of different dietary supplements (syrup and several pills) was evaluated and compared to the leaves infusion, aqueous and methanolic extracts. Furthermore, *G. biloba* was chemically characterized in nutritional and bioactive components namely, fatty acids, sugars, organic acids, tocopherols, phenolics and flavonoids. Palmitic, α-linolenic and oleic acids were the main fatty acids found; fructose was the most abundant sugar; quinic acid was the most abundant organic acid and α-tocopherol was, by far, the most abundant vitamer. Dietary supplements showed higher antioxidant activity than *G. biloba* infusion and extracts due to their higher phenolics and flavonoids concentration. The pills with the highest concentration of plant extract (100 mg) allow the intake of the highest antioxidants concentration.

1. Introduction

Ginkgo biloba L. (Ginkgoaceae) is an ancient tree growing in China for centuries; however, it was only during the last couple of decades that its true value was recognized, being considered sacred for its health-promoting properties (Smith et al., 1996; Singh et al., 2008).

The medical interest in western *G. biloba* has increased since the 1980s, due to its potent action on cardiovascular system and cerebral vascular activity. In recent decades, in Western countries, the concentrated extracts of the leaves have been marketed as herbal medicines due to the presence of bioactive components (*e.g.*, terpenoids, polyphenols, organic acids, carbohydrates, essential fatty acids, inorganic salts and amino acids), and to the capacity to increase microcirculation in brain (with the supply of oxygen and nutrients) and in body extremities (Beek, 2002; Singh et al., 2008). Thus, it can be useful in the improvement of symptoms of poor memory, impaired mental concentration, particularly in the elderly, for whom this function is sometimes lowered. It also has positive effects in certain situations such as tinnitus (ringing) and hearing capacity altered, bringing also cardiovascular protection due to the ability to prevent platelet aggregation and thrombus formation. Furthermore, due to the antioxidant properties it has been used in Alzheimer's patients (Smith et al., 1996; Diamond et al., 2000; Beek and Montoro, 2009).

Brain is particularly prone to damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) due to five main reasons: the high oxygen required by this organ; the abundance of redox-active metals; the relative deficit in antioxidant systems; the presence of great amount of oxidizable polyunsaturated fatty acids and catecholamines; and the fact that neurons are post-mitotic cells with relatively restricted replacement by progenitor cells during lifetime (Mangialasche et al., 2009; Wang and Michaelis, 2010).

Thus, a variety of mechanisms of neuronal degeneration in Alzheimer diseases has been proposed, including formation of free radicals, oxidative stress, mitochondrial dysfunction, inflammatory processes, genetic factors, environmental impact factors, apoptosis, among others (Zhi-you and Yong, 2007). Different studies reported that neuronal earliest changes and pathological features of this disease are related to oxidative damage (with a very high input of oxidative stress), mainly in the development of neuritic abnormalities. In fact, paired helical filaments are more often found in neurites with membrane abnormalities, which is indicative of extensive lipid peroxidation (Zhu et al., 2004; Zhi-you and Yong, 2007). Moreover, crosslinking of proteins by oxidative processes may lead to the resistance of the lesions to intracellular and extracellular removal even though when they are extensively ubiquitinated; this resistance of neurofibrillary tangles to proteolysis might play an important role in the progression of the degenerative disease (Zhu et al., 2004). In this context, the use of polyphenols may be useful, since they increase the cellular stress response and improve

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mitochondrial respiration, thus allowing the neuron to counteract free radical-induced damage and produce the ATP necessary to maintain the normal membrane potential (Mancuso et al., 2012).

Taking into account the described relation between antioxidants and Alzheimer's disease, and considering the use of *G. biloba* in the mentioned pathology, the antioxidant activity of different dietary supplements (syrup and several pills) was evaluated and compared to the leaves infusion, aqueous and methanolic extracts. Furthermore, *G. biloba* was chemically characterized in nutritional and bioactive components.

2. Materials and methods

2.1. Samples

G. biloba dry leaves and dietary supplements (syrup and different pills based on leaves standardized extract with 24% glycosides and 6% terpenes) (Table 1) were obtained from an herbalist shop and a pharmacy, respectively, located in Bragança, Portugal.

2.2. Standards and reagents

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(−)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose and D(+)-raffinose pentahydrate), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and (+)-catechin standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Chemical characterization of G. biloba

2.3.1. Macronutrients

G. biloba dry leaves were analyzed for chemical composition (moisture, proteins, fat, carbohydrates and ash) using the AOAC procedures (AOAC, 1995). The crude protein content ($N \times 6.25$) of the samples was estimated by the macro-Kjeldahl method; the crude fat was determined by extracting a known weight of powdered sample with petroleum ether, using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference.

2.3.2. Free sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by the authors (Rafael et al., 2011) using melezitoze as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco) and a RI detector (Knauer Smartline 2300). Data were analyzed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 mm × 250 mm, 5 mm, Knauer) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.3.3. Organic acids

Organic acids were determined following a procedure previously described by the authors (Pereira et al., 2013a). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation). Separation was achieved on a SphereClone (Phenomenex) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) thermostatted at 35 °C. The elution was performed with sulfuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight (dw).

2.3.4. Fatty acids

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC–FID)/capillary column as described previously by the authors (Rafael et al., 2011). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey–Nagel column (30 m × 0.32 mm ID × 0.25 μm d_f). The oven temperature program was as follows:

Table 1
Information about the studied *Ginkgo biloba* samples.

Code	Sample	Composition	Mode of consumption (minimal dose/day)	Concentration of the prepared solution
LI	Plant	Dry leaves	Infusion	20 mg/mL
LME		Methanolic extract from dry leaves	–	20 mg/mL
LAE		Aqueous extract from dry leaves	–	20 mg/mL
S	Syrup	40 mg of *Ginkgo biloba* standardized extract[a]/mL	1 mL diluted in a glass of water (200 mL); 2 or 3 times	200 μg/mL
P1	Pills	40 mg of *Ginkgo biloba* standardized extract[a]/pill	1 pill dissolved in ½ glass of water (100 mL); 2 or 3 times	400 μg/mL
P2		60 mg of *Ginkgo biloba* standardized extract[a]/pill	1 pill dissolved in a glass of water (200 mL); 2 times	300 μg/mL
P3		100 mg of *Ginkgo biloba* standardized extract[a]/pill	1 pill dissolved in a large glass of water (350 mL); 1 time	286 μg/mL

[a] Corresponds to *G. biloba* leaves extract with 24% glycosides and 6% terpenes (information available in the label).
Therapeutic indications: antioxidant properties, antiasthmatic, scavenge radicals, wound healing and neuroprotective properties as well as it improves mental capacities in Alzheimer's patients.

the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

2.3.5. Tocopherols

Tocopherols were determined following a procedure previously described by the authors (Rafael et al., 2011). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm) normal-phase column from YMC Waters operating at 30 °C. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight (dw).

2.4. Evaluation of antioxidant properties of G. biloba extracts and dietary supplements

2.4.1. Samples preparation

The samples were prepared as indicated in the label: for infusion preparation, 2 g of powdered dry leaves were added to 200 mL of boiling distilled water, left to stand at room temperature for 10 min, and then filtered under reduced pressure, frozen, lyophilized and redissolved in distilled water at a final concentration of 20 mg/mL; for dietary supplements, one pill was dissolved in distilled water. Information regarding the plant weight in each pill, distilled water volumes and final concentrations of the prepared solutions is provided in Table 1. Methanolic and aqueous extracts were also prepared from the dry leaves, stirring 1 g with 30 mL methanol or water, respectively, at 25 °C at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residues were then extracted with one additional 30 mL portion of the corresponding solvent. The combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland) and re-dissolved in the corresponding solvent at 20 mg/mL.

Several dilutions of all the prepared solutions were used in the antioxidant activity assays.

2.4.2. Phenolics and flavonoids content

Total phenolics were estimated by Folin-Ciocalteu colorimetric assay according to procedures previously described (Guimarães et al., 2010) and the results were expressed as mg of gallic acid equivalents (GAE) per g of sample.

Total flavonoids were determined by a colorimetric assay using aluminum trichloride, following procedures previously reported (Barros et al., 2010); the results were expressed as mg of (+)-catechin equivalents (CE) per g of sample.

2.4.3. DPPH radical-scavenging activity

This methodology was performed using an ELX800 microplate Reader (Bio-Tek Instruments, Inc.; Winooski, USA). The reaction mixture on 96 well plate consisted in the sample solutions (30 μL) and methanolic solution (270 μL) containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 30 min in the dark, and the absorption was measured at 515 nm (Barros et al., 2010). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA = [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution. The results were expressed in EC_{50} values (sample concentration providing 50% of radical scavenging activity). Trolox was used as positive control.

2.4.4. Reducing power

The sample solutions (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate, the same with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm in the Microplate Reader mentioned above (Barros et al., 2010). The results were expressed in EC_{50} values (sample concentration providing 0.5 of absorbance). Trolox was used as positive control.

2.4.5. Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Two milliliters of this solution were pipetted into a round-bottom flask. The chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing sample solutions (0.2 mL). The tubes were shaken and incubated at 50 °C in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (Barros et al., 2010). β-Carotene bleaching inhibition was measured by the formula: β-carotene absorbance after 2 h/initial absorbance) × 100. The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity). Trolox was used as positive control.

2.4.6. TBARS assay

Porcine (*Sus scrofa*) brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2, w/v brain tissue homogenate which was centrifuged at 3000 × *g* for10 min. An aliquot (100 μL) of the supernatant was incubated with the sample solutions (200 μL) in the presence of $FeSO_4$ (10 mM; 100 μL) and ascorbic acid (0.1 mM; 100 μL) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28%, w/v, 500 μL), followed by thiobarbituric acid (TBA, 2%, w/v, 380 μL), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000 × *g* for 10 min to remove the precipitated protein, the color intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm (Barros et al., 2010). The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = $[(A - B)/A] \times 100\%$, where *A* and *B* were the absorbance of the control and the sample solution, respectively. The results were expressed in EC_{50} values (sample concentration providing 50% of lipid peroxidation inhibition). Trolox was used as positive control.

2.5. Statistical analysis

For each formulation, three samples were used and all the assays were carried out in triplicate. The results were expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD

Table 2
Nutritional value of *Ginkgo biloba* dry leaves.

Parameter	Amount
Ash	10.01 ± 0.06 g/100 g dw
Proteins	12.27 ± 0.24 g/100 g dw
Fat	4.75 ± 0.22 g/100 g dw
Carbohydrates	72.98 ± 0.20 g/100 g dw

dw: dry weight.

Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results and discussion

3.1. Chemical characterization of G. biloba

The nutritional value of *G. biloba* leaves was evaluated, and the results are given in Table 2. Carbohydrates, calculated by difference, were the most abundant macronutrients (72.98 g/100 g dw). Otherwise, fat was the macronutrient present in lower amount, which confers a more healthy character to this medicinal plant (4.75 g/100 g dw). The levels of proteins and ash were 12.27 and 10.01 g/100 g dw, respectively.

Chemical composition in fatty acids, sugars, organic acids and tocopherols was also accessed and the results are shown in Table 3.

Palmitic (C16:0), α-linolenic (C18:3n3) and oleic (C18:1n9) acids were the most abundant fatty acids (35.90, 18.03 and 11.18%, respectively). The latter is nowadays considered as the preferred fatty acid for edible purposes, because it combines a hypocholesterolemic effect and a high oxidative stability (Mensink and Katan, 1989). In general, saturated fatty acids appeared in higher concentrations (59.15%), followed by polyunsaturated (28.85%) and lastly monounsaturated fatty acids (12%).

The sugars found were fructose, glucose and sucrose, being fructose (1.42 g/100 g dw) the main one and sucrose the least abundant sugar (0.23 g/100 g dw).

Oxalic, quinic, malic and shikimic acids were also identified and quantified, being quinic acid the most abundant organic acid (2.26 g/100 g dw). This acid is a very useful and versatile chiral pool starting material for natural product synthesis and many groups have developed elegant syntheses based on stereoselective reactions of quinic acid derivatives (Murray et al., 2004). Shikimic acid is also present in high quantity (2.24 g/100 g dw); it is used as key starting material for the synthesis of the neuramidase inhibitor GS4104 for treatment of antiviral infections (Krämer et al., 2003). On the other hand, malic acid was the organic acid found in lower quantity.

Table 3
Individual compounds present in *Ginkgo biloba* dry leaves.

Fatty acids (relative percentage)		**Free sugars** (g/100 g dw)	
C6:0	0.24 ± 0.01	Fructose	1.42 ± 0.05
C8:0	0.27 ± 0.04	Glucose	0.78 ± 0.01
C10:0	0.24 ± 0.01	Sucrose	0.23 ± 0.02
C12:0	0.61 ± 0.09	Total	2.43 ± 0.04
C14:0	6.13 ± 0.47	**Organic acids** (g/100 g dw)	
C15:0	0.68 ± 0.03	Oxalic acid	0.90 ± 0.00
C16:0	35.90 ± 0.97	Quinic acid	2.26 ± 0.09
C16:1	0.82 ± 0.12	Malic acid	0.58 ± 0.01
C17:0	1.28 ± 0.02	Shikimic acid	2.24 ± 0.01
C18:0	4.17 ± 0.24	Total	5.98 ± 0.10
C18:1n9	11.18 ± 1.23	**Tocopherols** (mg/100 g dw)	
C18:2n6c	10.53 ± 0.09	α-Tocopherol	124.88 ± 0.37
C18:3n3	18.03 ± 0.06	β-Tocopherol	0.36 ± 0.03
C20:0	2.70 ± 0.05	γ-Tocopherol	0.72 ± 0.06
C20:3n6	0.11 ± 0.07	δ-Tocopherol	0.28 ± 0.01
C20:3n3 + C21:0	0.19 ± 0.08	Total	126.23 ± 0.47
C22:0	2.19 ± 0.01		
C23:0	0.92 ± 0.00		
C24:0	3.82 ± 0.04		
SFA	59.15 ± 1.39		
MUFA	12.00 ± 1.35		
PUFA	28.85 ± 0.04		

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; dw: dry weight.

Regarding tocopherols, the isoforms α-, β-, γ- and δ- tocopherol were all detected. α-Tocopherol was, by far, the most abundant vitamer (124.88 mg/100 g dw in a total of 126.23 mg/100 g dw). Considering its antioxidant potential and various functions at the molecular level, this vitamer reduces the risk of cardiovascular diseases (eliminating reactive oxygen species, inhibiting lipid peroxidation and attenuating inflammatory reactions) and neurodegenerative disorders, particularly in Alzheimer's disease (Burton, 1994; Berman and Brodaty, 2004; Kontush and Schekatolina, 2004).

As far as we know, the present study is pioneer regarding chemical characterization of *G. biloba* in nutritional molecules, which is important considering that it is widely consumed as infusion and incorporated in dietary supplements.

3.2. Antioxidant properties of G. biloba extracts and dietary supplements

The antioxidant properties of different dietary supplements based on *G. biloba* (syrup and pills) and of extracts prepared from the leaves (infusion, methanolic and aqueous extracts) were compared. Dietary supplements showed higher antioxidant activity than extracts prepared from the dry leaves (Table 4). Among dietary supplements, pills gave higher antioxidant activity than syrup. Furthermore, it was observed an increase of antioxidant properties with the increase of *G. biloba* extract concentration in the pills. In fact, P3 (with 100 mg *G. biloba* standardized extract) was better than P2 (with 60 mg *G. biloba* standardized extract), and this one better than P1 (with 40 mg *G. biloba* standardized extract), for all the tested assays. The same behavior was observed for bioactive components namely, phenolics and flavonoids; the samples with highest antioxidant activity also gave the highest contents of the mentioned compounds, which pointed out for an involvement of phenolics and flavonoids in the observed activity.

Our research group reported opposing results in a study with *Cynara scolymus* L. (artichoke), *Silybum marianum* (L.) Gaertn (milk thistle) and *Cochlospermum angolensis* Welw. (borututu), in which infusions showed higher antioxidant activity than dietary supplements (Pereira et al., 2013b). This could be attributed to the very low amounts of phenolics found in those dietary supplements (3.35–30.70 mg GAE/g) in comparison with the ones obtained in the present study for *G. biloba* syrup and pills (396.98–553.41 mg GAE/g).

Regarding the extracts prepared from the dry leaves, methanolic extract (LME) showed higher DPPH radical scavenging activity, reducing power and lipid peroxidation inhibition, measured by β-carotene bleaching and TBARS inhibition. It also gave higher phenolics and flavonoids content than infusion or aqueous extract; a decrease in bioactive compounds was observed in infusion in comparison with aqueous extracts probably related to a degradation caused by heat. According to other authors, phenolic compounds are unstable and easily became non-antioxidative under heating and in the presence of antioxidants; thus, heat could destroy the structures of polyphenols and cause a decrease in their antioxidant activity (Yen and Hung, 2000).

Overall, dietary supplements containing plant extracts are complex mixtures whose therapeutic effect is often attributed to the

Table 4
Phenolics, flavonoids and antioxidant properties of *Ginkgo biloba* extracts and dietary supplements.

	LI	LME	LAE	S	P1	P2	P3
Phenolics (mg GAE/g)	37.71 ± 0.04g	129.5 ± 5.30e	61.58 ± 0.53f	461.45 ± 5.75c	396.98 ± 4.84d	501.86 ± 4.24b	553.41 ± 1.19a
Flavonoids (mg CE/g)	1.53 ± 0.01f	14.87 ± 0.84d	1.39 ± 0.32f	5.89 ± 0.90e	28.30 ± 1.61c	47.89 ± 0.55b	53.21 ± 0.59a
DPPH scavenging activity (EC_{50}, mg/mL)	1.52 ± 0.17a	0.74 ± 0.04b	1.58 ± 0.05a	0.14 ± 0.00cd	0.18 ± 0.01c	0.11 ± 0.01cd	0.07 ± 0.01d
Reducing power (EC_{50}, mg/mL)	0.83 ± 0.02a	0.36 ± 0.01c	0.73 ± 0.00b	0.14 ± 0.00d	0.13 ± 0.00e	0.08 ± 0.00f	0.06 ± 0.00g
β-Carotene bleaching inhibition (EC_{50}, mg/mL)	4.71 ± 0.35a	4.47 ± 0.08b	4.85 ± 0.10a	0.47 ± 0.05c	0.56 ± 0.07c	0.43 ± 0.03c	0.22 ± 0.02d
TBARS inhibition (EC_{50}, mg/mL)	1.29 ± 0.18a	0.13 ± 0.01cd	0.82 ± 0.09b	0.24 ± 0.06c	0.12 ± 0.01de	0.03 ± 0.00de	0.02 ± 0.01e

LI: infusion prepared from dry leaves; LME: methanolic extract prepared from dry leaves; LAE: aqueous extract prepared from dry leaves; S: syrup; P: pills. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GAE: gallic acid equivalents; CE: catechin equivalents. In each row different letters mean significant differences ($p < 0.05$).

cumulative effects of several components with bioactive properties. Thus, it is important to have an overview of all the elements present to evaluate product quality at nutraceutical and nutritional level. Particularly in *G. biloba* plant, several bioactive compounds were identified and quantified, such as tocopherols, mainly α-tocopherol, and phenolic compounds (potent antioxidants with an active role in relation to reducing the risk of atherosclerosis and attenuating neurological damage in patients with Alzheimer's disease). *G. biloba* pills (mainly P3, the dietary supplement with the highest concentration of plant extract) allow the intake of the highest antioxidants concentration.

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Annexes 2

Analytical Methods Applied to Assess the Effects of Gamma Irradiation on Color, Chemical Composition and Antioxidant Activity of *Ginkgo biloba* L.

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Analytical Methods Applied to Assess the Effects of Gamma Irradiation on Color, Chemical Composition and Antioxidant Activity of *Ginkgo biloba* L

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Abstract The extracts from the leaves of *Ginkgo biloba* are widely used in medicines and food supplements in order to overcome different health problems. To provide decontamination, irradiation is a safe and effective technique, particularly suitable to be integrated in quality control of the postharvest samples. In this study, different analytical methods were applied to assess the effects of gamma irradiation (1 and 10 kGy) in *G. biloba* color, chemical composition and antioxidant properties. Irradiation preserved macronutrients, fatty acids, γ- and δ-tocopherols, fructose, trehalose, quinic and shikimic acids. In particular, 1 kGy protected α-tocopherol, oxalic and malic acids contents, while 10 kGy decreased α-tocopherol, glucose, sucrose, oxalic and malic acids level. Nevertheless, this dose was the most effective for antioxidant activity. Overall, 1 kGy would be the recommended dose to maintain nutritional profile of *G. biloba*, protect specific molecules and also increase antioxidant activity of infusion and methanolic extracts prepared from its leaves.

Keywords Gamma irradiation · *Ginkgo biloba* · Analytical methods · Chemical characterization · Antioxidant activity

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Introduction

Ginkgo (*Ginkgo biloba* L.) is a very old tree widely used in traditional medicine, with its leaf extracts considered one of the most widely sold natural products (Kato-Noguchi et al. 2013). Ginkgo leaf extracts have been extensively studied in humans and animal models. In medicinal applications, ginkgo leaf infusions are used for treatment of asthma, bronchitis, memory, cognitive speed, edema, inflammation, and free-radical toxicity associated with traumatic brain injury (Smith et al. 1996; Diamond et al. 2000). EGb 761 is a standardized extract of *G. biloba* leaves, that contains approximately 24 % flavone glycosides (primarily quercetin, kaempferol and isorhamnetin) and 6 % terpene lactones (2.8–3.4 % ginkgolides A, B and C, and 2.6–3.2 % bilobalide), and these have been used experimentally as natural therapeutic agents in the treatment of Alzheimer's disease (Smith et al. 1996; Diamond et al. 2000; Annaházi et al. 2010). In fact, this plant is widely used the by pharmaceutical industry, which incorporates the leaf extracts in supplements and medicines (Pereira et al. 2013).

Nevertheless, the drying of plants outdoors exposes them to a high level of natural contamination, which may lead to the presence of microorganisms of great relevance to public health, such as *Salmonella* spp., *Escherichia coli*, *Clostridium perfringens*, *Bacillus cereus* and molds (Sádecká 2007).

In the pharmaceutical industry, the use of materials of good microbiological quality is one of the essential requirements, since the microorganisms can contaminate the final product, and could lead to diseases and deteriorate medications (Rosa et al. 1995). This is also important in the food industry, where microbiological decontamination provides a product with higher shelf life, while maintaining its quality (Kamat et al. 2003).

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Irradiation is a promising method for microbial safety (Sádecká 2007; Yordanov et al. 2009); because it is a safe and effective method, it is particularly suitable to be integrated in a comprehensive approach in the quality control of biological materials. It is a physical process in which the high-energy ionizing radiation passes through the target product improving their safety by inactivating microorganisms and without leaving chemical residues (Katusin-Razem et al. 2001; Shim et al. 2009; Khattak and Simpson 2010). One of the advantages of this treatment is its versatility in controlling a variety of microorganisms and insects, as well as the fact that the dietary macronutrients (carbohydrates, proteins and lipids) and micronutrients (e.g., vitamins) are not significantly affected using adequate doses of irradiation (Sádecká 2007; Khattak and Simpson 2010). Nevertheless, the technique should be tested for each particular plant, and only limited studies for gamma irradiation effects in *G. biloba* were performed (Soriani et al. 2005).

Therefore, the aim of this study was to use different analytical methods to evaluate the effects of gamma irradiation in *G. biloba* color, chemical composition and antioxidant properties, since these are related to its use in Alzheimer's disease, as previously noted.

Materials and Methods

Samples and Samples Irradiation

G. biloba L. samples were provided by Américo Duarte Paixão Lda., in Alcanede (Portugal), imported from China, as dry leaves material for infusion preparation (the taxonomical identification of the plant species mentioned in the label was confirmed). The samples were divided into three groups: control (non-irradiated, 0 kGy), group 1 and group 2, where 1 and 10 kGy were, respectively, the predicted doses.

The irradiation was performed in a Co-60 experimental chamber (Precisa 22; Graviner Manufacturing Company Ltd., UK) with a total activity of 177 TBq (4.78 kCi), in September 2013, and the estimated dose rate for the irradiation position was obtained with a Fricke dosimeter. During irradiation process, the dose was estimated using Amber Perspex routine dosimeters (batch V, from Harwell Company, Oxfordshire, UK), following the procedure previously described by Fernandes et al. (2013). The estimated doses, dose rates and dose uniformity ratios (D_{max}/D_{min}) were, respectively: 1.20±0.07 kGy, 2.57±0.15 kGy h^{-1}, 1.20 for sample 1 and 8.93±0.14 kGy, 1.91±0.03 kGy h^{-1}, 1.02 for sample 2. For simplicity, in the text and tables, we considered the values 0, 1 and 10 kGy, for the doses of non-irradiated and irradiated groups 1 and 2, respectively.

After irradiation, the samples were reduced to powder and mixed to obtain homogenized samples for subsequent analysis.

Standards and Reagents

Irradiation

To estimate the dose and dose rate of irradiation it was used a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM American Society for Testing and Materials 1992) and Amber Perspex dosimeters (batch V; Harwell Company). To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (model A10; Millipore, Billerica, MA, USA).

Chemical Analyses

Acetonitrile 99.9 %, *n*-hexane 95 % and ethyl acetate 99.8 % were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Racemic tocol (50 mg/ml) was purchased from Matreya (Pleasant Gap, PA, USA).

Antioxidant Activity Evaluation

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Sigma.

Color Measurement

A colorimeter (model CR-400; Konica Minolta Sensing, Inc., Japan), with an adapter for granular materials (model CR-A50) was used to measure the color of the samples. Using the illuminant C and diaphragm aperture of 8 mm, the CIE L^*, a^*, b^* color space values were registered using a data software "Spectra Magic Nx" (version CM-S100W 2.03.0006), from Konica Minolta Company (Japan). Before starting the measurements the instrument was calibrated against a standard white tile (Fernandes et al. 2012).

The color of three samples from each batch was measured in three different points, for each dose and at each time point, being considered the average value. The color difference or total color change for each sample was determined

using the three-dimensional color space coordinates: $\Delta E=(\Delta L^2+\Delta a^2+\Delta b^2)^{0.5}$.

Chemical Composition

Nutritional Value

Protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC 1995). The samples crude protein content (N×6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined using a Soxhlet apparatus by extracting a known weight of sample with petroleum ether; the ash content was determined by incineration at 600±15 °C. Total carbohydrates were calculated by difference and total energy was calculated according to the following equation: Energy (kcal)=4×(g protein+g carbohydrates)+9×(g fat).

Lipophilic Compounds

Fatty Acids

Fatty acids were determined after a transesterification procedure as described previously by the authors (Barros et al. 2013a), using a gas chromatographer (DANI 1000; Contone, Switzerland) equipped with a split/splitless injector and a flame ionization detector (GC-FID). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using CSW 1.7 software (DataApex 1.7, Prague, Czech Republic). The results were expressed in relative percentage of each fatty acid.

Tocopherols

Tocopherols were determined following a procedure previously optimized and described by the authors (Barros et al. 2013a). Analysis was performed by high-performance liquid chromatography (HPLC) system consisted of an integrated system with a pump (Knauer, Smartline system 1000; Berlin, Germany), degasser system (Smartline manager 5000) and autosampler (AS-2057; Jasco, Easton, MD, USA), coupled to a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

Hydrophilic Compounds

Sugars

Free sugars were determined following a procedure previously optimized and described by the authors (Barros et al. 2013a). Analysis was performed by HPLC (equipment described above) coupled to a refraction index detector (RI detector Knauer Smartline 2300; Berlin, Germany). Sugars identification was made by comparing the relative retention times of sample peaks with standards. Data were analyzed using Clarity 2.4 Software (DataApex). Quantification was based on the RI signal response of each standard, using the internal standard (IS, raffinose) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Organic Acids

Organic acids were determined following a procedure previously optimized and described by the authors (Barros et al. 2013b). Analysis was performed by ultra fast liquid chromatograph (UFLC) coupled to photodiode array detector (PDA), using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Detection was carried out in a PDA, using 215 nm and 245 as preferred wavelengths. The organic acids were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Evaluation of Antioxidant Activity

Preparation of the Extracts

Infusions Each sample (2 g) was added to 0.2 l of boiling distilled water, left to stand at room temperature for 5 min, and filtered through Whatman No. 4 paper.

Methanolic Extracts Each sample (1 g) were stirred with methanol (30 ml) at 25 °C at 150 rpm for 1 h and filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of methanol. The combined methanolic extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Flawil, Switzerland).

The infusion and methanolic extract were redissolved in water and methanol, respectively (final concentration 10 mg/ml). The final solutions were further diluted to different concentrations to be submitted to antioxidant activity evaluation and the results were expressed in

Table 1 CIE color L^* (lightness), a^* (redness) and b^* (yellowness) of non-irradiated and irradiated *G. biloba* samples

	0 kGy	1 kGy	10 kGy
L^*	46.43±1.42[a]	46.15±2.71[a]	42.94±1.58[b]
b^*	22.58±2.17[a]	22.18±1.64[a]	20.52±2.03[a]
ΔE	51.90±1.55[a]	51.37±2.75[a]	49.14±1.92[b]

The results are presented as mean ± SD. In each row, different letters mean significant differences, $p<0.05$

The value of ΔE, total color, was determined using the expression:

$$\Delta E = \sqrt{(L*)^2 + (a*)^2 + (b*)^2}$$

EC_{50} values (sample concentration providing 50 % of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as positive control.

Antioxidant Activity In Vitro Assays DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH}-A_S)/A_{DPPH}]\times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2 h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the color intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A-B)/A]\times 100$ %, where A and B are the absorbance of the control and the sample solution, respectively (Fernandes et al. 2013).

Table 2 Macronutrients and energetic value of non-irradiated and irradiated *G. biloba* samples (mean ± SD)

	0 kGy	1 kGy	10 kGy
Ash (g/100 g dw)	12.91±0.20[a]	12.74±0.65[a]	12.34±1.59[a]
Proteins (g/100 g dw)	15.32±0.16[a]	15.19±0.59[a]	12.79±0.15[b]
Fat (g/100 g dw)	4.42±0.13[a]	4.32±0.24[a]	4.56±0.42[a]
Carbohydrates (g/100 g dw)	67.36±0.28[b]	67.75±0.74[b]	70.31±0.88[a]
Energy (kcal/100 g dw)	370.44±0.09[a]	370.64±2.69[a]	373.44±5.98[a]

In each row, different letters mean significant differences ($p<0.05$)

dw dry weight

Statistical Analysis

Three samples from each group were analysed and all the assays were carried out in triplicate. The results are

Table 3 Lipophilic compounds (fatty acids and tocopherols) of non-irradiated and irradiated *G. biloba* samples (mean ± SD) Caproic acid (C6:0); caprylic acid (C8:0); capric acid (C10:0); undecanoic acid (C12:0); tridecanoic acid (C13:0); myristic acid (C14:0); myristoleic acid (C14:1); pentadecanoic acid (C15:0); *cis*-10-pentadecenoic acid (C15:1); palmitic acid (C16:0); palmitoleic acid (C16:1); heptadecanoic acid (C17:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6); α-linolenic acid (C18:3n3); stearic acid (20:0); eicosenoic acid (C20:1); *cis*-11,14,17-eicosatrienoic acid and heneicosanoic acid (C20:3n3+C21:0); behenic acid (C22:0); tricosanoic acid (C23:0); lignoceric acid (C24:0). In each row, different letters mean significant differences ($p<0.05$)

	0 kGy	1 kGy	10 kGy
C6:0	0.11±0.01	0.17±0.01	0.14±0.03
C8:0	0.14±0.02	0.32±0.01	0.19±0.04
C10:0	0.15±0.03	0.13±0.02	0.18±0.02
C12:0	0.95±0.06	0.88±0.06	1.09±0.14
C13:0	0.21±0.01	0.25±0.01	0.25±0.03
C14:0	9.58±0.08	9.10±0.48	10.19±1.00
C14:1	3.34±0.21	3.17±0.21	3.41±0.19
C15:0	0.52±0.05	0.67±0.03	1.01±0.10
C15:1	0.09±0.00	0.07±0.00	0.10±0.00
C16:0	24.84±0.51	23.50±0.66	25.15±0.57
C16:1	0.90±0.05	1.00±0.03	0.92±0.09
C17:0	0.85±0.03	0.85±0.02	0.90±0.03
C18:0	2.66±0.06	2.41±0.05	2.45±0.04
C18:1n9	7.03±0.06	6.74±0.06	6.66±0.23
C18:2n6	7.94±0.54	8.21±0.47	7.73±0.21
C18:3n3	28.64±2.12	31.63±0.87	28.85±2.31
C20:0	3.63±0.07	2.56±0.04	2.65±0.12
C20:1	0.19±0.02	0.17±0.01	0.27±0.03
C20:3n3+C21:0	1.21±0.01	1.48±0.06	1.29±0.23
C22:0	2.25±0.26	2.34±0.03	2.22±0.11
C23:0	0.87±0.01	0.82±0.06	0.71±0.02
C24:0	3.90±0.20	3.54±0.13	3.65±0.01
Total SFA (relative %)	50.65±2.83[a]	47.54±1.13[a]	50.79±2.24[a]
Total MUFA (relative %)	11.56±0.16[a]	11.16±0.15[a]	11.35±0.51[a]
Total PUFA (relative %)	37.79±2.67[a]	41.31±1.29[a]	37.86±2.74[a]
α-Tocopherol	58.77±0.74[b]	61.18±0.15[a]	52.64±0.92[c]
β-Tocopherol	28.96±0.74[b]	29.59±0.62[ab]	30.30±0.59[a]
γ-Tocopherol	0.92±0.02[a]	0.98±0.13[a]	0.95±0.01[a]
δ-Tocopherol	0.60±0.04[a]	0.54±0.03[a]	0.54±0.04[a]
Total tocopherols (mg/100 g)	89.25±1.53[b]	92.29±0.86[a]	84.43±1.56[c]

expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with α=0.05. This treatment was carried out using SPSS v. 22.0 program (IBM Corp.).

Results and Discussion

Color

The results of CIE color *L** (lightness), *a** (redness) and *b** (yellowness) of non-irradiated and irradiated *G. biloba* samples are presented in Table 1. It seems that for the highest dose, 10 kGy, there was a tendency for the samples to loose lightness, the *L** value diminishes, when compared to non-irradiated (0 kGy) and irradiated samples (1 kGy). For *b** value, which represents the yellowness–blueness tendency, no significant difference was observed with irradiation dose. Data values for *a** parameter are close to zero (data not shown). Color is a parameter of great importance not only for plants but also for other foods. For example, the cosmetics industry has a very stringent selection of the plants color level; the dark color of some natural matrices such as green tea or persimmon leaf makes very difficult their application in food or cosmetic products, and the process to remove

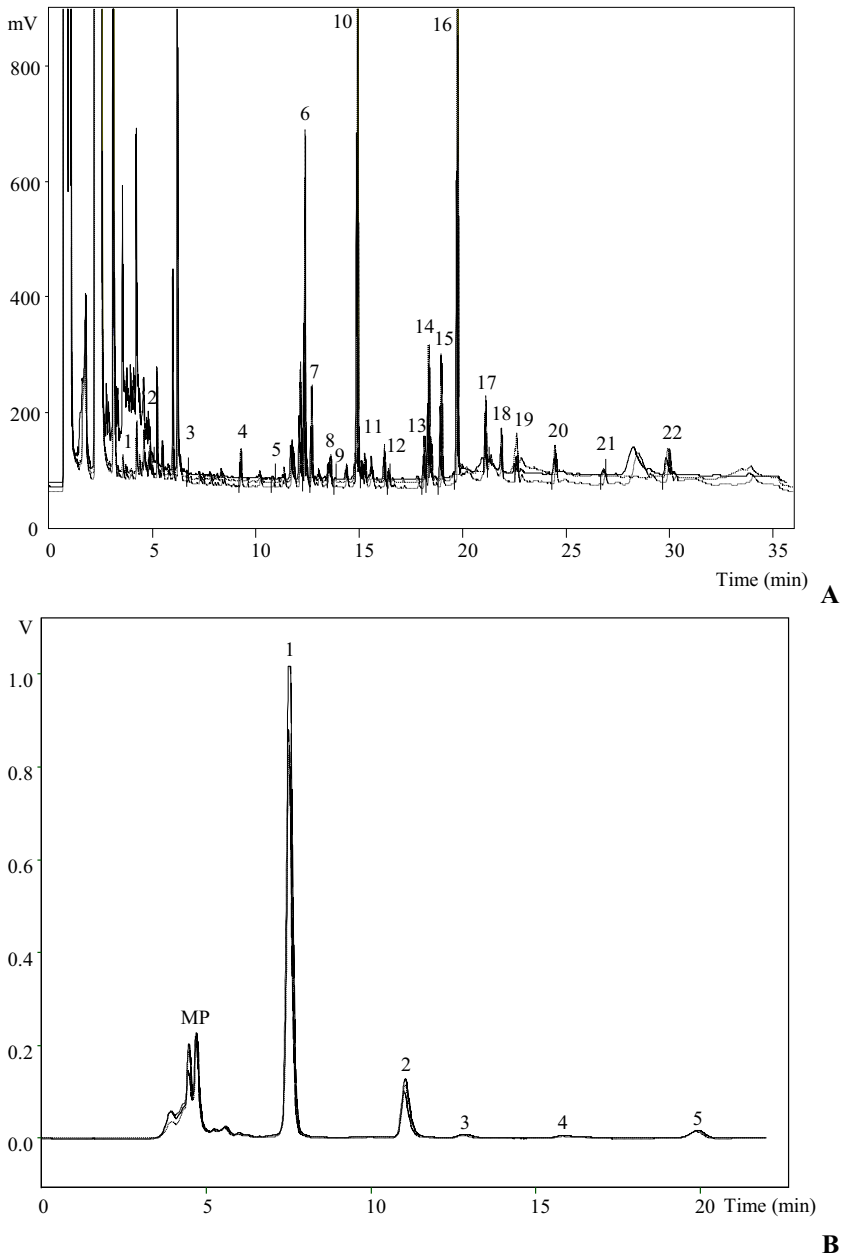


Fig. 1 Individual profile of 0 kGy (*dotted line*), 1 kGy (*solid line*) and 10 kGy (*broken line*) samples in **a** fatty acids: *1* C6:0, *2* C8:0, *3* C10:0, *4* C12:0, *5* C13:0, *6* C14:0, *7* C14:1, *8* C15:0, *9* C15:1, *10* C16:0, *11* C16:1, *12* C17:0, *13* C18:0, *14* C18:1n9, *15* C18:2n6, *16* C18:3n3, *17* 20:0, *18* C20:1, *19* C20:3n3+C21:0, *20* C22:0, *21* C23, *22* C24:0. **b** Tocopherols: *1* α-tocopherol, *2* β-tocopherol, *3* γ-tocopherol, *4* δ-tocopherol, *5* tocol (IS). *MP* mobile phase

color is a difficult, time-consuming, and costly procedure (Jo et al. 2003a,b).

Nutritional Profile

The nutritional profile of non-irradiated and irradiated *G. biloba* samples is shown in Table 2. The control sample showed values very similar to the ones obtained previously by the authors in a sample from different commercial origin (Pereira et al. 2013). Gamma irradiation did not alter significantly the nutritional profile of the samples, regarding ash, fat and energy since similar values were obtained in the control and in samples irradiated with 1 or 10 kGy, being in agreement with previous studies in other foods, such as hazelnuts, walnuts, almonds, and pistachios (Gecgel et al. 2011). Nevertheless, 10 kGy led to a decrease in proteins content (the same was observed in the study of Fernandes et al. 2013 on wild mushrooms) and, consequently, an increase in carbohydrates level (which were determined by difference). Protein values have been previously reported as having no significant changes after irradiation treatments (Fernandes et al. 2012; Kasera et al. 2012; Fernandes et al. 2014). The decrease of protein levels at 10 kGy could be explained by a possible degradation due to the high intensity applied. Proteins are known to be the most reliable irradiation indicators, especially due to degradation reactions such as scission of the C–N bonds in the backbone of the polypeptide chain or splitting of the disulfide bonds, and physical changes like unfolding and aggregation (Molins 2001). Nevertheless, the fact that irradiation induces alterations in the protein content, does not mean a significant problem in the nutritional point of view, since protected amino acids within the structure of the protein complex generally resists to this method (Kausar et al. 2013).

Composition in Lipophilic Compounds

Regarding fatty acids, 22 different molecules were identified (Table 3), which is in accordance with a previous study of the authors (Pereira et al. 2013). Irradiated and control samples revealed the same fatty acids profile, with α-linolenic acid as the major compound, followed by palmitic acid. Some studies showed that the lack of α-linolenic acid in the diet compromises the brain and heart function (Taha et al. 2006; Nguemeni et al. 2013), and therefore, it is important to preserve this and other compounds in irradiated samples. In all the samples, saturated fatty acids appeared in higher concentrations, followed by polyunsaturated and lastly monounsaturated fatty acids. No significant differences were observed between the control and the irradiated samples at two different doses (Table 3; Fig. 1a). Similar results were reported for cashew nuts (Mexis and Kontominas 2009) and lamb meat (Alfaia et al. 2007), where no significant differences were observed in the concentration of SFA, MUFA and PUFA.

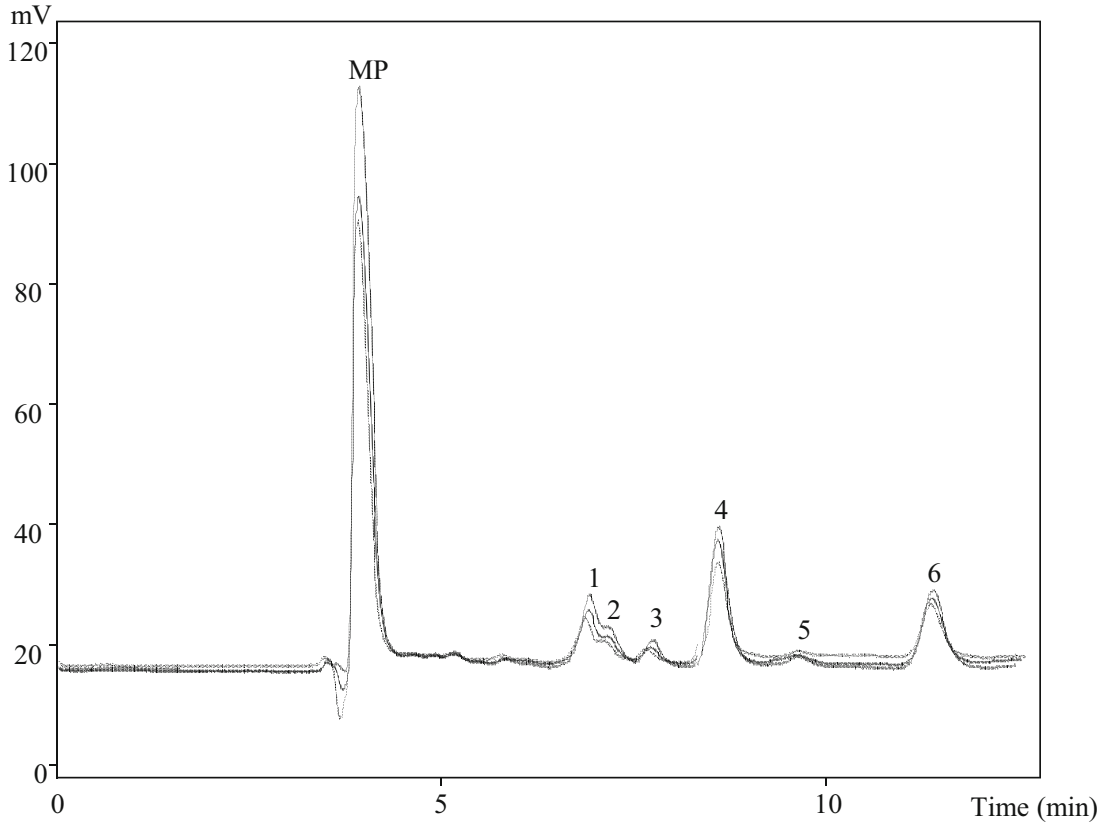
Table 4 Hydrophilic compounds (sugars and organic acids) of non-irradiated and irradiated *G. biloba* samples (mean ± SD) In each row, different letters mean significant differences ($p<0.05$)

Free sugars (g/100 g dw)	0 kGy	1 kGy	10 kGy
Fructose	1.86±0.12^{a}	1.87±0.17^{a}	1.81±0.01^{a}
Glucose	0.98±0.07^{a}	0.73±0.03^{b}	0.79±0.04^{b}
Sucrose	3.78±0.09^{a}	3.83±0.11^{a}	3.60±0.07^{b}
Threalose	0.38±0.04^{a}	0.41±0.00^{a}	0.40±0.02^{a}
Unknown	0.55±0.03^{a}	0.51±0.04^{a}	0.50±0.05^{a}
Total	7.55±0.07^{a}	7.35±0.34^{ab}	7.10±0.04^{b}
Organic acids (g/100 g)	0 kGy	1 kGy	10 kGy
Oxalic	0.82±0.00^{b}	0.89±0.00^{a}	0.80±0.00^{c}
Quinic	1.37±0.09^{a}	1.31±0.01^{a}	1.33±0.01^{a}
Malic	1.09±0.00^{b}	1.21±0.02^{a}	1.05±0.01^{c}
Shikimic	1.49±0.09^{a}	1.43±0.00^{a}	1.42±0.01^{a}
Total	4.78±0.17^{ab}	4.83±0.01^{a}	4.60±0.03^{b}

dw dry weight

Data concerning tocopherols (also lipophilic compounds) concentration are given in Table 3. The four vitamers were found in all the analyzed samples of *G. biloba*, with α-tocopherol as predominant form, as also stated by the authors in a sample from different commercial origin previously studied (Pereira et al. 2013), despite significant differences observed in the concentrations reported. In fact, tocopherols are very sensitive molecules that suffer rapid variation due to oxidation processes (Birringer et al. 2001; Luo et al. 2011). α-Tocopherol was the most susceptible isoform to irradiation process, decreasing with 10 kGy (Fig. 1b). Nevertheless, it should be pointed out that 1 kGy of irradiation dose protected degradation of this vitamer (Fig. 1b) (the same happened with the same irradiation dose, in a study with *Carya illinoensis* (Taipina et al. 2009)), which is very important as α-tocopherol holds several beneficial functions for humans, including antioxidant, anti-inflammatory, anti-carcinogenic and antiatherogenic properties (Manosso et al. 2013). In other studies performed in plants (sage, thyme, and oregano) irradiated with 10 kGy, there were no significant differences in the content of α- and γ-

Fig. 2 Individual profile of 0 kGy (*dotted line*), 1 kGy (*solid line*) and 10 kGy (*broken line*) samples in **a** sugars: *1* fructose, *2* glucose, *3* unknown, *4* sucrose, *5* trehalose, *6* melezitose (IS). **b** Organic acids: *1* oxalic acid, *2* quinic acid, *3* malic acid, *4* shikimic acid. *MP* mobile phase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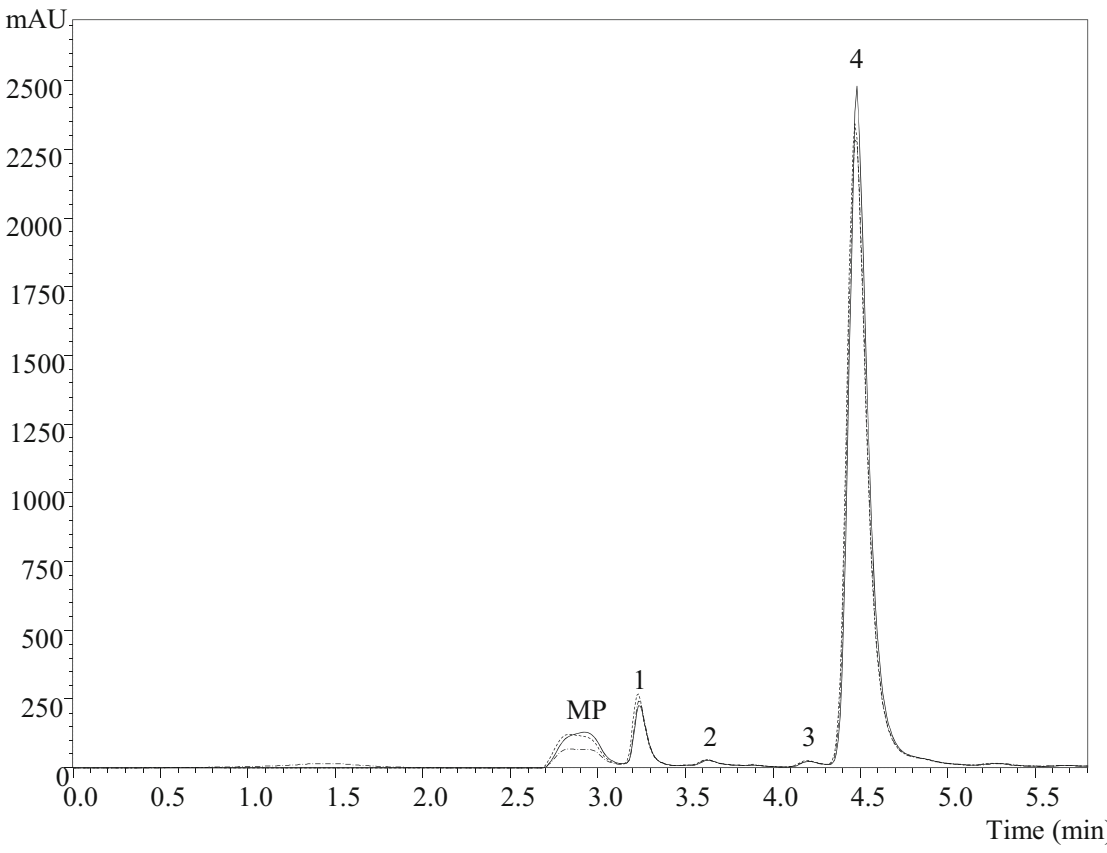
A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Time (min)

B

Table 5 Antioxidant activity (EC_{50} values, mg/ml) of infusions and methanolic extracts obtained from non-irradiated and irradiated *G. biloba* samples (mean ± SD)

Antioxidant activity	Infusion			Methanolic extract		
	0 kGy	1 kGy	10 kGy	0 kGy	1 kGy	10 kGy
DPPH scavenging activity	5.80±0.24^{a}	4.09±0.07^{b}	2.88±0.23^{c}	1.64±0.02^{a}	1.54±0.05^{ab}	1.49±0.16^{b}
Reducing power	4.58±0.06^{a}	3.41±0.01^{b}	2.37±0.02^{c}	0.65±0.00^{a}	0.63±0.00^{b}	0.49±0.00^{c}
β-Carotene bleaching inhibition	11.09±0.54^{a}	9.04±0.35^{b}	8.79±0.23^{b}	10.39±0.66^{a}	5.26±0.18^{b}	4.48±0.17^{c}
TBARS inhibition	0.15±0.01^{a}	0.13±0.01^{b}	0.10±0.01^{c}	0.24±0.01^{a}	0.16±0.03^{b}	0.08±0.00^{c}

tocopherol in control and irradiated samples (Brandstetter et al. 2009).

Composition in Hydrophilic Compounds

The composition in hydrophilic compounds was also assessed and the results are shown in Table 4. Fructose, glucose, sucrose and trehalose were identified and quantified in the samples, but with a slight different profile in relation to the one described for other samples of *G. biloba*, in which trehalose was not found (Pereira et al. 2013). In fact, sugar concentration depends on the maturity stage of the sample leaves and other environmental factors that influence the use of these primary metabolites for energy production (Apone et al. 2010). There was an observed decrease in glucose in both irradiated samples, while no significant differences were observed in regard to fructose and trehalose levels, and sucrose (the most abundant sugar) decreased only with 10 kGy of gamma radiation dose (Fig. 2a). Another study attributed the observed increase in sugar levels to a degradation of polysaccharides with the application of gamma irradiation (Kausar et al. 2013); in the present study, this did not occur.

Regarding organic acids (Table 4), oxalic, quinic, malic and shiquimic acids were identified and quantified in all the analyzed samples, which is in agreement with results reported by Pereira et al. (2013). Quinic and shikimic acids concentration was similar in all samples, which shows that gamma irradiation does not affect significantly these compounds. On the other hand, irradiation at 1 kGy protected oxalic and malic acids (higher values), while 10 kGy decreased their concentration (Fig. 2b). The decrease could be explain by a degradation process when 10 kGy is applied, being this doses much higher than 1 kGy, that showed a protective effect, maintaining the content found. According to a study performed by Wen et al. (2006) in irradiated lycium fruit, the concentration of malic and oxalic acids did not change significantly.

Antioxidant Properties

The results of antioxidant properties of infusions and methanolic extracts prepared form non-irradiated and irradiated samples, measured by four in vitro assays, are presented in Table 5. In general, methanolic extracts gave higher antioxidante activity (lower EC_{50} values) than the corresponding infusions (EC_{50} values ranging from 0.24 and 4.48 mg/ml when compared to the infusion 0.13–9.04 mg/ml), which is in agreement with results reported by Pereira et al. (2013). These results are also consistent with a previous study, where the alcoholic extracts showed better results than the corresponding infusions prepared from irradiated Korean medicinal plants (Byun et al. 1999).

For both infusion and methanolic extract, gamma irradiation at both doses increased DPPH scavenging activity, reducing power, β-carotene bleaching and lipid peroxidation inhibition of Ginkgo samples. In general, gamma irradiation at 10 kGy promotes more the antioxidant potential of *G. biloba* infusion and methanolic extract. This is in agreement with the results reported by the research group in a previous study with *Castanea sativa* fruits and skins (Antonio et al. 2011). Khattak et al. (2008) also reported an increase in DPPH scavenging properties of *Nigella sativa* seeds irradiated at 16 kGy).

The analytical methods used proved that irradiation can be a good alternative for *G. biloba* preservation since it maintained macronutrients, fatty acids, γ- and δ-tocpherols, fructose, trehalose, quinic and shikimic acids. Furthermore, 1 kGy protected α-tocopherol, oxalic and malic acids contents, while 10 kGy decreased α-tocopherol, glucose, sucrose, oxalic and malic acids level. Therefore, 1 kGy would be the recommended dose since it maintained the nutritional profile of *G. biloba*, protected specific molecules and increased antioxidant activity of infusion and methanolic extracts prepared from its leaves.

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Conflict of Interest Eliana Pereira declares that she has no conflict of interest. Lillian Barros declares that she has no conflict of interest. Amilcar L. Antonio declares that he has no conflict of interest. Albino Bento declares that he has no conflict of interest. Isabel C.F.R. Ferreira declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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Annexes 3

Gamma irradiation improves the extractability of phenolic compounds in *Ginkgo biloba* L.

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Gamma irradiation improves the extractability of phenolic compounds in *Ginkgo biloba* L.

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ABSTRACT

Irradiation has been increasingly recognized as an effective decontamination technique that ensures the chemical and organoleptic quality of medicinal and aromatic plants. The aim of the present study was to evaluate the effects of gamma irradiation in the phenolic compounds of *Ginkgo biloba* L. (infusion and methanol/water extract), widely used in traditional medicine and in dietary supplements. Twenty-five compounds were detected, eighteen of which were flavonoids, one phenolic acid, five terpene lactones and one unknown compound. Among the quantified phenolic compounds, flavonoids were the main group present, being two kaempferol derivatives the major compounds found: kaempferol-3-*O*-dirhamnosylglucoside and kaempferol-3-*O*-rutinoside. The irradiation with the highest dose (10 kGy) is sufficient to guarantee the product disinfestation and microbial decontamination, also contributing to an increase in the extractability of phenolic compounds, both in methanol/water and infusion preparations.

1. Introduction

The interest for natural antioxidants has been increasing over the years. Phenolic compounds comprise a very large group of biologically active molecules, being appreciated for their beneficial effects on health (physiologically active compounds with anti-allergic, anti-atherogenic, antimicrobial, antithrombotic, anti-inflammatory, antioxidant, cardioprotective and vasodilatory effects) (Mendel and Youdim, 2004; Balasundram et al., 2006; Martins et al., 2011). Their mechanism of action as antioxidants is considered essential regarding the reduction of the oxidation processes in the body, playing an important role in maintaining health, including protection of the cells and biomacromolecules and, therefore, intervening against certain human diseases (cancer, inflammatory diseases, neurological degeneration, heart disease, and many others) (Lan et al., 2007; Rawat et al., 2011; Acosta-Estrada et al., 2014). Thus, the most cited forms of intervention of antioxidant potential of phenolic compounds are their ability to scavenge reactive oxygen species and to chelate metal ions (Port's et al., 2013).

Plants are some of the most important sources of natural antioxidants including phenolic compounds (e.g. phenolic acids and flavonoids, phenolic diterpenes and tannins), which have been related with the bioactivity of several medicinal plants (Sati et al., 2013). One of those plants that has been highly studied is *Ginkgo biloba* L. due to its use in traditional medicine, but also by professionals in the medical field in order to treat problems typically associated with aging, such as intermittent claudication, decreased mental vitality in old age (mental confusion, memory loss, dementia praecox, concentration problems), poor circulation and tinnitus (Diamond et al., 2000). The extracts from *G. biloba*, such as EGb761 in Tebonin®, are also used as alternative therapy against Alzheimer's disease (van Beek and Montoro, 2009; Parimoo et al., 2014). The products with this plant are commercially available in various forms: leaves for infusions preparation, standardized extracts, pills, capsules or oral solutions (Liu et al., 2014).

The effectiveness of the therapeutic use of this plant leads to a strong demand from the pharmaceutical industry (Koch, 2005). However, due to the strict hygiene standards applied for raw materials to be incorporated into pharmaceuticals and/or dietary supplements, efficient decontamination methods are necessary, avoiding other alternatives that may leave chemical residues in the plant (Haleem et al., 2014).

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The use of irradiation has been increasingly accepted for decontamination and conservation, since it does not significantly affect (at specific doses) the organoleptic and physico-chemical properties of the irradiated matrices (Alothman et al., 2009; Kirkin et al., 2014), including *G. biloba* (Pereira et al., 2015). This method reduces reliance on chemical fumigants (ethylene oxide and methyl bromide), which have been pointed out as mutagens and carcinogens to humans, leave chemical residue on plants and destroy the ozone layer in the atmosphere (Migdal and Owczarczyk, 1998; Chmielewski and Migdał, 2005).

Therefore, this study aims to evaluate if gamma irradiation (at doses of 1 and 10 kGy) improves the extraction of phenolic compounds using *G. biloba* (infusion and methanolic extract) as source material.

2. Materials and methods

2.1. Samples and samples irradiation

G. biloba L. samples were supplied by Américo Duarte Paixão Lda. Alcanede (Portugal), imported from China, as dry leaves material. The botanical identification was confirmed by the biologist, Dr Carlos Aguiar of the Escola Superior Agrária of the Polytechnic Institute of Bragança (Trás-os-Montes, Portugal). The samples were divided into three groups: control (non-irradiated, 0 kGy), groups 1 and 2, where, 1 kGy and 10 kGy were, respectively, the predicted doses.

The irradiation was performed in a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with total activity 177 TBq (4.78 kCi), in September 2013, and the estimated dose rate for the irradiation position was obtained with Fricke dosimeter. During irradiation process, the dose was estimated using Amber Perspex routine dosimeters (batch V, from Harwell Company, U.K.), following the procedure previously described by Pereira et al. (2015). The estimated doses, dose rates and dose uniformity ratios (D_{max}/D_{min}) were, respectively: 1.20 ± 0.07 kGy, 2.57 ± 0.15 kGy h^{-1}, 1.20 for sample 1 and 8.93 ± 0.14 kGy, 1.91 ± 0.03 kGy h^{-1}, 1.02 for sample 2. For simplicity, in the text and tables, we considered the values 0, 1 and 10 kGy, for the doses of non-irradiated and irradiated groups 1 and 2, respectively.

After irradiation, the samples were reduced to powder and mixed to obtain homogenized samples for subsequent analysis.

2.2. Standards and reagents

For irradiation: to estimate the dose and dose rate of irradiation, a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Company, UK) were used. The acid aqueous Fricke dosimeter solution was prepared using ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA).

For chemical analyses: HPLC-grade acetonitrile was obtained from Merck KgaA (Darmstadt, Germany). Formic acid was purchased from Prolabo (VWR International, Fontenay-sous-Bois, France). Phenolic standards were from Extrasynthèse (Genay, France). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Phenolic compounds

2.3.1. Extraction procedure

Methanol/water extracts: each sample (1 g) was extracted with 30 mL of methanol/water 80:20 (v/v) at room temperature, 150 rpm, for 1 h. The extract was filtered through Whatman 4 paper. The residue was then re-extracted twice, with additional 30 mL portions of methanol/water 80:20 (v/v). The combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210), until complete removal of methanol. The aqueous phase was lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) (Barros et al., 2013).

Infusions preparation: each sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure; afterwards the obtained infusion was frozen and lyophilized (Barros et al., 2013).

Methanol/water extracts and lyophilized infusions were redissolved in 20% aqueous methanol and water, respectively, at 20 mg/mL and filtered through a 0.22 μm disposable LC filter disc for high performance liquid chromatography (HPLC) analysis.

2.3.2. Analysis of phenolic compounds

Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors (Barros et al., 2013). Double online detection was carried out in a DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system via the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. The phenolic compounds were identified by comparing their retention time, UV–vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compounds from the same phenolic group. The results were expressed in mg/g of methanol/water extract and lyophilized infusion.

2.4. Statistical analysis

Three samples from each group were analyzed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 program (IBM Corp.).

3. Results and discussion

The chromatographic profile of non-irradiated and irradiated at 10 kGy *G. biloba* samples, obtained after methanol/water extraction, and recorded at 370 nm is shown in Fig. 1; compound characteristics and tentative identities are presented in Table 1. Twenty-five compounds were detected, eighteen of which were flavonoids, one phenolic acid, five terpene lactones and one unknown compound.

Protocatechuic acid (compound **3**), myricetin-3-*O*-rutinoside (compound **11**), quercetin-3-*O*-rutinoside (compound **15**), quercetin-3-*O*-glucoside (compound **17**), kaempferol-3-*O*-rutinoside (compound **19**), isorhamnetin-3-*O*-rutinoside (compound **20**) and isorhamnetin-3-*O*-glucoside (compound **22**) were positively identified according to their retention, mass and UV–vis characteristics by comparison with commercial standards. All the compounds mentioned above, with the exception of protocatechuic acid were previously reported in leaves of *G. biloba*

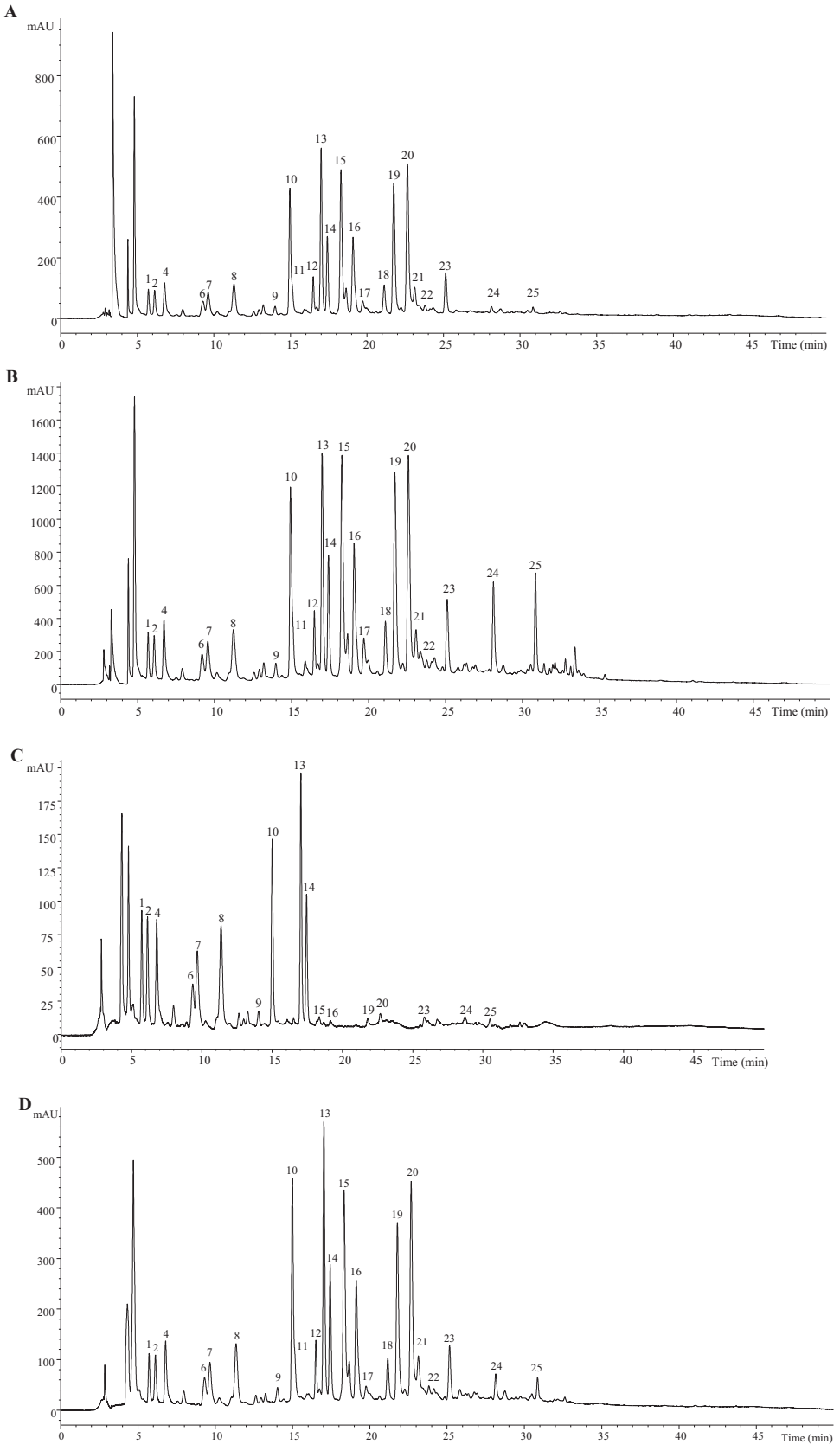

Fig. 1. Chromatographic profile of non-irradiated methanol/water (A), irradiated at 10 kGy methanol/water (B), non-irradiated infusion (C) and irradiated at 10 kGy infusion (D) of *G. biloba* samples, recorded at 370 nm.

Table 1
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and compound identification in *G. biloba* samples.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification
1	5.7	260,294,350	453	407 (100), 245 (3), 179 (7), 161 (3), 113 (5)	Ginkgolide A derivative
2	6.1	358	449	403 (16), 269 (4), 205 (8), 179 (5), 113 (4)	Unknown ginkgolide
3	6.2	262sh294	153	109 (100)	Protocatechuic acid
4	6.8	252,356	325	163 (100), 119 (87)	Bilobalide
5	7.1	274	407	245 (100)	Ginkgolide A
6	9.3	282sh336	423	221 (15), 179 (13), 161 (22), 131 (21), 113 (32)	Ginkgolide B
7	9.6	348	755	593 (100), 285 (22)	Kaempferol-3-*O*-rhamnosylhexoside-7-*O*-glucoside
8	11.3	358	785	623 (100), 315 (17)	Isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside
9	14.0	348	593	447 (23), 285 (58)	Kaempferol-*O*-rhamnosyl-glucoside
10	15.0	354	755	301 (100)	Quercetin 3-*O*-2″,6″-dirhamnosylglucoside
11	15.1	350	625	317 (100)	Myricetin-3-*O*-rutinoside
12	16.5	318	439[a]	411 (18), 383 (93), 365 (12), 322 (26), 304 (7), 277 (7), 259 (8)	Ginkgolide C derivative
13	17.0	348	739	285 (100)	Kaempferol-3-*O*-dirhamnosylglucoside
14	17.4	356	769	315 (100)	Isorhamnetin-3-*O*-dirhamnosylglucoside
15	18.3	356	609	301 (100)	Quercetin-3-*O*-rutinoside
16	19.1	360	639	331 (100)	Patuletin-3-*O*-rutinoside
17	19.7	358	463	301 (100)	Quercetin-3-*O*-glucoside
18	21.1	352	609	301 (100)	Quercetin-3-*O*-glucosyl-(1,2)-rhamnoside
19	21.7	348	593	285 (100)	Kaempferol-3-*O*-rutinoside
20	22.6	356	623	315 (100)	Isorhamnetin-3-*O*-rutinoside
21	23.1	352	447	301 (100)	Quercetin-3-*O*-rhamnoside
22	23.8	350	477	315 (100)	Isorhamnetin-3-*O*-glucoside
23	25.1	348	593	285 (100)	Kaempferol-3-*O*-glucosyl-(1,2)-rhamnoside
24	28.1	268,316	755	609 (46), 301 (21)	Quercetin-3-*O*-*p*-coumaroyl-rhamnosylhexoside
25	30.8	266,316	739	593 (51), 285 (21)	Kaempferol-3-*O*-*p*-coumaroyl-rhamnosylhexoside

[a] 879 $[2M-H]^-$.

Table 2
Quantification of the phenolic compounds (mg/g of extract/lyophilized infusion) identified in methanol/water extracts and infusions of *G. biloba* non-irradiated and irradiated samples.

Compounds	Methanol/water extracts			Infusions		
	0 kGy	1 kGy	10 kGy	0 kGy	1 kGy	10 kGy
Ginkgolide A derivative	nq	nq	nq	nq	nq	nq
Unknown ginkgolide	nq	nq	nq	nq	nq	nq
Protocatechuic acid	1.39 ± 0.08	1.22 ± 0.04	4.49 ± 0.05	0.43 ± 0.04	0.38 ± 0.01	1.95 ± 0.05
Bilobalide	nq	nq	nq	nq	nq	nq
Ginkgolide A	nq	nq	nq	nq	nq	nq
Ginkgolide B	nq	nq	nq	nq	nq	nq
Kaempferol-3-*O*-rhamnosylhexoside-7-*O*-glucoside	0.34 ± 0.02	0.49 ± 0.01	1.16 ± 0.06	0.24 ± 0.02	0.25 ± 0.01	0.34 ± 0.05
Isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside	0.32 ± 0.04	0.45 ± 0.04	1.09 ± 0.14	0.22 ± 0.02	0.24 ± 0.02	0.35 ± 0.05
Kaempferol-*O*-rhamnosyl-glucoside	0.12 ± 0.02	0.16 ± 0.01	0.53 ± 0.11	0.03 ± 0.01	0.04 ± 0.00	0.11 ± 0.02
Quercetin 3-*O*-2″,6″-dirhamnosylglucoside	0.55 ± 0.01	0.66 ± 0.04	1.67 ± 0.07	0.17 ± 0.01	0.19 ± 0.01	0.56 ± 0.03
Myricetin-3-*O*-rutinoside	0.11 ± 0.01	0.07 ± 0.01	0.59 ± 0.08	nd	nd	0.12 ± 0.03
Ginkgolide C derivative	nq	nq	nq	nd	nq	nq
Kaempferol-3-*O*-dirhamnosylglucoside	1.26 ± 0.01	1.48 ± 0.02	3.57 ± 0.03	0.44 ± 0.02	0.40 ± 0.02	1.29 ± 0.01
Isorhamnetin-3-*O*-dirhamnosylglucoside	0.42 ± 0.01	0.51 ± 0.03	1.30 ± 0.02	0.16 ± 0.01	0.15 ± 0.01	0.45 ± 0.01
Quercetin-3-*O*-rutinoside	0.84 ± 0.06	0.34 ± 0.02	2.74 ± 0.07	tr	0.07 ± 0.01	0.74 ± 0.03
Patuletin-3-*O*-rutinoside	0.52 ± 0.01	0.25 ± 0.01	1.89 ± 0.04	tr	0.04 ± 0.00	0.51 ± 0.03
Quercetin-3-*O*-glucoside	0.08 ± 0.01	tr	0.56 ± 0.05	nd	nd	0.05 ± 0.02
Quercetin-3-*O*-glucosyl-(1,2)-rhamnoside	0.20 ± 0.02	0.04 ± 0.01	0.78 ± 0.06	nd	tr	0.17 ± 0.01
Kaempferol-3-*O*-rutinoside	1.38 ± 0.04	0.57 ± 0.03	4.21 ± 0.02	0.02 ± 0.00	0.16 ± 0.02	1.15 ± 0.04
Isorhamnetin-3-*O*-rutinoside	1.06 ± 0.02	0.49 ± 0.03	3.05 ± 0.06	0.03 ± 0.00	0.16 ± 0.01	0.95 ± 0.01
Quercetin-3-*O*-rhamnoside	0.16 ± 0.01	0.09 ± 0.01	0.67 ± 0.05	nd	tr	0.17 ± 0.02
Isorhamnetin-3-*O*-glucoside	0.15 ± 0.04	0.12 ± 0.01	0.46 ± 0.05	nd	0.04 ± 0.00	0.16 ± 0.01
Kaempferol-3-*O*-glucosyl-(1,2)-rhamnoside	0.49 ± 0.02	0.15 ± 0.03	1.81 ± 0.15	0.02 ± 0.00	0.02 ± 0.00	0.38 ± 0.01
Quercetin-3-*O*-*p*-coumaroyl-rhamnosylhexoside	0.06 ± 0.01	0.04 ± 0.00	1.30 ± 0.08	tr	tr	0.10 ± 0.02
Kaempferol-3-*O*-*p*-coumaroyl-rhamnosylhexoside	0.11 ± 0.01	0.09 ± 0.01	1.83 ± 0.04	0.02 ± 0.00	0.01 ± 0.00	0.15 ± 0.01
Total phenolic acids	1.40 ± 0.08b	1.22 ± 0.04c	4.49 ± 0.05a	0.43 ± 0.04b	0.38 ± 0.01b	1.95 ± 0.05a
Total flavonoids	8.16 ± 0.04b	6.00 ± 0.18c	29.20 ± 0.74a	1.35 ± 0.01c	1.75 ± 0.06b	7.76 ± 0.16a
Total phenolic compounds	9.56 ± 0.06b	7.22 ± 0.22c	33.69 ± 0.69a	1.78 ± 0.06c	2.13 ± 0.04b	9.70 ± 0.11a

nd: not detected; nq: not quantified; tr: traces. In each row and for each extract (methanol/water extracts or infusions) different letters mean significant differences among total compounds ($p < 0.05$).

(Tang et al., 2001; Zhang et al., 2007; Ding et al., 2008 Lin et al., 2008).

Peaks 1, 2, 4–6 and 12 were associated to terpene trilactones. That type of compounds has low UV absorption and coexisting substances present in the complex matrix of *G. biloba* extracts make it difficult for their detection and quantification, using UV detection (Sloley et al., 2003; Mesbah et al., 2005). Nonetheless, they could be detected in the analyzed extracts and tentatively identified based on their MS characteristics and comparison with data reported in the bibliography (Bedir et al., 2002; van Beek, 2002;

Ding et al., 2008; Lin et al., 2008; van Beek and Montoro, 2009; Rossi et al., 2011; Liu et al., 2014); in particular, bilobalide (compound **4**), ginkgolide A (compound **5**), ginkgolide B (compound **6**) and ginkgolide C derivative (compound **12**). The latter compound should correspond to a derivative of ginkgolide C, since ginkgolide C would be expected to elute earlier than ginkgolide A and B (Ding et al., 2008; Lin et al., 2008; van Beek and Montoro, 2009). No identification could be assigned to compound **1** ($[M–H]^-$ at *m*/*z* 453), although the major *m*/*z* fragment at 407 might point to it was a derivative of ginkgolide A. No conclusions could be drawn about the identity and nature of compound **2** ($[M–H]^-$ at *m*/*z* 449). Three compounds with the same pseudomolecular ion were also detected by Ding et al. (2008) in *G. biloba* supplements, which were assigned as unknown glycosyl flavonoids. However, the flavonoid nature of the compound was not clear in our samples, but the presence of MS^2 fragments at *m*/*z* 179 and 113 also observed in compounds 1 and 6 rather suggested that it could be a ginkgolide, which remained unknown.

The rest of compounds showed UV and mass characteristics coherent with flavonol glycosides. Compounds **9**, **19** and **23** ($[M–H]^-$ at *m*/*z* 593) presented characteristics that match a structure of kaempferol bearing deoxyhexosyl and hexosyl residues. Ding et al. (2008) also detected three compounds with the same mass in *G. biloba* supplements, all of them identified as kaempferol-*O*-rhamnosyl-glucoside. Zhang et al. (2007) and Lin et al. (2008) also found two compounds with similar characteristics in *G. biloba* leaves, one of them kaempferol-3-*O*-rutinoside (positively identified as peak **19** in our samples) and the other one as kaempferol-3-*O*-glucosyl-(1,2)-rhamnoside. This latter identity was assumed for peak **23** owing to its delayed elution in relation to peak 19, as reported by those authors. As no further information was available for compound **9** it was just assigned as a kaempferol-*O*-rhamnosyl-glucoside. Similar reasoning was applied for the identification of compounds **15** and **18** showing the same pseudomolecular ion $[M–H]^-$ at *m*/*z* 609, which were respectively, identified as quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucosyl-(1,2)-rhamnoside, as also reported Zhang et al. (2007) and Lin et al. (2008).

Compounds **7**, **10** and **24** presented the same pseudomolecular ion $[M–H]^-$ at *m*/*z* 755. Their MS^2 spectra pointed to they are derived from different aglycones, i.e. kaempferol (**7**) and quercetin (**10** and **24**). Different compounds with similar UV and mass characteristics were reported by Lin et al. (2008) and Ding et al. (2008) in *G. biloba* leaves and supplements. Compound **7** would correspond to a kaempferol derivative bearing one deoxyhexosyl and two hexosyl residues; the observation of a main MS^2 fragment at *m*/*z* 593 from the lost of the hexosyl residue suggested that this latter was located at a different position of the other two glycosyl moieties that could be constituting a disaccharide. Although, no information about the actual nature and position of the sugar substituents can be obtained from the available data, based on the previous comments assumption and the identification made by Lin et al. (2008) the compound was tentatively assigned as kaempferol-3-*O*-rhamnosylhexoside-7-*O*-glucoside. Characteristics of compound **10** were consistent with a quercetin derivative possessing two deoxyhexosyl and one hexosyl residues. The fact that only one MS^2 fragment was released corresponding to the aglycone (i.e. *m*/*z* at 301, quercetin) would suggest that the three sugars constituted a trisaccharide; based on this assumption the compound was tentatively identified as quercetin 3-*O*-2″,6″-dirhamnosylglucoside reported in *G. biloba* leaves by Lin et al. (2008). Compound **24** showed different UV and MS^2 spectra than compound **10**, presenting maximum wavelength at 316 nm and an additional minor MS^2 fragment at *m*/*z* 609 (loss of 146 mu that may correspond to either a rhamnosyl or a *p*-coumaroyl moiety), due to its delayed retention time and previous identifications of similar compounds by Lin et al. (2008) and Ding et al. (2008), this compound was tentatively assigned as quercetin-3-*O*-*p*-coumaroyl-rhamnosylhexoside. Similar reasoning was applied for assigning compounds **13** and **25,** both showing pseudomolecular ions $[M–H]^-$ at *m*/*z* 739, which were tentatively identified, respectively, as kaempferol-3-*O*-dirhamnosylglucoside and kaempferol-3-*O*-*p*-coumaroyl-rhamnosylhexoside, as also proposed by Lin et al. (2008).

Mass spectra characteristics of compounds **8** ($[M–H]^-$ at *m*/*z* 785) and **14** ($[M–H]^-$ at *m*/*z* 769) were similar to those of compounds 7 and 13, respectively, but derived from an isorhamnetin aglycone as revealed by the MS^2 fragment produced at *m*/*z* 315. Based on this observation and previous identifications by Lin et al. (2008), these compounds were respectively assigned as isorhamnetin-3-*O*-rhamnosylhexoside-7-*O*-glucoside and isorhamnetin-3-*O*-dirhamnosylglucoside. Compounds **16** ($[M–H]^-$ at *m*/*z* 639) and **21** ($[M–H]^-$ at *m*/*z* 447) were assigned as patuletin-3-*O*-rutinoside and quercetin-3-*O*-rhamnoside, owing the identification of these compound in leaves from *G. biloba* by Lin et al. (2008) and the latter one also by Yao et al. (2013).

Among the twenty-five compounds detected, compounds 1, 2, 4–6 and 12, associated to ginkgolides, were not quantified due their low UV absorption and possible interferences in the complex matrix of *G. biloba* extracts, as well as the unavailability of commercial gingkolide standards. Flavonoids were the main group present, being two kaempferol derivatives the majority compounds found (Table 2). Thus, kaempferol-3-*O*-dirhamnosylglucoside (compound **13**) was the most abundant compound in all the infusion preparations and in the methanol/water extract irradiated at 1 kGy, whereas, kaempferol-3-*O*-rutinoside (compound **19**) was the most abundant one in the control and irradiated at 10 kGy methanol/water extracts. Protocatechuic acid was the only phenolic acid identified and the quantities present were in the same range as the major flavonoids.

This study intended to evaluate which irradiation dose would be the most efficient to improve the extractability of phenolic compounds in *G. biloba* samples (methanol/water extract and infusion oral solution). Infusions presented lower quantities than the methanol/water extracts, due to the high temperatures applied to obtain these preparations that could destroy some thermal sensitive compounds, but also due to the lower extraction time. Both methanol/water and infusion preparation irradiated at a dose of 10 kGy gave the highest content in phenolic compounds. The subproducts formed during food irradiation depend on the food matrix and dose (Stewart, 2001). The degradation of some molecules during irradiation occurs by complex mechanisms. Although it is considered that some bonds can be broken resulting in smaller molecules (Stewart, 2001), the use of high irradiation doses might also lead to higher compound extractability. This could explain the higher values of phenolic compounds concentration observed for the doses of 10 kGy compared with those found in non-irradiated and 1 kGy irradiated samples. A small decrease in the phenolic compounds content was observed for the dose of 1 kGy in the methanol/water extract, when compared with non-irradiated samples. Low doses up to 1 kGy are used for preservation of fresh samples (Molins, 2001), which are more sensitive than dried food, indicating that at this doses there are no effect or only slight changes in food main characteristics.

To our knowledge, this is the first report that describes the phenolic composition in irradiated samples of *G. biloba* using two different doses. The dose of 10 kGy is enough to guarantee product disinfestation and microbial decontamination (Molins, 2001), contributing also for an increase in the phenolic compounds extractability, both for methanol/water and infusion preparations. The use of irradiation to improve bioactive properties was also suggested by other authors as a result of an increase in the lev-

els of phenolic compounds in the extracts obtained from cooked and derived plant products (Zhu et al., 2010; Aouidi et al., 2011; Lee et al., 2013; Wanyo et al., 2014). Nevertheless, further research is needed to understand all the mechanisms involved in the irradiation processing effects in plant constituents.

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Annexes 4

Gamma irradiation as a practical alternative to preserve the chemical and bioactive wholesomeness of widely used aromatic plants

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Gamma irradiation as a practical alternative to preserve the chemical and bioactive wholesomeness of widely used aromatic plants

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ABSTRACT

Aromatic plants require effective conservation technologies to expand their use. Irradiation might ensure plant decontamination, while maintaining their chemical, organoleptic, nutritional and bioactive qualities. In this study, the effects of gamma irradiation (1 and 10 kGy) in chemical, nutritional and antioxidant properties of *Aloysia citrodora*, *Melissa officinalis*, *Melittis melissophyllum* and *Mentha piperita* were evaluated. Gamma irradiation (up to 10 kGy) caused some statistically significant changes. However, when analyzed under an integrated approach, unirradiated and irradiated samples were grouped indiscriminately, indicating that irradiation treatment did not cause sufficient changes to define a specific chemical profile. Interestingly, each species was differentially affected by irradiation treatment. Overall, it might be considered that gamma irradiation (up to 10 kGy) is a feasible conservation technology for the assayed *Lamiaceae* and *Verbenaceae* species. This is an interesting result because the 10 kGy dose guarantees disinfested and decontaminated samples.

1. Introduction

Aloysia citrodora P., *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha piperita* L. are widely consumed in infusions and other beverages, being also included as ingredients in many other food products (e.g., salads, sauces, marinades, ice-creams, flavoring jams and jellies, cheese, etc.) (Small, 1996). Besides aromatic and culinary purposes, their infusions are used for gastrointestinal and nervous system disorders, displaying antioxidant, antimicrobial and anti-inflammatory properties (Kapp et al., 2013; Ragone, Sella, Conforti, Volonté, & Consolini, 2007; Skrzypczak-Pietraszeka & Pietraszek, 2012).

Currently, the plants used in food products or dietary supplements gather special interest. Their inclusion in food formulations requires stringent regulations, starting by an irreproachable microbiological quality of raw materials (Haleem, Salem, Fatahallah, & Abdelfattah, 2014; Ibrahim, Mohammed, Isah, & Aliyu, 2014). This might be achieved by decontamination methods that should be safe, fast and effective against microorganisms, without changing the organoleptic and chemical characteristics of the plant (Migdal & Owczarczyk, 1998). Hence, it is important to verify the maintenance of individual compounds such as fatty acids, tocopherols, organic acids or free sugars, besides ensuring that physical parameters are kept unchanged in the samples submitted to the decontamination treatments. Likewise, the bioactive properties of the final products should at least maintain the effectiveness of the starting materials (Nagy, Solar, Sontag, & Koenig, 2011).

One of the decontamination techniques used for plants with food applications is irradiation. This method, besides being recommended for dry ingredients, reduces reliance on chemical fumigants (which are carcinogens and mutagens to humans, leave chemical residue on plant and destroy the ozone layer in the atmosphere) (Chmielewski & Migdał, 2005; Migdal & Owczarczyk, 1998). It is also characterized for its efficiency in storage, reducing losses caused by natural physiological processes (budding, maturation and aging), and eliminating or reducing microorganisms, parasites and pests without causing significant changes (chemical or organoleptic), making the plants safer for consumers (Byun, Yook, Kim, & Chung, 1999; Nagy et al., 2011).

The aim of this work is to evaluate the effects of gamma irradiation (at 1 and 10 kGy doses) on chemical, nutritional and antioxidant properties of *A. citrodora*, *M. officinalis*, *M. melissophyllum* and *M. piperita*.

2. Materials and methods

2.1. Samples and sample irradiation

Samples of *A. citrodora* P. (Verbenaceae; lemon verbena), *M. officinalis* L. (Lamiaceae; lemon balm), *M. melissophyllum* L. (Lamiaceae; bastard balm) and *M. piperita* L. (Lamiaceae; peppermint) were provided as dry leaves by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal). After confirmation of the taxonomical identification,

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the samples were divided into three groups: control (unirradiated, 0 kGy), group 1 and group 2, where 1 kGy and 10 kGy were, respectively, the predicted doses.

The irradiation was performed in a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with total activity 177 TBq (4.78 kCi), in September 2013 (Fernandes et al., 2013). The estimated doses, dose rates and dose uniformity ratios (D_{max}/D_{min}) were, respectively: 1.20 ± 0.07 kGy, 2.57 ± 0.15 kGy h^{-1}, 1.20 for sample 1 and 8.93 ± 0.14 kGy, 1.91 ± 0.03 kGy h^{-1}, 1.02 for sample 2. For simplicity, the values 0, 1 and 10 kGy were considered as the doses of unirradiated and irradiated groups 1 and 2, respectively.

After irradiation, the samples were grinded to powder (20 mesh) and mixed to obtain homogenized samples for subsequent analysis.

2.2. Standards and reagents

2.2.1. For irradiation

A Fricke dosimeter (chemical solution sensitive to ionizing radiation) prepared in the lab following the standards (ASTM, 1992) and Amber Perspex dosimeters (batch V, from Harwell Company, UK) were used to estimate the dose and dose rate of irradiation. To prepare the acid aqueous Fricke dosimeter solution, the following reagents were used: ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA).

2.2.2. For chemical analyses

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA).

2.3. Proximate analysis

Protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 1995). The crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined using a Soxhlet apparatus by extracting (during 12 h) a known weight (≈5 g) of sample with petroleum ether; the ash content was determined by incineration at 600 ± 15 °C, until a whitish ash was formed. Total carbohydrates were calculated by difference and total energy was calculated according to the following equation:

$$\text{Energy (kcal)} = 4 \times \left(g_{protein} + g_{carbohydrates}\right) + 9 \times (g_{fat}).$$

2.4. Color Measurement

A colorimeter (model CR-400, from Konica Minolta Sensing, Inc., Japan), with an adapter for granular materials (model CR-A50) was used to measure the color of the samples. Using the illuminant C and diaphragm aperture of 8 mm, the CIE $L^*a^*b^*$ color space values were registered using a data software "Spectra Magic Nx" (version CM-S100W 2.03.0006), from Konica Minolta company (Japan). Before starting the measurements the instrument was calibrated against a standard white tile (Fernandes et al., 2012).

The color of three samples from each batch was measured in three different points, for each dose and at each time point, being considered the average value.

Table 1
Proximate composition and color parameters (L*: lightness, a*: redness, b*: yellowness) of the four assayed species submitted to gamma irradiation (GI).[1]

		Fat (g/100 g fw)	Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)	L^*	a^*	b^*
Aloysia citrodora									
GI	0 kGy	1.6 ± 0.1^b	3.0 ± 0.1^a	8.2 ± 0.1^b	87.1 ± 0.1^b	375 ± 1^b	49 ± 1^b	−8.4 ± 0.2	27.2 ± 0.3^b
	1 kGy	2.1 ± 0.1^a	1.8 ± 0.1^b	8.5 ± 0.3^a	87.6 ± 0.4^a	377 ± 1^a	50 ± 1^a	−8.8 ± 0.3	28.0 ± 0.4^a
	10 kGy	1.7 ± 0.1^b	3.0 ± 0.2^a	8.6 ± 0.2^a	86.7 ± 0.1^c	374 ± 1^c	48 ± 1^b	−8 ± 1	26.4 ± 0.4^c
p-values	Homoscedasticity[2]	0.471	0.323	0.001	0.003	0.074	0.495	0.031	0.951
	Normal distribution[3]	0.001	<0.001	0.016	0.033	0.125	0.110	<0.001	0.612
	1-way ANOVA[4]	<0.001	<0.001	0.004	<0.001	<0.001	<0.001	0.100	<0.001
Melissa officinalis									
GI	0 kGy	1.2 ± 0.1^b	2.5 ± 0.3^b	8.4 ± 0.4	88 ± 1^a	372 ± 2^c	48 ± 1	−5.1 ± 0.5	20.9 ± 0.4^a
	1 kGy	1.9 ± 0.1^a	7 ± 1^a	8.1 ± 0.3	83 ± 1^b	377 ± 1^a	48 ± 1	−5.1 ± 0.5	20.9 ± 0.4^a
	10 kGy	1.8 ± 0.1^a	6 ± 1^a	8.4 ± 0.2	83 ± 1^b	376 ± 1^b	47 ± 1	−5.0 ± 0.5	20.3 ± 0.5^b
p-values	Homoscedasticity[2]	0.113	0.003	0.054	0.002	0.004	0.191	0.926	0.412
	Normal distribution[3]	<0.001	0.005	0.145	0.002	0.037	0.346	0.703	0.096
	1-way ANOVA[4]	<0.001	<0.001	0.072	<0.001	<0.001	0.269	0.926	0.022
Melittis melissophyllum									
GI	0 kGy	1.8 ± 0.1^a	4.6 ± 0.2^b	7.6 ± 0.1^c	86.0 ± 0.4^b	378 ± 1^a	42 ± 2	−8.4 ± 0.5	18 ± 3
	1 kGy	1.6 ± 0.1^b	2.6 ± 0.1^c	8.1 ± 0.1^b	87.7 ± 0.2^a	376 ± 1^b	44 ± 2	−8.2 ± 0.5	17 ± 1
	10 kGy	1.5 ± 0.1^b	5.6 ± 0.5^a	8.6 ± 0.2^a	84 ± 1^c	373 ± 1^c	41 ± 2	−8.0 ± 0.5	16 ± 1
p-values	Homoscedasticity[2]	0.007	<0.001	0.108	<0.001	0.002	0.811	0.555	0.053
	Normal distribution[3]	0.056	0.004	0.124	0.057	0.291	0.090	0.588	<0.001
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	0.055	0.311	0.381
Mentha piperita									
GI	0 kGy	2.4 ± 0.1^b	5.1 ± 0.3^b	9.2 ± 0.2^a	83.3 ± 0.5^b	375 ± 1^b	40 ± 1^a	−5.9 ± 0.1^a	23.9 ± 0.3^a
	1 kGy	2.7 ± 0.2^a	3.1 ± 0.1^c	8.4 ± 0.1^c	85.8 ± 0.3^a	380 ± 1^a	39 ± 1^a	−5.7 ± 0.2^a	23.2 ± 0.5^a
	10 kGy	2.0 ± 0.2^c	10.5 ± 0.3^a	8.6 ± 0.1^b	78.9 ± 0.4^c	375 ± 1^b	37 ± 1^b	−4.8 ± 0.4^b	20.7 ± 0.5^b
p-values	Homoscedasticity[2]	0.169	<0.001	<0.001	0.379	0.006	0.515	0.072	0.036
	Normal distribution[3]	0.448	<0.001	0.010	0.001	<0.001	0.406	0.008	0.005
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1] The results are presented as the mean ± SD. [2]Homoscedasticity among GI doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro–Wilk test. [4]$p < 0.05$ indicates that the mean value of the evaluated parameter of at least one GI dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

Table 2
Hydrophilic compounds (free sugars and organic acids) composition (g/100 g dw) of the four assayed species submitted to gamma irradiation (GI). The results are presented as mean ± SD[1].

		Fructose	Glucose	Sucrose	Trehalose	Unknown	Total sugars	Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Citric acid	Total organic acids
Aloysia citrodora													
GI	0 kGy	1.0 ± 0.1	1.3 ± 0.1	7.1 ± 0.3^{a}	1.2 ± 0.1	nd	10.7 ± 0.4^{a}	1.1 ± 0.1	nd	0.14 ± 0.03^{b}	1.4 ± 0.1^{c}	1.4 ± 0.1^{c}	4.1 ± 0.1^{c}
	1 kGy	1.0 ± 0.1	1.2 ± 0.1	6.4 ± 0.3^{b}	1.2 ± 0.1	nd	9.8 ± 0.4^{b}	1.1 ± 0.1	nd	0.17 ± 0.02^{a}	1.8 ± 0.1^{a}	2.0 ± 0.2^{a}	5.1 ± 0.3^{a}
	10 kGy	1.0 ± 0.1	1.2 ± 0.1	6.6 ± 0.3^{b}	1.2 ± 0.1	nd	10.0 ± 0.5^{b}	1.1 ± 0.1	nd	0.13 ± 0.02^{b}	1.6 ± 0.1^{b}	1.7 ± 0.1^{b}	4.6 ± 0.3^{b}
p-values	Homoscedasticity[2]	0.115	0.072	0.818	0.011	–	0.944	0.401	–	0.190	0.625	0.034	0.154
	Normal distribution[3]	0.672	0.333	0.308	0.319	–	0.799	0.288	–	0.481	0.281	0.184	0.140
	1-way ANOVA[4]	0.882	0.065	<0.001	0.843	–	0.001	0.233	–	0.007	<0.001	<0.001	<0.001
Melissa officinalis													
GI	0 kGy	1.2 ± 0.1^{b}	1.0 ± 0.1	4.8 ± 0.2^{c}	0.49 ± 0.05^{c}	nd	7.5 ± 0.2^{c}	0.5 ± 0.1	0.26 ± 0.04	0.4 ± 0.1	4.1 ± 0.2	nd	5.3 ± 0.3
	1 kGy	1.4 ± 0.1^{a}	1.0 ± 0.1	5.4 ± 0.2^{b}	0.67 ± 0.03^{b}	nd	8.4 ± 0.3^{b}	0.5 ± 0.1	0.23 ± 0.03	0.4 ± 0.1	4.1 ± 0.4	nd	5.3 ± 0.4
	10 kGy	1.3 ± 0.1^{ab}	1.0 ± 0.1	5.6 ± 0.2^{a}	0.85 ± 0.05^{a}	nd	8.8 ± 0.4^{a}	0.5 ± 0.1	0.24 ± 0.04	0.4 ± 0.1	4.1 ± 0.4	nd	5.3 ± 0.4
p-values	Homoscedasticity[2]	0.045	0.051	0.931	0.009	–	0.680	0.836	0.745	0.393	0.059	–	0.540
	Normal distribution[3]	0.357	0.167	0.361	0.440	–	0.684	0.179	0.140	0.121	0.115	–	0.073
	1-way ANOVA[4]	0.004	0.832	<0.001	<0.001	–	<0.001	0.818	0.185	0.540	0.986	–	0.929
Melittis melissophyllum													
GI	0 kGy	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.28 ± 0.03^{c}	2.5 ± 0.1^{b}	5.5 ± 0.3^{b}	1.4 ± 0.1^{a}	0.17 ± 0.01^{ab}	6.0 ± 0.3^{a}	0.97 ± 0.05^{a}	0.022 ± 0.001^{b}	8.6 ± 0.4^{a}
	1 kGy	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.53 ± 0.05^{b}	2.7 ± 0.1^{a}	5.9 ± 0.4^{b}	1.2 ± 0.1^{b}	0.15 ± 0.02^{b}	4.5 ± 0.2^{b}	0.86 ± 0.05^{b}	0.019 ± 0.001^{c}	6.6 ± 0.3^{b}
	10 kGy	1.0 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.63 ± 0.05^{a}	2.8 ± 0.1^{a}	6.3 ± 0.3^{a}	1.4 ± 0.1^{a}	0.19 ± 0.01^{a}	5.9 ± 0.3^{a}	0.95 ± 0.05^{a}	0.026 ± 0.002^{a}	8.5 ± 0.4^{a}
p-values	Homoscedasticity[2]	0.495	0.954	0.040	<0.001	0.709	0.431	0.921	0.630	0.269	0.902	0.058	0.378
	Normal distribution[3]	0.270	0.759	0.005	0.012	0.799	0.681	0.054	0.839	0.002	0.998	0.113	0.005
	1-way ANOVA[4]	0.052	0.055	0.072	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001	<0.001
Mentha piperita													
GI	0 kGy	0.47 ± 0.05^{a}	0.30 ± 0.05	0.7 ± 0.1	1.0 ± 0.1^{a}	nd	2.4 ± 0.2	1.1 ± 0.1^{a}	0.040 ± 0.003^{a}	0.9 ± 0.1^{a}	nd	8.5 ± 0.2^{a}	10.6 ± 0.3^{a}
	1 kGy	0.42 ± 0.03^{b}	0.29 ± 0.03	0.8 ± 0.1	1.0 ± 0.1^{a}	nd	2.5 ± 0.2	1.2 ± 0.1^{a}	0.036 ± 0.004^{ab}	0.9 ± 0.1^{a}	nd	6.5 ± 0.2^{c}	8.7 ± 0.2^{c}
	10 kGy	0.47 ± 0.04^{ab}	0.31 ± 0.03	0.7 ± 0.1	0.8 ± 0.1^{b}	nd	2.3 ± 0.2	1.0 ± 0.1^{b}	0.035 ± 0.003^{b}	0.7 ± 0.1^{b}	nd	7.7 ± 0.2^{b}	9.5 ± 0.2^{b}
p-values	Homoscedasticity[2]	0.665	0.061	0.131	0.320	–	0.573	0.934	0.880	0.880	–	0.559	0.039
	Normal distribution[3]	0.767	0.240	0.818	0.626	–	0.681	0.178	0.196	0.016	–	0.046	<0.001
	1-way ANOVA[4]	0.030	0.507	0.060	<0.001	–	0.094	<0.001	0.013	<0.001	–	<0.001	<0.001

[1] The results are presented as the mean ± SD. [2]Homoscedasticity among GI doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro–Wilk test. [4]$p < 0.05$ indicates that the mean value of the evaluated parameter of at least one GI dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

2.5. Chemical composition of hydrophilic compounds

2.5.1. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI). Dried sample powder (1.0 g) was spiked with melezitose as internal standard (IS, 5 mg/mL), and extracted with 40 mL of 80% aqueous ethanol at 80 °C for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000 *g* for 10 min. The supernatant was concentrated at 60 °C under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 °C, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 μm Whatman nylon filters. Chromatographic conditions were applied as previously defined (Barros et al., 2013). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g/100 g of dry weight (dw).

2.5.2. Organic acids

Organic acids were determined following a procedure previously described by the authors. Samples (≈2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25 °C at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 μm nylon filters. Chromatographic conditions were applied as previously defined (Barros et al., 2013). Detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound.

2.6. Chemical composition in lipophilic compounds

2.6.1. Tocopherols

Tocopherols were determined following a procedure previously described by the authors (Pereira, Barros, & Ferreira, 2013). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound.

2.6.2. Fatty acids

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Pereira et al., 2013). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7, Prague, Czech Republic).

2.7. Evaluation of bioactivity

2.7.1. Samples preparation

The methanolic extracts were obtained from the dried plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

The infusions were also obtained from the dried plant material. The sample (1 g) was added to 200 mL of boiling distilled water (after being taken out from the heating source) and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusions were frozen and lyophilized.

Table 3
Tocopherols composition (mg/100 g dw) of the four assayed species submitted to gamma irradiation (GI). The results are presented as mean ± SD[1].

		α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols
Aloysia citrodora						
GI	0 kGy	15.3 ± 0.4^{b}	0.41 ± 0.04^{a}	1.8 ± 0.1^{ab}	nd	17.5 ± 0.4^{b}
	1 kGy	17.5 ± 0.4^{a}	0.44 ± 0.05^{a}	1.9 ± 0.1^{a}	nd	19.8 ± 0.4^{a}
	10 kGy	13.4 ± 0.3^{c}	0.29 ± 0.04^{b}	1.7 ± 0.1^{b}	nd	15.4 ± 0.3^{c}
p-values	Homoscedasticity[2]	0.831	0.012	0.341	–	0.412
	Normal distribution[3]	0.024	0.378	0.352	–	0.020
	1-way ANOVA[4]	<0.001	<0.001	0.002	–	<0.001
Melissa officinalis						
GI	0 kGy	29 ± 1^{b}	1.3 ± 0.1^{a}	1.5 ± 0.1^{b}	0.37 ± 0.05^{b}	32 ± 1^{b}
	1 kGy	33 ± 1^{a}	1.1 ± 0.1^{b}	1.8 ± 0.1^{a}	0.38 ± 0.05^{b}	37 ± 1^{a}
	10 kGy	29 ± 1^{b}	0.9 ± 0.1^{c}	1.7 ± 0.1^{a}	0.49 ± 0.05^{a}	33 ± 1^{b}
p-values	Homoscedasticity[2]	0.646	0.017	0.264	0.215	0.671
	Normal distribution[3]	0.001	0.139	0.553	0.151	0.003
	1-way ANOVA[4]	<0.001	<0.001	<0.001	0.001	<0.001
Melittis melissophyllum						
GI	0 kGy	0.88 ± 0.05^{a}	13.4 ± 0.3^{b}	0.18 ± 0.02^{a}	0.14 ± 0.02^{a}	14.6 ± 0.4^{b}
	1 kGy	0.81 ± 0.05^{b}	13.2 ± 0.2^{b}	0.16 ± 0.02^{a}	0.14 ± 0.02^{a}	14.3 ± 0.2^{b}
	10 kGy	0.46 ± 0.04^{c}	28.9 ± 0.3^{a}	0.11 ± 0.02^{b}	0.08 ± 0.01^{b}	29.5 ± 0.2^{a}
p-values	Homoscedasticity[2]	0.073	0.501	0.423	0.245	0.481
	Normal distribution[3]	0.001	<0.001	0.386	0.180	<0.001
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001
Mentha piperita						
GI	0 kGy	16.5 ± 0.4^{a}	1.1 ± 0.1^{a}	1.8 ± 0.1	0.23 ± 0.03^{b}	19.7 ± 0.5^{a}
	1 kGy	15.7 ± 0.2^{b}	0.8 ± 0.1^{b}	1.8 ± 0.1	0.28 ± 0.04^{a}	18.6 ± 0.2^{b}
	10 kGy	13.2 ± 0.2^{c}	0.9 ± 0.1^{b}	1.8 ± 0.1	0.30 ± 0.03^{a}	16.2 ± 0.4^{c}
p-values	Homoscedasticity[2]	0.002	0.064	0.778	0.427	0.001
	Normal distribution[3]	0.001	0.012	0.187	0.559	0.021
	1-way ANOVA[4]	<0.001	<0.001	0.797	0.001	<0.001

[1] The results are presented as the mean ± SD. [2]Homoscedasticity among GI doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro–Wilk test. [4]$p < 0.05$ indicates that the mean value of the evaluated parameter of at least one GI dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

Table 4A
A. Minor fatty acids (values < 1% in all species) of the four assayed species submitted to gamma irradiation (GI). The results are presented in relative percentage as mean ± SD[1].

		C6:0	C8:0	C11:0	C12:0	C13:0	C15:0	C15:1	C17:0	C20:1n9	C20:2n6	C20:3n3 + C21:0	C22:1n9
Aloysia citrodora													
GI	0 kGy	0.30 ± 0.01^{a}	0.11 ± 0.01^{b}	0.26 ± 0.02^{a}	0.26 ± 0.02^{b}	0.32 ± 0.01^{c}	0.58 ± 0.02^{b}	0.10 ± 0.01^{a}	0.22 ± 0.01^{c}	0.25 ± 0.03^{b}	0.21 ± 0.01^{b}	0.30 ± 0.01^{a}	0.27 ± 0.02^{b}
	1 kGy	0.28 ± 0.04^{a}	0.10 ± 0.01^{b}	0.21 ± 0.01^{b}	0.29 ± 0.02^{b}	0.46 ± 0.03^{a}	0.61 ± 0.05^{b}	0.09 ± 0.01^{b}	0.24 ± 0.01^{b}	0.39 ± 0.04^{a}	0.17 ± 0.01^{c}	0.27 ± 0.01^{c}	0.37 ± 0.01^{a}
	10 kGy	0.23 ± 0.02^{b}	0.13 ± 0.01^{a}	0.24 ± 0.03^{a}	0.37 ± 0.03^{a}	0.35 ± 0.02^{b}	0.71 ± 0.02^{a}	0.10 ± 0.01^{a}	0.27 ± 0.01^{a}	0.22 ± 0.02^{b}	0.27 ± 0.01^{a}	0.28 ± 0.01^{b}	0.19 ± 0.01^{c}
p-values	Homoscedasticity[2]	<0.001	0.008	0.008	0.100	0.004	0.003	0.002	0.038	0.001	0.008	<0.001	<0.001
	Normal distribution[3]	0.015	0.163	0.210	0.071	0.003	0.010	0.038	0.002	0.001	0.001	0.001	0.001
	1-way ANOVA[4]	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Melissa officinalis													
GI	0 kGy	0.22 ± 0.01^{a}	0.40 ± 0.02^{a}	0.13 ± 0.01^{b}	0.46 ± 0.01^{a}	0.14 ± 0.01^{b}	0.44 ± 0.03^{a}	0.55 ± 0.01^{a}	0.81 ± 0.01^{b}	0.18 ± 0.02^{a}	nd	0.28 ± 0.01^{c}	nd
	1 kGy	0.15 ± 0.01^{b}	0.30 ± 0.02^{b}	0.13 ± 0.01^{b}	0.34 ± 0.01^{b}	0.16 ± 0.01^{a}	0.42 ± 0.01^{a}	0.49 ± 0.01^{c}	0.87 ± 0.01^{a}	0.15 ± 0.01^{b}	nd	0.35 ± 0.01^{b}	nd
	10 kGy	0.14 ± 0.01^{c}	0.29 ± 0.01^{b}	0.17 ± 0.01^{a}	0.30 ± 0.01^{c}	0.14 ± 0.01^{b}	0.36 ± 0.01^{b}	0.51 ± 0.01^{b}	0.80 ± 0.01^{c}	0.12 ± 0.03^{b}	nd	0.36 ± 0.01^{a}	nd
p-values	Homoscedasticity[2]	0.002	0.672	0.089	0.002	<0.001	<0.001	<0.001	0.007	0.039	–	<0.001	–
	Normal distribution[3]	0.001	0.001	<0.001	<0.001	0.058	0.006	0.001	<0.001	0.500	–	<0.001	–
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–	<0.001	–
Melittis melissophyllum													
GI	0 kGy	0.18 ± 0.01^{a}	0.07 ± 0.01^{b}	0.04 ± 0.01^{b}	0.18 ± 0.01^{b}	0.05 ± 0.01^{c}	0.90 ± 0.02^{b}	0.09 ± 0.01^{b}	0.24 ± 0.02	0.16 ± 0.01^{c}	0.09 ± 0.02^{c}	0.24 ± 0.01^{b}	nd
	1 kGy	0.06 ± 0.01^{c}	0.07 ± 0.01^{b}	0.04 ± 0.01^{b}	0.24 ± 0.02^{a}	0.06 ± 0.01^{b}	0.83 ± 0.03^{c}	0.08 ± 0.01^{c}	0.24 ± 0.01	0.20 ± 0.01^{a}	0.15 ± 0.01^{b}	0.27 ± 0.01^{a}	nd
	10 kGy	0.08 ± 0.01^{b}	0.09 ± 0.01^{a}	0.08 ± 0.01^{a}	0.25 ± 0.01^{a}	0.07 ± 0.01^{a}	0.96 ± 0.02^{a}	0.10 ± 0.01^{a}	0.24 ± 0.01	0.18 ± 0.01^{b}	0.17 ± 0.01^{a}	0.24 ± 0.01^{b}	nd
p-values	Homoscedasticity[2]	0.025	0.004	<0.001	<0.001	0.034	0.828	<0.001	0.005	0.001	0.001	0.003	–
	Normal distribution[3]	<0.001	0.117	<0.001	<0.001	0.005	0.547	0.037	0.277	0.024	0.002	<0.001	–
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.507	<0.001	<0.001	<0.001	–
Mentha piperita													
GI	0 kGy	0.15 ± 0.02^{a}	1.0 ± 0.1^{a}	0.12 ± 0.01^{b}	0.14 ± 0.01^{b}	0.15 ± 0.01^{a}	0.59 ± 0.05^{a}	0.04 ± 0.01	0.44 ± 0.01^{b}	0.25 ± 0.01^{b}	0.19 ± 0.01^{a}	0.45 ± 0.04^{b}	0.11 ± 0.01^{c}
	1 kGy	0.16 ± 0.02^{a}	1.0 ± 0.1^{a}	0.17 ± 0.02^{a}	0.15 ± 0.02^{b}	0.12 ± 0.01^{b}	0.48 ± 0.01^{b}	0.05 ± 0.01	0.47 ± 0.01^{a}	0.28 ± 0.05^{b}	0.18 ± 0.01^{b}	0.47 ± 0.02^{b}	0.21 ± 0.04^{b}
	10 kGy	0.10 ± 0.03^{b}	0.9 ± 0.1^{b}	0.11 ± 0.01^{b}	0.20 ± 0.01^{a}	0.09 ± 0.01^{c}	0.53 ± 0.04^{b}	0.04 ± 0.01	0.45 ± 0.02^{b}	0.52 ± 0.02^{a}	0.16 ± 0.01^{c}	0.54 ± 0.02^{a}	0.28 ± 0.02^{a}
p-values	Homoscedasticity[2]	0.437	0.002	0.021	0.992	<0.001	<0.001	0.260	<0.001	<0.001	0.207	0.036	0.016
	Normal distribution[3]	0.118	0.022	<0.001	0.035	0.011	<0.001	0.218	0.084	<0.001	0.885	0.604	0.006
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.135	0.001	<0.001	<0.001	<0.001	<0.001

2.7.2. Antioxidant activity

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH} - A_S) / A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2 h of assay/initial absorbance) × 100% (Pereira et al., 2013).

2.8. Statistical analysis

For each irradiation dose and plant species, three independent samples were analyzed. Each of the samples was taken after pooling the plants treated in the same conditions together. Data were expressed as mean ± standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Principal component analysis (PCA) was applied as a pattern recognition unsupervised classification method. The number of dimensions to keep for data analysis was assessed by the respective eigenvalues (which should be greater than one), by Cronbach's alpha parameter (that must be positive) and also by the total percentage of variance (that should be as high as possible) explained by the number of components selected. The number of plotted dimensions was chosen in order to allow meaningful interpretations.

3. Results and discussion

3.1. Effects on chemical parameters

The proximate composition and color parameters (Table 1) of *A. citrodora* (lemon verbena), *M. officinalis* (lemon balm), *M. melissophyllum* (bastard balm) and *M. piperita* (peppermint) showed some similarity, with carbohydrates as predominant component, followed by ash, protein and fat contents. Except for lemon balm, the proximate composition of these species is described for the first time. The nutritional profile detected for lemon balm is coherent to that reported in previous works (Dias, Barros, Sousa, & Ferreira, 2012). Regarding the effect of gamma irradiation (GI), all these parameters showed to be relatively susceptible ($p < 0.05$), except ash content in lemon balm ($p = 0.072$). Despite the detected variations, it was not possible to identify overall tendencies, with the exception of protein content, which tended to be higher in samples irradiated with 10 kGy for all species. The increase in protein content might be related to chemical processes (scission of the carbon-nitrogen bonds in the backbone of the polypeptide chain or splitting of the disulfide bonds) or to physical changes (like unfolding), which are commonly associated to irradiation treatment (Molins, 2001).

Color parameters are assessed in the quality control of post-harvest preservation processes (Hsu, Simonne, Jitareerat, & Marshall, 2010). Herein, these parameters were also similar, with higher lightness values in lemon verbena (≈49) and lemon balm (≈49), lower redness in lemon verbena (≈−8.4) and bastard balm (≈−8.2) and higher yellowness (≈27) in lemon verbena. Color parameters proved to be less susceptible to irradiation than those evaluated in the proximate analysis, since the detected differences had no statistical significance ($p > 0.050$) in most cases. Considering the cases where a statistically significant difference was found, it might be said that lightness, redness and yellowness leaned toward lower values in samples irradiated with 10 kGy. That is similar with the decrease of a* and b* observed in gamma irradiated green tea extracts (Jo, Son, Shin, & Byun, 2003). The results for peppermint are in agreement with those reported in North American samples, showing no variation in color parameters when irradiated with low doses (Hsu et al., 2010).

Concerning free sugar composition (Table 2), fructose, glucose, sucrose and trehalose were quantified in all species. A fifth sugar was also quantified in bastard balm, but its identity could not be determined. Sucrose was the main sugar in lemon verbena (≈6.7 g/100 g dw) and lemon balm (≈5.3 g/100 g dw), while the unidentified sugar (≈2.7 g/100 g dw) and trehalose (≈0.9 g/100 g dw) were the most abundant in bastard balm and peppermint, respectively. Lemon verbena showed the highest content (≈10.2 g/100 g dw) in total sugars. The 10 kGy dose seemed to increase sugars content in lemon balm and bastard balm, while lemon verbena and peppermint tended to present higher values in unirradiated samples. The increase in free sugars, which was previously reported in soybean (Byun, Kang, & Mori, 1996), ginseng (Byun, Yook, Kwon, & Kang, 1997), green, black and oolong teas (Kausar, Akram, & Kwon, 2013) and plan waste materials (Tissot, Grdanovska, Barkatt, Silverman, & Al-Sheikhly, 2013) as a result of gamma irradiation, might be explained by the shortening or depolymerization of polysaccharide molecules. Other verified changes might be explained by variations in the optical rotation of sugars, which is a common occurrence under irradiation treatment (Molins, 2001).

Peppermint gave the highest content in organic acids (Table 2), mainly due to the citric acid amounts (≈7.6 g/100 g dw). Malic acid (≈5.5 g/100 g dw) was the predominant form in bastard balm, while shikimic acid (≈4.1 g/100 g dw) and citric acid (≈1.7 g/100 g dw) were the organic acids quantified in highest amounts in lemon balm and lemon verbena, respectively. Oxalic acid and quinic acid (except in lemon verbena) were also quantified. In general, the highest changes were detected in samples irradiated with 1 kGy dose, indicating that some degradation processes commonly triggered by the molecular oxygen inside the polyethylene bag might decrease due to an oxygen ionizing effect produced when using the 10 kGy dose.

The four tocopherol isoforms (α, β, γ and δ) were detected in all species, except for δ-tocopherol in lemon verbena (Table 3). α-Tocopherol was the main isoform in lemon balm (≈30.3 mg/100 g dw), lemon verbena (≈15.4 mg/100 g dw) and peppermint (≈15.1 mg/100 g dw), while β-tocopherol predominated in bastard balm (≈18.5 mg/100 g dw). In line with previous results (Taipina, Lamardo, Rodas, & Mastro, 2009), the tocopherol contents were significantly changed in response to irradiation treatment (especially for the 1 kGy dose) in all the assayed samples, except for γ-tocopherol in peppermint ($p = 0.797$). These differences are mainly linked to α-and β-tocopherol contents, which are not as stable to irradiation as γ-tocopherol, and are also recognized as having higher oxidative stability (Warner, Miller, & Demurin, 2008).

Tables 4A and 4B present the individual fatty acids (FA) divided as those quantified below 1% in all species (Table 4A) and those quantified above 1% at least in one species (Table 4B). The predominant FA in the four species were linolenic acid (C18:3n3), followed by palmitic (C16:0) and linoleic (C18:2n6) acids in lemon verbena and lemon balm, linoleic and palmitic acids in bastard balm, and arachidic and palmitic acids in peppermint. The FA profile detected for lemon balm is similar to that reported previously in the same species (Dias et al., 2012). Despite the individual differences, polyunsaturated fatty acids (PUFA) were predominant in all species (52.6 to 69.5%), followed by saturated fatty acids (SFA, 28.1 to 41.2%) and monounsaturated fatty acids (MUFA, 2.07 to 16.6%) (Table 4B). The detected percentages were significantly changed by irradiation treatment with the exceptions of C23:0 in lemon balm ($p = 0.110$), C17:0 ($p = 0.507$), C24:0 ($p = 0.124$) and SFA ($p = 0.214$) in bastard balm and C15:1 ($p = 0.135$) and C16:0 ($p =$

Table 4B
Major fatty acids (values > 1%, at least in one species) of the four assayed species submitted to gamma irradiation (GI). The results are presented in relative percentage as mean ± SD[1].

		C10:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1n9	C18:2n6
Aloysia citrodora									
GI	0 kGy	nd	1.1 ± 0.1[b]	nd	15.7 ± 0.2[b]	0.50 ± 0.02[b]	1.17 ± 0.01[b]	0.95 ± 0.02[b]	12.6 ± 0.1[a]
	1 kGy	nd	1.3 ± 0.1[a]	nd	15.8 ± 0.4[b]	0.62 ± 0.01[a]	1.10 ± 0.01[c]	0.95 ± 0.02[b]	12.4 ± 0.1[b]
	10 kGy	nd	0.9 ± 0.1[c]	nd	16.6 ± 0.5[a]	0.64 ± 0.03[a]	1.31 ± 0.01[a]	1.13 ± 0.03[a]	12.6 ± 0.1[a]
p-values	Homoscedasticity[2]	–	0.273	–	0.071	0.008	0.002	0.225	<0.001
	Normal distribution[3]	–	0.080	–	0.025	0.001	<0.001	<0.001	<0.001
	1-way ANOVA[4]	–	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001
Melissa officinalis									
GI	0 kGy	0.29 ± 0.02[a]	2.9 ± 0.1[a]	0.53 ± 0.01[b]	22.7 ± 0.3[a]	nd	3.6 ± 0.1[a]	4.9 ± 0.2[a]	15.3 ± 0.4[ab]
	1 kGy	0.25 ± 0.01[b]	2.6 ± 0.1[b]	0.52 ± 0.01[b]	20.9 ± 0.1[c]	nd	3.6 ± 0.1[a]	4.8 ± 0.1[a]	15.0 ± 0.1[b]
	10 kGy	0.22 ± 0.01[c]	2.4 ± 0.1[c]	0.62 ± 0.02[a]	21.5 ± 0.1[b]	nd	3.2 ± 0.1[b]	4.3 ± 0.1[b]	15.5 ± 0.1[a]
p-values	Homoscedasticity[2]	0.001	<0.001	<0.001	<0.001	–	0.048	<0.001	<0.001
	Normal distribution[3]	0.061	0.002	<0.001	0.002	–	0.002	0.001	0.062
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	0.001
Melittis melissophyllum									
GI	0 kGy	nd	0.58 ± 0.03[c]	nd	14.3 ± 0.2[b]	1.29 ± 0.05[a]	2.41 ± 0.05[b]	11.5 ± 0.3[c]	14.8 ± 0.4[c]
	1 kGy	nd	0.81 ± 0.05[b]	nd	14.2 ± 0.5[b]	1.14 ± 0.03[b]	2.43 ± 0.01[b]	13.0 ± 0.4[b]	16.2 ± 0.4[b]
	10 kGy	nd	0.92 ± 0.03[a]	nd	15.1 ± 0.1[a]	1.25 ± 0.04[a]	2.76 ± 0.01[a]	15.1 ± 0.5[a]	18.2 ± 0.4[a]
p-values	Homoscedasticity[2]	–	0.022	–	<0.001	0.005	0.004	<0.001	0.964
	Normal distribution[3]	–	0.004	–	0.006	0.214	<0.001	0.029	0.049
	1-way ANOVA[4]	–	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001
Mentha piperita									
GI	0 kGy	0.07 ± 0.01[a]	1.4 ± 0.1[b]	1.2 ± 0.1[a]	10.4 ± 0.3	0.88 ± 0.05[b]	2.47 ± 0.03[b]	1.62 ± 0.05[b]	7.3 ± 0.1[b]
	1 kGy	0.04 ± 0.01[b]	1.5 ± 0.1[a]	1.2 ± 0.1[a]	10.4 ± 0.3	0.97 ± 0.01[a]	2.55 ± 0.01[a]	1.61 ± 0.01[b]	7.5 ± 0.1[a]
	10 kGy	0.02 ± 0.01[c]	1.6 ± 0.1[a]	1.0 ± 0.1[b]	10.1 ± 0.5	0.81 ± 0.05[b]	2.60 ± 0.05[a]	1.91 ± 0.05[a]	7.2 ± 0.1[c]
p-values	Homoscedasticity[2]	0.160	0.062	0.001	0.036	0.001	0.005	0.001	0.001
	Normal distribution[3]	0.008	0.660	0.179	0.103	0.017	0.509	<0.001	0.006
	1-way ANOVA[4]	<0.001	<0.001	<0.001	0.313	<0.001	<0.001	<0.001	<0.001

[1]The results are presented as the mean ± SD. [2]Homoscedasticity among GI doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$. [3]Normal distribution of the residuals was evaluated using Shapiro–Wilk test. [4]$p < 0.05$ indicates that the mean value of the evaluated parameter of at least one GI dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

0.313) in peppermint. The differences verified for irradiated samples might be explained by mechanisms of lipid radiolysis, involving primary ionization, followed by migration of the positive charge either toward the carboxyl carbonyl group or double bonds (Molins, 2001).

3.2. Effects on antioxidant parameters

In order to compare the effects of gamma irradiation on the antioxidant activity, three in vitro assays were applied: scavenging effects on DPPH radicals (measures the decrease in DPPH radical absorption after exposure to radical scavengers), reducing power (conversion of a Fe^{3+}/ferricyanide complex to Fe^{2+}) and inhibition of β-carotene bleaching (measures the capacity to neutralize the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models). Moreover, a preliminary quantification of total phenols and flavonoids subgroup was also performed; the results are expressed in Table 5. Among the assayed species, lemon balm showed the highest antioxidant activity on all the assays, especially concerning the infusions, presenting values similar to those published in Iranian (Dastmalchi et al., 2008) and Brazillian (Kamdem et al., 2013) samples. The EC_{50} values are close to those reported in previous studies. Nevertheless, the infusions prepared in this study gave lower amounts of bioactive compounds (Dias et al., 2012). On the other hand, bastard balm proved to be the least effective in terms of antioxidant activity, as well as phenols and flavonoids content. The methanolic extracts gave higher activities than the corresponding infusions, showing to be correlated with the amounts of bioactive compounds quantified in each case.

Changes induced by gamma irradiation proved to be statistically significant in almost all cases, except for DPPH scavenging activity in methanolic extracts ($p = 0.996$) of bastard balm. Likewise, changes in bioactive compound amounts were always significant except for phenols content in the infusions of bastard balm ($p = 0.474$). Despite the significant changes found within these parameters, it was not possible to identify unequivocal tendencies common to all assays and/or plant species.

3.3. Principal component analysis (PCA)

In the former section, the differences resulting from gamma irradiation were compared considering the individual effect within each species. Despite the high number of statistically significant changes, it was not possible to identify overall trends, which might characterize the effects of gamma irradiation. Furthermore, it was intended to validate this technology independently of the treated plant species. Accordingly, in the present section the results were evaluated considering data for all species and parameters simultaneously.

Hence, to verify if irradiation maintains the chemical profile, principal component analysis (PCA) was applied. In this analysis, instead of evaluating individual changes caused in each parameter, the effects in all parameters were considered at once. Due to the great variation (in some parameters) among species, the values were normalized by subtracting the value corresponding to unirradiated samples to those from 1 and 10 kGy irradiations. The obtained differences were further divided by the value of the respective control. In this way, the classification procedure was applied to the differences caused by irradiation and not to the absolute values measured for each parameter. Due to practical reasons, only the parameters detected in the four species were included in this study.

The plot of object scores (Fig. 1**A**) for gamma irradiation dose, indicated that the first two dimensions (first: Cronbach's α, 0.941; eigenvalue, 13.031; second: Cronbach's α, 0.915; eigenvalue, 9.819) account for most of the variance of all quantified variables (34.1% and 28.1%, respectively). The included variance would ideally be higher,

Table 4b (*continued*)

C18:3n6	C18:3n3	C20:0	C20:5n3	C22:0	C23:0	C22:6n3	C24:0	SFA	MUFA	PUFA
Aloysia citrodora										
nd	56.2 ± 0.3[a]	0.87 ± 0.02[b]	nd	1.00 ± 0.02[a]	5.4 ± 0.1[b]	nd	1.4 ± 0.1[c]	28.6 ± 0.2[b]	2.07 ± 0.03[c]	69.3 ± 0.3[a]
nd	56.6 ± 0.5[a]	0.99 ± 0.03[a]	nd	0.82 ± 0.01[c]	4.2 ± 0.1[c]	nd	1.7 ± 0.1[b]	28.1 ± 0.5[c]	2.42 ± 0.03[a]	69.5 ± 0.5[a]
nd	54.3 ± 0.4[b]	0.59 ± 0.04[c]	nd	0.93 ± 0.04[b]	5.9 ± 0.4[a]	nd	1.8 ± 0.1[a]	30.3 ± 0.5[a]	2.27 ± 0.03[b]	67.4 ± 0.5[b]
–	0.259	0.265	–	0.001	<0.001	–	<0.001	0.158	0.742	0.231
–	0.007	0.001	–	0.004	0.001	–	0.003	0.045	0.033	0.005
–	<0.001	<0.001	–	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001
Melissa officinalis										
nd	33.2 ± 0.5[c]	3.4 ± 0.1[c]	3.9 ± 0.1[b]	1.3 ± 0.1[b]	3.3 ± 0.2	nd	1.2 ± 0.2[ab]	41.2 ± 0.5[a]	6.2 ± 0.2[a]	52.6 ± 0.5[c]
nd	34.4 ± 0.1[b]	3.9 ± 0.1[a]	4.5 ± 0.1[a]	1.5 ± 0.1[a]	3.2 ± 0.1	nd	1.3 ± 0.1[a]	39.7 ± 0.2[b]	6.0 ± 0.1[b]	54.3 ± 0.1[b]
nd	36.3 ± 0.2[a]	3.5 ± 0.1[b]	3.5 ± 0.1[c]	1.5 ± 0.1[a]	3.1 ± 0.1	nd	1.1 ± 0.1[b]	38.7 ± 0.2[c]	5.6 ± 0.1[c]	55.7 ± 0.2[a]
–	0.003	0.002	0.437	<0.001	0.005	–	0.107	0.005	<0.001	0.007
–	0.012	<0.001	0.002	<0.001	0.033	–	0.411	0.041	0.020	0.029
–	<0.001	<0.001	<0.001	<0.001	0.110	–	0.004	<0.001	<0.001	<0.001
Melittis melissophyllum										
5.8 ± 0.1[b]	36 ± 1[a]	0.88 ± 0.02[b]	nd	1.3 ± 0.1[b]	6.2 ± 0.2[a]	nd	3.0 ± 0.1	30.4 ± 0.2	13.1 ± 0.2[c]	56.5 ± 0.2[a]
5.8 ± 0.1[b]	33 ± 1[b]	0.96 ± 0.02[a]	nd	1.3 ± 0.1[b]	5.9 ± 0.4[a]	nd	2.9 ± 0.2	30.1 ± 0.4	14.4 ± 0.3[b]	55.5 ± 0.5[b]
6.3 ± 0.1[a]	28 ± 1[c]	0.97 ± 0.03[a]	nd	1.4 ± 0.1[a]	4.1 ± 0.1[b]	nd	3.1 ± 0.2	30.2 ± 0.3	16.6 ± 0.5[a]	53.2 ± 0.5[c]
0.009	0.010	0.497	–	<0.001	<0.001	–	0.002	0.186	<0.001	0.001
<0.001	0.003	0.454	–	0.001	<0.001	–	0.491	0.532	0.013	0.005
<0.001	<0.001	<0.001	–	0.003	<0.001	–	0.124	0.214	<0.001	<0.001
Mentha piperita										
nd	46 ± 1[a]	15.8 ± 0.5[c]	2.8 ± 0.2[c]	2.6 ± 0.1[b]	0.24 ± 0.0[b]	1.4 ± 0.1[c]	2.1 ± 0.1[a]	38 ± 1[c]	4.1 ± 0.1[c]	58 ± 1[a]
nd	44 ± 1[b]	16.7 ± 0.5[b]	3.0 ± 0.1[b]	2.8 ± 0.1[a]	0.21 ± 0.0[c]	1.5 ± 0.1[a]	1.9 ± 0.1[b]	39 ± 1[b]	4.3 ± 0.1[b]	57 ± 1[b]
nd	43 ± 1[c]	17.9 ± 0.1[a]	3.3 ± 0.1[a]	2.9 ± 0.1[a]	0.26 ± 0.0[a]	1.6 ± 0.1[a]	1.9 ± 0.1[b]	40 ± 1[a]	4.6 ± 0.2[a]	56 ± 1[c]
–	0.151	0.001	<0.001	0.237	<0.001	<0.001	0.058	0.134	0.361	0.050
–	0.246	0.012	0.057	0.904	0.002	<0.001	0.262	0.381	0.815	0.247
–	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 5
Antioxidant properties of extracts from the species submitted to gamma irradiation (GI).[1] EC_{50} values (μg/mL) are presented for all assays except phenols and flavonoids, expressed as mg GAE/g extract and mg CE/g extract, respectively.

		DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		Phenols		Flavonoids	
		Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
Aloysia citrodora											
GI	0 kGy	232 ± 8[a]	39 ± 4[c]	169 ± 1[b]	22.8 ± 0.3[c]	580 ± 31[c]	208 ± 9[b]	134 ± 8[c]	665 ± 13[a]	92 ± 1[a]	369 ± 5[a]
	1 kGy	237 ± 5[a]	90 ± 6[b]	184 ± 2[a]	49.2 ± 0.4[b]	1004 ± 23[a]	235 ± 5[a]	188 ± 2[b]	531 ± 34[b]	60 ± 2[c]	359 ± 9[b]
	10 kGy	205 ± 16[b]	109 ± 4[a]	170 ± 1[b]	62 ± 1[a]	829 ± 36[b]	198 ± 6[c]	205 ± 3[a]	455 ± 12[c]	76 ± 3[b]	277 ± 2[c]
p-values	Homoscedasticity[b]	0.002	0.238	0.031	0.005	0.340	0.200	0.002	<0.001	<0.001	<0.001
	Normal distribution[c]	0.002	<0.001	<0.001	<0.001	0.005	0.033	<0.001	0.002	0.001	<0.001
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Melissa officinalis											
GI	0 kGy	101 ± 3[b]	67 ± 1[b]	80 ± 1[b]	44 ± 1[c]	165 ± 4[a]	125 ± 3[a]	100 ± 1[c]	829 ± 6[a]	63 ± 1[c]	448 ± 4[b]
	1 kGy	101 ± 1[b]	73 ± 3[a]	75 ± 1[c]	48 ± 1[b]	130 ± 5[c]	113 ± 2[b]	108 ± 2[a]	786 ± 22[b]	69 ± 1[a]	498 ± 11[a]
	10 kGy	107 ± 2[a]	73 ± 2[a]	103 ± 1[a]	55 ± 1[a]	135 ± 2[b]	109 ± 2[c]	104 ± 2[b]	742 ± 8[c]	65 ± 1[b]	417 ± 4[c]
p-values	Homoscedasticity[b]	<0.001	0.010	0.037	0.397	0.028	0.224	<0.001	<0.001	<0.001	0.023
	Normal distribution[c]	0.097	0.029	<0.001	<0.001	<0.001	0.008	0.029	0.002	0.016	0.006
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Melittis melissophyllum											
GI	0 kGy	583 ± 24[c]	354 ± 39	512 ± 16[b]	249 ± 2[b]	1648 ± 154[c]	447 ± 66[b]	70 ± 4	160 ± 3[a]	29 ± 2[a]	108 ± 4[a]
	1 kGy	696 ± 92[b]	355 ± 19	605 ± 29[a]	198 ± 3[c]	2105 ± 139[b]	538 ± 61[a]	73 ± 5	100 ± 3[c]	16 ± 1[b]	73 ± 1[c]
	10 kGy	843 ± 28[a]	354 ± 23	457 ± 12[c]	290 ± 2[a]	2299 ± 187[a]	595 ± 37[a]	70 ± 3	135 ± 2[b]	15 ± 1[b]	83 ± 5[b]
p-values	Homoscedasticity[b]	0.171	0.005	0.017	0.300	0.359	0.082	0.233	0.199	<0.001	<0.001
	Normal distribution[c]	0.008	0.007	0.054	0.001	0.286	0.060	0.007	0.001	<0.001	<0.001
	1-way ANOVA[d]	<0.001	0.996	<0.001	<0.001	<0.001	<0.001	0.474	<0.001	<0.001	<0.001
Mentha piperita											
GI	0 kGy	184 ± 5[b]	83 ± 7[b]	119 ± 2[c]	52 ± 2[a]	597 ± 44[b]	184 ± 5[a]	218 ± 2[c]	591 ± 19[a]	117 ± 2[a]	319 ± 6[b]
	1 kGy	192 ± 6[b]	98 ± 5[a]	136 ± 2[b]	43 ± 1[b]	465 ± 5[c]	137 ± 2[b]	276 ± 4[a]	572 ± 25[a]	95 ± 3[b]	354 ± 3[a]
	10 kGy	225 ± 9[a]	86 ± 3[b]	146 ± 4[a]	53 ± 1[a]	715 ± 67[a]	95 ± 4[c]	242 ± 4[b]	527 ± 13[b]	78 ± 2[c]	266 ± 8[c]
p-values	Homoscedasticity[b]	0.039	0.055	0.007	<0.001	<0.001	0.048	0.006	0.032	0.114	0.001
	Normal distribution[c]	0.002	0.316	0.002	<0.001	0.009	0.002	0.001	0.018	0.002	<0.001
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MeOH—methanol; GAE—gallic acid equivalents; CE—catechin equivalents.

[a] The results are presented as the mean ± SD.

[b] Homoscedasticity among GI doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

[c] Normal distribution of the residuals was evaluated using Shapiro–Wilk test.

[d] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one GI dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

to each gamma irradiation dose (0 kGy, 1 kGy and 10 kGy) were not shaped, as it could have been anticipated from Tables 1–5. In fact, and as it can be concluded by comparing the plots of object scores (Fig. 1**A**) and component loadings (Fig. 1**B**), the four defined groups include unirradiated samples, but also samples irradiated with 1 and 10 kGy, making impossible to point out which parameter variations characterize better each of the studied groups (0, 1 and 10 kGy). This result clearly indicates that, when considered from a global point of view, the changes resulting from irradiation treatment are not enough to separate each of the corresponding groups.

Nevertheless, gamma irradiation seemed to have caused changes in a species-dependent manner. In fact, the object scores corresponding to each plant species were clearly separated (Fig. 1**C**), especially for *A. citrodora*. The defined dimensions had, off course, the same Cronbach's α and eigenvalues, including also the same percentage of variance. By comparing Fig. 1**B** and **C**, it is evident that the major differences in lemon verbena were caused on carbohydrates, physical parameters, malic acid, oxalic acid, total organic acids, C17:0, TBARS formation inhibition, reducing power and DPPH scavenging activity (all in methanolic extracts) and phenol content in infusions; on the other hand, energy, reducing sugars, C11:0, C22:0 and C20:3n3 + C21:0 suffer minor changes. The main differences on lemon balm were observed for protein, phenols (methanolic extracts) and reducing power (infusions), while ash, carbohydrates, C8:0, C13:0, C15:0, C16:0, SFA, and β-carotene bleaching inhibition remain almost unchanged. Since the object scores of peppermint are in symmetric position in relation to lemon balm, the main characteristic alterations for peppermint are exactly the inverse to those verified in lemon balm. Lastly, the most sensitive parameters of bastard balm samples were C11:0, C14:0, C18:2n6 and DPPH scavenging activity (infusion), whereas fat, α-tocopherol, γ-tocopherol, C6:0, C18:3n3 and flavonoids were less sensitive in this species.

4. Conclusion

When considered individually, the effects of gamma-irradiation (up to 10 kGy) in the chemical/nutritional and antioxidant properties of lemon verbena, lemon balm, bastard balm and peppermint proved to have statistical significance in particular cases. Nonetheless, when analyzed under an integrated approach, unirradiated and irradiated samples were grouped indiscriminately (as it might be deduced from the PCA plots), indicating that irradiation treatment did not cause sufficient changes to define a specific chemical profile. Interestingly, the way by which each species was affected by irradiation seemed to be characterized by some specificity, as revealed by the PCA plot of object scores. Overall, it might be considered that gamma irradiation treatment (up to 10 kGy) is a feasible conservation technology for the assayed *Lamiaceae* and *Verbenaceae* species. This is an interesting result because the 10 kGy dose allows obtaining disinfested and decontaminated samples.

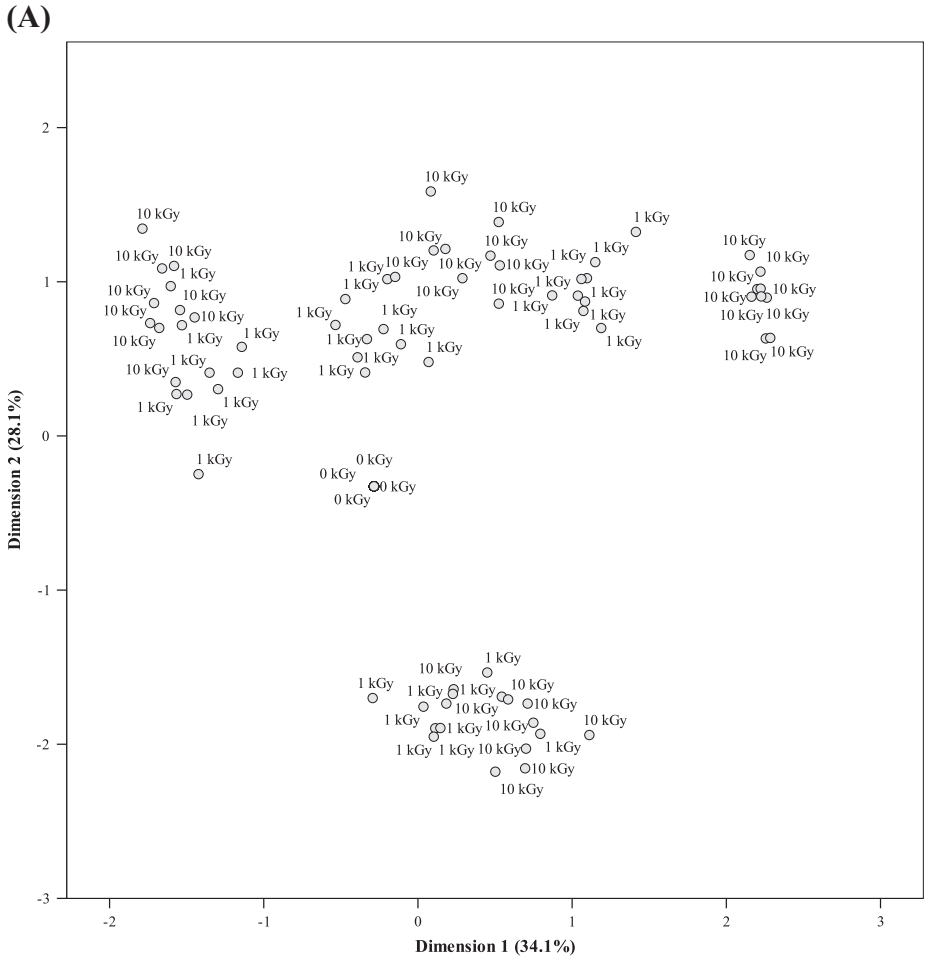

Fig. 1. Plots of objects scores and component loadings. A: using gamma irradiation doses as objects; B: using the differences in the evaluated parameters as component loadings. C: using the assayed *Lamiaceae* and *Verbenaceae* species as objects.

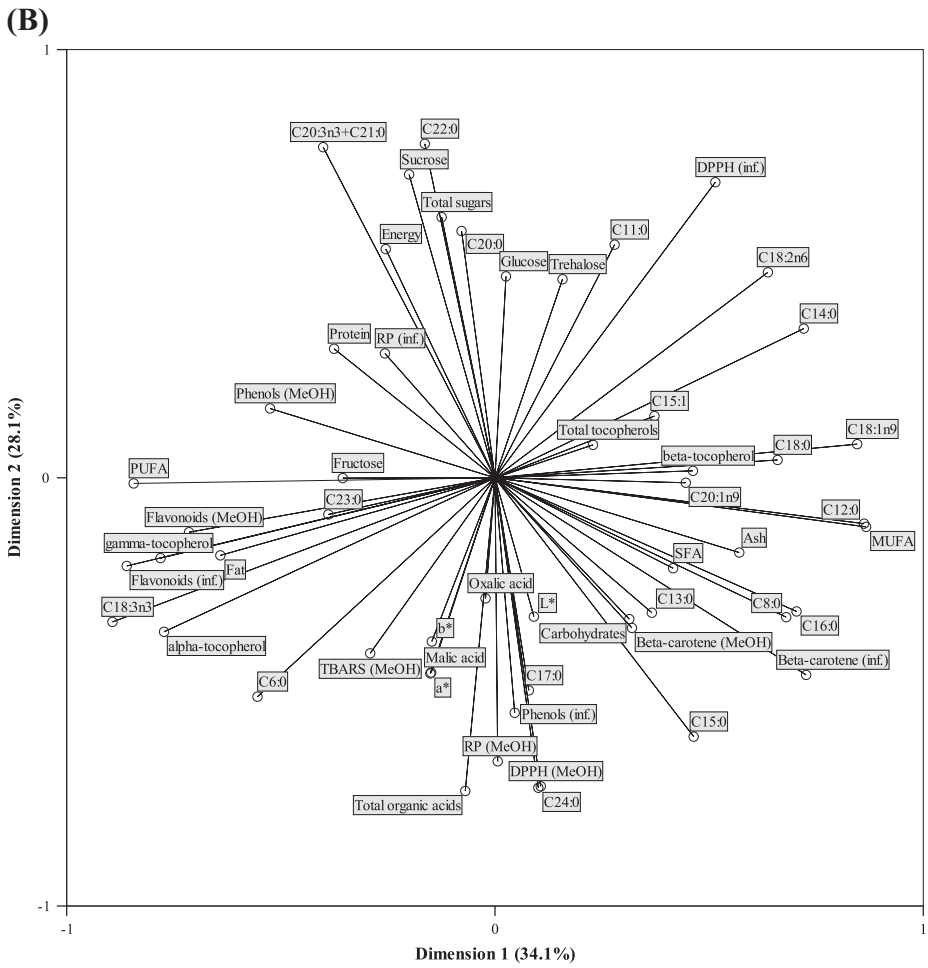

Fig. 1 (*continued*).

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Conflict of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2014.11.047.

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Annexes 5

Effects of gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Mentha x piperita* L.

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Effects of gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Mentha* x *piperita* L.

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ABSTRACT

The aim of the present study was to evaluate the effects of gamma irradiation on cytotoxicity and phenolic compounds of *Thymus vulgaris* L. and *Menta* × *piperita* L. (methanolic extracts), used in traditional medicine. Thirteen and fourteen phenolic compounds, including caffeoyl derivatives and flavonoid glycosides, were detected in *T. vulgaris* and *Mentha piperita*, respectively, none of which was affected by the irradiation dose used (10 kGy). Furthermore, the irradiation up to 10 kGy did not change the cytotoxic properties of peppermint samples on tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2), whereas thyme samples irradiated at 10 kGy increased their cytotoxicity in the assayed tumor cell lines compared with samples submitted to 2 and 5 kGy. All in all, the dose of 10 kGy was considered as suitable to be applied for the purpose of disinfestation and microbial decontamination of these plants without modifying their phenolic composition and bioactive properties.

1. Introduction

Phytochemicals present in medicinal plants have revealed to be beneficial for the prevention of various diseases due to its capacity to act in multiple biological mechanisms (Zhang et al., 2011). Its antioxidant potential acts in the neutralization of reactive oxygen species (ROS) that cause damaging changes in the cells (Bajpai, Agrawal, Bang, & Park, 2015; Jain, Jain, Jain, Jain, & Balekar, 2010). The increasing interest in the use of phytochemicals is due to their natural origin, the possibility to be ingested through the diet, easy acquisition and also due to their reduced adverse effects (Dillard & German, 2000). The use of medicinal plants for the treatment of different diseases dates back to ancient times, revealing satisfactory results as anti-inflammatory, antimicrobial, antimutagenic, anticancer and antioxidant agents.

These effects are in part explained by the presence of phytochemicals such as phenolic compounds (Wojdyło, Oszmiański, & Czemerys, 2007).

Phenolic compounds are secondary metabolites ubiquitously distributed in plants (Jabri-Karoui, Bettaieb, Msaada, Hammami, & Marzouk, 2012; Wojdyło et al., 2007). They include a large group of biologically active compounds, with over 8000 individual molecules described, having at least one aromatic ring with one or more hydroxyl groups attached, being able to vary from small molecules to large and complex ones. These compounds usually appear in their natural sources as esters and glycosides (Roby, Sarhan, Selim, & Khalel, 2013; Vallverdú-Queralt et al., 2014).

Many species of plants recognized for their medicinal properties and beneficial impact on health contain these metabolites, such as e.g., *Thymus vulgaris* L. (thyme) and *Mentha* × *piperita* L. (peppermint) (Zgorka & Glowniak, 2001; Kapp et al., 2013), belonging to the Lamiaceae family. Thyme is a plant widely used in folk medicine and its essential oil has shown bioactive properties because of its composition in bioactive metabolites (mixture of monoterpenes, being the main compounds thymol), such as antimicrobial, anti-

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inflammatory, expectorant, spasmolytic, antioxidant and hepatoprotective activities (Fecka & Turek, 2008; Fachini-Queiroz et al., 2012; Martins et al., 2015; Nikolić et al., 2014; Gavarić et al., 2015). Similarly, peppermint is a perennial herb that is also commonly used in traditional medicine, mainly consumed as teas. It has been associated to antioxidant, antitumor, antimicrobial, hypoallergenic and immunomodulatory effects, as well as benefits for the digestive tract (Grigoleit & Grigoleit, 2005; McKay & Blumberg, 2006; Singh, Shushni, & Belkheir, 2015). Its essential oil is also well known and widely used in food, pharmaceutical and cosmetic industries, because of presenting biological activity against several organisms (Moghaddam, Pourbaige, Tabar, Farhadi, & Hosseini, 2013; Sharma & Sharma, 2013).

The commercialization and use of medicinal plants must accomplish certain rules regarding to decontamination (Haleem, Salem, Fatahallah, & Abdelfattah, 2015). Irradiation is increasingly recognized as a suitable approach to decontaminate natural matrices and preserve their components (Kume, Furuta, Todoriki, Uenoyama, & Kobayashi, 2009). In particular, gamma irradiation was approved for disinfection and microbial control in various food products and supplements by the Food and Drug Administration (FDA), being a procedure technically and economically viable and physically safe with a powerful antimicrobial effect (Mizani, Sheikh, Ebrahimi, Gerami, & Tavakoli, 2009). This decontamination method has interesting advantages that present it as a good alternative to other methods, namely for aromatic herbs (Pereira et al., 2015a). The majority of the studies with gamma irradiated herbs are related with the effects on nutritional composition and antioxidant properties. In this work we aimed at evaluating the effects on cytotoxicity and phenolic compounds using two Lamiaceae herbs (thyme and peppermint) as case-studies.

2. Materials and methods

2.1. Herbs and samples irradiation

Samples of *T. vulgaris* L. (thyme) and *Mentha* × *piperita* L. (peppermint) were provided as dry leaves by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal). After confirmation of the taxonomical identification, the samples were divided into four groups: control (non-irradiated, 0 kGy), and samples irradiated with different doses (2, 5 and 10 kGy).

A Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with total activity 140 TBq (3.77 kCi), was used for sample irradiations, in May 2015. During the irradiation procedure, the dose and dose rate were estimated using a chemical solution sensitive to ionizing radiation, with an Amber Perspex dosimeters (Batch X, from Harwell Company, Didcot, Oxfordshire, UK), and a Fricke dosimeter, respectively (ASTM, 1992; Pereira et al., 2015a).

The estimated doses for thyme samples were 2.4 ± 0.1 kGy, 5.5 ± 0.2 kGy and 10.4 ± 0.5 kGy; and 2.2 ± 0.3 kGy, 5.7 ± 0.21 kGy and 10.3 ± 0.4 kGy for peppermint samples. The dose rates and dose uniformity ratios (D_{max}/D_{min}) were, 1.2 kGy/h and 1.1 respectively. For simplicity, the values 0, 2, 5 and 10 kGy were considered for the doses of non-irradiated and irradiated groups, respectively.

2.2. Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic compound standards (apigenin-6-*C*-glucoside, caffeic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside and rosmarinic acid) were from Extrasynthese (Genay, France). Fetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, UT, USA). Acetic acid, formic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Ferrous ammonium sulfate(II) hexahydrate, sodium chloride and sulfuric acid, all with PA purity, were purchased from Panreac S.A. (Barcelona, Spain) (proanalysis), in order to prepare the acid aqueous Fricke dosimeter solution.

2.3. Preparation of the extracts

The dried samples of *T. vulgaris* and *Mentha* × *piperita* were reduced to a fine dried powder (20 mesh) and mixed to obtain homogeneity. To prepare the methanolic extracts, each sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and then filtered through Whatman No. 4 paper. The residue was then extracted with an additional portion of 25 mL of methanol (25 °C at 150 rpm) for another 1 h. Subsequently, the combined extracts were evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) until dryness.

2.4. Evaluation of cytotoxic properties

2.4.1. General

For cytotoxicity evaluation the extracts were redissolved in water, with a final solution of 8 mg/mL and diluted to different concentrations, and ellipticine was used as positive control. The results were calculated as GI_{50} values (sample concentration that inhibited 50% of the net cell growth).

2.4.2. In tumor cell lines

The human tumor cell lines used were: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines were plated in a 96-well plate, at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 and 1.0×10^4 cells/well for HeLa and HepG2) and were allowed to attach for 24 h. Afterwards, various extract concentrations were added to the cells and incubated during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100 μL) was used in order to bind the adherent cells and further incubated for 60 min at 4 °C. After the incubation period, the plates were washed with deionised water and dried and sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 μL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Guimarães et al., 2013).

2.4.3. In non-tumor cells

A freshly harvested porcine liver, obtained from a local slaughter house, was used in order to obtain the cell culture, designated as PLP2. The liver tissues were rinsed in Hank's balanced salt solution containing penicillin (100 U/mL), streptomycin (100 μg/mL) and divided into 1 × 1 mm^3 explants. A few of these explants were transferred to a tissue flasks (25 cm^2) containing DMEM medium supplemented with fetal bovine serum (FBS, 10%), nonessential amino acids (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL) and incubated at 37 °C with a humidified atmosphere (5% CO_2). The medium was changed every two days and the cell cultivation was continuously monitored using a phase contrast

microscope. When confluence was reached, the cells were subcultured and plated in 96-well plate (density of 1.0×10^4 cells/well) containing DMEM medium supplemented with FBS (10%), penicillin (100 U/mL) and streptomycin (100 μg/mL). The growth inhibition was evaluated using the SRB assay, previously described (Guimarães et al., 2013).

2.5. Analysis of phenolic compounds

The extracts obtained above were analysed using a HPLC chromatograph (Hewlett–Packard 1100, Agilent Technologies, Santa Clara, CA, US) with a double online detection using a diode array detector (DAD) with 280, 330 and 370 nm as preference wavelengths, and a mass spectrometer (MS) equipped with an ESI source and a triple quadrupole-ion trap mass analyser, which was connected to the HPLC system via the DAD cell outlet (Barros et al., 2013). The DAD was coupled to an HP Chem Station (rev. A.05.04) and the MS was controlled by the Analyst 5.1 software. The separation was achieved using a Spherisorb S3 ODS-2 C_{18} column (3 μm, 4.6 × 150 mm) thermostatted at 35 °C, using a gradient elution with the following solvents: 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient established was 15% B for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% B for 10 min, and re-equilibration of the column (10 min), using a flow rate of 0.5 mL/min. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS), employed to show full scan spectra, and enhanced product ion (EPI) analysis. Air (zero graded) was used as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen functioned as the curtain (20 psi) and collision gas (medium). The ion spray voltage was set at −4500 V and spectras were recorded in negative ion mode between *m/z* 100 and 1700. The settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V. Compounds were tentatively identified comparing the obtained information (retention times, UV–vis and mass spectra) with available data reported in the literature and by comparison with standard compounds, when available. For the quantification a calibration curve for each available phenolic standard (apigenin-6-*C*-glucoside, caffeic acid, chlorogenic acid, hesperetin, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside, rosmarinic acid) was constructed based on the UV signal (maximum absorption of each standard compound lambda max). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of another compound from the same phenolic group. The results were expressed as mg per g of extract.

2.6. Statistical analysis

For each one of the species two samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. In the case of phenolic composition, a Student's *t*-test was used to determine the significant difference among two different samples, with $\alpha = 0.05$. This analysis was carried out using IBM SPSS Statistics for Windows, Version 22.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

3.1. Cytotoxic properties of extracts from non-irradiated and irradiated samples

The results obtained in the evaluation of the cytotoxic properties of thyme and peppermint (extracts prepared from non-irradiated and irradiated samples) against four human tumor cell lines (MCF-7, NCI-H460, HeLa and HepG2) and against non-tumor cells are given in Table 1. All samples showed cytotoxicity, and these results are in agreement with a previous study performed by Berdowska et al. (2013) that used dried aqueous extracts of *T. vulgaris* from Poland, reporting its cytotoxicity in MCF-7 (breast carcinoma) tumor cell line. Lv et al. (2012) also evaluated the antiproliferative activity of a peppermint extract against the human tumor cell line HT-29, describing promising results.

The thyme sample irradiated at 10 kGy showed cytotoxicity for MCF-7, HeLa and HepG2 cell lines similar to the control sample, and higher (lower GI_{50} values) than the toxicity revealed by the samples irradiated at 2 and 5 kGy. Regarding peppermint, no significant differences were observed when different doses were applied, with the exception of MCF-7 cell line, where a higher cytotoxicity was observed in the control sample (0 kGy). In relation to the toxicity for non-tumor cells, none of the samples presented hepatotoxicity ($GI_{50} > 400$ μg/mL).

3.2. Comparative analysis of the phenolic compounds in non-irradiated and irradiated samples

As, in general, no significant differences were found in the cytotoxic properties of the extracts prepared from samples irradiated at three different doses (2, 5 and 10 kGy). Nonetheless, control samples and samples irradiated at the highest dose (10 kGy) were used for phenolic compounds analysis, due to the higher efficiency of higher doses in the decontamination process.

Thirteen and fourteen phenolic compounds were identified in the methanolic extracts of thyme and peppermint, respectively. Tables 2 and 3 present data related to the phenolic compounds identification (retention time, λ_{max} in the visible region, molecular ion, main fragment ions in MS^2, tentative identification and quantification) obtained by HPLC-DAD-ESI/MS analysis for both species. Fig. 1 shows the phenolic compounds profile in thyme and peppermint, recorded at 280 nm.

Compounds 2 (apigenin 6,8-di-*C*-glucoside), 3 (caffeic acid), 6 (rosmarinic acid hexoside), 7 (luteolin-7-*O*-glucuronide), 8 (luteolin-7-*O*-glucoside), 10 (rosmarinic acid), 12 (lithospermic acid A) and 13 (eriodictyol) in thyme were already described and tentatively identified in a previous study carried out by our research group, but using a different commercial sample (Martins et al., 2015). Moreover, the majority of the mentioned compounds have been previously cited in thyme samples (Boros et al., 2010; Costa et al., 2012; Dapkevicius et al., 2002; Fecka & Turek, 2008; Hossain, Rai, Brunton, Martin-Diana, & Barry-ryan, 2010; Roby et al., 2013; Vallverdú-Queralt et al., 2014; Vergara-Salinas, Perez-Jiménez, Torres, Agosin & Pérez-Correa, 2012). Compounds 1, 4, 5, 9 and 11 were not described in our previous study (Martins et al., 2015), which may be due to the existence of different composition and/or distribution of phenolic compounds depending on the part tissue, the origin of the plant and the edaphoclimatic conditions (Boros et al., 2010; Costa et al., 2012). Based on their UV and mass spectra, compounds were tentatively identified as caffeic acid hexoside (peak 1), methyleriodictyol *O*-pentosylhexoside (peak 4) and quercetin-*O*-glucuronide (peak 5). Peak 9 ($[M-H]^-$ at *m/z* 537) showed similar UV and mass spectra characteristics as lithospermic acid A (peak 12), although it was eluted at a different retention

Table 1
Cytotoxicity of thyme and peppermint extracts prepared from non-irradiated and irradiated samples.

	Doses			
	0 kGy	2 kGy	5 kGy	10 kGy
Thyme				
MCF-7 (breast carcinoma)	88 ± 7^{b}	104 ± 6^{a}	106 ± 10^{a}	83 ± 9^{b}
NCI-H460 (non-small cell lung cancer)	294 ± 12^{a}	276 ± 16^{ab}	297 ± 11^{a}	262 ± 4^{b}
HeLa (cervical carcinoma)	161 ± 6^{b}	189 ± 13^{a}	191 ± 9^{a}	160 ± 13^{b}
HepG2 (hepatocellular carcinoma)	103 ± 10^{a}	110 ± 13^{a}	106 ± 8^{a}	100 ± 10^{a}
Hepatotoxicity PLP2 (non-tumor cells)	>400	>400	>400	>400
Peppermint				
MCF-7 (breast carcinoma)	114 ± 12^{b}	175 ± 15^{a}	150 ± 4^{ab}	154 ± 7^{ab}
NCI-H460 (non-small cell lung cancer)	226 ± 11^{a}	224 ± 2^{a}	213 ± 20^{a}	229 ± 16^{a}
HeLa (cervical carcinoma)	221 ± 13^{a}	206 ± 11^{a}	211 ± 21^{a}	214 ± 12^{a}
HepG2 (hepatocellular carcinoma)	98 ± 9^{a}	115 ± 9^{a}	106 ± 11^{a}	111 ± 12^{a}
Hepatotoxicity PLP2 (non-tumor cells)	>400	>400	>400	>400

Positive control (Ellipticine) - MCF-7: 1.21 ± 0.02; NCI-H460: 1.03 ± 0.09; HeLa: 0.91 ± 0.11; HepG2: 1.10 ± 0.09; PLP2: 2.29 ± 0.18. GI_{50} values (μg/mL) correspond to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

Table 2
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in thyme extracts prepared from non-irradiated and irradiated samples (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Type of identification	Quantification (mg/g)		*t*-Students test *p*-value
							0 kGy	10 kGy	
1	7.3	320	341	179(100),135(88)	Caffeic acid hexoside	References 1,2,3,4/DAD/MS	1.7 ± 0.1	1.5 ± 0.1	0.988
2	10.8	338	593	473(20),383(33),353(27),297(5)	Apigenin 6,8-di-*C*-glucoside	Reference 5/ DAD/MS	3.45 ± 0.04	3.31 ± 0.04	0.212
3	11.3	326	179	135(100)	Caffeic acid	Reference 5/ Standard/DAD/ MS	2.69 ± 0.01	2.5 ± 0.4	0.258
4	17.4	284,336sh	595	301(47),286(100)	Methyleriodictyol-*O*-pentosylhexoside	DAD/MS	3.12 ± 0.01	1.9 ± 0.4	0.005
5	18.1	350	461	301(100)	Quercetin-*O*-glucuronide	DAD/MS	0.4 ± 0.1	0.35 ± 0.03	0.742
6	18.8	322	521	359(100),197(13),179(36),161(62),135(21)	Rosmarinic acid hexoside	Reference 5/ DAD/MS	16.8 ± 0.1	14.7 ± 0.2	0.378
7	20.0	348	461	285(100)	Luteolin-7-*O*-glucuronide	Reference 5/ DAD/MS	8.4 ± 0.2	7.1 ± 0.2	0.381
8	20.8	350	447	285(100)	Luteolin-7-*O*-glucoside	Reference 5/ Standard/DAD/ MS	3.34 ± 0.01	3.2 ± 0.1	0.003
9	23.4	286,320sh	537	493(20),359(70),295(5),197(13),179(28),161(100), 135(63)	Caffeic acid trimer	DAD/MS	9.1 ± 0.1	8.0 ± 0.1	0.027
10	24.0	330	359	197(17),179(35),161(100),135(29)	Rosmarinic acid	Reference 5/ Standard/DAD/ MS	12.7 ± 0.4	10.4 ± 0.2	0.038
11	25.6	282	567	535(23),493(49),387(32),285(25),197(13)	Caffeic acid derivative	DAD/MS	2.3 ± 0.1	1.68 ± 0.05	0.001
12	27.5	290,326sh	537	493(50),359(17),295(33),179(75),135(100)	Lithospermic acid A	Reference 5/ DAD/MS	2.25 ± 0.01	1.9 ± 0.1	0.999
13	30.6	288,334sh	287	151(35),135(100)	Eriodictyol	Reference 5/ Standard/DAD/ MS	0.87 ± 0.01	0.53 ± 0.04	0.001
					Total phenolic acids		48 ± 1	47 ± 1	0.251
					Total flavonoids		19.5 ± 0.3	19.0 ± 0.1	0.010
					Total phenolic compounds		67 ± 1	66 ± 1	0.097

References: (1) Hossain et al. (2010); (2) Nagy et al. (2011); (3) Vergara-Salinas et al. (2012); (4) Vallverdú-Queralt et al. (2014); (5) Martins et al. (2015).

time. The presence of salvianolic acid I with the same molecular weight was reported in thyme by Dapkevicius et al. (2002) and Nagy, Solar, Sontag, and Koenig (2011), although no sufficient elements for assigning that identity to the compound detected herein, so that the compound was just identified as a caffeic acid trimer. Compound 11 ($[M-H]^-$ at *m/z* 567) should also correspond to a caffeic acid derivative, owing to its UV spectrum and the observation of an MS^2 fragments at *m/z* 493, coherent with salvianolic acid A, furthermore, the fragment at *m/z* 197 could be attributed to dihydroxyphenyl-lactic acid (danshensu); however, no definite structure could be matched for the compound, so that it remains as an unidentified caffeic acid derivative. The presence of caffeic acid

Table 3
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in peppermint extracts prepared from non-irradiated and irradiated samples (mg/g extract).

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Type of identification	Quantification (mg/g)		*t*-Students test *p*-value
							0 kGy	10 kGy	
1′	5.1	328	353	191(100),179(27),173(5),161(15),135(30)	3-*O*-Caffeoylquinic acid	Reference 1/DAD/MS	0.87 ± 0.02	0.76 ± 0.01	0.001
2′	7.1	328	353	191(100),179(90),173(50),161(20),135(57)	5-*O*-Caffeoylquinic acid	References 2,3/ Standard/DAD/MS	1.4 ± 0.1	1.2 ± 0.1	0.025
3′	11.1	326	179	135(100)	Caffeic acid	References 2,3,4,5/ Standard/DAD/MS	0.44 ± 0.01	0.5 ± 0.1	0.134
4′	14.5	348	637	285(100)	Luteolin-*O*-diglucuronide	References 2,3,7/DAD/ MS	7.1 ± 0.2	6.22 ± 0.01	0.001
5′	15.7	288,330sh	537	493(45),313(18),295(36),269(55),197(36),179(64),135(100)	Caffeic acid trimer	DAD/MS	3.1 ± 0.2	2.9 ± 0.2	0.115
6′	16.1	284,332sh	595	287(100)	Eriodictyol-*O*-rutinoside	References 3,7/DAD/MS	100 ± 1	102.47 ± 0.01	0.005
7′	17.1	286,336sh	449	287(100)	Eriodictyol-*O*-hexoside	DAD/MS	2.2 ± 0.2	2.1 ± 0.1	0.157
8′	19.0	350	593	285(100)	Luteolin-7-*O*-rutinoside	References 2,3,6/ Standard/DAD/MS	30.2 ± 0.1	30.3 ± 0.6	0.677
9′	19.8	348	461	285(100)	Luteolin-7-*O*-glucuronide	References 2,3,7/DAD/ MS	11.2 ± 0.2	10.0 ± 0.4	0.002
10′	20.0	282,330sh	579	271(100)	Naringenin-*O*-rutinoside	References 2,3/DAD/MS	3.1 ± 0.1	3.0 ± 0.2	0.362
11′	21.5	278,338sh	717	537(34),519(50),493(39),339(29),321(37),313(6),295(100),197(3),179(11),161(5),135(11)	Salvianolic acid B/E/L	References 2,3/DAD/MS	13 ± 1	13.4 ± 0.3	0.276
12′	22.6	286,338sh	609	301(100)	Hesperetin-*O*-rutinoside	DAD/MS	5.5 ± 0.3	5.6 ± 0.2	0.302
13′	23.6	330	359	197(13),179(20),161(100),135(21)	Rosmarinic acid	References 2,3,4,5/ Standard/DAD/MS	25 ± 1	25.1 ± 0.1	0.291
14′	24.0	288,340sh	493	313(5),295(100),279(3),197(14),179(8),135(5)	Salvianolic acid A	DAD/MS	10.3 ± 0.5	9.72 ± 0.01	0.065
					Total phenolic acids		53 ± 3	54 ± 1	0.939
					Total flavonoids		159 ± 2	159.7 ± 0.1	0.248
					Total phenolic compounds		212 ± 4	213.7 ± 0.5	0.607

References: (1) Clifford et al. (2003); (2) Kapp et al. (2013); (3) Riachi and De Maria (2015); (4) Pérez et al. (2014); (5) Lv et al. (2012); (6) Areias et al. (2001); (7) Krzyzanowska et al. (2011).

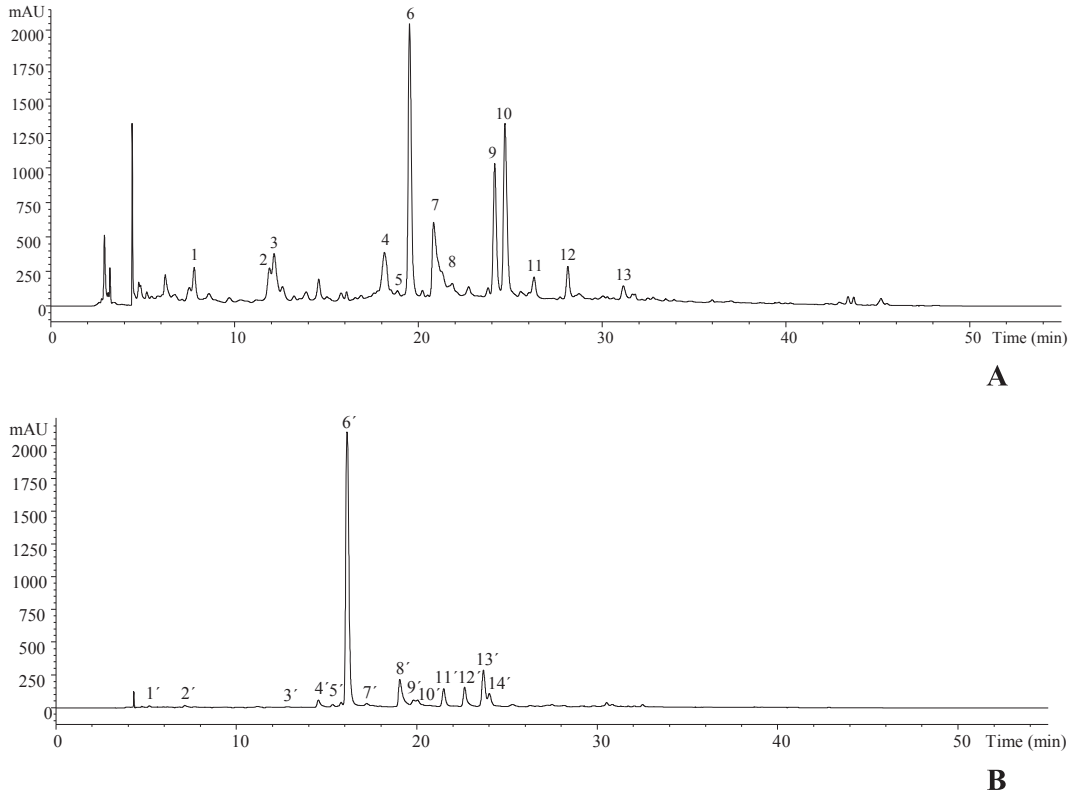

Fig. 1. Individual profile of thyme (A) and peppermint (B) irradiated with 10 kGy recorded at 280.

hexoside has been already reported in thyme by Hossain et al. (2010), Nagy et al. (2011), Vergara-Salinas et al. (2012) and Vallverdú-Queralt et al. (2014). To the best of our knowledge the other three compounds tentatively identified herein have not been previously cited in *T. vulgaris*.

Regarding peppermint, caffeic acid (compound 3′), chlorogenic acid (i.e., 5-*O*-caffeoylquinic acid; compound 2′), luteolin-7-*O*-rutinoside (compound 8′) and rosmarinic acid (compound 13′) were positively identified according to their retention, mass spectra and UV–vis characteristics in comparison with commercial standards. These compounds were also described in *Mentha piperita* leaves (using petroleum ether, chloroform, ethyl ether, ethyl acetate, acetone, methanol, ethanol, ethanol 80% and ethanol 30% and a boiling water extract) of commercial and non-commercial samples (Areias, Valentão, Andrade, Ferreres, & Seabra, 2001), in *M. piperita* infusions (Kapp et al., 2013; Pérez, Rocha-Guzmán, Mercado-Silva, Loarca-Piña, & Reynoso-Camacho, 2014), in extracts from conventional and organically grown peppermint samples (soluble free phenolics, soluble conjugated phenolics, insoluble bound phenolics) (Lv et al., 2012) and in a revision of the literature performed by Riachi and De Maria (2015).

Compound 1′ was identified as 3-*O*-caffeoylquinic acid based on its MS^2 fragmentation, yielding the base peak at *m/z* 191 and the ion at *m/z* 179 with an intensity of 72% relative to the base peak, considered characteristic of 3-acylchlorogenic acids as reported by Clifford, Johnston, Knight, and Kuhnert (2003). The sample also presented other caffeic acid derivatives, namely compounds 5′, 11′ and 14′. The pseudomolecular ion ($[M-H]^-$ at *m/z* 537) and fragmentation pattern of peak 5′ were consistent with a caffeic acid trimer, although it eluted at an earlier retention time than lithospermic acid A. As above discussed for peak 9 in the thyme sample (Table 2), besides lithospermic acid A, the molecular weight of the compound would also match that of salvianolic acid H/I, reported by Kapp et al. (2013) in peppermint teas, although no further support for that identity could be obtained, so that in our case the compound has been just assigned as a caffeic acid trimer. The molecular weight of compound 14′ ($[M-H]^-$ at *m/z* 493, compound 14′) might correspond to salvianolic acid A, whereas that of compound 11′ ($[M-H]^-$ at *m/z* 717) might correspond to salvianolic acids B or E, whose presence was reported in peppermint teas by Kapp et al. (2013), or salvianolic acid L, cited by Krzyzanowska, Janda, Pecio, Stochmal, and Oleszek (2011) in the aerial parts of *Mentha* species. Similar caffeoyl derivatives have been cited in mentha samples by other authors as reviewed by Riachi and De Maria (2015). All these compounds were quantified based on caffeic and rosmarinic acid calibration curves.

The remaining compounds were identified as flavonoids. Peaks 4′ ($[M-H]^-$ at *m/z* 637) and 9′ ($[M-H]^-$ at *m/z* 461) were assigned as luteolin glycosides, based on their UV spectra (λ_{max} around 350 nm) and the production of an MS^2 fragment ion at *m/z* 285. Compound 9′ can be assumed as luteolin-7-*O*-glucuronide by comparison with a commercial standard, whereas compound 4′ was tentatively assigned as luteolin-*O*-diglucuronide. The presence of luteolin glucuronides in *M. piperita* samples was also reported by Krzyzanowska et al. (2011), Kapp et al. (2013) and Riachi and De Maria (2015). Compounds 6′ ($[M-H]^-$ at *m/z* 595) and 7′ ($[M-H]^-$ at *m/z* 449) were tentatively identified as eriodictyol-*O*-rutinoside and eriodictyol-*O*-hexoside, respectively, previously described in *M. piperita* by Krzyzanowska et al. (2011) and Riachi and De Maria (2015). Based on the mass spectra, compounds 10′ ($[M-H]^-$ at *m/z* 579) and 12′ ($[M-H]^-$ at *m/z* 609) were identified as rutinosyl derivatives of the flavanones naringenin and hesperetin, respectively. The presence of narirutin (i.e., naringenin-7-*O*-rutinoside) in peppermint samples was cited by Kapp et al. (2013) and Riachi and De Maria (2015).

In a previous study rosmarinic acid and luteolin-7-*O*-

glucuronide were found as the most abundant compounds in thyme (Martins et al., 2015). These compounds were also relevant components in the sample analysed herein, although in this case rosmarinic acid hexoside appeared as the most abundant phenolic compound. These differences could be related with the natural variability inherent to plants grown under different environmental conditions that influence their secondary metabolism (Riachi & De Maria, 2015). Eriodictyol-7-*O*-rutinoside was the most abundant compound in peppermint, in agreement with a previous study performed by Areias et al. (2001). The irradiation at 10 kGy did not affect the phenolic composition in both plant samples in relation to non irradiated control samples, which could be explained by the reduced water activity of this matrix. The molecules preservation by irradiation mostly depends on the food composition in water content, temperature and the presence or absence of oxygen in the process, thus the dried food has a reduced effect of radiolytic products production (Sadecká, 2007). However, a different effect was observed in a study performed by Pereira et al. (2015b), where the irradiated sample at 10 kGy showed the highest content in phenolic compounds. This could be explained by the usage of a high dose of radiation that leads to an increase in the extractability of certain phenolic compounds. Another reason that could justify this effect is the water activity that remains after the drying process. Thereby, *Ginkgo biloba* sample might have higher water content, which triggered a higher formation of radiolytic compounds, leading to the changes verified in the phenolic contents (Tezotto-Uliana, Silva, Kluge, & Spoto, 2015). Therefore, it can be concluded that gamma irradiation does not conduct to a linear behaviour towards the conservation of compounds in plants, and depends also on other factors (e.g., water composition, different compounds present in plants, dose applied). Nonetheless, this radiation dose could be recommended as adequate to decontaminate these plants without affecting their contents on phenolic compounds. The same dose was also recommended by Machhour, Hadrami, Imziln, Mouhib, and Mahrouz (2011) with similar purposes.

In order to correlate the sample's cytotoxic effects with the phenolic composition, correlation factors were obtained between total phenolic acids and total flavonoids, and the GI_{50} values obtained for the four cell lines. The results showed high correlations in both plant samples for three of the cell lines, with the exception of MCF-7, where no correlation was found between the total contents of these phenolic groups. Nevertheless, the thyme cytotoxic activity obtained in MCF-7 cell line was highly correlated with caffeic acid ($R^2 = 0.7100$), caffeic acid trimer ($R^2 = 0.7709$), methyleriodictyol-*O*-pentosylhexoside ($R^2 = 0.4375$), rosmarinic acid hexoside ($R^2 = 0.4247$), luteolin-7-*O*-glucoside ($R^2 = 0.4305$), and lithospermic acid A ($R^2 = 0.4272$), while in peppermint the main contributor compounds were caffeic acid ($R^2 = 0.8586$), caffeic acid trimer ($R^2 = 0.7667$), luteolin-7-*O*-rutinoside ($R^2 = 0.6649$) and luteolin-7-*O*-glucuronide ($R^2 = 0.6466$).

The cytotoxic effects of thyme extracts for the other three cell lines, also presented high correlation factors, being NCI-H460 cell line highly correlated with total flavonoids ($R^2 = 0.9991$) and HeLa and HepG2 cell lines with total phenolic acids ($R^2 = 0.7483$ and 0.8139, respectively). The individual flavonoids that were highly correlated with NCI-H460 cell line were apigenin 6,8-di-C-glucoside ($R^2 = 0.8294$), methyleriodictyol-*O*-pentosylhexoside ($R^2 = 0.7416$), luteolin-7-O-glucoside ($R^2 = 0.7708$) and eriodictyol ($R^2 = 0.9235$), while the phenolic acids were caffeic acid hexoside ($R^2 = 0.9226$, for HeLA), caffeic acid ($R^2 = 0.5037$, for HeLA), rosmarinic acid hexoside ($R^2 = 0.7211$ and 0.5939, for HeLA and HepG2, respectively), rosmarinic acid ($R^2 = 0.5748$, for HepG2) and caffeic acid trimer ($R^2 = 0.8894$ and 0.5696, for HeLA and HepG2, respectively). In relation to peppermint extracts an opposite effect was observed in relation to thyme, being NCI-H460 cell line correlated with the total phenolic acids ($R^2 = 0.5319$), while HeLa and HepG2 cell lines were correlated with total flavonoids ($R^2 = 0.6946$ and 0.6214, respectively). The individual compounds that were responsible for these correlations were caffeic acid trimer ($R^2 = 0.5246$, for NCI-H460), rosmarinic acid ($R^2 = 0.5917$, for NCI-H460), luteolin-*O*-diglucuronide ($R^2 = 0.4148$ and 0.6462, for HeLA and HepG2, respectively), eriodictyol-*O*-rutinoside ($R^2 = 0.4075$ and 0.6986, for HeLA and HepG2, respectively), luteolin-7-*O*-rutinoside ($R^2 = 0.6409$ and 0.5638, for HeLA and HepG2, respectively) and luteolin-7-*O*-glucuronide ($R^2 = 0.4207$ and 0.7956, for HeLA and HepG2, respectively). Most of the mentioned phenolic compounds were not the main molecules present in the sample, meaning that synergistic effects are probably observed between the compounds, in order to enhance these activities.

Overall, all samples showed cytotoxic properties in human tumor cell lines, but with no toxicity for non-tumor cells. The different irradiation doses did not affect these properties or the phenolic composition of the peppermint samples. However, thyme samples irradiated at 10 kGy showed higher cytotoxicity for tumor cell lines in comparison with the other doses applied. Therefore, for the studied species, it is confirmed that 10 kGy can be applied because it did not affect the bioactive properties of these plants. The studied plants can represent a rich source of antioxidant compounds of phenolic nature: flavonols, flavones, flavanones and phenolic acid derivatives, which might contribute to the prevention and control of diseases through their incorporation into a normal diet or as supplements. Therefore, irradiation can be considered as a decontamination and preservation process (as described and tested by other authors), because when analysing the influence of this technology in compounds that provide the bioactive potential, it was found that it can be applied up to the maximum dose recommended in legislation (10 kGy) because it does not affect their concentration. This technology represents an added-value solution to meet the requirements of the food and pharmaceutical industries in the acquisition of high quality raw materials.

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Annexes 6

Infusions from *Thymus vulgaris* L. treated at different gamma radiation doses: effects on antioxidant activity and phenolic composition

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Infusions from *Thymus vulgaris* L. treated at different gamma radiation doses: Effects on antioxidant activity and phenolic composition

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ABSTRACT

The use of ionizing radiation dates back to many years ago, and is accredited for application in different foods with several purposes. It has been increasingly used in many countries for the treatment of aromatic plants. *Thymus vulgaris* L. (thyme) is a plant commonly used by food, pharmaceutical and cosmetic industries representing a natural source of several bioactives such as phenolic compounds. The aim of this work was to evaluate the effects of gamma radiation on the antioxidant activity (measured through the free radical scavenging activity, reducing power and lipid peroxidation inhibition) and phenolic compounds profile (obtained by HPLC-DAD-ESI/MS) of infusions prepared from irradiated thyme. The results showed that gamma irradiation at the dose of 10 kGy improved the free radical scavenging activity, reducing power and lipid peroxidation inhibition capacity of the studied infusions, while increasing significantly the concentrations of methyleriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside, eriodictyol and total flavonoids content. Thus, gamma radiation could be considered as a suitable treatment to be used in *Thymus vulgaris* L., herein validated for its bioactive parameters.

1. Introduction

Irradiation is a physical decontamination and preservation method, which processes the food at room temperature (Alothman, Bhat & Karim, 2009). It is used by the industry for different purposes, such as disinfestation, shelf life extension, decontamination and improvement of product quality (Singh & Datta, 2010). Despite being an increasingly popular technology, the number of irradiated foods is very low, even in countries where this preservation process is permitted. The main reasons are not only related with the high cost of the equipment but, especially, with the lack of acceptance by consumers (Roberts, 2014; Sádecká, 2007). The irradiation can be applied by using gamma rays, electron beams or X-rays, each one with different properties and comprising technological advantages and disadvantages (Alothman et al., 2009).

Gamma radiation has been applied to several matrices, including mushrooms and aromatic plants, being verified that guarantees the quality of the products for the authorized radiation doses (Fernandes et al., 2012; Kausar, Akram, & Kwon, 2013; Pereira et al., 2015b). It is also characterized by its high penetration ability, being effective in irradiation of large volume foods (IAEA, 2002). Irradiation has been applied to aromatic and medicinal plants with a maximum permitted dose of 10 kGy in Europe; this treatment allows the decontamination of such matrices, while maintaining their chemical, nutritional and organoleptic properties (EU, 1999).

For a long period of time, the plants were almost the only source of therapeutic agents for humans. Indeed plants are natural sources of phytochemicals, especially polyphenols, which have been related to the prevention of chronic diseases and improved health quality (Hayta, Polat, & Selvi, 2014; Zhang et al., 2011). Presently, plants continue being the main sources of substances for drug development by the pharmaceutical industry (Caleja et al., 2015; Carvalho, Costa, & Carnelossi, 2010). Several studies have been performed in order to test the effects of irradiation on phytochemicals present in dried plants, and in favorable conditions (irradiation source, dose,

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humidity, etc.) the antioxidant potential of the species can be increased (Alothman et al., 2009). The intention of these studies is to follow the process from the irradiation until the consumer and to study the impact of this industrial process on the main components of herbal infusions (DeRuiter & Dwyer, 2002).

Thyme (*Thymus vulgaris* L.) is an herbaceous and perennial aromatic plant belonging to the Lamiaceae family, commonly consumed as herbal infusion and as a condiment and spice (flavoring agent) (Balladin & Headley, 1999; Helmy, Farrag, & Hasaballah, 2015). It is one of the most cultivated herbs of *Thymus* genus, being used in food, pharmaceutical and cosmetic industries. It is listed in current editions of the European Pharmacopoeia, US Pharmacopeia and other official papers (Damianova, Tasheva, Stoyanova, & Damianov, 2008; Gavarić et al., 2015), exhibiting carminative, antispasmodic, antitussive, expectorant, bactericidal, antihelmintic and astringent effects. Traditionally, the herb has been used for the treatment of dyspepsia, chronic gastritis and diseases of the upper respiratory tract (Fecka & Turek, 2008; Helmy et al., 2015). It has also been reported that thyme infusions are low caloric beverages (Pereira, Barros, & Ferreira, 2015a), while containing high levels of phenolic compounds with antioxidant properties (Martins et al., 2015).

The aim of the present work was to go further on the study of antioxidant activity and phenolic composition of thyme, previously reported (Brandstetter, Berthold, Isnardy, Solar, & Elmadfa, 2009; Martins et al., 2015; Pereira et al., 2016), evaluating the effects of gamma irradiation on antioxidant potential and phenolic composition of infusions prepared from non-irradiated (0 kGy) and irradiated samples of thyme with 1 and 10 kGy. These doses where chosen since 1 kGy guarantees insects' disinfestation and 10 kGy is the highest dose permitted by the EU legislation that allows also microbiological decontamination (EU, 1999). The obtained results will assess if the expected benefits of these beverages are maintained after the application of this decontamination/preservation technique.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile 99.9% was of HPLC grade from Fisher Scientific (Lisbon, Portugal). Phenolic compound standards (apigenin-6-*C*-glucoside, caffeic acid, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside and rosmarinic acid) were from Extrasynthese (Genay, France). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma (St. Louis, MO, USA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH• was obtained from Alfa Aesar (Ward Hill, MA, USA). β-Carotene and linoleic acid were acquired from Sigma-Aldrich (St. Louis, Missouri, USA) and Tween 80 from Panreac (Barcelona, Spain). All other solvents and reagents were acquired from scientific retailers. Ferrous ammonium sulfate (II) hexahydrate, sodium chloride and sulfuric acid, all with PA purity, were purchased from Panreac S.A. (Barcelona, Spain), in order to prepare the acid aqueous Fricke dosimeter solution. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Samples and samples irradiation

The samples (dry leaves of *Thymus vulgaris* L.) were provided by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal), and divided in three groups: control (non-irradiated), 1 (irradiated at 1 kGy) and 2 (10 kGy). Each group consisted of three samples, each one with 40 g of dry material, with a total amount of 120 g for each group.

A gamma radiation equipment (Precisa 22, Graviner Manufacturing Company Ltd., UK) with four ^{60}Co sources, with a total activity 140 TBq (3.77 kCi), was used for sample irradiations, in May 2015. During the irradiation procedure, the dose and dose rate were estimated using Amber Perspex (Batch X, from Harwell Company, Didcot, Oxfordshire, UK) and Fricke reference dosimeters, respectively (ASTM, 1992; Pereira et al., 2015b).

The estimated radiation doses for groups 1 and 2 were 1.2 ± 0.1 kGy and 10.4 ± 0.9 kGy, respectively. For simplicity, the values 1 kGy, and 10 kGy are considered for presentation and discussion of the results. The dose rate and dose uniformity ratio (D_{max}/D_{min}) were 1.7 and 1.2 kGy/h, respectively.

2.3. Infusions preparation

The infusions were prepared according to Pereira et al. (2015b) and were obtained from dried plants irradiated. Briefly, 1 g of the sample was added to 200 mL of boiling distilled water (after being taken out from the heating source) and left to stand at room temperature for 5 min, and then filtered under reduced pressure.

2.4. Total phenolic content, total flavonoid content and in vitro antioxidant activity

The Folin-Ciocalteu method was used to estimate total phenolic content following a method explained by Wolfe, Wu, and Liu (2003) and total flavonoid content were determined by a colorimetric assay using aluminum trichloride, as previously described by Jia, Tang, and Wu (1999). The results were expressed as mg of gallic acid equivalents (GAE) per mL of infusion for total phenolic content and as mg of (+)-catechin equivalents (CE) per mL of infusion for total flavonoid content.

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution (Pereira, Barros, & Ferreira, 2013).

Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader (Pereira et al., 2013). Inhibition of β-carotene bleaching was evaluated through the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, the reaction was measured at 470 nm in a spectrophotometer (AnalytikJena, Jena, Germany) and β-carotene bleaching inhibition (%) was calculated using the equation: (β-carotene absorbance after 2 h of assay/initial absorbance) × 100 (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004).

Lipid peroxidation inhibition in porcine brain homogenates was evaluated by the reduction of thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively (Fernandes et al., 2013).

The results were then converted to EC_{50} values (mg/mL) by using the graphs of the antioxidant activity percentage or absorbance at 690 nm (in the case of reducing power assay) against the extract concentration. Trolox was used as positive control.

2.5. Analysis of individual phenolic compounds

The aqueous preparations (infusions) obtained above were

filtered through a 0.45 μm Whatman syringe filter and transferred to an amber HPLC vial for chromatographic injection. The analysis was performed in an HPLC equipment (Agilent Technologies, Santa Clara, CA, USA) with a double online detection in a diode array detector (DAD), using with 280, 330 and 370 nm as preference wavelengths, and a mass spectrometer (MS) equipped with an ESI source and a triple quadrupole-ion trap mass analyser, which was connected to the HPLC system via the DAD cell outlet. The DAD was coupled to an HP Chem Station (rev. A.05.04) and the MS was controlled by the Analyst 5.1 software. The separation was achieved using a Spherisorb S3 ODS-2 C_{18} column (3 μm, 4.6 × 150 mm) thermostatted at 35 °C, using a gradient elution with the following solvents: 0.1% formic acid in water (A) and acetonitrile (B). The elution gradient was 15% B for 5 min, 15% B to 20% B over 5 min, 20–25% B over 10 min, 25–35% B over 10 min, 35–50% B for 10 min, and re-equilibration of the column (10 min), using a flow rate of 0.5 mL/min, as previously described by Barros et al. (2013). The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS), employed to record full scan spectra, and enhanced product ion (EPI) analysis. Air (zero graded) was used as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 °C, 40 psi). Nitrogen functioned as the curtain (20 psi) and collision gas (medium). The ion spray voltage was set at −4500 V and spectra were recorded in negative ion mode between *m/z* 100 and 1700. The settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP −50 V, EP −6 V, CE −25 V, and collision energy spread (CES) 0 V. Compounds were tentatively identified comparing the obtained information (retention times, UV–vis and mass spectra) with available data reported in the literature and by comparison with standard compounds, when available. For the quantification a calibration curve for each available phenolic standard (apigenin-6-*C*-glucoside, caffeic acid, luteolin-7-*O*-glucoside, naringenin, quercetin-3-*O*-rutinoside, rosmarinic acid) was constructed based on the UV signal (maximum wavelength of absorption of each standard compound). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of another compound from the same phenolic group. The results were expressed in μg per mL of infusion.

2.6. Statistical analysis

Three independent samples were analysed for each dose of irradiation with three aqueous extractions performed for each sample, and all the assays were carried out in triplicate (n = 27). The results for control (non-irradiated) and irradiated samples were expressed as mean values ± standard deviation (SD). The results were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $p = 0.05$. In the case of phenolic composition, a Student's *t*-test was used to determine the significant difference among two different samples, with $p = 0.05$. When the p value was lower than 0.05, significant differences between samples were considered. Furthermore, a Pearson's correlation analysis between the antioxidant activity and all the analysed compounds was carried out, with a 95% confidence level. Analyses were carried out using IBM SPSS Statistics for Windows, version 23.0. (IBM Corp., Armonk, New York, USA).

3. Results and discussion

The results for the total phenolic content and total flavonoid content of infusions prepared from non-irradiated and irradiated samples of *T. vulgaris* and the evaluation of the antioxidant properties tested by four *in vitro* assays (DPPH scavenging activity, reducing power, β-carotene bleaching and lipid peroxidation inhibition) are presented in Table 1. Significantly higher values of total phenolic and flavonoid contents were found in the samples irradiated at 10 kGy (0.168 mg GAE/mL of infusion and 0.06 mg CE/mL of infusion, respectively). The increase in total phenolic and flavonoid contents with the irradiation dose could be related with the release of these compounds from the matrix structures, increasing extractability of certain compounds and the degradation of larger compounds into smaller ones (Polovka & Suhaj, 2010; Taheri, Abdullah, Karimi, Oskoueian, & Ebrahimi, 2014). Statistically significant differences were observed in the EC_{50} values of all the antioxidant assays among samples subjected to different radiation doses, with 10 kGy leading to the highest antioxidant potential (lowest EC_{50} values). The increase in the antioxidant capacity of the *T. vulgaris* samples submitted to 10 kGy is in agreement with previous findings in infusions of irradiated *Ginkgo biloba* L. (Pereira et al., 2015c), Borututu (a folk medicine plant obtained from the African tree *Cochlospermum angolense* Welw.) (Pereira et al., 2014) and ethanolic extracts of *Hizikia fusiformis* Harvey (edible brown seaweed consumed in Korea and Japan) (Kim et al., 2009).

Table 2 presents data related to the phenolic compounds identification (retention time, λ_{max} in the visible region, pseudomolecular ion, main fragment ions in MS^2, and tentative identities) obtained by HPLC-DAD-ESI/MS analysis. The phenolic profile of *T. vulgaris* infusions revealed the presence of thirteen phenolic compounds, from which seven were phenolic acids derivatives and six were flavonoid glycosides (Table 2). The phenolic profile of thyme irradiated with 10 kGy was shown in Fig. 1. In this study, the phenolic profile was only evaluated for samples irradiated with the dose of 10 kGy, and compared with the control sample; this is because 10 kGy showed higher antioxidant potential comparatively with 1 kGy, i.e., lower EC_{50} values. A similar phenolic profile was previously observed by the authors in methanolic extracts obtained from irradiated samples of *T. vulgaris* (Pereira et al., 2016). Similarly, Martins et al. (2015) reported apigenin 6,8-di-*C*-glucoside (peak 2), caffeic acid (3), rosmarinic acid hexoside (6), luteolin-7-*O*-glucuronide (7), luteolin-7-*O*-glucoside (8), rosmarinic acid (10), lithospermic acid A (12) and eriodictyol (13) in non-irradiated samples of *T. vulgaris*. The presence of caffeic acid, luteolin-7-*O*-glucuronide, rosmarinic acid and eriodictyol was also found in thyme hydrophilic extracts by Fecka and Turek (2008), whereas Boros et al. (2010) detected apigenin 6,8-di-*C*-glucoside, caffeic acid, rosmarinic acid and eriodictyol in hydroalcoholic extracts of different *Thymus* species (*Thymus pannonicus*, *Thymus glabrescens*, *Thymus pulegioides*, *Thymus praecox*, *Thymus serpyllum*).

The majority phenolic compounds found in *T. vulgaris* infusions were rosmarinic acid hexoside (peak 6), luteolin-7-*O*-glucuronide (7), caffeic acid trimer (9) and rosmarinic acid (10), as previously found in methanolic extracts of irradiated thyme samples (Pereira et al., 2016). Also, the presence of luteolin-7-*O*-glucuronide and rosmarinic acid as major compounds in thyme infusions was reported by Martins et al. (2015).

In general, gamma radiation at a dose of 10 kGy causes statistically relevant changes in the concentration of the phenolic compounds. Small but significant increases were observed in the levels of methyleriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside and eriodictyol, and consequently in total flavonoid content. This is in agreement with Pereira et al. (2015c) and Alothman et al. (2009) who also observed that irradiation at 10 kGy dose caused an increase in some individual phenolic compounds in the infusions of *Ginkgo biloba*. These results could be explained by an increase in compounds extractability due to depolymerization and release from cell wall polysaccharides produced by the irradiation process,

Table 1
Total phenolic content, total flavonoid content and *in vitro* antioxidant properties of infusions prepared from *Thymus vulgaris* subjected to gamma radiation.

Dose (kGy)	0	1	10
Total phenolic content (mg GAE/mL of infusion)	0.138 ± 0.001c	0.150 ± 0.001b	0.168 ± 0.001a
Total flavonoid content (mg CE/mL of infusion)	0.048 ± 0.001c	0.053 ± 0.001b	0.060 ± 0.001a
DPPH scavenging activity (EC_{50} value, mg/mL)	0.87 ± 0.05a	0.76 ± 0.02b	0.66 ± 0.02c
Reducing power (EC_{50} value, mg/mL)	0.48 ± 0.01a	0.43 ± 0.01b	0.41 ± 0.01c
β-carotene bleaching inhibition (EC_{50} value, mg/mL)	1.63 ± 0.01a	1.66 ± 0.04a	1.25 ± 0.01b
TBARS inhibition (EC_{50} value, mg/mL)	0.22 ± 0.01a	0.22 ± 0.01a	0.13 ± 0.01b

GAE-Gallic acid equivalents; CE-Catechin equivalents. In each row different letters mean significant differences ($p < 0.05$).

Table 2
Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in infusion prepared from *Thymus vulgaris* subjected to gamma irradiation (μg/mL of infusion).

Peak	Rt (min)	λ_{max} (nm)	Pseudomolecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Infusion		*t*-Students test *p*-value
						0 kGy	10 kGy	
1	7.3	320	341	179(100), 135(88)	Caffeic acid hexoside	3.1 ± 0.1	2.5 ± 0.2	0.003
2	10.8	338	593	473(20), 383(33), 353(27), 297(5)	Apigenin 6,8-di-*C*-glucoside	6.6 ± 0.4	6.2 ± 0.1	0.122
3	11.3	326	179	135(100)	Caffeic acid	4.54 ± 0.1	4.19 ± 0.4	0.091
4	17.4	284,336sh	595	301(47), 286(100)	Methyleriodictyol-*O*-pentosylhexoside	5.03 ± 0.01	6.34 ± 0.04	<0.001
5	18.1	350	461	301(100)	Quercetin-*O*-glucuronide	0.21 ± 0.01	0.13 ± 0.02	0.002
6	18.8	322	521	359(100), 197(13), 179(36), 161(62), 135(21)	Rosmarinic acid hexoside	33 ± 1	32 ± 1	0.035
7	20.0	348	461	285(100)	Luteolin-7-*O*-glucuronide	24.0 ± 0.1	23.8 ± 0.1	0.062
8	20.8	350	447	285(100)	Luteolin-7-*O*-glucoside	4.7 ± 0.1	5.57 ± 0.01	<0.001
9	23.4	286,320sh	537	493(20), 359(70) ,295(5), 197(13), 179(28), 161(100), 135(63)	Caffeic acid trimer	19.36 ± 0.02	17.8 ± 0.1	<0.001
10	24.0	330	359	197(17), 179(35), 161(100), 135(29)	Rosmarinic acid	22.3 ± 0.4	21 ± 1	0.012
11	25.6	282	567	535(23), 493(49), 387(32), 285(25), 197(13)	Caffeic acid derivative	3.0 ± 0.1	2.6 ± 0.3	0.034
12	27.5	290,326sh	537	493(50), 359(17), 295(33), 179(75), 135(100)	Lithospermic acid A	3.53 ± 0.04	2.1 ± 0.4	0.004
13	30.6	288,334sh	287	151(35), 135(100)	Eriodictyol	1.51 ± 0.02	1.6 ± 0.1	0.019
					Total phenolic acids	89 ± 1	82 ± 2	0.001
					Total flavonoids	42.0 ± 0.4	43.7 ± 0.4	0.002
					Total phenolic compounds	131 ± 1	125 ± 1	0.001

as stated above. On the contrary, small but statistically significant decreases were produced in the levels of caffeic acid hexoside, quercetin-*O*-glucuronide, rosmarinic acid hexoside, caffeic acid trimer, rosmarinic acid, caffeic acid derivative and lithospermic acid A, after irradiation at 10 kGy, being also, in total phenolic acids and total phenolic compounds. Otherwise, non-significant changes were observed in other phenolic compounds, such as apigenin 6,8-di-*C*-glucoside (peak 2), caffeic acid (peak 3) and luteolin-7-*O*-glucuronide (peak 7). It could be supposed that phenolic acids have lower stability against gamma irradiation and, therefore, they are degraded to some extent at higher irradiation doses. Thus, radiation may contribute to increase compounds extractability, on the other hand, it may also lead to degradation of some less stable compounds.

In order to try to relate antioxidant capacity and phenolic composition of the samples, correlation factors were obtained between the sums of phenolic acid derivatives, flavonoids and total phenolic compounds. A Pearson's correlation between all antioxidant activities tested was performed, because the normality was verified through a Shapiro-Wilk test. The correlations of all antioxidant assays (DPPH scavenging activity, reducing power, β-carotene bleaching and lipid peroxidation inhibition) and total phenolic compounds are presented in Table 3. In general, the majority of the compounds showed correlations statistically significant with the antioxidant activity assays. Methyleriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside and eriodictyol (peaks 4, 8 and 13) presented highly significant correlations with all antioxidant activities tested but these correlations were negative, meaning that the antioxidant activity increased (lower EC_{50} values), for irradiated samples with 10 kGy, with increasing concentrations of these compounds at the same dose; therefore, inducing a similar behavior in the total flavonoid content. However, the compounds corresponding to peaks 1, 5, 7, 6, 10 and 12 also revealed statistically significant correlations, presenting *p-values* < 0.05. Otherwise, apigenin 6,8-di-*C*-glucoside (peak 2) and caffeic acid (peak 3) were the only compounds that did not statistically significantly relate to any of the antioxidant assays, presenting *p-value* > 0.05. The major individual phenolic compound (peak 6 – rosmarinic acid hexoside) also had a high correlation with all the antioxidant activity assays, showing a variation between 0.815 and 0.865. Overall, total phenolic acids, total flavonoids and total phenolic compounds showed statistically significant correlations with all assays, displaying p-values ≤ 0.05.

4. Conclusion

Infusions are a form of consumption of thyme leaves and evaluation of their bioactive potential depending on the type of plant

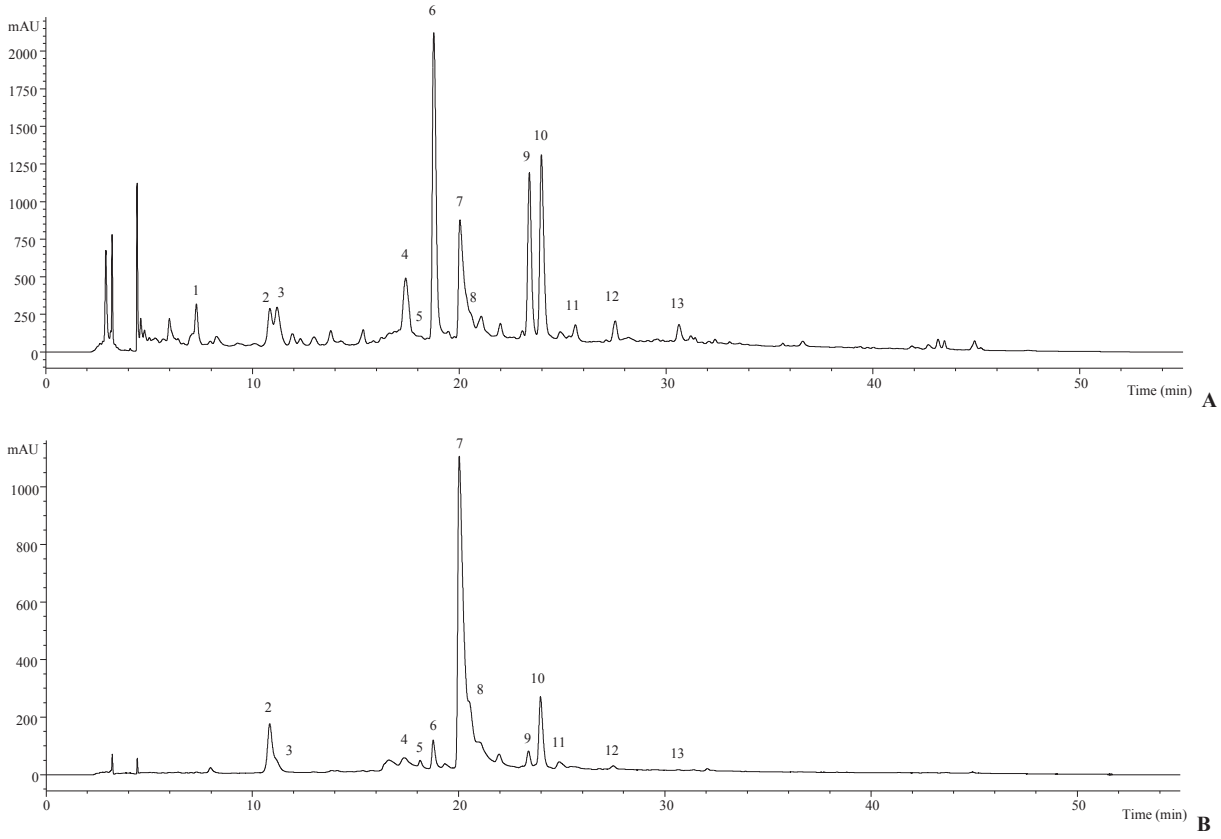

Fig. 1. Phenolic compounds profile in infusions prepared from thyme samples irradiated with 10 kGy, recorded at 280 nm (A) and 370 nm (B).

Table 3
Correlation coefficients of phenolic compounds with *in vitro* antioxidant activity of infusions prepared from *Thymus vulgaris* subjected to gamma radiation.

Peaks	Compounds	DPPH scavenging activity		Reducing power		β-Carotene bleaching inhibition		TBARS inhibition	
		Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*	Correlation factor	*p-value*
1	Caffeic acid hexoside	0.878	0.021	0.969	0.001	0.952	0.003	0.956	0.003
2	Apigenin 6,8-di-*C*-glucoside	0.530	0.280	0.742	0.091	0.689	0.137	0.698	0.123
3	Caffeic acid	0.719	0.107	0.724	0.104	0.750	0.086	0.742	0.091
4	Methyleriodictyol-*O*-pentosylhexoside	−0.954	0.003	−0.997	<0.001	−1.000	<0.001	−1.000	<0.001
5	Quercetin-*O*-glucuronide	0.945	0.004	0.962	0.002	0.965	0.002	0.967	0.002
6	Rosmarinic acid hexoside	0.865	0.026	0.815	0.048	0.851	0.032	0.843	0.035
7	Luteolin-7-*O*-glucuronide	0.679	0.138	0.817	0.047	0.778	0.068	0.788	0.063
8	Luteolin-7-*O*-glucoside	−0.926	0.008	−0.998	<0.001	−0.992	<0.001	−0.993	<0.001
9	Caffeic acid trimer	0.956	0.003	0.997	<0.001	0.999	<0.001	1.000	<0.001
10	Rosmarinic acid	0.906	0.013	0.890	0.017	0.916	0.010	0.911	0.012
11	Caffeic acid derivative	0.791	0.061	0.856	0.029	0.837	0.038	0.844	0.035
12	Lithospermic acid A	0.906	0.013	0.941	0.005	0.953	0.003	0.950	0.004
13	Eriodictyol	−0.808	0.052	−0.885	0.019	−0.887	0.018	−0.884	0.011
	Total phenolic acids	0.945	0.004	0.961	0.002	0.975	0.001	0.972	0.001
	Total flavonoids	−0.955	0.003	−0.943	0.005	−0.965	0.002	−0.961	0.002
	Total phenolic compounds	0.940	0.005	0.964	0.002	0.977	0.001	0.974	0.001

processing is extremely important. According to the results, it was evident that gamma radiation had an influence on the antioxidant activity of the samples, with treatment at 10 kGy leading to higher antioxidant potential (lower EC_{50} values in all the assays). This irradiation dose (10 kGy) also evidenced higher total phenolic and flavonoid contents. The composition in phenolic compounds was determined and thirteen compounds were detected in the infusions prepared either from irradiated or non-irradiated samples and, in general, the gamma irradiation treatment at a dose of 10 kGy caused relevant changes in the concentration of several compounds. Methyleriodictyol-*O*-pentosylhexoside, luteolin-7-*O*-glucoside, eriodictyol and total flavonoid content were the only ones that increase with the irradiation dose applied (10 kGy), and apigenin 6,8-di-*C*-glucoside, caffeic acid and luteolin-7-O-glucuronide were the only compounds with no statistically significant changes observed. The results show that, for the legally permitted maximum radiation dose in EU (10 kGy), gamma radiation may even improve the antioxidant potential and total flavonoid content of *T. vulgaris* infusion without changing its chemical profile.

Conflict of interest

The authors declare they have no conflict of interest.

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Annexes 7

Extending the use of irradiation to preserve chemical and bioactive properties of aromatic plants: a case study with four species submitted to electron beam

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Extending the use of irradiation to preserve chemical and bioactive properties of medicinal and aromatic plants: A case study with four species submitted to electron beam

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ABSTRACT

The effects of gamma irradiation on *Aloysia citrodora*, *Melissa officinalis*, *Melittis melissophyllum* and *Mentha piperita* were previously evaluated. Herein, the same species were treated with electron-beam irradiation (EB) and the same parameters were evaluated. Instead of presenting absolute values for each studied parameter, data were evaluated as percentage of induced variation. Besides the newly obtained results, data from a previous work was recalled and normalized in the same manner. Several examples of percentage variations specific to a plant species or irradiation condition were found. Nevertheless, it was not possible to identify unequivocal trends. Even so, when evaluated in an integrative way, the parameters with highest discriminating ability among irradiation conditions or plant species were fatty acids and bioactive indicators. Comparing the effects of gamma and EB irradiations, it might be concluded that the most suitable solution to irradiate aromatic plants would be EB, independently of the used dose.

1. Introduction

Food irradiation is a non-thermal processing technique, which has been increasingly applied with several purposes. Nowadays, it is highlighted as a preservation and decontamination technique, ensuring the elimination of pathogenic microorganisms, parasites and pests, without changing the nutritional and organoleptic characteristics of the targeted food product (Molins, 2001; Villavicencio et al., 2007; Wen et al., 2010).

Despite the irradiation concept is often misunderstood by most consumers, it is a safe process that exposes food (pre-packaged or unpackaged) to a predetermined dose of radiation according to the food type to be treated, plant-derived products (such as vegetables, fruits and cereals) or even derived from animals, such as meat or fish (Sádecká, 2007; Nagy et al., 2011; Kanatt et al., 2015). It is characterized as a versatile, efficient, safe, secure and highly effective technique, *i.e.*, it is a process that fully satisfies the objective of providing stability to nutritious foods, health conditions and longer storage period (Hunter, 2000; Roberts, 2014). There are several processes of irradiation for food preservation using ionizing radiations, being gamma and electron beam the more well established for industrial purposes (Van Calenberg et al., 1998; Roberts, 2014). Electron beam irradiation is mainly used for food products with low density; the sources can be easily connected/disconnected, whereas the gamma sources are continuously decaying.

Aromatic and medicinal herbs are among the products submitted to decontamination assays based on irradiation treatment. The fact that these matrices are quite popular in the pharmaceutical and food industries requires specific criteria in terms of microbiological safety (Katuŝin-Razĕm et al., 2001; Haleem et al., 2014). *Aloysia citrodora* P., *Melissa officinalis* L., *Melittis melissophyllum* L. and *Mentha piperita* L. are among the studied plants, namely submitted to gamma radiation (Pereira et al., 2015). All of them are characterized by being culinary and medicinal herbs, consumed usually as infusions and used since ancient times as medicinal plants for different diseases, especially in healing and treatment of gastrointestinal and nervous system disorders, displaying antioxidant, antimicrobial and anti-inflammatory properties, due to the presence of bioactive compounds (Ragone et al., 2007; Skrzypczak-Pietraszek and Pietraszek, 2012; Barros et al., 2013; Pereira et al., 2014; Skalicka-Woźniak and Walasek, 2014).

In this study the objective was to compare the effects of gamma irradiation and electron beam irradiation in the chemical parame-

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ters and bioactive indicators of aromatic plants in order to find the most suitable technology in each case.

2. Materials and methods

2.1. Samples and samples irradiation

Samples of *A. citrodora* P. (Verbenaceae; lemon verbena), *M. officinalis* L. (Lamiaceae; lemon balm), *M. melissophyllum* L. (Lamiaceae; bastard balm) and *M. piperita* L. (Lamiaceae; peppermint) were provided as dry leaves by a local producer (Pragmático Aroma Lda, Alfândega da Fé, Bragança, Portugal). After confirmation of the taxonomical identification, the samples were divided into three groups: control (non-irradiated, 0 kGy), groups 1 and 2, where 1 kGy and 10 kGy were, respectively, the predicted doses.

The irradiation was performed at the INCT—Institute of Nuclear Chemistry and Technology, in Warsaw, Poland. To estimate the dose during the irradiation process three types of dosimeters were used: a standard dosimeter, a graphite calorimeter, and two routine Gammachrome YR and Amber Perspex dosimeters, from Harwell Company (UK). The irradiation took place in an e-beam irradiator of 10 MeV of energy with pulse duration of 5.5 ms, pulse frequency of 440 Hz and average beam current of 1.1 mA; the scan width was 68 cm, the conveyer speed was settled to the range 20–100 cm/min and the scan frequency was 5 Hz. The estimated absorbed dose for irradiated samples was 0.83 kGy for group 1 and 10.09 kGy for group 2, with a maximum uncertainty of 20%. To read the Amber Perspex and Gammachrome YR dosimeters, spectrophotometric methods were used at 603 nm and at 530 nm, respectively, to estimate the dose from the value of absorbance according to a previous calibration curve. For the graphite calorimeter dosimeter the electrical resistance was read and converted in dose according to a calibrated curve, obtained following the standards during the Quality Control procedures of the irradiation equipment and facility.

2.2. Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47,885-U) was purchased from Sigma (St. Louis, MO, USA), as well as other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA).

2.3. Nutritional value

Protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 1995). The crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined using a Soxhlet apparatus; the ash content was determined by incineration at 600 ± 15 °C, until a whitish ash was formed. Total carbohydrates were calculated by difference. The results were expressed in g/100 g of dry weight (dw). Total energy was calculated according to the following equation: Energy (kcal) = $4 \times (g_{protein} + g_{carbohydrates}) + 9\ (g_{fat})$, and the results were expressed in kcal/100 g dw.

2.4. Color measurement

A colorimeter (model CR-400, from Konica Minolta Sensing, Inc., Japan), with an adapter for granular materials (model CR-A50) was used to measure the color of the samples. Using the illuminant C and diaphragm aperture of 8 mm, the CIE $L^*a^*b^*$ color space values were registered using a data software "Spectra Magic Nx" (version CM-S100W 2.03.0006), from Konica Minolta company (Japan). Before starting the measurements the instrument was calibrated against a standard white tile (Pereira et al., 2015). The colour of three samples from each batch was measured in three different points, for each dose and at each time point, being considered the average value.

2.5. Chemical composition of hydrophilic compounds

2.5.1. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), using a previously described procedure (Pereira et al., 2015). Data were analysed using Clarity 2.4 Software (DataApex). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard (melezitose) method and the results were expressed in g/100 g dw.

2.5.2. Organic acids

Organic acids were determined following a procedure previously described by the authors (Pereira et al., 2015). Detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound, and the results were expressed in g/100 g dw.

2.6. Chemical composition in lipophilic compounds

2.6.1. Tocopherols

Tocopherols were determined following a procedure previously described by the authors (Pereira et al., 2015). The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg/100 g dw.

2.6.2. Fatty acids

Fatty acids were determined by gas–liquid chromatography with flame ionization detection (GC–FID)/capillary column as described previously by the authors (Pereira et al., 2015). Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded, processed using the CSW 1.7 Software (DataApex 1.7, Prague, Czech Republic) and expressed in relative percentages.

2.7. Evaluation of bioactivity

2.7.1. Samples preparation

The methanolic extracts were obtained from the dried plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

The infusions were also obtained from the dried plant material. The sample (2 g) was added to 200 mL of boiling distilled water (after being taken out from the heating source) and left to stand at room temperature for 5 min, and then filtered under reduced pressure.

Table 1
Proximate composition (g/100 g dw), energy (kcal/100 g dw) and color parameters (L*: lightness, a*: redness, b*: yellowness) of the aromatic species (controls; non-irradiated samples). Values for irradiated samples are presented as percentage of variation in comparison to the control.[a]

		Fat	Protein	Ash	Carbohydrates	Energy	L^*	a^*	b^*
Dose	Irradiation type								
Aloysia citrodora (Lemon verbena)									
0 kGy	Control	1.6 ± 0.1	3.0 ± 0.1	8.2 ± 0.1	87.1 ± 0.1	375 ± 1	49 ± 1	-8.4 ± 0.2	27.2 ± 0.3
1 kGy	Electron beam	20 ± 4^{b}	1 ± 2^{b}	-1 ± 2^{b}	-1 ± 1^{b}	1 ± 1	1 ± 2	-8 ± 8	7 ± 7
	Gamma rays	32 ± 5^{a}	-42 ± 5^{c}	3 ± 2^{a}	1 ± 1^{a}	1 ± 1	3 ± 2	5 ± 5	3 ± 1
10 kGy	Electron beam	19 ± 8^{b}	45 ± 10^{a}	-1 ± 1^{b}	-2 ± 1^{c}	1 ± 1	2 ± 3	-10 ± 10	3 ± 2
	Gamma rays	6 ± 3^{c}	-2 ± 3^{b}	4 ± 2^{a}	-1 ± 1^{b}	-1 ± 1	-2 ± 2	-1 ± 3	-3 ± 1
p-values	Homoscedasticity*	0.012	0.172	0.073	0.016	0.008	0.310	0.003	0.030
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	0.081	0.104	0.087	0.117
Melissa officinalis (Lemon balm)									
0 kGy	Control	1.2 ± 0.1	2.5 ± 0.3	8.4 ± 0.4	88 ± 1	372 ± 2	48 ± 1	-5.1 ± 0.5	20.9 ± 0.4
1 kGy	Electron beam	-7 ± 4^{c}	4 ± 2^{b}	-3 ± 2	1 ± 1^{a}	1 ± 1	1 ± 1^{b}	-10 ± 4^{b}	-2 ± 1^{c}
	Gamma rays	65 ± 5^{a}	167 ± 11^{a}	-3 ± 2	-5 ± 1^{b}	1 ± 1	-1 ± 1^{bc}	-1 ± 2^{a}	-1 ± 1^{b}
10 kGy	Electron beam	11 ± 3^{b}	5 ± 2^{b}	-1 ± 2	-1 ± 1^{a}	1 ± 1	4 ± 1^{a}	-13 ± 2^{b}	6 ± 1^{a}
	Gamma rays	60 ± 2^{a}	156 ± 20^{a}	1 ± 1	$-5 \pm 1^{*}$	1 ± 1	-2 ± 1^{c}	-2 ± 4^{a}	-3 ± 1^{d}
p-values	Homoscedasticity*	0.731	0.002	0.045	0.009	0.003	0.850	0.180	0.261
	1-way ANOVA**	<0.001	<0.001	0.082	<0.001	0.071	<0.001	<0.001	<0.001
Melittis melissophyllum (Bastard balm)									
0 kGy	Control	1.8 ± 0.1	4.6 ± 0.2	7.6 ± 0.1	86.0 ± 0.4	378 ± 1	42 ± 2	-8.4 ± 0.5	18 ± 3
1 kGy	Electron beam	-7 ± 7	-7 ± 5^{b}	-2 ± 4^{bc}	1 ± 1^{a}	1 ± 1	-1 ± 3	36 ± 11^{a}	1 ± 2
	Gamma rays	-8 ± 5	-45 ± 4^{c}	7 ± 2^{ab}	2 ± 1^{a}	1 ± 1	3 ± 3	-3 ± 4^{b}	-1 ± 2
10 kGy	Electron beam	-13 ± 8	2 ± 4^{b}	-4 ± 5^{c}	1 ± 1^{a}	-1 ± 1	-2 ± 4	28 ± 13^{a}	-4 ± 4
	Gamma rays	-13 ± 5	22 ± 5^{a}	13 ± 3^{a}	-2 ± 1^{b}	-1 ± 1	-3 ± 4	-4 ± 4^{b}	-5 ± 5
p-values	Homoscedasticity*	0.064	<0.001	0.059	0.053	0.012	0.111	0.188	0.962
	1-way ANOVA**	0.400	<0.001	<0.001	<0.001	0.082	0.743	<0.001	0.698
Mentha piperita (Peppermint)									
0 kGy	Control	2.4 ± 0.1	5.1 ± 0.3	9.2 ± 0.2	83.3 ± 0.5	375 ± 1	40 ± 1	-5.9 ± 0.1	23.9 ± 0.3
1 kGy	Electron beam	-5 ± 2^{b}	19 ± 8^{b}	-4 ± 3^{a}	1 ± 1^{b}	1 ± 1	-1 ± 2^{a}	17 ± 8	3 ± 2^{a}
	Gamma rays	13 ± 5^{a}	-45 ± 10^{d}	-10 ± 2^{b}	3 ± 1^{a}	1 ± 1	-3 ± 3^{a}	-5 ± 2	-3 ± 2^{b}
10 kGy	Electron beam	-4 ± 5^{b}	3 ± 4^{c}	-3 ± 2^{a}	1 ± 1^{b}	1 ± 1	1 ± 2^{a}	-26 ± 15	2 ± 2^{ab}
	Gamma rays	-21 ± 6^{c}	91 ± 3^{a}	-6 ± 3^{ab}	-6 ± 1^{c}	1 ± 1	-7 ± 3^{b}	-25 ± 14	-16 ± 5^{c}
p-values	Homoscedasticity*	0.056	0.045	0.306	0.544	0.053	0.376	0.064	0.580
	1-way ANOVA**	<0.001	<0.001	0.002	<0.001	0.082	<0.001	0.077	<0.001

[a] The results are presented as the mean ± SD.
* Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
** $p < 0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

2.7.2. Antioxidant activity

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH}\text{-}A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance) × 100% (Pereira et al., 2013). The results were expressed as EC_{50} values (μg/mL).

2.7.3. Phenolics and flavonoids content

Total phenolics were estimated by Folin–Ciocalteu colorimetric assay, while total flavonoids were determined by a colorimetric assay using aluminum trichloride, according to procedures previously described (Pereira et al., 2013). The results were expressed in mg GAE (gallic acid equivalents)/g of extract and mg CE (catechin equivalents)/g of extract for phenolics and flavonoids, respectively.

2.8. Statistical analysis

For each irradiation dose and plant species, three independent samples were analysed. Each of the samples was taken after pooling the plants treated in the same conditions together. Data for control (non-irradiated) samples were expressed as mean ± standard deviation. Data for irradiated samples were presented as the normalized difference ((irradiated sample value-control value)/control value × 00) among the values obtained for each irradiated sample and the respective control.

The obtained values were evaluated using 1-way ANOVA. The homogeneity of variance, was tested by means of the Levene's tests. All dependent variables were compared using Tukey's honestly significant difference (HSD) or Tamhane's T2 multiple comparison tests, when homoscedasticity was verified or not, respectively.

Owing the high number of evaluated parameters, a LDA was used to evaluate the association of variations in the measured parameters with, sequentially, irradiation condition and plant species. A stepwise technique, using the Wilks' λ method with the usual probabilities of F (3.84 to enter and 2.71 to remove), was applied for variable selection. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable, it is verified whether all variables previously selected remain significant (Palacios-Morillo et al., 2013). With this approach, it is also possible to identify the significant variables that contribute most to the possible discrimination of a determined irradiation treatment or plant species. To verify which canonical discriminant functions were significant, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., Armonk, NY, USA).

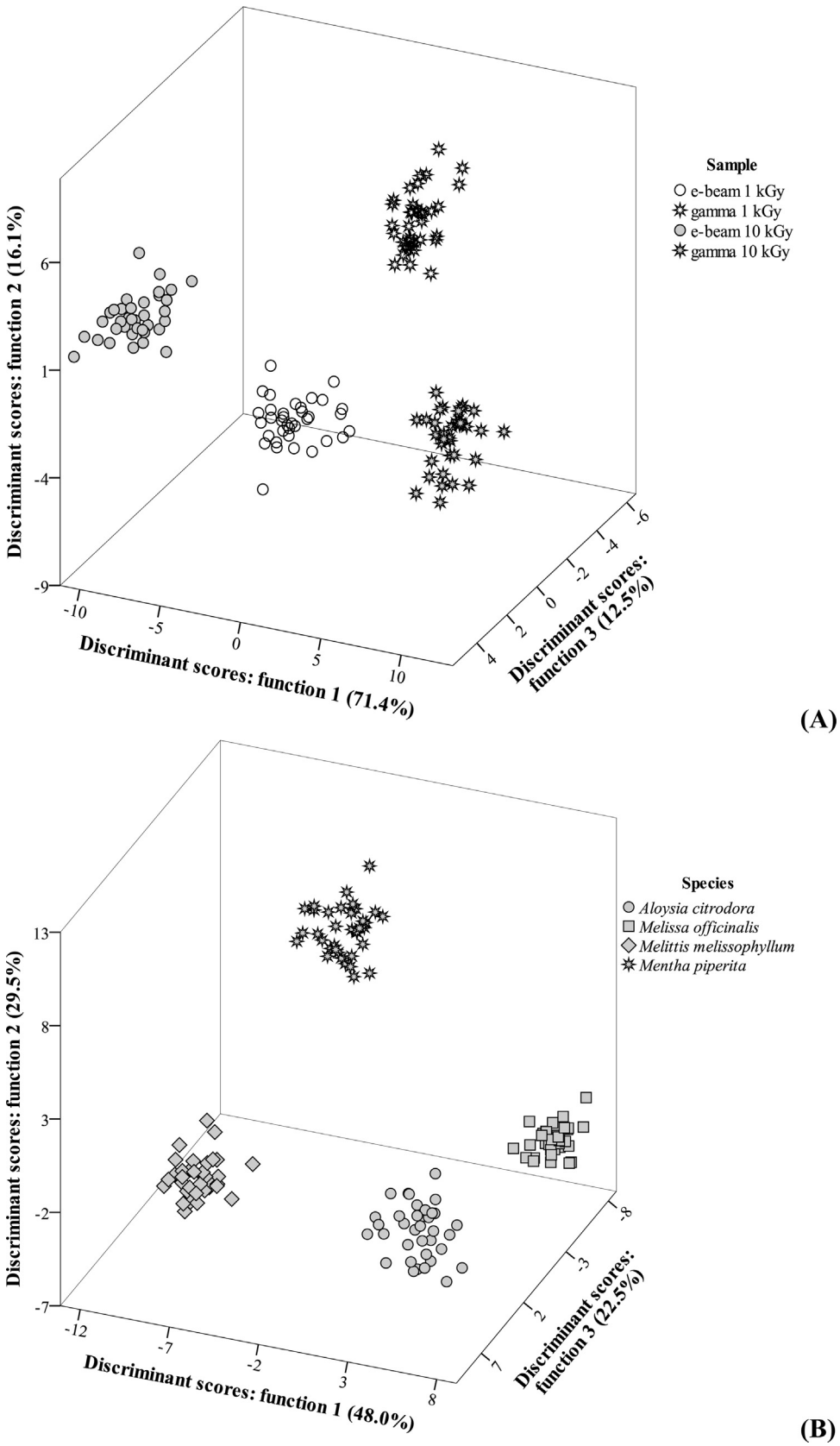

Fig. 1. Mean scores of different irradiation conditions (A) and different plant species (B) projected for the three discriminant functions defined variations measured in all evaluated parameters.

Table 2
Hydrophilic compounds (free sugars and organic acids) composition (g/100 g dw) of the aromatic species (controls; non-irradiated samples). Values for irradiated samples are presented as percentage of variation in comparison to the control.[a]

		Fructose	Glucose	Sucrose	Trehalose	Total sugars	Oxalic acid	Quinic acid	Malic acid	Shikimic acid	Citric acid	Organic acids
Dose	Irradiation type											
Aloysia citrodora (Lemon verbena)												
0 kGy	Control	1.0 ± 0.1	1.3 ± 0.1	7.1 ± 0.3	1.2 ± 0.1	10.7 ± 0.4	1.1 ± 0.1	nd	0.14 ± 0.03	1.4 ± 0.1	1.4 ± 0.1	4.1 ± 0.1
1 kGy	Electron beam	1 ± 5	−7 ± 6	16 ± 6^{b}	12 ± 8^{ab}	3 ± 5^{b}	−3 ± 3^{ab}	–	−5 ± 7^{ab}	-6 ± 5^{b}	−4 ± 3^{b}	−4 ± 2^{c}
	Gamma rays	−2 ± 4	−9 ± 7	−10 ± 7^{c}	−1 ± 2^{b}	−8 ± 2^{c}	−2 ± 2^{ab}	–	29 ± 16^{a}	29 ± 5^{a}	40 ± 9^{a}	24 ± 9^{a}
10 kGy	Electron beam	18 ± 13	−2 ± 6	28 ± 9^{a}	18 ± 7^{a}	13 ± 6^{a}	−9 ± 6^{b}	–	−10 ± 6^{b}	−11 ± 4^{c}	−3 ± 4^{c}	−8 ± 4^{c}
	Gamma rays	−1 ± 3	−5 ± 5	−8 ± 4^{c}	−2 ± 5^{b}	−6 ± 3^{c}	5 ± 7^{a}	–	3 ± 7^{ab}	12 ± 3^{b}	20 ± 7^{b}	12 ± 5^{b}
p-values	Homoscedasticity*	0.023	0.029	0.003	0.012	0.002	0.354	–	0.056	0.390	0.059	0.459
	1-way ANOVA**	0.131	0.726	<0.001	0.007	<0.001	0.035	–	0.044	<0.001	<0.001	<0.001
Mellissa officinalis (Lemon balm)												
0 kGy	Control	1.2 ± 0.1	1.0 ± 0.1	4.8 ± 0.2	0.49 ± 0.05	7.5 ± 0.2	0.5 ± 0.1	0.26 ± 0.04	0.4 ± 0.1	4.1 ± 0.2	nd	5.3 ± 0.3
1 kGy	Electron beam	9 ± 5^{a}	21 ± 9^{a}	−11 ± 7^{b}	5 ± 3^{c}	11 ± 4^{ab}	−48 ± 3^{c}	−24 ± 5^{b}	−27 ± 3^{b}	−30 ± 3^{c}	–	−36 ± 2^{c}
	Gamma rays	9 ± 3^{a}	1 ± 1^{b}	12 ± 5^{a}	37 ± 17^{b}	11 ± 4^{ab}	−2 ± 3^{a}	−12 ± 5^{c}	−8 ± 8^{b}	1 ± 2^{b}	–	−1 ± 2^{b}
10 kGy	Electron beam	1 ± 2^{b}	8 ± 6^{b}	−59 ± 16^{c}	16 ± 4^{c}	4 ± 4^{b}	−10 ± 3^{b}	25 ± 5^{a}	8 ± 4^{a}	16 ± 4^{a}	–	6 ± 3^{a}
	Gamma rays	5 ± 3^{ab}	1 ± 2^{b}	17 ± 1^{a}	72 ± 8^{a}	17 ± 1^{a}	−3 ± 5^{a}	−4 ± 4^{b}	−1 ± 4^{b}	−1 ± 2^{b}	–	−1 ± 2^{b}
p-values	Homoscedasticity*	0.030	0.026	<0.001	0.004	0.095	0.188	0.934	0.009	0.306	–	0.160
	1-way ANOVA**	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–	<0.001
Melittis melissophyllum (Bastard balm)												
0 kGy	Control	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.28 ± 0.03	5.5 ± 0.3	1.4 ± 0.1	0.17 ± 0.01	6.0 ± 0.3	0.97 ± 0.05	0.022 ± 0.001	8.6 ± 0.4
1 kGy	Electron beam	−11 ± 5^{b}	5 ± 5^{ab}	4 ± 3^{a}	−11 ± 8^{b}	−1 ± 2^{b}	−11 ± 4^{b}	−31 ± 8^{c}	8 ± 5^{a}	−24 ± 9^{b}	41 ± 17^{a}	−3 ± 2^{a}
	Gamma rays	−8 ± 6^{ab}	−3 ± 5^{b}	4 ± 4^{a}	84 ± 20^{a}	6 ± 4^{b}	−16 ± 5^{b}	−10 ± 5^{b}	−26 ± 4^{b}	−12 ± 6^{ab}	−12 ± 6^{c}	−22 ± 2^{b}
10 kGy	Electron beam	−24 ± 4^{c}	−26 ± 3^{c}	−17 ± 4^{b}	−21 ± 10^{b}	−21 ± 3^{c}	−12 ± 2^{b}	−45 ± 9^{d}	12 ± 4^{a}	−13 ± 7^{ab}	18 ± 7^{b}	1 ± 2^{a}
	Gamma rays	1 ± 2^{a}	9 ± 6^{a}	8 ± 6^{a}	119 ± 32^{a}	17 ± 4^{a}	1 ± 2^{a}	10 ± 4^{a}	−1 ± 1^{b}	−3 ± 4^{a}	16 ± 6^{b}	−1 ± 1^{a}
p-values	Homoscedasticity*	0.040	0.030	0.017	0.511	0.338	0.575	0.055	0.064	0.364	0.369	0.032
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.045	<0.001	<0.001
Mentha piperita (Peppermint)												
0 kGy	Control	0.47 ± 0.05	0.30 ± 0.05	0.7 ± 0.1	1.0 ± 0.1	2.4 ± 0.2	1.1 ± 0.1	0.040 ± 0.003	0.9 ± 0.1	nd	8.5 ± 0.2	10.6 ± 0.3
1 kGy	Electron beam	−3 ± 5	5 ± 5^{a}	10 ± 6^{a}	−6 ± 6^{ab}	3 ± 3^{a}	−11 ± 8	−4 ± 5	9 ± 5^{a}	–	7 ± 7^{a}	6 ± 6^{a}
	Gamma rays	−12 ± 8	−1 ± 2^{ab}	12 ± 8^{a}	3 ± 4^{a}	3 ± 4^{a}	6 ± 5	−10 ± 8	−2 ± 4^{a}	–	−30 ± 4^{c}	−20 ± 4^{b}
10 kGy	Electron beam	−1 ± 4	−11 ± 5^{b}	−26 ± 10^{b}	−29 ± 10^{c}	−11 ± 5^{b}	4 ± 5	−21 ± 10	8 ± 8^{a}	–	11 ± 7^{a}	9 ± 8^{a}
	Gamma rays	−1 ± 2	5 ± 5^{a}	5 ± 5^{a}	−24 ± 8^{bc}	−6 ± 4^{b}	−11 ± 5	−17 ± 8	−32 ± 12^{b}	–	−10 ± 3^{b}	−10 ± 2^{b}
p-values	Homoscedasticity*	0.742	0.199	0.065	0.011	0.660	0.311	0.720	0.255	–	0.033	0.164
	1-way ANOVA**	0.157	0.004	<0.001	<0.001	<0.001	0.052	0.118	<0.001	–	<0.001	0.062

[a] The results are presented as the mean ± SD.
* Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
** $p < 0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

3. Results and discussion

In a previous study, the effect of gamma irradiation was evaluated by measuring changes in the same parameters as those assayed herein. The values obtained in non-irradiated samples are recalled for each plant species and assayed parameter. To allow a more immediate comparison of the effects of gamma and electron-beam irradiations the percentages of variances (calculated as explained in Section 2) are indicated for both types of irradiation. These percentages were obtained from previously published results for gamma irradiation (GI) (Pereira et al., 2015) and from the newly assessed values resulting from applying electron-beam irradiation (EB) in the same doses as those used for gamma-irradiation. In every cases where the variation laid below 5% (either representing an increase or a decrease), it was assumed that the irradiation had no identifiable effect.

3.1. Effects on chemical parameters

Regarding the proximate composition and color parameters (Table 1), it became obvious that fat and protein are the ones suffering higher changes with irradiation treatment. Nevertheless, the observed effect was highly dependent on the plant species. Fat content, for instance, tended to increase in *A. citrodora* (lemon verbena) and *M. officinalis* (lemon balm), but an opposite effect was produced in *M. melissophyllum* (bastard balm) and *M. piperita* (peppermint). Likewise, no general trend could be identified for the effect on protein content, despite the similar variation in lemon verbena, bastard balm and peppermint obtained with 1 kGy of GI. Furthermore, the effects on the remaining parameters, despite lower in magnitude, were significantly different ($p < 0.05$) for each of the applied conditions in most occasions (21 out of 32 cases). Nevertheless, the 10 kGy dose tended to have a more pronounced effect than the 1 kGy dose, independently of the irradiation technology (except for a^* in all plants and fat content in bastard balm).

In this first approach, it is important to highlight the slight effects caused on L^* and b^*, since colour parameters are usually used in the quality control of post-harvest preservation processes (Hsu et al., 2010). In the case of a^*, the results are even better, since a general decrease was observed in response to irradiation treatment, which should be interpreted as an increase of samples greenness, resulting more appealing to the consumers. The variation in colour parameters is in general agreement with those available from similar reports (Jo et al., 2003; Hsu et al., 2010).

Concerning free sugars composition (Table 2), the induced variations were more pronounced, despite the specificity of effect towards the plant species. Sucrose and trehalose seemed to be the most susceptible sugars to irradiation, as they suffered significant ($p < 0.05$) changes in all cases. Fructose, on the other hand, showed significant changes only in in lemon balm and bastard balm, while glucose remained nearly unchanged in lemon verbena. This result might be an indicator of the vulnerability of the glycosidic bond, since the monosaccharides presented higher resistance. Whereas total sugars, only minor variations were detected, which could be anticipated from the changes in individual sugars, since the decrease in sucrose and trehalose contribute to an increase in fruc-

Table 3
Tocopherols composition (mg/100 g dw) of the aromatic species (controls; non-irradiated samples). Values for irradiated samples are presented as percentage of variation in comparison to the control.[a]

	α-Tocopherol	β-Tocopherol	γ-Tocopherol	δ-Tocopherol	Total tocopherols	
Dose	Irradiation type					
Aloysia citrodora (Lemon verbena)						
0 kGy	Control	15.3 ± 0.4	0.41 ± 0.04	1.8 ± 0.1	nd	17.5 ± 0.4
1 kGy	Electron beam	22 ± 5^{a}	2 ± 4^{a}	5 ± 5	–	17 ± 3^{a}
	Gamma rays	14 ± 4^{a}	7 ± 9^{a}	4 ± 5	–	13 ± 4^{a}
10 kGy	Electron beam	5 ± 5^{b}	−12 ± 10^{ab}	−5 ± 6	–	2 ± 2^{b}
	Gamma rays	−12 ± 4^{c}	−29 ± 10^{b}	−5 ± 5	–	−12 ± 3^{c}
p-values	Homoscedasticity*	0.053	0.279	0.168	–	0.426
	1-way ANOVA**	<0.001	0.004	0.050	–	<0.001
Melissa officinalis (Lemon balm)						
0 kGy	Control	29 ± 1	1.3 ± 0.1	1.5 ± 0.1	0.37 ± 0.05	32 ± 1
1 kGy	Electron beam	−10 ± 2^{d}	−22 ± 5^{b}	−15 ± 3^{d}	1 ± 1^{b}	−10 ± 2^{d}
	Gamma rays	16 ± 1^{a}	−15 ± 4^{a}	18 ± 5^{a}	2 ± 4^{b}	14 ± 1^{a}
10 kGy	Electron beam	−2 ± 2^{c}	−30 ± 3^{c}	−7 ± 3^{c}	2 ± 2^{b}	−3 ± 1^{c}
	Gamma rays	2 ± 1^{b}	−25 ± 3^{bc}	12 ± 6^{b}	31 ± 9^{a}	1 ± 1^{b}
p-values	Homoscedasticity*	<0.001	0.148	0.802	<0.001	0.304
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001
Melittis melissophyllum (Bastard balm)						
0 kGy	Control	0.88 ± 0.05	13.4 ± 0.3	0.18 ± 0.02	0.14 ± 0.02	14.6 ± 0.4
1 kGy	Electron beam	1 ± 3^{b}	−22 ± 5^{d}	−25 ± 10^{bc}	−34 ± 10^{b}	−21 ± 4^{d}
	Gamma rays	−8 ± 5^{b}	−1 ± 1^{c}	−8 ± 5^{b}	3 ± 3^{a}	−2 ± 1^{c}
10 kGy	Electron beam	60 ± 24^{a}	21 ± 5^{b}	14 ± 8^{a}	−39 ± 7^{b}	21 ± 5^{b}
	Gamma rays	−48 ± 6^{c}	115 ± 6^{a}	−40 ± 9^{c}	−44 ± 6^{b}	102 ± 6^{a}
p-values	Homoscedasticity*	0.002	0.559	0.749	0.098	0.363
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001
Mentha piperita (Peppermint)						
0 kGy	Control	16.5 ± 0.4	1.1 ± 0.1	1.8 ± 0.1	0.23 ± 0.03	19.7 ± 0.5
1 kGy	Electron beam	18 ± 6^{a}	27 ± 10^{a}	8 ± 10	−4 ± 2^{b}	18 ± 6^{a}
	Gamma rays	−5 ± 3^{c}	−42 ± 12^{b}	−3 ± 5	15 ± 6^{a}	−6 ± 3^{c}
10 kGy	Electron beam	7 ± 4^{b}	15 ± 10^{a}	−2 ± 5	5 ± 7^{b}	6 ± 4^{b}
	Gamma rays	−25 ± 4^{d}	−29 ± 10^{b}	−1 ± 4	22 ± 7^{a}	−21 ± 4^{d}
p-values	Homoscedasticity*	0.648	0.229	0.097	<0.001	0.906
	1-way ANOVA**	<0.001	<0.001	0.278	<0.001	<0.001

[a] The results are presented as the mean ± SD.

tose and especially glucose. Other less coherent variations might be explained by changes in the optical rotation, which is a common occurrence under irradiation treatment (Molins, 2001).

Significant variations were also detected in the organic acids (Table 2), with quinic and citric acids as the compounds more prone to suffer quantitative changes. It could also be observed that the species with the highest contents in organic acids (bastard balm and peppermint) were the ones with higher number of significant variations. Another interesting observation was the higher propensity of lemon verbena and peppermint to have increased levels of organic acids when GI was applied, while lemon balm and bastard balm showed a general trend to lower amounts of organic acids when irradiated with EB.

Among tocopherols (Table 3), α and β isoforms were the ones presenting higher number of significant variations, but the produced effect was once again highly dependent on the assayed plant species. α-and β-Tocopherols are known for being less stable to irradiation than γ-tocopherol (Warner et al., 2008). Regarding total tocopherols, this dissimilarity among effects was also observed. For instance, lemon verbena present higher amounts in samples irradiated with 1 kGy, while the 10 kGy had a very positive effect on bastard balm (independently of irradiation technology in both case) and peppermint' tocopherols were increased when EB was applied. The significant changes in tocopherols profile in response to irradiation treatment had already been published in different species (Taipina et al., 2009).

Due to the high number of individual fatty acids (FA), these compounds were divided as those quantified below 1% in all species (Table 4A) and those above 1% at least in one species (Table 4B). Like it was verified for the previous parameters, the variations in FA were highly dependent on the analyzed plant species. Nevertheless, it is easily observable that irradiated samples (except for bastard balm) presented higher percentages of monounsaturated fatty acids (MUFA), which represents an interesting result. A similar result was also obtained for some particular polyunsaturated fatty acids (PUFA), such as C18:2n6, C18:3n6 (bastard balm), C18:3n3 (lemon balm) and C20:5n3 (peppermint). Besides C18:2n6, the variations for the remaining predominant FA (C16:0 and C18:3n3) were not particularly noticeable (exempting the decrease of C18:3n3 in bastard balm samples irradiated with 10 kGy). Among the studied plants, lemon balm was the one showing less variation in the FA profiles, especially those samples irradiated with EB. The higher effect in the remaining species might be related with their higher fat contents (Table 1), that might have boosted mechanisms of lipid radiolysis, involving primary ionization, followed by migration of the positive charge toward the carbonyl group or double bonds (Molins, 2001).

3.2. Effects on antioxidant parameters

The effects on the antioxidant activity, namely the scavenging effects on DPPH radicals, reducing power and inhibition of β-carotene bleaching, as well as the amounts of total phenols and flavonoids were also compared (gamma irradiation). In general, EB produced an increase in the ability to scavenge DPPH radicals and in the reducing power (especially the 10 kGy dose), while GI caused the opposite effect. On the other hand, the effect of irradiation on β-carotene bleaching inhibition did not seem to be ruled by any overall trend, being highly dependent on the extract type (aqueous or methanolic) and on the plant species. Regarding bioactive com-

Table 4A
Minor (<1% in all species) fatty acids of the aromatic species. The results are presented in relative percentage (controls; non-irradiated samples). Values for irradiated samples are presented as percentage of variation in comparison to the control.[a]

	C6:0		C8:0	C11:0	C12:0	C13:0	C15:0	C15:1	C17:0	C20:1n9	C20:2n6	C20:3n3+C21:0	C22:1n9
Dose	Irradiation type												
Aloysia citrodora (Lemon verbena)													
0 kGy	Control	0.30 ± 0.01	0.11 ± 0.01	0.26 ± 0.02	0.26 ± 0.02	0.32 ± 0.01	0.58 ± 0.02	0.10 ± 0.01	0.22 ± 0.01	0.25 ± 0.03	0.21 ± 0.01	0.30 ± 0.01	0.27 ± 0.02
1 kGy	Electron beam	−37 ± 6^{c}	−49 ± 12^{c}	-23 ± 8^{c}	10 ± 6^{b}	−50 ± 3^{d}	−19 ± 5^{c}	−26 ± 6^{c}	2 ± 4^{bc}	−16 ± 7^{b}	−14 ± 6^{b}	28 ± 9^{a}	83 ± 18^{b}
	Gamma rays	−7 ± 7^{a}	−4 ± 5^{b}	-19 ± 5^{bc}	9 ± 5^{b}	41 ± 9^{b}	5 ± 5^{b}	−14 ± 3^{b}	10 ± 5^{b}	62 ± 20^{a}	−19 ± 3^{b}	−12 ± 1^{bc}	36 ± 11^{c}
10 kGy	Electron beam	−22 ± 8^{b}	−42 ± 9^{c}	15 ± 6^{a}	5 ± 4^{b}	83 ± 6^{a}	−13 ± 8^{c}	−13 ± 3^{b}	−6 ± 6^{c}	−18 ± 9^{b}	−46 ± 6^{c}	−17 ± 4^{c}	181 ± 43^{a}
	Gamma rays	−24 ± 8^{b}	17 ± 6^{a}	-6 ± 8^{b}	40 ± 11^{a}	9 ± 5^{c}	23 ± 7^{a}	2 ± 4^{a}	27 ± 4^{a}	−11 ± 7^{b}	27 ± 5^{a}	−8 ± 4^{bc}	−32 ± 5^{d}
p-values	Homoscedasticity*	0.104	0.836	0.374	0.055	0.021	0.272	0.007	0.097	0.147	0.078	<0.001	<0.001
	1-way ANOVA[4]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Melissa officinalis (Lemon balm)													
0 kGy	Control	0.22 ± 0.01	0.40 ± 0.02	0.13 ± 0.01	0.46 ± 0.01	0.14 ± 0.01	0.44 ± 0.03	0.55 ± 0.01	0.81 ± 0.01	0.18 ± 0.02	nd	0.28 ± 0.01	nd
1 kGy	Electron beam	1 ± 2^{a}	−3 ± 2^{a}	1 ± 2^{b}	1 ± 2^{a}	−1 ± 5^{a}	−3 ± 4^{a}	−2 ± 2^{a}	2 ± 4	−1 ± 3^{a}	–	−1 ± 2^{b}	–
	Gamma rays	−30 ± 4^{b}	−25 ± 12^{b}	−2 ± 1^{bc}	−27 ± 1^{b}	15 ± 2^{a}	−4 ± 4^{a}	−12 ± 1^{b}	7 ± 1	−18 ± 2^{b}	–	25 ± 1^{a}	–
10 kGy	Electron beam	−3 ± 4^{a}	1 ± 2^{a}	−10 ± 5^{c}	−45 ± 6^{d}	−40 ± 10^{b}	−8 ± 6^{ab}	−30 ± 4^{c}	8 ± 10	−20 ± 12^{bc}	–	−8 ± 5^{b}	–
	Gamma rays	−36 ± 2^{b}	−27 ± 1^{b}	27 ± 2^{a}	−36 ± 2^{c}	1 ± 2^{a}	−19 ± 6^{b}	−7 ± 2^{a}	−1 ± 1	−33 ± 12^{c}	–	28 ± 1^{a}	–
p-values	Homoscedasticity*	<0.001	0.008	0.001	0.008	0.002	0.006	0.025	0.006	0.026	–	0.001	–
	1-way ANOVA[d]	<0.001	<0.001	<0.001	<0.001	<0.001	0.015	<0.001	0.578	<0.001	–	<0.001	–
Melittis melissophyllum (Bastard balm)													
0 kGy	Control	0.18 ± 0.01	0.07 ± 0.01	0.04 ± 0.01	0.18 ± 0.01	0.05 ± 0.01	0.90 ± 0.02	0.09 ± 0.01	0.24 ± 0.02	0.16 ± 0.01	0.09 ± 0.02	0.24 ± 0.01	nd
1 kGy	Electron beam	78 ± 24^{a}	79 ± 34^{b}	1 ± 2^{b}	5 ± 5^{b}	26 ± 5^{b}	−10 ± 4^{b}	14 ± 4^{a}	−20 ± 6^{b}	−18 ± 7^{c}	−20 ± 3^{b}	−10 ± 7^{b}	–
	Gamma rays	−64 ± 2^{c}	4 ± 5^{c}	10 ± 7^{b}	32 ± 6^{a}	35 ± 10^{b}	−7 ± 2^{b}	−9 ± 5^{b}	−2 ± 2^{a}	26 ± 7^{a}	68 ± 16^{a}	10 ± 4^{a}	–
10 kGy	Electron beam	29 ± 13^{b}	118 ± 13^{a}	3 ± 5^{b}	−23 ± 5^{c}	−11 ± 2^{c}	−29 ± 7^{c}	−3 ± 3^{b}	−18 ± 7^{b}	−12 ± 6^{c}	−24 ± 3^{b}	−36 ± 9^{b}	–
	Gamma rays	−58 ± 4^{c}	33 ± 8^{c}	127 ± 12^{a}	37 ± 2^{a}	48 ± 7^{a}	7 ± 3^{a}	17 ± 5^{a}	1 ± 2^{a}	10 ± 2^{b}	93 ± 21^{a}	1 ± 1^{a}	–
p-values	Homoscedasticity*	0.001	0.002	0.130	0.005	0.078	0.038	<0.001	0.143	0.023	<0.001	0.022	–
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	–
Mentha piperita (Peppermint)													
0 kGy	Control	0.15 ± 0.02	1.0 ± 0.1	0.12 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.59 ± 0.05	0.04 ± 0.01	0.44 ± 0.01	0.25 ± 0.01	0.19 ± 0.01	0.45 ± 0.04	0.11 ± 0.01
1 kGy	Electron beam	−23 ± 4^{b}	−22 ± 6^{b}	−14 ± 2^{b}	−8 ± 2^{d}	−72 ± 5^{c}	−15 ± 4^{b}	−43 ± 2^{c}	−6 ± 5^{b}	7 ± 7^{b}	32 ± 9^{a}	−18 ± 6^{c}	35 ± 5^{d}
	Gamma rays	2 ± 4^{ab}	−8 ± 5^{b}	27 ± 8^{a}	7 ± 5^{c}	−19 ± 6^{b}	−23 ± 6^{b}	6 ± 6^{a}	7 ± 2^{ab}	7 ± 7^{b}	−6 ± 6^{b}	6 ± 5^{b}	48 ± 10^{c}
10 kGy	Electron beam	21 ± 2^{a}	37 ± 9^{a}	−13 ± 2^{b}	53 ± 9^{a}	28 ± 7^{a}	16 ± 4^{a}	15 ± 1^{a}	8 ± 8^{a}	26 ± 13^{b}	−8 ± 8^{b}	16 ± 5^{a}	79 ± 7^{a}
	Gamma rays	−60 ± 16^{c}	−19 ± 6^{b}	−9 ± 4^{b}	29 ± 5^{b}	−71 ± 12^{c}	−12 ± 5^{b}	−9 ± 7^{b}	2 ± 2^{ab}	52 ± 2^{a}	−20 ± 7^{b}	18 ± 5^{a}	61 ± 3^{b}
p-values	Homoscedasticity*	<0.001	0.229	0.136	0.011	<0.001	0.017	<0.001	<0.001	<0.001	<0.001	0.316	0.018
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.030	<0.001	<0.001	<0.001	<0.001

[a] The results are presented as the mean ± SD.

* Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

** $p < 0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

Table 4B
Major (>1%, at least in one species) fatty acids of the aromatic species. The results are presented in relative percentage (controls; non-irradiated samples). Values for irradiated samples as presented as percentage of variation in comparison to the control.[a]

		C10:0	C14:0	C14:1	C16:0	C16:1	C18:0	C18:1n9	C18:2n6	C18:3n6	C18:3n3	C20:0	C20:5n3	C22:0	C23:0	C22:6n3	C24:0	SFA	MUFA	PUFA
Dose	Irradiation type																			
Aloysia citrodora (Lemon verbena)																				
0 kGy	Control	nd	1.1 ± 0.1	nd	15.7 ± 0.2	0.50 ± 0.02	1.17 ± 0.01	0.95 ± 0.02	12.6 ± 0.1	nd	56.2 ± 0.3	0.87 ± 0.02	nd	1.00 ± 0.02	5.4 ± 0.1	nd	1.4 ± 0.1	28.6 ± 0.2	2.07 ± 0.03	69.3 ± 0.3
1 kGy	Electron beam	–	-20 ± 5^{c}	–	5 ± 4^{a}	-18 ± 5^{b}	12 ± 4^{a}	8 ± 6^{b}	5 ± 2^{a}	–	-3 ± 1^{c}	10 ± 5^{a}	–	29 ± 9^{a}	15 ± 5^{a}	–	9 ± 7^{bc}	4 ± 2^{b}	9 ± 5^{b}	-2 ± 1^{b}
	Gamma rays	–	26 ± 7^{a}	–	1 ± 2^{ab}	25 ± 5^{a}	-5 ± 1^{b}	1 ± 2^{bc}	-1 ± 1^{b}	–	1 ± 1^{b}	13 ± 2^{a}	–	-18 ± 2^{c}	-22 ± 1^{c}	–	22 ± 2^{ab}	-2 ± 2^{c}	17 ± 3^{a}	1 ± 1^{a}
10 kGy	Electron beam	–	10 ± 8^{b}	–	-3 ± 2^{b}	35 ± 12^{a}	-8 ± 4^{b}	-3 ± 4^{c}	-8 ± 4^{c}	–	3 ± 2^{a}	-11 ± 5^{b}	–	11 ± 8^{b}	-4 ± 2^{b}	–	-7 ± 7^{c}	-4 ± 2^{c}	18 ± 6^{a}	1 ± 1^{a}
	Gamma rays	–	-15 ± 6^{c}	–	5 ± 4^{a}	27 ± 5^{a}	13 ± 1^{a}	19 ± 3^{a}	-1 ± 1^{b}	–	-3 ± 1^{c}	-33 ± 6^{c}	–	-7 ± 5^{c}	10 ± 4^{a}	–	32 ± 6^{a}	6 ± 2^{a}	10 ± 2^{b}	-3 ± 1^{b}
p-values	Homoscedasticity*	–	0.051	–	0.620	0.012	0.001	0.002	0.001	–	0.129	0.038	–	0.001	<0.001	–	<0.001	0.600	0.002	0.470
	1-way ANOVA**	–	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	–	<0.001	<0.001	–	<0.001	<0.001	0.002	<0.001
Melissa officinalis (Lemon balm)																				
0 kGy	Control	0.29 ± 0.02	2.9 ± 0.1	0.53 ± 0.01	22.7 ± 0.3	nd	3.6 ± 0.1	4.9 ± 0.2	15.3 ± 0.4	nd	33.2 ± 0.5	3.4 ± 0.1	3.9 ± 0.1	1.3 ± 0.1	3.3 ± 0.2	nd	1.2 ± 0.2	41.2 ± 0.5	6.2 ± 0.2	52.6 ± 0.5
1 kGy	Electron beam	1 ± 2^{a}	-3 ± 4^{a}	-2 ± 4^{c}	1 ± 1^{a}	–	1 ± 2^{a}	-1 ± 2^{a}	-1 ± 1^{bc}	–	-1 ± 1^{c}	1 ± 2^{bc}	-1 ± 2^{b}	1 ± 2^{b}	1 ± 2^{b}	–	-10 ± 5^{b}	1 ± 1^{a}	-1 ± 2^{a}	-1 ± 1^{c}
	Gamma rays	-13 ± 3^{b}	-9 ± 2^{ab}	-1 ± 2^{c}	-8 ± 1^{c}	–	1 ± 1^{a}	-2 ± 2^{a}	-1 ± 1^{c}	–	4 ± 1^{b}	17 ± 2^{a}	16 ± 1^{a}	12 ± 5^{a}	-1 ± 2^{b}	–	12 ± 5^{a}	-4 ± 1^{b}	-3 ± 1^{a}	3 ± 1^{b}
10 kGy	Electron beam	-18 ± 5^{b}	-48 ± 6^{c}	39 ± 10^{a}	-15 ± 2^{d}	–	-2 ± 2^{a}	-4 ± 4^{a}	5 ± 2^{a}	–	8 ± 2^{a}	-1 ± 2^{c}	-16 ± 7^{c}	-5 ± 5^{b}	29 ± 8^{a}	–	-8 ± 4^{b}	-9 ± 2^{d}	1 ± 2^{a}	7 ± 1^{a}
	Gamma rays	-26 ± 3^{c}	-15 ± 1^{b}	18 ± 2^{b}	-5 ± 1^{b}	–	-11 ± 2^{b}	-12 ± 3^{b}	2 ± 2^{b}	–	9 ± 1^{a}	4 ± 2^{b}	-9 ± 1^{c}	11 ± 5^{a}	-5 ± 5^{b}	–	-8 ± 6^{b}	-6 ± 1^{c}	-10 ± 2^{b}	6 ± 1^{a}
p-values	Homoscedasticity*	0.106	<0.001	<0.001	0.196	–	0.045	0.005	0.621	–	0.080	0.177	<0.001	0.093	0.274	–	0.072	0.581	0.010	0.659
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	<0.001
Melittis melissophyllum (Bastard balm)																				
0 kGy	Control	nd	0.58 ± 0.03	nd	14.3 ± 0.2	1.29 ± 0.05	2.41 ± 0.05	11.5 ± 0.3	14.8 ± 0.4	5.8 ± 0.1	36 ± 1	0.88 ± 0.02	nd	1.3 ± 0.1	6.2 ± 0.2	nd	3.0 ± 0.1	30.4 ± 0.2	13.1 ± 0.2	56.5 ± 0.2
1 kGy	Electron beam	–	36 ± 7^{b}	–	-7 ± 3^{c}	12 ± 6^{a}	5 ± 4^{ab}	16 ± 4^{c}	10 ± 5^{c}	33 ± 10^{a}	-7 ± 3^{a}	5 ± 5^{a}	–	-13 ± 4^{b}	-10 ± 4^{a}	–	1 ± 2	-5 ± 2^{b}	15 ± 3^{c}	-2 ± 1^{a}
	Gamma rays	–	39 ± 5^{b}	–	-1 ± 1^{b}	-11 ± 4^{bc}	1 ± 1^{b}	13 ± 2^{c}	9 ± 3^{c}	1 ± 1^{b}	-7 ± 1^{a}	8 ± 2^{a}	–	3 ± 3^{a}	-4 ± 2^{a}	–	-5 ± 4	-1 ± 1^{a}	10 ± 2^{c}	-2 ± 1^{a}
10 kGy	Electron beam	–	-2 ± 4^{c}	–	-7 ± 4^{c}	-25 ± 8^{c}	1 ± 2^{b}	51 ± 5^{a}	31 ± 6^{a}	18 ± 6^{ab}	-21 ± 3^{b}	-26 ± 8^{b}	–	-35 ± 7^{c}	-11 ± 8^{a}	–	-5 ± 4	-10 ± 3^{c}	46 ± 4^{a}	-7 ± 1^{b}
	Gamma rays	–	59 ± 12^{a}	–	6 ± 1^{a}	-3 ± 3^{ab}	14 ± 2^{a}	31 ± 6^{b}	23 ± 1^{b}	9 ± 4^{b}	-21 ± 2^{b}	10 ± 4^{a}	–	11 ± 4^{a}	-33 ± 2^{b}	–	1 ± 2	-1 ± 1^{a}	27 ± 5^{b}	-6 ± 1^{b}
p-values	Homoscedasticity*	–	0.463	–	0.014	0.012	0.008	0.024	0.003	<0.001	0.003	0.001	–	0.802	<0.001	–	0.993	0.045	0.007	0.053
	1-way ANOVA**	–	<0.001	–	<0.001	<0.001	0.001	<0.001	<0.001	0.005	<0.001	<0.001	–	<0.001	<0.001	–	0.216	<0.001	<0.001	<0.001
Mentha piperita (Peppermint)																				
0 kGy	Control	0.07 ± 0.01	1.4 ± 0.1	1.2 ± 0.1	10.4 ± 0.3	0.88 ± 0.05	2.47 ± 0.03	1.62 ± 0.05	7.3 ± 0.1	nd	46 ± 1	15.8 ± 0.5	2.8 ± 0.2	2.6 ± 0.1	0.24 ± 0.0	1.4 ± 0.1	2.1 ± 0.1	38 ± 1	4.1 ± 0.1	58 ± 1
1 kGy	Electron beam	-34 ± 2^{a}	-2 ± 4^{c}	6 ± 6^{a}	-5 ± 3^{b}	-12 ± 5^{b}	8 ± 4^{b}	33 ± 6^{b}	3 ± 3^{b}	–	1 ± 1^{a}	2 ± 4^{b}	-17 ± 4^{b}	1 ± 2^{b}	2 ± 4^{ab}	-6 ± 4^{b}	-5 ± 2^{ab}	-2 ± 2^{c}	8 ± 4^{b}	1 ± 1^{a}
	Gamma rays	-76 ± 9^{a}	11 ± 5^{bc}	-1 ± 2^{a}	-1 ± 1^{b}	9 ± 5^{a}	3 ± 1^{b}	-1 ± 2^{d}	2 ± 1^{b}	–	-4 ± 2^{b}	5 ± 3^{b}	8 ± 4^{a}	7 ± 2^{ab}	-20 ± 8^{b}	9 ± 3^{a}	-12 ± 5^{b}	2 ± 2^{b}	4 ± 2^{b}	-2 ± 1^{b}
10 kGy	Electron beam	-20 ± 3^{a}	30 ± 9^{a}	4 ± 4^{a}	13 ± 4^{a}	8 ± 8^{a}	21 ± 6^{a}	42 ± 4^{a}	8 ± 3^{a}	–	-6 ± 2^{bc}	4 ± 2^{b}	8 ± 6^{a}	10 ± 5^{a}	-5 ± 8^{ab}	-17 ± 3^{c}	4 ± 4^{a}	10 ± 2^{a}	20 ± 3^{a}	-5 ± 1^{c}
	Gamma rays	-376 ± 53^{b}	16 ± 4^{ab}	-20 ± 6^{b}	-3 ± 3^{b}	-9 ± 4^{b}	5 ± 3^{b}	15 ± 3^{c}	-2 ± 1^{c}	–	-7 ± 2^{c}	12 ± 2^{a}	15 ± 4^{a}	9 ± 4^{a}	9 ± 2^{a}	10 ± 3^{a}	-12 ± 3^{b}	5 ± 2^{b}	10 ± 2^{b}	-4 ± 1^{c}
p-values	Homoscedasticity*	<0.001	0.001	0.265	0.104	0.179	0.014	0.014	0.001	–	0.143	0.007	0.013	0.093	<0.001	0.090	0.124	0.787	0.007	0.092
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001	–	<0.001	<0.001	<0.001	0.002	0.042	<0.001	<0.001	<0.001	<0.001	<0.001

[a] The results are presented as the mean ± SD.

* Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

** $p < 0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

Table 5
Antioxidant properties of extracts from the aromatic species.[1] EC_{50} values (μg/mL) for the controls (non-irradiated samples) are presented for all assays except phenols and flavonoids, which are expressed as mg GAE/g extract and mg CE/g extract, respectively. Values for irradiated samples are presented as percentage of variation in comparison to the control.[a]

		DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		Phenols		Flavonoids	
Dose	Irradiation type	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
Aloysia citrodora (Lemon verbena)											
0 kGy	Control	232 ± 8	39 ± 4	169 ± 1	22.8 ± 0.3	580 ± 31	208 ± 9	134 ± 8	665 ± 13	92 ± 1	369 ± 5
1 kGy	Electron beam	−1 ± 2^{a}	13 ± 8^{c}	−13 ± 2^{d}	−9 ± 1^{c}	−10 ± 5^{c}	254 ± 63^{a}	4 ± 4^{d}	5 ± 2^{a}	3 ± 6^{a}	7 ± 2^{a}
	Gamma rays	2 ± 1^{a}	130 ± 16^{b}	9 ± 1^{a}	115 ± 1^{b}	73 ± 7^{a}	14 ± 7^{c}	41 ± 11^{b}	−20 ± 6^{b}	−35 ± 2^{c}	-3 ± 3^{b}
10 kGy	Electron beam	−8 ± 5^{b}	−13 ± 5^{d}	−11 ± 1^{c}	−10 ± 1^{c}	67 ± 25^{a}	60 ± 28^{b}	30 ± 5^{c}	6 ± 1^{a}	12 ± 6^{a}	7 ± 2^{a}
	Gamma rays	−12 ± 6^{b}	177 ± 15^{a}	1 ± 1^{b}	172 ± 2^{a}	43 ± 6^{b}	−5 ± 3^{c}	54 ± 8^{a}	−31 ± 3^{c}	−18 ± 4^{b}	−25 ± 1^{c}
p-values	Homoscedasticity*	<0.001	<0.001	<0.001	0.014	<0.001	<0.001	0.038	<0.001	<0.001	0.001
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Melissa officinalis (Lemon balm)											
0 kGy	Control	101 ± 3	67 ± 1	80 ± 1	44 ± 1	165 ± 4	125 ± 3	100 ± 1	829 ± 6	63 ± 1	448 ± 4
1 kGy	Electron beam	−7 ± 5^{c}	17 ± 8^{a}	1 ± 1^{c}	20 ± 1^{b}	86 ± 8^{b}	−14 ± 3^{b}	−5 ± 2^{c}	−12 ± 1^{c}	8 ± 8	−12 ± 1^{d}
	Gamma rays	1 ± 1^{b}	9 ± 3^{b}	−6 ± 1^{d}	8 ± 1^{c}	−21 ± 2^{c}	−10 ± 1^{a}	8 ± 1^{a}	−5 ± 2^{b}	9 ± 1	11 ± 1^{a}
10 kGy	Electron beam	−14 ± 5^{d}	−9 ± 3^{c}	9 ± 1^{b}	1 ± 1^{d}	118 ± 15^{a}	−14 ± 4^{b}	−6 ± 2^{c}	1 ± 1^{a}	5 ± 5	4 ± 1^{b}
	Gamma rays	7 ± 2^{a}	8 ± 2^{b}	28 ± 1^{a}	25 ± 1^{a}	-18 ± 1^{c}	−13 ± 1^{ab}	4 ± 1^{b}	−10 ± 1^{c}	4 ± 1	−7 ± 1^{c}
p-values	Homoscedasticity*	<0.001	<0.001	0.075	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	<0.001	<0.001	0.499	<0.001
Melittis melissophyllum (Bastard balm)											
0 kGy	Control	583 ± 24	354 ± 39	512 ± 16	249 ± 2	1648 ± 154	447 ± 66	70 ± 4	160 ± 3	29 ± 2	108 ± 4
1 kGy	Electron beam	−12 ± 4^{c}	36 ± 7^{a}	−7 ± 2^{c}	35 ± 1^{a}	−13 ± 2^{c}	−22 ± 4^{b}	10 ± 1^{a}	6 ± 2^{a}	12 ± 1^{a}	1 ± 1^{b}
	Gamma rays	19 ± 7^{b}	2 ± 4^{b}	18 ± 4^{a}	−20 ± 2^{c}	28 ± 5^{b}	21 ± 5^{a}	3 ± 4^{ab}	−37 ± 2^{d}	−45 ± 5^{c}	−32 ± 3^{d}
10 kGy	Electron beam	−1 ± 2^{c}	−24 ± 2^{c}	6 ± 1^{b}	−38 ± 2^{d}	−14 ± 4^{c}	−15 ± 4^{b}	−1 ± 1^{c}	−5 ± 1^{b}	-3 ± 2^{b}	15 ± 4^{a}
	Gamma rays	45 ± 8^{a}	1 ± 2^{b}	−11 ± 2^{c}	16 ± 2^{b}	40 ± 10^{a}	35 ± 7^{a}	−1 ± 2^{c}	−16 ± 2^{c}	−49 ± 3^{c}	−23 ± 2^{c}
p-values	Homoscedasticity*	<0.001	<0.001	<0.001	0.487	<0.001	0.081	<0.001	<0.001	0.002	0.001
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.011	<0.001	<0.001	<0.001
Mentha piperita (Peppermint)											
0 kGy	Control	184 ± 5	83 ± 7	119 ± 2	52 ± 2	597 ± 44	184 ± 5	218 ± 2	591 ± 19	117 ± 2	319 ± 6
1 kGy	Electron beam	−12 ± 4^{c}	18 ± 3^{a}	16 ± 1^{c}	1 ± 1^{a}	−27 ± 4^{c}	92 ± 19^{b}	−1 ± 1^{a}	−6 ± 1^{a}	−1 ± 1^{a}	−8 ± 1^{b}
	Gamma rays	4 ± 2^{b}	15 ± 3^{b}	13 ± 1^{a}	−22 ± 3^{c}	−28 ± 8^{b}	−35 ± 4^{a}	21 ± 1^{ab}	−4 ± 1^{d}	−23 ± 3^{c}	10 ± 1^{d}
10 kGy	Electron beam	−14 ± 3^{c}	−7 ± 2^{c}	35 ± 1^{b}	15 ± 1^{d}	63 ± 15^{c}	32 ± 6^{b}	1 ± 1^{c}	−6 ± 1^{b}	−1 ± 2^{b}	−11 ± 1^{a}
	Gamma rays	18 ± 3^{a}	4 ± 2^{b}	18 ± 3^{c}	1 ± 2^{b}	15 ± 5^{a}	−64 ± 10^{a}	10 ± 1^{c}	−12 ± 2^{c}	−51 ± 4^{c}	−20 ± 2^{c}
p-values	Homoscedasticity*	0.140	0.086	0.002	0.066	0.003	<0.001	0.006	<0.001	0.499	0.001
	1-way ANOVA**	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MeOH- Methanol; GAE- Gallic acid equivalents; CE- Catechin equivalents.

[a] The results are presented as the mean ± SD.

* Homoscedasticity among obtained ratios was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.

** $p < 0.05$ indicates that the mean value of at least one ratio differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

pounds, irradiation tended to increase the levels of total phenols in infusions, while methanolic extracts suffered the opposite effect. Flavonoids tended to diminish with irradiation, independently of plant species, extract type or irradiation technology.

3.3. Linear discriminant analysis (LDA)

In the former sections, the differences resulting from irradiation treatment at different doses were compared for each individual parameter within each species. Despite the significant variations verified in several cases, it was not possible to identify unequivocal tendencies. Accordingly, the results were evaluated considering data for all irradiation conditions and evaluated parameters simultaneously. In the performed LDA, irradiation conditions and plant species were sequentially used as grouping factors. All those parameters not detected in the four species were not used in the analysis.

The significant independent variables (evaluated parameters) were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only those with a statistical significant classification performance ($p < 0.050$) were kept in the analysis.

In the discriminant model obtained to verify if the different irradiation treatments (EB, 1 kGy; EB 10 kGy; GI, 1 kGy; GI 10 kGy) exerted variations in the evaluated parameters in a specific way, the three defined functions (plotted in Fig. 1A) integrated 100% of the observed variance (first: 71.4%; second: 16.1%; third: 12.5%). Among the tested variables 26 were selected as having discriminant ability: fat, carbohydrates, energy, sucrose, organic acids, C6:0, C11:0, C13:0, C14:0, C15:0, C18:0, C18:2n6, C20:0, C20:1, C20:3n3 + C21:0, MUFA and all those in Table 5, which indicates that the fatty acids profile and the antioxidant activity were the most affected variables considering the overall results of the different irradiation treatments. The groups corresponding to each condition were completely individualized, thereby indicating that its effects are highly specific. Function 1 (more correlated with DPPH scavenging activity in infusions, total phenols and flavonoids in methanolic extracts) separated mainly the groups corresponding to the 10 kGy dose of both types of irradiation; function 2 (more correlated with C13:0, β-carotene bleaching inhibition in methanolic extracts and flavonoids in infusions) separated mainly EB at 1 kGy dose, while function 3 (more correlated with C20:0, carbohydrates, β-carotene bleaching inhibition in infusions and MUFA) was more effective in separating the doses of 1 kGy and 10 kGy for both irradiation sources.

In the assessment of the interaction with the plant species the three defined functions included also 100% of the observed variance (first: 48.0%; second: 29.5%; third: 22.5%), selecting 30 variables (fat, protein, ash, fructose, sucrose, trehalose, oxalic acid, organic acids, α-tocopherol, tocopherols, C6:0, C8:0, C13:0, C14:0, C16:0, C18:1n9, C18:3n3, C20:0, C20:1, C23:0, C24:0, SFA, MUFA and all the variables in Table 5, except DPPH scavenging activity and flavonoids content in infusions). Likewise, the defined functions separate the markers corresponding to each of the assayed species (Fig. 1B). Function 1 (highly correlated to C18:3n3, C8:0, C18:1n9, C14:0 and fat) separated mainly bastard balm (*M. melissophyllum*); function 2 (more correlated to reducing power in infusions, trehalose and C13:0) contributed mainly to discriminate peppermint (*M. piperita*); finally, function 3 (closely correlated to phenols in infusions, MUFA, protein and β-carotene bleaching inhibition in methanolic extracts) allowed to separate lemon verbena (*M. officinalis*).

Overall, when analyzed individually, the chemical parameters and bioactive indicators of the tested aromatic plants showed that the effects of EB and GI irradiation were highly dependent on the plant species. After, when evaluated together it became evident that changes in fatty acids profiles and antioxidant activity were those showing the highest differences, either when discriminating among irradiation conditions, as well as plant species. Combining this information with that obtained in Tables 1–5, that highlight irradiated samples as having higher MUFA (and some PUFA) percentages and a beneficial effect of EB irradiation on the antioxidant activity, it might be concluded that the most suitable solution to irradiate aromatic plants would be EB. Nevertheless, the dependence on the plant species and irradiation dose was strongly demonstrated, advising for accurate studies of any plant species to be considered for irradiation.

Conflict of interest

The authors declare no conflict of interest.

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Annexes 8

Bioactivity and phytochemical characterization of *Arenaria montana* L.

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Bioactivity and phytochemical characterization of *Arenaria montana* L.

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The bioactivity (antioxidant and cytotoxic activities) of the aqueous and methanolic extracts of *Arenaria montana* L., a plant commonly used in Portuguese folk medicine, was evaluated and compared. Furthermore, the phytochemical composition was determined based on hydrophilic (sugars, organic acids and phenolic compounds) and lipophilic (fatty acids and tocopherols) compounds, in order to valorize this plant material as a functional food/nutraceutical. Fructose, oxalic acid, methyl-luteolin 2″-*O*-feruloylhexosyl-*C*-hexoside, α-tocopherol, and linoleic acid were the main individual compounds found in *A. montana*. In general, the aqueous extract showed higher antioxidant and cytotoxic activities than the methanolic extract; the latter showed activity only against HeLa and HepG2 cell lines. Both aqueous and methanolic extracts showed some hepatotoxicity but at higher doses than the ones active for tumor cell lines. Moreover, the aqueous extract of *A. montana* may be used as a functional food or nutraceutical due to the high antioxidant and cytotoxic activities, and due to the presence of bioactive compounds. As far as we know, this is the first report on the phytochemical composition and bioactivity of *A. montana*.

Introduction

The study of plants used in folk medicine has progressively increased over the last few decades.[1] Some of their putative therapeutic benefit arise from a diverse phytochemical composition, which confers them antioxidant potential along with other bioactive properties namely, anticarcinogenic/antimutagenic, antibacterial, antiviral or anti-inflammatory properties.[2,3] Among the various biologically active molecules, phenolic compounds are major contributors to the antioxidant activity of those plants.[4–10] The antioxidant activity of phenolic compounds is influenced by the number and position of phenolic hydroxyls and other substituents, and glycosylation of the molecules.[11,12] Furthermore, antitumor properties have also been attributed to different phenolic compounds, including flavones.[13]

Other important antioxidant molecules are tocopherols, which are considered as one of the most important antioxidants to combat oxidative stress, because they inhibit the production of peroxyl radicals, protecting cells of oxidative damage to low density lipoproteins, proteins and DNA, and of membrane degeneration due to peroxidation of polyunsaturated fatty acids.[14,15] Some organic acids are also excellent antioxidants; for example, ascorbic acid, being a potent reducing agent, has the capacity to reduce the most reactive species of oxygen and nitrogen protecting against lipid peroxidation.[16] The reducing sugars, due to the same capacity, could also display antioxidant activity.[17] Different health benefits of polyunsaturated fatty acids (PUFA) have also been described. For example, it was reported that PUFA could be used to sensitize breast cancer cell lines and mammary tumors to anticancer drugs, increasing survival and chemotherapy efficacy.[18,19] The mentioned phytochemicals are common in medicinal plants and often responsible for their bioactive effects.

Arenaria montana L. (Mountain sandwort) is an herbaceous plant native to mountainous regions of southwestern Europe, being usually gathered in woodlands. The infusion of the dried plant (stems, leaves and flowers) is used in Portuguese traditional medicine for its anti-inflammatory and diuretic properties.[20,21] Nevertheless, as far as we know, there are no previous reports on the phytochemical composition of this plant.

The aim of the current study was to characterize the chemical composition of *A. montana* and to assess the antioxidant and cytotoxic properties of their aqueous and methanol extracts.

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Experimental

Sample

Arenaria montana L. (Caryophyllaceae) flowers and leafy stems (approximately the upper 15 cm of the dense clumps produced in spring) are commonly wild gathered in Bragança (North-eastern Portugal). Then these plant materials are dried, prepared in infusion, recommended and used as homemade remedies.[21] Considering the availability and local consumers' criteria for its medicinal use, the species was collected in full bloom, in spring along paths through the oak trees, in Oleiros, Bragança. A sample for analysis was made by putting together the material from different plants. Voucher specimens are deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The sample was lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain homogeneity.

Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers, L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(−)-fructose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose and D(+)-raffinose pentahydrate), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), gallic acid and (+)-catechin standards. Racemic tocol, 50 mg mL^{-1}, was purchased from Matreya (Plesant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Fetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin–EDTA (ethylenediaminetetraacetic acid), penicillin–streptomycin solution (100 U mL^{-1} and 100 mg mL^{-1}, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, Utah, USA). Acetic acid, ellipticine, sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (Saint Louis, MO, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

Evaluation of bioactivity

Sample preparation. The methanolic extract was obtained from the lyophilized plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through the Whatman no. 4 paper. The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

The aqueous extract (infusion) was also obtained from the lyophilized plant material. The sample (1 g) was added to 200 mL of boiling distilled water, left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained extract was frozen and lyophilized.

Methanolic and aqueous extracts were redissolved in (i) methanol and water, respectively (final concentration: 2.5 mg mL^{-1}) for antioxidant activity evaluation, or (ii) water (final concentration: 8 mg mL^{-1}) for cytotoxicity evaluation. The final solutions were further diluted to different concentrations to be submitted for distinct bioactivity evaluation *in vitro* assays. The results were expressed in (i) EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or (ii) GI_{50} values (the sample concentration that inhibited 50% of the net cell growth) for cytotoxicity. Trolox and ellipticine were used as positive controls in antioxidant and cytotoxic activity evaluation assays, respectively.

Antioxidant activity. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde–thiobarbituric acid (MDA–TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively.[22]

Cytotoxicity for tumor cell lines. Five human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin (HeLa and HepG2 cells), at 37 °C, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells per well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells per well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed by adding cold 10% trichloroacetic acid (TCA, 100 μL) and incubated for 60 min at 4 °C. Plates were then washed with deionized water and dried; sulforhodamine B solution (0.1% in 1% acetic acid, 100 μL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 μL) and the absorbance was measured at 540 nm in the microplate reader mentioned above.[23]

Hepatotoxicity. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designated as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and divided into 1 × 1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin and incubated at 37 °C with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.[23]

Phytochemical composition in hydrophilic compounds

Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by the authors[22] using melezitose as the internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), a degasser system (Smartline manager 5000), an auto-sampler (AS-2057 Jasco, Easton, MD, USA) and a RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile–deionized water, 70 : 30 (v/v) at a flow rate of 1 mL min^{-1}. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

Organic acids. Organic acids were determined following a procedure previously described by the authors.[24] The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Coperation, Kyoto, Japan). Separation was achieved using a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d) thermostatted at 35 °C. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL min^{-1}. Detection was carried out using a DAD, at 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g of dry weight.

Phenolic compounds. Phenolic compounds were determined by HPLC (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) as previously described by the authors.[25] Double online detection was carried out using a DAD at 280 nm and 370 nm as preferred wavelengths and using a mass spectrometer (API 3200 Qtrap, Applied Biosystems, Darmstadt, Germany) connected to the HPLC system *via* the DAD cell outlet. The phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. The phenolic compounds were identified by comparing their retention time, UV-vis and mass spectra with those obtained from standard solutions, when available. Otherwise, peaks were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve (2.5–100 μg mL^{-1}) for each available phenolic standard was constructed based on the UV signal: apigenin 6-*C*-glucoside ($y = 223.22x + 60.915$; $R^2 = 1$); luteolin 6-*C*-glucoside ($y = 508.54x - 152.82$; $R^2 = 0.997$). For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of other compounds from the same phenolic group. The results were expressed in mg per 100 g of dry weight (dw).

Phytochemical composition in lypophilic compounds

Fatty acids. Fatty acids were determined by gas–liquid chromatography with a flame ionization detection (GC-FID)/capillary column as described previously by the authors.[22] The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey-Nagel (Duren, Germany) column (50% cyanopropyl-methyl-50% phenyl-methylpolysiloxane, 30 m × 0.32 mm ID × 0.25 μm d_f). The oven temperature program is as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C min^{-1} ramp to 125 °C, 5 °C min^{-1} ramp to 160 °C, 20 °C min^{-1} ramp to 180 °C, 3 °C min^{-1} ramp to 200 °C, 20 °C min^{-1} ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL min^{-1} (0.61 bar), measured at 50 °C. Split injection (1 : 40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

Tocopherols. Tocopherols were determined following a procedure previously described by the authors.[22] Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco, Easton, MD, USA) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column from YMC Waters operating at 30 °C. The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70 : 30, v/v) at a flow rate of 1 mL min^{-1}, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

Statistical analysis

For each one of the species three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using the SPSS v. 18.0 program.

Results and discussion

Evaluation of antioxidant activity

The results obtained from the antioxidant activity evaluation of the aqueous and methanolic extracts of *A. montana* are given in Table 1. The aqueous extract showed higher antioxidant activity than the methanolic extract in all the assays, with the exception of DPPH scavenging activity assay in which both samples showed similar results.

The effects of *A. montana* methanolic and aqueous extracts on the growth of five human tumor cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are also summarized in Table 1. The aqueous extract showed activity against all the tumor cell lines, while the methanolic extract only presented some activity for HeLa and HepG2 cell lines. In general, it may be concluded that the bioactive compounds involved in growth inhibition of the other cell lines are preferentially extracted in the aqueous preparation. Curiously, HeLa and HepG2 were the most susceptible (lowest GI_{50} values) cell lines to the aqueous extract. Both the aqueous and methanolic extracts showed some hepatotoxicity but at high doses (332.18 μg mL^{-1} and 350.25 μg mL^{-1}, respectively). However, it should be highlighted that at the doses active against tumor cell lines, the aqueous extract did not show hepatotoxicity.

Trolox and ellipticine were used as positive controls in antioxidant and cytotoxic activities evaluation assays, respectively, but the comparison with the samples should be avoided, because they are individual compounds and not mixtures as the studied extracts.

To the best of our knowledge, no reports are available on the bioactivity of the aqueous or methanolic extracts of the aforementioned plant.

Composition in hydrophilic compounds

The chemical composition of the samples in sugars and organic acids was also analyzed and the results are shown in Table 2. The sugars found were fructose, glucose, sucrose trehalose and raffinose, fructose being the most abundant; as a reducing sugar it has antioxidant capacity.

Oxalic, quinic, malic, ascorbic, citric, succinic and fumaric acids were also identified and quantified (Table 2), oxalic and malic acids being the most abundant organic acids. Some of these acids (*e.g.*, ascorbic and citric acids) have been reported as having antioxidant capacity and health benefits.[26,27] Oppositely, several studies indicate that oxalic acid causes acute oxalate nephropathy and neurotoxicity in humans and animals.[28]

Ten phenolic compounds were identified in the methanolic extract of *A. montana* (flowers and leafy stems) being all of them flavone derivatives. The HPLC phenolic profile recorded at 370

Table 2 Composition in hydrophilic compounds of wild *Arenaria montana*.[a]

Free sugars	g per 100 g dw	Organic acids	g per 100 g dw
Fructose	5.46 ± 0.53	Oxalic acid	1.93 ± 0.09
Glucose	2.05 ± 0.33	Quinic acid	0.06 ± 0.00
Sucrose	1.41 ± 0.32	Malic acid	1.48 ± 0.00
Trehalose	0.80 ± 0.01	Ascorbic acid	0.02 ± 0.00
Raffinose	0.43 ± 0.00	Citric acid	0.30 ± 0.03
Total sugars	10.15 ± 0.99	Succinic acid	0.28 ± 0.03
		Fumaric acid	0.01 ± 0.00
		Total organic acids	4.07 ± 0.08

[a] dw – dry weight.

Table 1 Bioactivity of *Arenaria montana* methanolic and aqueous extracts

	Methanolic extract	Aqueous extract	Positive control[a]
Antioxidant activity			
DPPH scavenging activity (EC_{50}, mg mL^{-1})	0.90 ± 0.01[a]	0.93 ± 0.02[a]	0.04 ± 0.00
Reducing power (EC_{50}, mg mL^{-1})	0.82 ± 0.01[a]	0.77 ± 0.02[b]	0.03 ± 0.00
β-Carotene bleaching inhibition (EC_{50}, mg mL^{-1})	6.25 ± 0.31[a]	1.71 ± 0.02[b]	0.003 ± 0.00
TBARS inhibition (EC_{50}, mg mL^{-1})	0.90 ± 0.08[a]	0.20 ± 0.02[b]	0.004 ± 0.00
Cytotoxic activity			
MCF-7 (breast carcinoma) (GI_{50}, μg mL^{-1})	>400[a]	130.05 ± 8.05[b]	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer) (GI_{50}, μg mL^{-1})	>400[a]	231.08 ± 5.86[b]	1.42 ± 0.00
HCT-15 (colon carcinoma) (GI_{50}, μg mL^{-1})	>400[a]	183.51 ± 15.54[b]	1.91 ± 0.06
HeLa (cervical carcinoma) (GI_{50}, μg mL^{-1})	329.46 ± 12.46[a]	80.21 ± 6.29[b]	1.14 ± 0.21
HepG2 (hepatocellular carcinoma) (GI_{50}, μg mL^{-1})	308.68 ± 13.25[a]	58.57 ± 6.59[b]	3.22 ± 0.67
Hepatotoxicity PLP2 (GI_{50}, μg mL^{-1})	350.25 ± 5.70[a]	332.18 ± 3.61[b]	2.06 ± 0.03

[a] Trolox and ellipticine for antioxidant and cytotoxic activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p < 0.05$).

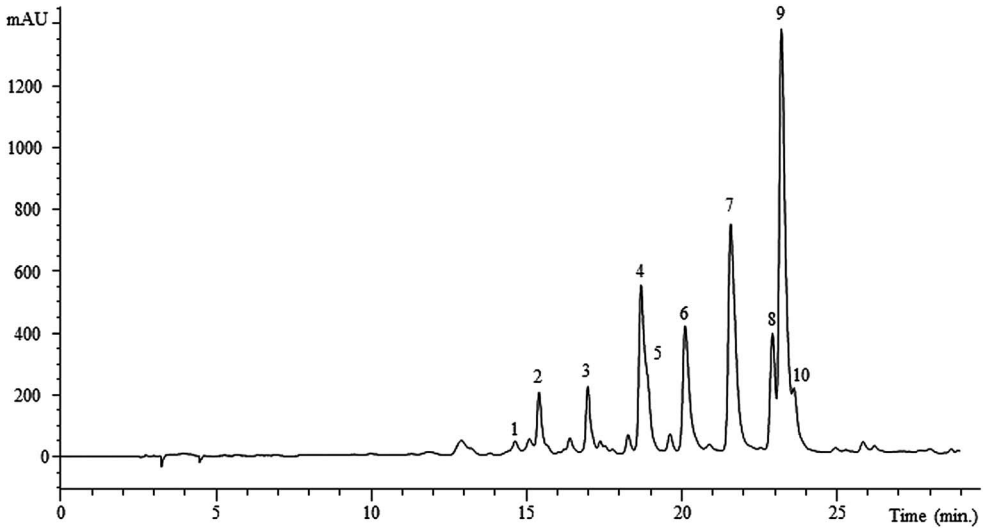

Fig. 1 HPLC phenolic profile of wild *Arenaria montana*, obtained at 370 nm.

Table 3 Retention time (R_t), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), tentative identification and quantification of phenolic compounds in wild *Arenaria montana*

Peak	R_t (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (m/z)	MS^2 (m/z)	Tentative identification	Quantification (mg per 100 g dw)
1	14.7	330	593	473(4), 353(6), 341(19), 311(65), 283(12)	Apigenin 6-*C*-hexoside-8-*C*-hexoside	8.71 ± 0.32
2	15.4	336	563	443(29), 383(33), 353(34), 311(4), 297(6)	Apigenin 6-*C*-hexoside-8-*C*-pentoside	41.63 ± 0.27
3	16.9	350	447	429(12), 357(50), 327(58), 285(12)	Luteolin-6-*C*-glucoside	22.57 ± 0.84
4	18.7	338	563	443(7), 413(62), 341(22), 311(22), 293(72)	Apigenin 2″-*O*-pentosyl-6-*C*-hexoside	123.74 ± 2.13
5	18.9	348	785	665(11), 609(12), 489(4), 429(26), 339(8), 285(4)	Luteolin 2″-*O*-feruloylhexosyl-6-*C*-hexoside	37.95 ± 0.50
6	20.1	336	431	341(72), 311(100)	Apigenin-6-*C*-glucoside	159.91 ± 1.83
7	21.6	336	605	563(4), 431(6), 413(44), 341(13), 311(13)	Apigenin 2″-*O*-acetylpentosyl-6-*C*-hexoside	179.84 ± 0.88
8	22.9	350	635	593(4), 515(7), 461(5), 443(50), 371(19), 323(68)	Methyl-luteolin 2″-*O*-acetylpentosyl-*C*-hexoside	88.36 ± 1.44
9	23.2	330	799	679(7), 623(21), 443(64), 371(6), 323(33)	Methyl-luteolin 2″-*O*-feruloylhexosyl-*C*-hexoside	450.26 ± 3.50
10	23.6	332	769	649(14), 593(19), 443(18), 413(60), 341(7), 311(5)	Apigenin 2″-*O*-feruloylhexosyl-6-*C*-hexoside	91.68 ± 2.54
					Total flavones	1204.63 ± 5.76

nm is shown in Fig. 1, and peak characteristics, identities and quantification are presented in Table 3.

Luteolin-6-*C*-glucoside (peak 3) and apigenin 6-*C*-glucoside (peak 6) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards.

Peaks 2, 4 and 7 presented similar UV spectra to peak 6 with a λ_{max} at 336–338 nm, suggesting that may derive from apigenin. All these peaks showed fragments ions at m/z 311 and 341, corresponding to the aglycone (apigenin) +41 mu and +71 mu, respectively, that are characteristic of *C*-glycosylated flavones.[29] Peaks 2 and 4 had the same pseudomolecular ion $[M-H]^-$ at m/z 563 pointing to apigenin bearing pentose and hexose sugar substituents, but yielded different MS^2 fragment ions. According to Ferreres *et al.*,[29] the ions aglycone + 83 mu and aglycone + 113 mu would typify di-*C*-glycosylated flavones. Thus, the observation of the ions at m/z 383 (agl + 113) and 353 (agl + 83) in the case of peak 2 would indicate that both sugars are *C*-attached, which is supported by the losses of −120 mu (ion at m/z 443), −180 mu (90 + 90; m/z at 383), and 210 mu (120 + 90; m/z at 353), characteristic of *C*-glycosylated flavones.[30] The loss of −120 mu is typical of *C*-attached hexoses, whereas that of −90 mu is observed for *C*-attached pentoses and it is also usual in the case of 6-*C*-hexoses but less common in the case of

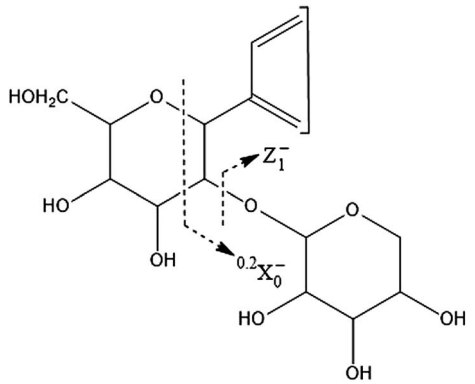

Fig. 2 Fragmentation of *x*″-glycosyl-*C*-glycosylflavones (adapted from Ferreres *et al.*[29]).

8-*C*-hexoses.[31] These observations allowed the tentative identification of peak 2 as apigenin 6-*C*-hexoside-8-*C*-pentoside.

The fragmentation of peak 4 would be more coherent with an *O*,*C*-diglycoside. The loss of −120 mu leading to the ion at *m*/*z* 443 ($^{0,2}X_0^-$ in Fig. 2) supported the presence of a *C*-attached hexose, while the absence of an ion $[(M - H) - 90]^-$ pointed to a 6-*C* attachment. The lack of an ion $[(M - H) - 132]^-$ from the loss of the pentosyl residue suggested that this sugar was not linked to the aglycone but to the other sugar; this was confirmed by the presence of an abundant $[(M - H) - 150]^-$ ion (Z_1^- in Fig. 2) at *m*/*z* 413, which according to Ferreres *et al.*[29] would be characteristic of an *O*-attached pentose on the *C*-glycosylating hexose. The *O*-glycosylation should not take place in the positions 6″, 4″ or 3″ of the hexose, otherwise the fragment $[(M - H) - 120]^-$ would not be produced. Finally, the ion at *m*/*z* 293 would result from the fragment at *m*/*z* 413 by further loss of a fragment of 120 mu (partial loss of the *C*-attached hexose). All in all, peak 4 could be tentatively identified as apigenin 2″-*O*-pentosyl-6-*C*-hexoside.

Peak 7 ($[M - H]^-$ at *m*/*z* 605) was 42 mu greater than peak 4 and showed a similar fragmentation pattern, so that it can be assigned to an acetyl derivative of peak 4. The observation of an abundant ion at *m*/*z* 413 ($[(M - H) - 42 - 150]^-$) from the loss of the pentose after release of the acetyl residue would confirm that this sugar was *O*-linked to the *C*-hexose. The observation of an ion at *m*/*z* 431 ($[(M - H) - 42 - 132]^-$) might indicate that the acetyl moiety is attached to the pentose. Further losses of −120 mu and −90 mu from that ion suggested that the hexose was 6-*C*-attached. Thus, the peak was tentatively assigned as apigenin 2″-*O*-acetylpentosyl-6-*C*-hexoside.

Peaks 1 and 10 would also derive from apigenin owing to the presence of the fragments at *m*/*z* 311 and 341. In the case of peak 1 ($[M - H]^-$ at *m*/*z* 593) the observation of the ions at *m*/*z* 473 and 353 from two consecutive losses of −120 mu would point to a di-*C*-hexosyl derivative, so that it could be tentatively associated with apigenin 6-*C*-hexoside-8-*C*-hexoside.

Peak 10 ($[M - H]^-$ at *m*/*z* 769) had a mass 176 mu greater than apigenin di-hexoside suggesting acylation with ferulic acid, which is coherent with its delayed elution. The cleavage of the feruloyl residue yielded the ion at *m*/*z* 593, which would give rise to the formation of the abundant ion at *m*/*z* 413 by loss of a fragment of 180 mu, which, according to Ferreres *et al.*,[29] would be characteristic of an *O*,*C*-dihexoside. On the other hand, the loss of −120 mu to produce the ion at *m*/*z* 649 confirmed the existence of a hexose *C*-attached to the aglycone, and also that the feruloyl residue was linked to the second *O*-attached hexose. In similarity with the other apigenin *O*,*C*-diglycosides observed in the sample, a 6-*C* attachment might be supposed. Thus, peak 10 was tentatively assigned as apigenin 2″-*O*-feruloylhexosyl-6-*C*-hexoside.

The fragmentation pattern of peak 5 ($[M - H]^-$ at *m*/*z* 769) would also be coherent with a structure similar to peak 10 although having luteolin as the aglycone. Thus, the losses of −120 mu (ion at *m*/*z* 489) and −176 mu (ion at *m*/*z* 609), and further −180 mu (ion at *m*/*z* 429) would point to a *O*,*C*-dihexoside. The fragment at *m*/*z* 489 (loss of −120 mu from the ion at *m*/*z* 609) would confirm the presence of the *C*-attached hexose, and the ion at *m*/*z* 339 (loss of −90 mu from the ion at *m*/*z* 429) would suggest a 6 C attachment. Thus, the peak was tentatively identified as luteolin 2″-*O*-feruloylhexosyl-6-*C*-hexoside.

A pseudomolecular ion of peak 9 ($[M - H]^-$ at *m*/*z* 799) was 14 mu greater than peak 5 and showed a similar fragmentation pattern, with characteristic product ions resulting from the losses of fragments of 120 mu (ion at *m*/*z* 679), 176 mu (ion at *m*/*z* 623), 176 + 180 mu (ion at *m*/*z* 609) and 176 + 180 + 120 mu (ion at *m*/*z* 323). The observation of fragments at *m*/*z* 371 (aglycone + 71 mu) and 323 (aglycone + 41–18 mu) would support the presence of methyl-luteolin as the aglycone.[29] Therefore, the compound might be tentatively assigned as methyl-luteolin 2″-*O*-feruloylhexosyl-*C*-hexoside.

Finally, peak 8 presented a pseudomolecular ion $[M - H]^-$ at *m*/*z* 635 and fragment ions at *m*/*z* 593 (−42 mu), 515 (−120 mu), indicating the presence of an acetyl residue and a *C*-attached hexose. The abundant ion at *m*/*z* 443 by loss of a fragment of 150 mu from the ion at *m*/*z* 515 would indicate the presence of an *O*-attached pentose on the *C*-glycosylating hexose.[29] The fragment at *m*/*z* 461 would result from the loss of the pentosyl residue (−132 mu) from the ion at *m*/*z* 515, and the ions at *m*/*z* 371 and 323 would also support methyl-luteolin as the aglycone. Thus, the compound was tentatively identified as methyl-luteolin 2″-*O*-acetylpentosyl-*C*-hexoside.

Methyl-luteolin 2″-*O*-feruloylhexosyl-*C*-hexoside was the main flavone found (450.26 mg per 100 g dw) in *A. montana* (Table 3), the total amount of flavones being 1204.63 mg per 100 g dw. As far as we know, there are no data regarding the phenolic composition in this plant, and thus these values cannot be compared to the literature. It should also be highlighted that little is known about phenolic compound bioactive forms *in vivo* (achievable concentrations in the circulation after ingestion as well as the possibility of metabolism) and the mechanisms by which they may contribute toward disease prevention.[32]

Composition in lipophilic compounds

The results of lipophilic compounds (fatty acids and tocopherols) are shown in Table 4. Up to 28 fatty acids were identified

Table 4 Chemical composition in lipophilic compounds of wild *Arenaria montana*[a]

Fatty acids	Relative percentage	Fatty acids	Relative percentage
C6:0	1.32 ± 0.01	C18:3n3	15.94 ± 0.14
C8:0	0.30 ± 0.01	C20:0	3.84 ± 0.67
C10:0	0.12 ± 0.03	C20:1	0.52 ± 0.29
C12:0	0.66 ± 0.21	C20:2	0.70 ± 0.09
C13:0	0.15 ± 0.00	C20:3n6	1.99 ± 0.04
C14:0	1.37 ± 0.27	C20:4n6	1.75 ± 0.06
C14:1	0.55 ± 0.04	C20:3n3 + C21:0	0.50 ± 0.00
C15:0	0.93 ± 0.17	C20:5n3	0.31 ± 0.08
C15:1	0.09 ± 0.00	C22:0	3.58 ± 0.23
C16:0	22.18 ± 0.40	C22:1n9	0.08 ± 0.00
C16:1	0.36 ± 0.18	C23:0	0.20 ± 0.06
C17:0	0.68 ± 0.03	C24:0	3.45 ± 0.46
C18:0	4.38 ± 0.10	SFA	43.16 ± 0.38
C18:1n9	8.57 ± 0.28	MUFA	10.16 ± 0.43
C18:2n6	23.39 ± 0.66	PUFA	46.68 ± 0.82
C18:3n6	2.11 ± 0.02		
Tocopherols	mg per 100 g dw		
α-Tocopherol	1.22 ± 0.21		
γ-Tocopherol	0.23 ± 0.02		
δ-Tocopherol	0.84 ± 0.08		
Total tocopherols	2.29 ± 0.31		

[a] dw – dry weight; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.

and quantified. Polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). Linoleic (C18:2n6) acid was the major fatty acid and contributes to the high levels of PUFA observed. The interest of linoleic acid has increased over time, since it is an essential fatty acid for human development and plays an active role in good general health.[33] Among others, it has been shown to have a role on the prevention of cancer diseases.[34] PUFA, besides being endogenous mediators of cell signaling and being involved in regulating gene expression, are also precursors of eicosanoids, such as prostaglandins and leukotrienes, as well as docosanoids as protectins or resolvins.[33]

α-Tocopherol was the most abundant tocopherol in *A. montana*, which is also found in the isoforms γ- and δ-; Table 4. Tocopherols are very important natural antioxidants and can be used to delay rancidity in fatty materials in manufactured foods; they may also reduce the effects of aging and help to prevent oxidative stress-related diseases such as cancer, neurodegenerative and heart diseases.[35,36]

Conclusion

In summary, bioactive phytochemicals such as phenolic compounds and tocopherols were identified and quantified in *A. montana*, as also omega-3 and omega-6 families, constituting another important class of phytochemicals due to their generalised beneficial health effects. The aqueous extract revealed higher antioxidant and cytotoxic activities than the methanolic extract. Therefore, the aqueous extract of *A. montana* may be used as a functional food, due to the high antioxidant activity, and as a nutraceutical, by presenting bioactive compounds, such as flavones and tocopherols, that can be used as cytotoxic agents. Moreover, this study supports the documented medicinal effect of this species and opens up the possibilities of food and pharmaceutical applications.

Competing interests

The authors declare no competing financial interest.

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Annexes 9

Electron beam and gamma irradiation as feasible conservation technologies for wild *Arenaria montana* L.: effects on chemical and antioxidant parametrs

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Electron beam and gamma irradiation as feasible conservation technologies for wild *Arenaria montana* L.: Effects on chemical and antioxidant parameters

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ABSTRACT

Wild plants are widely recognized as high-potential sources of several bioactive compounds. Nevertheless, these natural matrices require effective decontamination steps before they might be considered for different industrial purposes. Irradiation techniques are being progressively acknowledged as feasible conservation methodologies, either for their high decontamination effectiveness, as well as for their innocuousness on most chemical and bioactive parameters of the material to be treated. *Arenaria montana* L. (Caryophyllaceae) is recognized for its phytochemical richness, having a relevant geographical distribution in the Southern Europe. Herein the effects of irradiation (gamma and electron beam up to 10 kGy) were evaluated by comparing the nutritional, chemical and antioxidant profiles in *A. montana* extracts. In general, the assayed parameters showed statistically significant variations in response to irradiation treatment. Furthermore, the performed LDA allowed identifying the antioxidant indicators as the most affected parameters in irradiated samples, especially when using the 10 kGy dose and e-beam irradiation.

Industrial relevance: Wild plants are recognized as high-potential sources of several bioactive compounds. Nevertheless, they require effective decontamination steps before being considered for different industrial purposes. Irradiation techniques are being progressively acknowledged as feasible decontamination methodologies, but several options are available. The present study, using *Arenaria montana* as a case-study, reports important clues for choosing a specific irradiation type or dose according to the need of maintain a specific chemical or bioactive profile.

1. Introduction

Traditional medicine is world-widely used for several disorders and is based on natural products with specific physiological actions on the human body (Adebayo, Dzoyem, Shai, & Eloff, 2015; Ibrahim, Mohammed, Isah, & Aliyu, 2014). The rich composition of plants in tocopherols, alkaloids, tannins, flavonoids and other phenolic compounds, terpenoids and saponins makes them effective and beneficial on lipid metabolism, stimulating digestion, acting as anti-diabetics, and also as antioxidant and anti-inflammatory agents (Skotti, Anastasaki, Kanellou, Polissiou, & Tarantilis, 2014; Rawat, Bhatt, & Rawal, 2011).

The growing demands for natural sources of bioactive compounds have stimulated various studies with the purpose of discover new pharmacological compounds with lower toxicity (Haleem, Salem, Fatahallah, & Abdelfattah, 2014; Lubbe & Verpoorte, 2011). However, the fact that the pharmaceutical industry is very strict regarding microbiological quality of raw materials makes necessary the application of decontamination techniques (Haleem et al., 2014; Katušin-Ražem, Novak, & Ražema, 2001).

Irradiation is a methodology accredited for dry ingredients and can be performed using various radiation sources (*e.g.*, gamma rays, electron beam and X-rays) and doses, in accordance with the objectives to be achieved. This technique is increasingly recognized throughout the world and is characterized as eliminating or being reducing microorganisms, parasites and pests without causing any change (chemical or organoleptic) in food, being safe for the consumer and also allowing a reduction of the use of chemical fumigants (Jung et al., 2015; Owczarczyk, Migdal, & Kędzia, 2000; Roberts, 2014; Shim et al., 2009; Supriya, Sridhar, & Ganesh, 2014; Van Calenberg et al., 1998).

Electron beam irradiation is used mainly for food products with low density and the equipment can be easily connected/disconnected. Otherwise, gamma irradiation is mainly used for large volumes (Fernandes et al., 2014; Van Calenberg et al., 1998).

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Arenaria montana L. is a flowering plant belonging to the Caryophyllaceae family, native to mountainous regions of southwestern Europe. It is used in the Portuguese traditional medicine, acting therapeutically as an anti-inflammatory and diuretic, being mainly ingested in the form of infusion, prepared from the leaves, stems and flowers (Timité et al., 2011; Carvalho, & Morales, 2013). Moreover, we previously described its antioxidant potential and richness in bioactive phytochemicals (Pereira et al., 2014).

In the present study, the objective was to evaluate the effects of irradiation (gamma and electron beam) at different doses (1 and 10 kGy) in nutritional, chemical and antioxidant parameters of *A. montana*.

2. Materials and methods

2.1. Samples and samples irradiation

Arenaria montana L. (Caryophyllaceae) flowers and leafy stems (approximately the upper 15 cm of the dense clumps produced in Spring) are commonly gathered in the wild regions of Northeastern Portugal. These plant materials are usually dried and kept in dark, to be prepared in infusion and used as homemade remedies. Considering the availability and local consumers' criteria for its medicinal use, *A. montana* was collected in full bloom, in Spring along paths through the oak forest, in Oleiros, Bragança, Portugal. The sample for analysis was made by putting together the vegetative material from different plants randomly selected. A voucher specimen was deposited at the Herbarium of the School of Agriculture of Bragança, Portugal. Then, the sampled material was divided for gamma and for e-beam irradiation, in control (non-irradiated, 0 kGy), group 1 (1 kGy) and group 2 (10 kGy).

2.1.1. Gamma irradiation

The irradiation was performed in a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with total activity 177 TBq (4.78 kCi), in September 2013, and the estimated dose rate for the irradiation position was obtained with the Fricke dosimeter. During irradiation process, the dose was estimated using Amber Perspex routine dosimeters (batch V, from Harwell Company, U.K.), following the procedure previously described by Pereira et al. (2015). The estimated doses were, respectively: 0.92 ± 0.01 kGy, 1.9 kGy h^{-1}, 1.1 for sample 1 and 8.97 ± 0.35 kGy, 1.2 for sample 2, both at a dose rate of 1.9 kGy $h^{-1}$and 1.2 dose uniformity ratio (D_{max}/D_{min}). For simplicity, in the text and tables we considered the values 0, 1 and 10 kGy, for the doses of non-irradiated and irradiated groups 1 and 2, respectively.

2.1.2. Electron beam irradiation

The irradiation was performed at the INCT-Institute of Nuclear Chemistry and Technology, in Warsaw, Poland. To estimate the dose during the irradiation process three types of dosimeters were used: a standard dosimeter, a graphite calorimeter, and two routine Gammachrome YR and Amber Perspex dosimeters, from Harwell Company (UK). The irradiation took place in an e-beam irradiator of 10 MeV of energy with pulse duration of 5.5 ms, pulse frequency of 440 Hz and average beam current of 1.1 mA; the scan width was 68 cm, the conveyer speed was settled to the range 20–100 cm/min and the scan frequency was 5 Hz. The absorbed dose for e-beam irradiated *A. montana* were, 0.83 and 10.09 kGy, for group 1 and group 2 respectively, measured with a maximum uncertainty of 20%. To read the Amber and Gammachrome YR dosimeters, spectrophotometric methods were used at 603 nm and at 530 nm, respectively, to estimate the dose from the value of absorbance according to a previous calibration curve. For the graphite calorimeter dosimeter the electrical resistance was read and converted in dose according to a calibrated curve, available at the facility and made during equipment routine calibrations.

2.2. Standards and reagents

2.2.1. For irradiation

To estimate the dose and dose rate for gamma irradiation it was used a chemical solution sensitive to ionizing radiation, Fricke dosimeter, prepared in the lab following the standards (ASTM, 1992) and during irradiations Amber Perspex routine dosimeters (batch V, from Harwell Dosimeters Ltd., Oxfordshire, UK) were used, previously calibrated against the standard dosimeter. To prepare the acid aqueous Fricke dosimeter solution the following reagents were used: ferrous ammonium sulphate(II) hexahydrate, sodium chloride and sulfuric acid, all purchased from Panreac S.A. (Barcelona, Spain) with purity PA (proanalysis), and water treated in a Milli-Q water purification system (Millipore, model A10, USA). For e-beam routine irradiation were used Gammachrome YR and Amber Perspex routine dosimeters (batch V, from Harwell Dosimeters Ltd., Oxfordshire, UK) and a graphite calorimeter as standard dosimeter.

2.2.2. For chemical analyses

Acetonitrile 99.9%, n-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA), as also were other individual fatty acid isomers, L-ascorbic acid, tocopherol, sugar and organic acid standards, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA).

2.3. Nutritional value

Protein, fat, carbohydrates and ash were determined following the AOAC procedures (AOAC, 1995). The samples crude protein content (N × 6.25) was estimated by the macro-Kjeldahl method; the crude fat was determined using a Soxhlet apparatus by extracting a known weight of sample with petroleum ether; the ash content was determined by incineration at 600 ± 15 °C, until a whitish ash was formed. Total carbohydrates were calculated by difference and total energy was calculated according to the following equations: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

2.4. Phytochemical composition in hydrophilic compounds

2.4.1. Sugars

Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by the authors (Pereira et al., 2015) using melezitose as internal standard (IS). The equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD, USA) and a RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (v/v) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.4.2. Organic acids

Organic acids were determined following a procedure previously described by the authors (Pereira et al., 2015). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu

Cooperation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 μm, 250 mm × 4.6 mm i.d) thermostatted at 35 °C. The elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL/min. Detection was carried out in a DAD, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 nm with calibration curves obtained from commercial standards of each compound. The results were expressed in g per 100 g dw.

2.5. Phytochemical composition in lipophilic compounds

2.5.1. Fatty acids

Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column as described previously by the authors (Pereira et al., 2015). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 °C) and a Macherey-Nagel (Duren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm ID × 0.25 μm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

2.5.2. Tocopherols

Tocopherols were determined following a procedure previously described by the authors (Pereira et al., 2015). Analysis was performed by HPLC (equipment described above), and a fluorescence detector (model FP-2020, Jasco International Co., Tokyo, Japan) programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 × 4.6 mm) normal-phase column (model YMC, Waters Corporation, Milford Massachusetts, USA) operating at 30 °C. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, v/v) at a flow rate of 1 mL/min, and the injection volume was 20 μL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the Internal Standard (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.6. Evaluation of antioxidant activity

2.6.1. Samples preparation

Two different extracts were prepared to evaluate their antioxidant activity.

The methanolic extract was obtained from the dried plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 °C at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper (Sigma-Aldrich, St. Louis, Missouri, USA). The residue was then extracted with 25 mL of methanol (25 °C at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 °C (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

The infusion was also obtained from the dried plant material. The sample (2 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure.

2.6.2. Antioxidant activity

DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc.; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: $[(A_{DPPH} - A_S) / A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2 h of (assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decreasing in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: [(A − B) / A] × 100%, where A and B were the absorbance of the control and the sample solution, respectively (Pereira et al., 2015).

2.7. Statistical analysis

Within each irradiation type and for each dose, three independent samples were analysed. Each of the samples was taken after pooling the plants treated in the same conditions together. Data were expressed as mean ± standard deviation. All statistical tests were performed at a 5% significance level using IBM SPSS Statistics for Windows, version 22.0. (IBM Corp., USA).

An analysis of variance (ANOVA), followed by Tukey's test (homoscedastic distributions) or Tamhane's T2 test (heteroscedastic distributions) was used to classify the statistical differences induced by the irradiation dose in each of the assayed parameters. The fulfillment of the one-way ANOVA requirements, specifically the normal distribution of the residuals and the homogeneity of variance, was tested by means of the Shapiro Wilk's and the Levene's tests, respectively.

Linear discriminant analysis (LDA) was used to identify the parameters undergoing the most significant changes for i) each irradiation dose and ii) each irradiation type. A stepwise technique was applied, based on the Wilks' Λ test with the usual probabilities of *F* (3.84 to enter and 2.71 to be removed) for variable selection. This procedure combines a series of forward selection and backward elimination steps, where the inclusion of any new variable is preceded by verifying the significance of all previously selected variables (Zielinski et al., 2014). In the present study, the purposes of the performed LDA were identifying the relationship between a single categorical dependent variable (irradiation dose or irradiation type) and the set of quantitative independent variables (studied parameters). With this method, it is possible to determine which of the independent variables contributed more for the differences in the average score profiles of *A. montana* samples submitted to each irradiation type and dose. To verify the significance of the canonical discriminating functions, Wilk's Λ test was used. A leaving-one-out cross validation procedure was carried out to assess the model performance.

3. Results and discussion

As previous consideration, it should be highlighted that there were no available reports (at the moment of preparation of this manuscript) on the chemical composition or antioxidant activity of *A. montana*, except for our work on samples gathered in a different year (Pereira et al., 2014), which described the cytotoxicity and phenolic composition of this species, but in non-irradiated samples.

3.1. Effects on chemical parameters

The proximate composition (in dry basis) of *A. montana* was dominated by the carbohydrates content, followed by ash, protein and fat levels (Table 1). Except for the fat content in gamma irradiated samples ($p = 0.051$), all the nutritional components showed significant changes in response to irradiation treatment, either for electron beam (e-beam), as well as in gamma irradiated samples. In the first case (e-beam), fat and protein contents decreased in irradiated samples, while ash, carbohydrates and energy values only showed slight variations. The effect produced by gamma irradiation on the nutritional parameters was similar to the one observed for electron beam irradiation, except for the already pointed out lack of variation in fat content and the less pronounced decrease observed in proteins.

Regarding the free sugars composition (Table 2), fructose was quantified as the main compound (4.2–4.7 g/100 g dw), followed by glucose (1.5–1.7 g/100 g dw), sucrose (0.4–1.0 g/100 g dw), trehalose (0.19–0.23 g/100 g dw) and raffinose (0.09–0.13 g/100 g dw). The detected profile and individual proportions are similar to those presented in a previous report (Pereira et al., 2014), despite the overall quantities detected in this work are slightly lower. A good result was obtained for the effect of irradiation treatment, since almost none of the characterized molecules suffer significant changes (except for sucrose with both irradiation types and total sugars when samples were treated with e-beam irradiation). This is particularly valuable because sugars are often pointed out as good indicators of an adequate conservation technology (Barreira, Pereira, Oliveira, & Ferreira, 2010).

Concerning the organic acids profile, oxalic acid was the prevalent form (2.2–2.6 g/100 g dw), followed by malic acid (0.9–1.1 g/100 g dw), succinic acid (0.5–0.7 g/100 g dw), citric acid (0.29–0.38 g/100 g dw), quinic acid and fumaric acid (which were detected below the limit of quantification); this result is also closely related to the mentioned previous report (Pereira et al., 2014). The dissimilarity observed among the effect produced by each type of irradiation is quite interesting. In fact, while e-beam irradiation did not cause statistically significant changes in any case, gamma irradiation produced exactly the opposite effect, *i.e.*, all the quantified organic acids presented significant changes, with a clear tendency to increase with irradiation. This result is in agreement with a previous study conducted to evaluate the effects of gamma irradiation and accelerated electrons on organic acids (Semelová, Čuba, John, & Múčka, 2008).

The individual fatty acids (FA) profiles are depicted in Table 4. Besides the presented FA, C6:0, C8:0, C10:0, C13:0, C16:1, C20:2, C20:3n3 + C21:0 and C23:0 were also detected, but their relative percentages laid below 0.5%. In general, the detected profile is highly similar to the one reported before (Pereira et al., 2014).

The main saturated fatty acid (SFA) was palmitic acid (22–26%), while oleic acid (10.1–13.4%) and α-linolenic acid (17.4–22.7%) were the predominant monounsaturated and polyunsaturated forms, respectively. Nearly half of the detected forms are polyunsaturated fatty acids (PUFA), among which ALA (α-linolenic acid) and GLA (γ-linolenic acid) deserve special attention. Despite the significant changes induced by both irradiation types in all FA, the effect of e-beam treatment was more pronounced. Considering that the results are presented in relative percentage, if some FA decrease significantly, others will, inevitably, increase. Nevertheless, when evaluated as grouped SFA, MUFA and PUFA, it became clear that e-beam treatment increased the relative percentages of SFA and MUFA, while reducing those of PUFA.

The tocopherol profile was similar to the previously characterized (Pereira et al., 2014), despite the higher quantities detected herein. α-Tocopherol was the main isoform, showing nearly 20-fold higher amounts than the remaining vitamers. In what concerns the main subject of this work, the irradiation treatment caused statistically significant changes in the tocopherols contents, especially in samples irradiated with 10 kGy. The significant effect of irradiation over the tocopherols content was previously reported (Pereira et al., 2015; Taipina, Lamardo, Rodas, & Mastro, 2009), being probably associated to their oxidative instability (Warner, Miller, & Demurin, 2008).

3.2. Effects on the antioxidant activity

Besides comparing the chemical parameters described in the previous section, the effects induced by gamma and e-beam irradiation on the antioxidant activity of *A. montana* were also compared in its aqueous and methanolic extracts. Four *in vitro* assays were applied: scavenging effects on DPPH radicals (measures the decrease in DPPH radical absorption after exposure to radical scavengers), reducing power (conversion of a Fe^{3+}/ferricyanide complex to Fe^{2+}), inhibition of β-carotene bleaching (measures the capacity to neutralize the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models) and thiobarbituric acid reactive substances (TBARS) formation inhibition. Likewise, an overall quantification of total phenols was also performed (Table 5). The methanolic

Table 1
Proximate composition of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

		Fat (g/100 g fw)	Protein (g/100 g dw)	Ash (g/100 g dw)	Carbohydrates (g/100 g dw)	Energy (kcal/100 g dw)
E-beam irradiation						
Dose	0 kGy	1.4 ± 0.1^{a}	4.9 ± 0.3^{a}	8.4 ± 0.3^{ab}	85.4 ± 0.3^{b}	373 ± 1^{a}
	1 kGy	1.2 ± 0.1^{b}	3.9 ± 0.3^{b}	8.2 ± 0.2^{b}	86.7 ± 0.4^{a}	373 ± 1^{a}
	10 kGy	1.2 ± 0.1^{b}	3.6 ± 0.2^{c}	8.6 ± 0.2^{a}	86.7 ± 0.4^{a}	372 ± 1^{b}
p-Values	Homoscedasticity[2]	0.451	0.891	0.111	0.231	0.058
	Normal distribution[3]	0.373	0.080	0.346	0.102	0.794
	1-way ANOVA[4]	<0.001	<0.001	0.007	<0.001	0.004
Gamma irradiation						
Dose	0 kGy	1.7 ± 0.1	4.4 ± 0.5^{b}	9.6 ± 0.2^{a}	84.3 ± 0.5^{b}	370 ± 1^{b}
	1 kGy	1.7 ± 0.1	5.1 ± 0.3^{a}	9.1 ± 0.2^{b}	84.1 ± 0.3^{b}	372 ± 1^{a}
	10 kGy	1.6 ± 0.1	3.8 ± 0.2^{c}	9.5 ± 0.2^{a}	85.1 ± 0.2^{a}	370 ± 1^{b}
p-Values	Homoscedasticity[2]	0.824	0.011	0.851	0.004	0.760
	Normal distribution[3]	0.448	0.020	0.621	0.106	0.148
	1-way ANOVA[4]	0.051	<0.001	<0.001	<0.001	<0.001

[1] The results are presented as the mean ± SD.
[2] Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[3] Normal distribution of the residuals was evaluated using Shapiro-Wilk test.
[4] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

Table 2
Hydrophilic compounds (free sugars and organic acids) composition (g/100 g dw) of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

		Free sugars						Oxalic acids				
		Fructose	Glucose	Sucrose	Trehalose	Raffinose	Total	Oxalic acid	Malic acid	Citric acid	Succinic acid	Total
E-beam irradiation												
Dose	0 kGy	4.5 ± 0.3	1.6 ± 0.1	0.6 ± 0.1^{b}	0.22 ± 0.03	0.11 ± 0.02	7.1 ± 0.3^{ab}	2.3 ± 0.1	0.9 ± 0.1	0.30 ± 0.05	0.5 ± 0.1	4.0 ± 0.3
	1 kGy	4.2 ± 0.3	1.5 ± 0.1	0.9 ± 0.1^{a}	0.19 ± 0.02	0.12 ± 0.01	6.9 ± 0.4^{b}	2.2 ± 0.2	0.9 ± 0.1	0.29 ± 0.04	0.5 ± 0.1	3.8 ± 0.2
	10 kGy	4.4 ± 0.4	1.7 ± 0.2	1.0 ± 0.1^{a}	0.21 ± 0.04	0.13 ± 0.02	7.3 ± 0.3^{a}	2.2 ± 0.2	0.9 ± 0.1	0.31 ± 0.05	0.5 ± 0.1	3.9 ± 0.3
p-Values	Homoscedasticity[2]	0.435	0.462	0.119	0.221	0.842	0.560	0.579	0.669	0.795	0.072	0.737
	Normal distribution[3]	0.123	0.712	0.150	0.206	0.818	0.390	0.762	0.688	0.360	0.377	0.852
	1-way ANOVA[4]	0.195	0.135	<0.001	0.217	0.082	0.034	0.182	0.369	0.743	0.988	0.278
Gamma irradiation												
Dose	0 kGy	4.7 ± 0.3	1.7 ± 0.1	0.6 ± 0.1^{b}	0.23 ± 0.05	0.09 ± 0.01	7.3 ± 0.4	2.3 ± 0.1^{b}	0.9 ± 0.1^{b}	0.30 ± 0.04^{b}	0.5 ± 0.1^{b}	4.0 ± 0.3^{b}
	1 kGy	4.5 ± 0.3	1.7 ± 0.2	0.4 ± 0.1^{c}	0.20 ± 0.03	0.10 ± 0.02	7.0 ± 0.5	2.6 ± 0.1^{a}	1.1 ± 0.1^{a}	0.37 ± 0.05^{a}	0.7 ± 0.1^{a}	4.8 ± 0.3^{a}
	10 kGy	4.3 ± 0.3	1.6 ± 0.2	0.9 ± 0.1^{a}	0.21 ± 0.02	0.09 ± 0.02	7.2 ± 0.4	2.6 ± 0.2^{a}	1.0 ± 0.1^{a}	0.38 ± 0.04^{a}	0.6 ± 0.1^{a}	4.6 ± 0.3^{a}
p-Values	Homoscedasticity[2]	0.993	0.685	0.202	0.086	0.510	0.514	0.557	0.737	0.700	0.526	0.987
	Normal distribution[3]	0.331	0.445	0.069	0.711	0.044	0.747	0.587	0.657	0.404	0.574	0.800
	1-way ANOVA[4]	0.157	0.105	<0.001	0.102	0.337	0.198	<0.001	0.002	0.001	<0.001	<0.001

[1] The results are presented as the mean ± SD.
[2] Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[3] Normal distribution of the residuals was evaluated using Shapiro-Wilk test.
[4] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

extracts showed higher (approximately threefold) antioxidant activity than the corresponding infusions in all performed assays. In agreement with these results, the quantities of phenolic compounds were nearly threefold lower in the infusions. Furthermore, *A. montana* extracts were particularly active as inhibitors of TBARS formation, as indicated by the lowest EC_{50} values detected in this case.

Independently of irradiation type, the antioxidant activity decreased in the infusions along the irradiation treatment, in line with the observed among the methanolic extracts when treated with gamma irradiation. Nevertheless, the effect observed in the methanolic extracts submitted to e-beam irradiation showed a general tendency to increased antioxidant activity, except for the β-carotene bleaching inhibition.

Table 3
Fatty acids profile (relative percentage) of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

	E-beam irradiation			*p*-Values			Gamma irradiation			*p*-Values		
	0 kGy	1 kGy	10 kGy	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]	0 kGy	1 kGy	10 kGy	Homoscedasticity[2]	Normal distribution[3]	1-way ANOVA[4]
C12:0	0.9 ± 0.1^{b}	0.7 ± 0.1^{c}	1.0 ± 0.1^{a}	0.050	0.435	<0.001	1.0 ± 0.1^{b}	1.3 ± 0.1^{a}	1.0 ± 0.1^{b}	0.219	0.809	<0.001
C14:0	1.4 ± 0.1^{b}	1.3 ± 0.1^{b}	2.8 ± 0.2^{a}	0.496	<0.001	<0.001	1.9 ± 0.1^{a}	1.5 ± 0.1^{b}	1.6 ± 0.2^{b}	0.636	0.661	<0.001
C15:0	0.9 ± 0.1^{b}	0.8 ± 0.1^{b}	1.0 ± 0.1^{a}	0.740	0.142	<0.001	1.0 ± 0.1^{a}	0.7 ± 0.1^{b}	0.8 ± 0.1^{b}	0.680	0.101	<0.001
C16:0	26 ± 1^{a}	24 ± 1^{b}	23 ± 1^{b}	0.410	0.344	<0.001	22 ± 1^{b}	23 ± 1^{ab}	24 ± 1^{a}	0.576	0.670	0.001
C17:0	1.1 ± 0.1^{b}	1.3 ± 0.1^{a}	1.3 ± 0.1^{a}	0.316	0.377	<0.001	1.2 ± 0.1^{a}	1.1 ± 0.1^{b}	1.3 ± 0.1^{a}	0.278	0.312	0.001
C18:0	5.0 ± 0.3^{c}	5.7 ± 0.2^{b}	6.6 ± 0.3^{a}	0.210	0.185	<0.001	6.0 ± 0.2^{a}	5.5 ± 0.2^{b}	5.6 ± 0.3^{b}	0.654	0.624	<0.001
C18:1n9	10.1 ± 0.4^{b}	13.4 ± 0.3^{a}	13.2 ± 0.5^{a}	0.563	<0.001	<0.001	12.2 ± 0.3^{a}	11.4 ± 0.2^{b}	10.7 ± 0.3^{c}	0.518	0.287	<0.001
C18:2n6	17.2 ± 0.5^{a}	17.5 ± 0.4^{a}	14.7 ± 0.4^{b}	0.627	0.001	<0.001	14.8 ± 0.4^{b}	16.6 ± 0.3^{a}	16.3 ± 0.4^{a}	0.496	0.011	<0.001
C18:3n6	2.7 ± 0.1^{a}	2.8 ± 0.2^{a}	2.4 ± 0.2^{b}	0.389	0.631	<0.001	2.6 ± 0.2^{b}	3.1 ± 0.2^{a}	2.8 ± 0.3^{b}	0.765	0.593	0.002
C18:3n3	21.6 ± 0.5^{a}	19.6 ± 0.5^{b}	17.4 ± 0.5^{c}	0.998	0.107	<0.001	22.0 ± 0.4^{b}	22.4 ± 0.4^{ab}	22.7 ± 0.3^{a}	0.504	0.331	0.002
C20:0	2.2 ± 0.1^{b}	2.3 ± 0.2^{b}	2.5 ± 0.2^{a}	0.110	0.776	0.001	2.0 ± 0.1^{a}	1.7 ± 0.2^{b}	1.9 ± 0.2^{a}	0.453	0.471	0.001
C20:1	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.004	0.206	0.218	0.6 ± 0.1^{b}	0.8 ± 0.1^{a}	0.6 ± 0.1^{b}	0.579	0.599	<0.001
C20:3n6	1.4 ± 0.1^{a}	1.1 ± 0.1^{b}	1.1 ± 0.1^{b}	0.177	0.335	<0.001	1.5 ± 0.1^{a}	1.4 ± 0.2^{a}	1.2 ± 0.2^{b}	0.466	0.170	0.002
C20:4n6	1.9 ± 0.1^{b}	1.9 ± 0.1^{b}	2.9 ± 0.2^{a}	0.004	<0.001	<0.001	3.6 ± 0.2^{a}	2.6 ± 0.2^{b}	2.4 ± 0.2^{b}	0.740	0.002	<0.001
C20:5n3	0.8 ± 0.1^{b}	0.5 ± 0.1^{c}	1.1 ± 0.1^{a}	0.002	0.008	<0.001	0.7 ± 0.1^{a}	0.6 ± 0.1^{b}	0.5 ± 0.1^{c}	0.089	0.114	<0.001
C22:0	3.2 ± 0.2^{a}	2.9 ± 0.1^{b}	2.9 ± 0.2^{b}	0.038	0.210	<0.001	3.1 ± 0.3^{a}	1.9 ± 0.2^{c}	2.4 ± 0.3^{b}	0.309	0.195	<0.001
C24:0	1.3 ± 0.1^{b}	1.4 ± 0.1^{b}	1.6 ± 0.2^{a}	<0.001	0.020	<0.001	1.7 ± 0.1^{a}	1.2 ± 0.2^{c}	1.4 ± 0.1^{b}	0.121	0.962	<0.001
SFA	42.7 ± 0.5^{b}	41.8 ± 0.5^{c}	45.4 ± 0.5^{a}	0.881	0.017	<0.001	41 ± 1^{a}	39 ± 1^{b}	41 ± 1^{a}	0.358	0.583	0.001
MUFA	10.9 ± 0.3^{b}	14.2 ± 0.2^{a}	14.0 ± 0.5^{a}	0.193	<0.001	<0.001	13.1 ± 0.3^{a}	13.0 ± 0.3^{a}	12.0 ± 0.2^{b}	0.467	0.014	<0.001
PUFA	46.4 ± 0.5^{a}	44.0 ± 0.5^{b}	40.6 ± 0.5^{c}	0.709	0.030	<0.001	46 ± 1^{b}	48 ± 1^{a}	47 ± 1^{ab}	0.107	0.330	0.002

[1] The results are presented as the mean ± SD.
[2] Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[3] Normal distribution of the residuals was evaluated using Shapiro-Wilk test.
[4] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

Table 4
Tocopherols composition (mg/100 g dw) of *Arenaria montana* submitted do different doses of electron-beam or gamma irradiation.[1]

		α-Tocopherol	γ-Tocopherol	δ-Tocopherol	Tocopherols
E-beam irradiation					
Dose	0 kGy	4.6 ± 0.3^{a}	0.26 ± 0.03^{a}	0.29 ± 0.03^{a}	5.2 ± 0.3^{a}
	1 kGy	3.9 ± 0.2^{b}	0.23 ± 0.03^{ab}	0.28 ± 0.03^{ab}	4.4 ± 0.3^{b}
	10 kGy	3.7 ± 0.3^{b}	0.21 ± 0.04^{b}	0.25 ± 0.03^{b}	4.2 ± 0.3^{b}
p-Values	Homoscedasticity[2]	0.375	0.374	0.895	0.595
	Normal distribution[3]	0.123	0.138	0.247	0.231
	1-way ANOVA[4]	<0.001	0.040	0.029	<0.001
Gamma irradiation					
Dose	0 kGy	4.8 ± 0.2^{a}	0.28 ± 0.03^{a}	0.16 ± 0.02^{ab}	5.3 ± 0.2^{a}
	1 kGy	4.8 ± 0.1^{a}	0.29 ± 0.03^{a}	0.15 ± 0.01^{b}	5.2 ± 0.2^{a}
	10 kGy	3.5 ± 0.2^{b}	0.20 ± 0.03^{b}	0.19 ± 0.03^{a}	3.8 ± 0.2^{b}
p-Values	Homoscedasticity[2]	0.435	0.691	0.005	0.342
	Normal distribution[3]	<0.001	0.526	0.120	<0.001
	1-way ANOVA[4]	<0.001	<0.001	0.004	<0.001

[1] The results are presented as the mean ± SD.
[2] Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[3] Normal distribution of the residuals was evaluated using Shapiro-Wilk test.
[4] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one irradiation dose differs from the others (in this case multiple comparison tests were performed). For each species, means within a column with different letters differ significantly ($p < 0.05$).

3.3. Linear discriminant analysis

As indicated by the results presented in Tables 1–5, most of the characterized parameters (with the exceptions of the majority of free sugars and organic acids) showed statistically significant differences when submitted to gamma or e-beam irradiation treatment. To take this comparative study a step further, the overall significance of the detected differences was also evaluated by verifying which statistically significant differences maintain their relevance when compared globally. Accordingly, the results were evaluated simultaneously by applying two sequential LDA: initially the results were grouped according to irradiation type, while in the second case the grouping criterion was based on the irradiation dose. The significant independent variables were selected using the stepwise procedure of the LDA, according to the Wilks' λ test, which maintains only those with a statistically significant ($p < 0.05$) classification ability.

The two discriminant functions plotted in Fig. 1A, included 100.0% of the observed variance (first: 80.0%, second: 20.0%). As an initial result, the reduction in the variables number was noteworthy. From the initial 58 parameters, only 11 (fat, carbohydrates, raffinose, C6:0, C20:4n6, C20:5n3, C24:0, reducing power in infusions, TBARS formation inhibition and β-carotene bleaching inhibition in methanolic extracts, phenols content in infusions) were selected as having discriminant ability. Concerning the correlation between the selected discriminating variables and the canonical discriminant functions, function 1 was more correlated with TBARS formation inhibition in methanolic extracts (which present higher EC_{50} values in gamma irradiated samples) and fat (higher values in gamma irradiated samples), separating mostly gamma irradiated samples from the remaining groups. Function 2, in turn, was more correlated to the reducing power (lower in e-beam irradiated samples) and phenolic content in the *A. montana* infusions (lower in e-beam irradiated samples), clearly separating the markers

Table 5
Antioxidant properties of extracts from *Arenaria montana* submitted to electron beam or gamma irradiation (GI).[1] EC_{50} values (μg/mL) are presented for all assays except phenols, expressed as mg GAE/g extract. The results are presented as the mean ± SD.

		DPPH scavenging activity		Reducing power		β-carotene bleaching inhibition		TBARS formation inhibition		Phenols	
		Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH	Infusion	MeOH
Electron beam											
	0 kGy	3532 ± 175^{b}	988 ± 20^{a}	1592 ± 15^{c}	528 ± 3^{a}	3658 ± 120^{c}	1772 ± 52^{b}	310 ± 4^{c}	97 ± 2^{a}	40 ± 1^{a}	102 ± 5^{c}
	1 kGy	3998 ± 147^{a}	813 ± 80^{b}	1816 ± 12^{b}	509 ± 4^{b}	4230 ± 227^{b}	1450 ± 131^{c}	365 ± 35^{b}	60 ± 2^{c}	35 ± 1^{b}	109 ± 1^{b}
	10 kGy	3945 ± 338^{a}	631 ± 34^{c}	1954 ± 8^{a}	441 ± 3^{c}	7210 ± 517^{a}	1906 ± 70^{a}	427 ± 29^{a}	72 ± 2^{b}	33 ± 1^{c}	119 ± 1^{a}
p-Values	Homoscedasticity[1]	0.011	<0.001	0.198	0.604	<0.001	0.001	0.003	0.684	0.113	<0.001
	Normal distribution[2]	0.750	0.003	0.001	<0.001	<0.001	0.054	0.003	0.001	0.001	0.008
	1-way ANOVA[3]	0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Gamma irradiation											
	0 kGy	3475 ± 228^{b}	972 ± 55^{b}	1613 ± 12^{b}	520 ± 8^{a}	2895 ± 173^{b}	665 ± 9^{c}	487 ± 41^{b}	164 ± 3^{c}	37 ± 1^{a}	116 ± 2^{c}
	1 kGy	3619 ± 109^{b}	958 ± 26^{b}	1619 ± 18^{b}	498 ± 13^{b}	4489 ± 325^{a}	875 ± 18^{b}	579 ± 22^{a}	230 ± 2^{b}	37 ± 1^{a}	122 ± 1^{a}
	10 kGy	3942 ± 86^{a}	1962 ± 160^{a}	1709 ± 18^{a}	507 ± 2^{b}	4324 ± 144^{a}	1134 ± 21^{a}	633 ± 107^{a}	245 ± 3^{a}	35 ± 1^{b}	119 ± 1^{b}
p-Values	Homoscedasticity[1]	<0.001	<0.001	0.420	0.003	0.052	0.012	0.003	0.013	<0.001	0.001
	Normal distribution[2]	0.043	<0.001	0.002	0.102	0.001	0.001	0.027	<0.001	0.008	0.014
	1-way ANOVA[3]	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

MeOH - methanol; GAE - gallic acid equivalents.
[1] Homoscedasticity among irradiation doses was tested by the Levene test: homoscedasticity, $p > 0.05$; heteroscedasticity, $p < 0.05$.
[2] Normal distribution of the residuals was evaluated using Shapiro-Wilk test.
[3] $p < 0.05$ indicates that the mean value of the evaluated parameter of at least one dose differs from the others. For each species, means within a column with different letters differ significantly ($p < 0.05$).

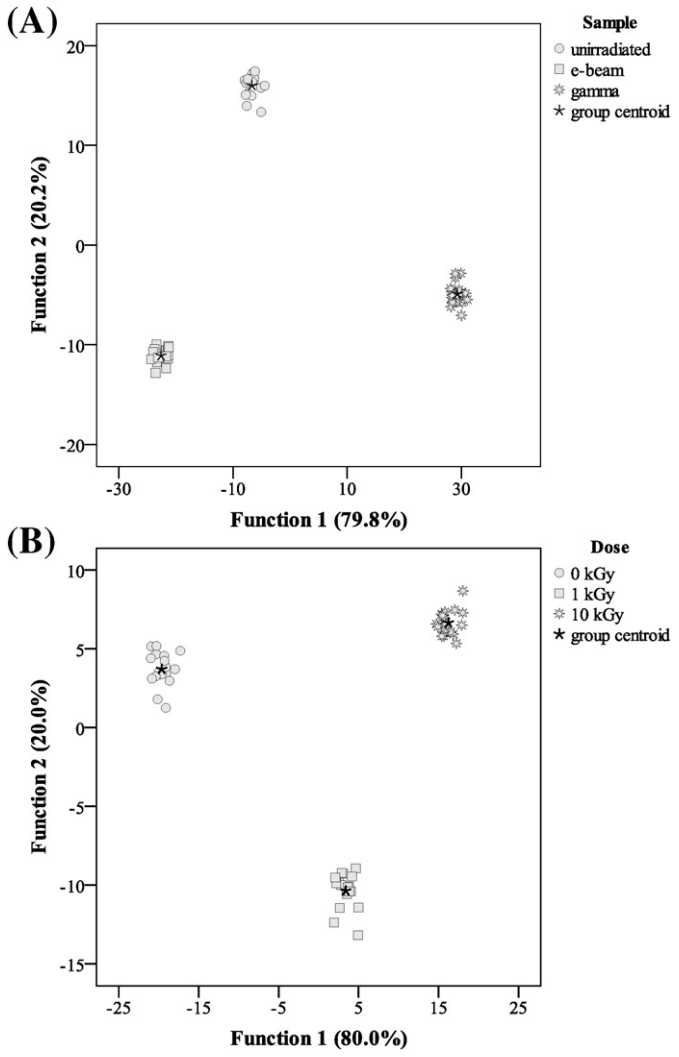

Fig. 1. Canonical discriminant functions coefficients defined from the evaluated parameters plotted to highlight differences among irradiation technologies (A) and irradiation doses (B).

corresponding to e-beam irradiated samples from those belonging to unirradiated ones. The classification performance was 100% accurate, either for the originally grouped cases, as well as for the cross-validated cases.

When a similar assay was conducted to evaluate the variables undergoing the most relevant changes once submitted to different irradiation doses (independently of being generated by gamma rays or accelerated electrons), the two discriminant functions (Fig. 1B) also included 100.0% of the observed variance (first: 78.8%, second: 20.2%).

The reduction in the variables number was significant again, indicating carbohydrates, C6:0, C8:0, C20:4n6, C20:5n3, SFA, γ-tocopherol, reducing power EC_{50} values in infusions, DPPH scavenging activity, reducing power, β-carotene bleaching inhibition and TBARS formation inhibition in methanolic extracts and phenols content in the infusions as the variables with the highest changes. Function 1 was highly correlated with reducing power (which present higher EC_{50} values for the 10 kGy dose) and phenolic content (lower in samples irradiated with 10 kGy), clearly separating the markers corresponding to the 10 kGy dose. Function 2, on the other hand, was more correlated to C6:0 (higher in samples irradiated with 1 kGy) and SFA (lower in samples with 1 kGy), particularly contributing to separate the markers corresponding to samples irradiated with 1 kGy (independently of irradiation source). The classification performance was 100% accurate, either for the originally grouped cases, as well as for the cross-validated cases.

4. Conclusions

Most of the assayed parameters (except for the majority of sugars and organic acids) showed statistically significant variations in response to irradiation treatment. Nevertheless, the performed LDA allowed defining which of the studied parameters were mostly affected by gamma or e-beam irradiation, as well as by using 1 or 10 kGy. In fact, the antioxidant parameters proved to be the ones suffering the most significant changes, especially when using the 10 kGy dose and e-beam irradiation. In general, the obtained results might be a good guidance to choose irradiation type or dose according to the need of maintain a specific chemical or bioactive profile.

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Annexes 10

VNiVERSiDAD
D SALAMANCA

FACULTAD DE FARMACIA

DEPARTAMENTO DE QUÍMICA ANALÍTICA, NUTRICIÓN Y BROMATOLOGÍA

Evaluación de los efectos de la irradiación en la composición química y bioactividad de ~~las~~ plantas utilizadas en la industria farmacéutica o alimentaria

TESIS DOCTORAL

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Dr. Albino António Bento

ÍNDICE

1.1 Introducción

1.1.1 Una breve introducción a la irradiación de alimentos

Hoy en día es esencial que los alimentos lleguen al consumidor cumpliendo todas las normas de seguridad a fin de prevenir riesgos para la salud. Otra preocupación es mantener una alta calidad en los alimentos no sólo en su temporada de cosecha o después de su transformación, sino también por períodos prolongados de tiempo. Los procesos utilizados para descontaminar y preservar alimentos deben no sólo eliminar insectos, microorganismos y toxinas con el fin de garantizar la seguridad alimentaria, sino también respetar al máximo posible el aspecto y las características organolépticas originales, así como evitar la presencia de residuos químicos que puedan derivar de los mismos y minimizar el impacto ambiental (Migdal and Owczarczyk, 1998). De ahí que en las muestras sometidas a los tratamientos de descontaminación es importante verificar el mantenimiento de nutrientes y otros componentes importantes, tales como ácidos grasos, tocoferoles, ácidos orgánicos o azúcares, además de asegurar que las características físicas se mantienen sin cambios. Del mismo modo, las propiedades bioactivas de los productos finales deben al menos mantener la eficacia de los productos originales (Nagy et al., 2011).

Una de las técnicas de descontaminación que pueden ser utilizadas en el caso de las plantas medicinales y alimenticias es la irradiación. Este método, además de ser recomendado para varios tipos de alimentos, y especialmente productos desecados, reduce la dependencia de sustancias químicas fitosanitarias, que pueden quedar como residuos en las plantas y llegar a causar efectos indesables tanto en los seres humanos como los ecosistemas (Migdal and Owczarczyk, 1998; Chmielewski and Migdal, 2005). También se caracteriza por su eficiencia para asegurar la vida útil dureante el almacenamiento, limitar las pérdidas causadas por los procesos fisiológicos naturales (brotación, maduración y envejecimiento), y eliminar o reducir microorganismos, parásitos y plagas sin causar cambios significativos en las características químicas u organolépticas (Byun, et al., 1999; Molins, 2001; Villavicencio, et al., 2007; Wen et al., 2010; Nagy et al., 2011).

La irradiación es una técnica de procesado no térmico, también denominada "pasteurización en frío", ya que no aumenta significativamente la temperatura de los productos sometidos a la misma. De esta manera, esta tecnología está indicada para componentes de los alimentos que sean particularmente sensibles a tratamientos térmicos, como pueden ser los compuestos volátiles y aromáticos en plantas medicinales o comestibles (Alothman et al., 2009).

A pesar de que el fundamento y repercusiones de la irradiación son a menudo mal entendidos por la mayoría de los consumidores, se trata de un proceso seguro que expone el producto (pre-embalado o sin embalar) a una dosis predeterminada de radiación de acuerdo con el tipo de alimento a ser tratado, ya sea de origen vegetal (como hortalizas, frutas y cereales) o animal (carnes y pescados) o productos derivados (Sádecká, 2007; Nagy et al., 2011; Kanatt et al., 2015). Se trata, en definitiva, de una técnica versátil, segura y altamente eficaz, que satisface plenamente el objetivo de proporcionar mayor estabilidad y seguridad a los alimentos, manteniendo sus propiedades nutritivas y características saludables y asegurando períodos de almacenamiento más largos (Hunter, 2000; Roberts, 2014).

1.1.2 Radiación gamma y haces de electrones - Diferencias, aplicaciones y detección

Hay tres tipos de radiaciones ionizantes permitidas para el procesado de alimentos, la radiación gamma, haces de electrones y rayos X, teniendo cada uno diferentes características (EU, 1999; Kim et al., 2009b; Jung et al., 2015). La radiación gamma es el tipo de radiación más energética dentro del espectro electromagnético, y está constituida por fotones de logitud de onda muy corta, lo que permite que penetren profundamente en el alimento. Los rayos gamma utilizados en la industria alimentaria provienen de la descomposición espontánea de elementos radiactivos, siendo las fuentes más utilizadas los isótopos ^{60}Co y ^{137}Cs, con períodos de semidesintegración de 5,27 años y 30,1 años, respectivamente. Además de su elevado poder de penetración, este tipo de radiación cuenta con las ventajas de ser económicamente viable, físicamente segura y poseer un alto potencial antimicrobiano (DeRuiter and Dwyer, 2002; Mexis and Kotominas, 2009).

Los haces de electrones son un tipo de radiación ionizante producida por la aceleración de electrones hasta una energía de 10 MeV (mega electron volt). Entre sus ventajas está que no se generan a partir de materiales radiactivos, su producción se puede detener y se pueden dirigir y, por tanto, hacer incidir de manera directa sobre el material a irradiar. Sin embargo, poseen baja capacidad de penetración en comparación con otros tipos de radiación ionizante, estando limitada a unos 3,8 cm en tratamientos por una sola cara del alimento, o de unos 8,9 cm para tratamientos de doble cara.

Los rayos X son producidos por el impacto de electrones acelerados sobre un blanco metálico, con la consiguiente emisión de radiación (fotones), por un fenómeno físico denominado

'Bremsstrahlung' o "radiación de frenado" (Farkas, 2006). Se trata de un tipo de radiación electromagnética de energía intermedia entre la radiación ultravioleta y los rayos gamma, y con menor capacidad de penetración que estos últimos. Esta radiación ionizante fue la primera que se empleó para la conservación de alimentos, aunque fue luego desplazada por otras debido a la baja eficiencia de conversión de los rayos de electrones de baja energía en rayos X; el empleo de éstos sólo recuperó interés recientemente debido al desarrollo de nuevos equipos mas eficientes (Farkas and Mohácsi-Farka, 2011; Antonio, 2014).

Los tres tipos de radiación difieren, especialmente, en la profundidad de penetración; sin embargo, todos se pueden utilizar para el tratamiento de alimentos mediante la configuración adecuada adaptada al tipo o el volumen de los alimentos a procesar.

1.1.3 Legislación e inquietudes del consumidor

Las radiaciones ionizantes se utilizan como una tecnología de procesamiento de alimentos no sólo en Europa sino también en otros continentes. Los requisitos y procedimientos están bien definidos y legislados, considerándose que bajo esas circunstancias se trata de una opción segura y viable.

Hay varias organizaciones nacionales e internacionales que han defendido y preconizado el uso de la irradiación, entre ellas la OMS/WHO (Organización Mundial de la Salud), FAO (Food and Agriculture Organization), AIEA (Agencia Internacional de la Energía Atómica), CDC (Centers for Disease Control and Prevention), ACSH (American Council on Science and Health), CAST (Council for Agricultural Science and Technology), ADA (American Dietetic Association), AMA (American Medical Association), IFT (Institute of Food Technologists) o CAC (Codex Alimentarius Commission) (DeRuiter and Dwyer, 2002; Ihsanullah and Rashid, 2016).

En la Unión Europea, la irradiación de alimentos está regulada por las Directivas 1999/2/CE y 1999/3/CE, del Parlamento Europeo y del Consejo de 22 de febrero de 1999, donde se describen las técnicas y requisitos generales de los procesos de irradiación, las condiciones para su uso en los alimentos, las excepciones y las normas para el etiquetado de alimentos y productos irradiados. Se establece, además, una lista comunitaria de alimentos e ingredientes alimentarios que pueden tratarse con radiaciones ionizantes, donde se incluyen una amplia variedad de productos, como frutas y verduras; cereales y harina de arroz; especias y

condimentos; pescados, mariscos; carnes, aves, ancas de rana; queso camembert de leche cruda; enzimas, caseína y caseinatos, goma arábiga o clara de huevo (EU, 2013).

De acuerdo con las normas establecidas, todos los alimentos vendidos sometidos a este tipo de procesamiento debe identificarse claramente en la etiqueta con el símbolo "radura" (Figura 1) y con la información "alimento tratado por proceso de irradiación". Esta simbología debe estar presente no sólo en los productos irradiados, sino también en los productos en los cuales la radiación se ha aplicado en sus ingredientes, que deben incluir la declaración anterior entre paréntesis a continuación del nombre del ingrediente irradiado (Tezotto-Uliana et al., 2015).

Figure 1. Imagen representativa del símbolo "Radura".

1.1.4 Componentes bioactivos y contaminantes en plantas secas

Desde los tiempos más antiguos, las plantas secas se han utilizado no sólo como alimento sino también para aplicaciones terapéuticas. Algunas de estas plantas tienen propiedades medicinales con potencial terapéutico en el tratamiento de diversas enfermedades, infecciosas y no infecciosas, ya que son una fuente natural de compuestos bioactivos, como pueden ser polifenoles, vitaminas anioxidantes, carotenoides o ácidos grasos insaturados. Esta diversidad de biomoléculas permite su uso en diversas áreas, especialmente como aditivos alimentarios y la producción de fitoquímicos, que representan ya una parte significativa del mercado farmacéutico mundial y pueden ser utilizados como ingredientes en la formulación de alimentos funcionales y nutracéuticos para la promoción de la salud (Pal et al., 2010; Ramarathnam et al., 1995; Skerget et al., 2005). En los últimos años, el interés por las plantas medicinales ha aumentado en todo el mundo y sido objeto de numerosos estudios científicos.

Esto es debido al hecho de que la acción de estos productos sobre el organismo es suave, generalmente sin los efectos secundarios asociados a los medicamentos, y porque son fuente para la extracción de biológicamente activas, lo que resulta en ocasiones más fácil y conveniente que recurrir a su síntesis (Marques and Farah, 2009; Yordanov et al., 2009).

Además de las plantas medicinales, las especias y condimentos, que aportan gusto y sabor de los alimentos, también se utilizan habitualmente como aditivos alimentarios naturales como conservantes en la industria alimentaria y con fines terapéuticos en la industria farmacéutica (Gahukar, 2012; Pal et al., 2010). A algunos de estos productos se le asocian efectos beneficiosos sobre el metabolismo lipídico, eficacia como antidiabéticos, capacidad para estimular la digestión y potencial antioxidante o anti-inflamatorio, dándoles el carácter de nutracéuticos (Srinivasan, 2005). Tanto en la industria alimentaria como en la farmacéutica, es requisito indispensable el empleo de materias primas de buena calidad microbiológica, ya que los microorganismos que vehiculan pueden contaminar el producto final conduciendo a su deterioro o a efectos indeseables para la salud (Rosa et al., 1995; Kamat et al., 2003).

1.1.4.1 Antioxidantes comunes en plantas secas

Las moléculas antioxidantes presentes en los alimentos son sustancias capaces de eliminar o inhibir la producción de especies reactivas de oxígeno (ROS) y contribuir a la regulación de las defensas antioxidantes del organismo. Estos compuestos pueden actuar a través de diversos mecanismos, entre ellos mediante su capacidad para formar nuevos radicales estables después de la cesión de hidrógeno o electrones para la eliminación de radicales dañinos (Benzie, 2003; Embuscado, 2015). Como ejemplos destacados se encuentran algunas vitaminas (p.ej., A, C ó E), carotenoides (licopeno, β-caroteno, xantofilas), flavonoides (flavonoles, flavonas, flavanonas, flavanoles, antocianos o isoflavonas) y otros compuestos fenólicos (alcoholes fenólicos, ácidos fenólicos, taninos, estilbenos y lignanos) (Carocho and Ferreira, 2013). Estas moléculas se pueden encontrar formando parte de la composición de muy diversos tipos de productos alimenticios, especialmente frutas, verduras, especias y hierbas aromáticas, hongos, pescados o productos lácteos, entre otros. Entre ellos, destaca el grupo de las hierbas y especias como fuentes particularmente ricas en antioxidante.

1.1.4.2 Detección y determinación de moléculas antioxidantes

Los antioxidantes naturales se han convertido en un tema de gran interés en las últimas décadas. Las características químicas y bioactividad mostradas por estas moléculas tienen un papel muy importante no sólo en la estabilidad de los alimentos sino también en la prevención de enfermedades. Por lo tanto, su caracterización es importante para la adecuada comprensión de sus mecanismos de acción y establecer la relación entre su estructura química y la bioactividad mostrada.

Diversos autores han desarrollado diferentes metodologías para identificar y determinar moléculas específicas en diferentes matrices después de la optimización del método de extracción más adecuado. Para la determinación de compuestos fenólicos, como primera aproximación, se suelen realizar ensayos colorimétricos tales como el método de Folin-Ciocalteu que se utiliza para determinar "compuestos fenólicos totales" en una muestra, aunque no se trata de un método específico, ya que el reactivo empleado también reacciona con otras moléculas reductoras. No obstante, las técnicas más comunes para la identificación y determinación de moléculas individuales son las separativas, como la cromatografía líquida de alta eficacia (HPLC), cromatografía de gases (GC) y electroforesis capilar (CE), asociadas a distintos sistemas de detección y especialmente la espectrofotometría de arreglo de diodos (DAS) y la espectrometría de masas (MS). La HPLC es la técnica más utilizada, por su versatilidad para el análisis de compuestos con diferentes características de solubilidad y volatilidad. En la mayoría de los casos, las separaciones se llevan a cabo usando columnas C18 de fase inversa, estando la fase móvil por lo general compuesta de dos disolventes: una fase orgánica apolar (habitualmente metanol o acetonitrilo) y una fase acuosa acidificada polar (Barros et al., 2009; Carocho and Ferreira, 2013; Gouveia-Figueira and Castilho, 2015). La HPLC es también la técnica de elección para el análisis de vitaminas, generalmente acoplada a detectores UV o de diodos (Langer and Lodge, 2014). Con respecto a tocoferoles, se prefiere la HPLC acoplada a un detector de fluorescencia, que proporciona mayor sensibilidad que los detectores fotométricos, sobre todo cuando los contenidos de compuestos a cuantificar son bajos (Barros et al., 2008).

1.1.4.3 Descontaminación de plantas secas

Las plantas se contaminan desde el suelo, el agua, el aire y el polvo durante su proceso de crecimiento, la cosecha y el secado (Shim et al., 2009). El secado de las plantas aromáticas y

medicinales para su almacenamiento durante un largo período de tiempo es una práctica común en muchas partes del mundo. Sin embargo, el deterioro microbiano o infestación de insectos durante el almacenamiento y el transporte reducen la calidad y la vida útil de estas plantas, que pueden llegar a suponer un peligro para la salud pública, ya que están expuestas a un alto nivel de contaminación natural. Así, en este tipo de productos pueden encontrarse microorganismos de gran relevancia para la salud, como *Salmonella*, *Escherichia coli*, *Clostridium perfringens*, *Bacillus cereus* y diversos tipos de mohos (Sádecká, 2007; Pal et al., 2010). Hay más de 400 compuestos clasificados como micotoxinas y, entre ellas, aflatoxinas (AFs) y ocratoxina A (OTA) son las más estudiadas. Las AFs son producidas por *Aspergillus flavus* y algunos hongos cercanos. La aflatoxina B1 (AFB1) es una de las micotoxinas más comunes en productos alimenticios contaminados, está considerada como el compuesto con mayor potencial tóxico y cancerígeno producido de forma natural y clasificada como carcinógeno del Grupo 1 (IARC, 2002; Rodrigues et al., 2012). Los efectos mutagénicos y carcinogénicos de la AFB1 en modelos animales están bien documentados, y diferentes estudios epidemiológicos han puesto de manifiesto la existencia de una correlación entre cáncer de hígado humano y los niveles de esta micotoxina en la dieta (Lee et al., 2015a; Romagnoli, et al., 2007). La OTA es producida por varias especies de *Aspergillus* y *Penicillium* y está reconocida como un agente nefrotóxico, hepatotóxico, neurotóxico, teratogénico e inmunotóxico. Su presencia en la dieta se ha asociado con una enfermedad renal humana fatal, conocida como "Nefropatía Balcánica endémica" ('Balkan Endemic Nephropathy', BEN), y también se ha relacionado con una mayor incidencia de tumores del tracto urinario superior (Harris and Mantle, 2001; Majeed et al., 2013; Waśkiewicz et al., 2013). Está clasificada como carcinógeno del Grupo 2B (IARC, 1993). La presencia natural de micotoxinas en las plantas se ha informado con frecuencia, siendo las hierbas medicinales y aromáticas tradicionales de varios países asiáticos y africanos algunos ejemplos conocidos por poseer niveles elevados de aflatoxinas y ocratoxina A (Ashiq et al., 2014; Santos et al., 2009; Waśkiewicz et al., 2013).

Existen varias técnicas para la descontaminación de plantas secas tales como la fumigación con óxido de etileno o bromuro de metilo, el tratamiento térmico y la irradiación (Pal et al., 2010). El óxido de etileno se utiliza comúnmente para la descontaminación de especias, con diferentes grados de éxito. No obstante, a pesar de su eficacia descontaminante, su uso está prohibido en diversos países, incluyendo Japón, la UE, el Reino Unido y Brasil, ya que se

considera carcinógeno humano y mutágeno cuando se inhala, y deja residuos químicos en la planta (Chmielewski & Migdal, 2005; Migdal & Owczarczyk, 1998).

La irradiación es otro proceso utilizado para la descontaminación de especias y plantas secas. Se caracteriza por ser eficiente en la conservación frente a las pérdidas causadas por procesos fisiológicos naturales y el deterioro microbiano, además mediante la eliminación o reducción de los microorganismos hace el producto más seguro para el consumidor. Por otra parte, permite reducir la dependencia de los fumigantes químicos y conservantes utilizados por la industria alimentaria (Byun et al., 1999; Nagy et al., 2011). Los beneficios y descripción de esta tecnología están descritos anteriormente.

1.2 Objetivos

Las plantas medicinales y aromáticas, debido a su composición en compuestos bioactivos han sido utilizadas como auxiliares en el mantenimiento de la salud, e incluso como productos terapéuticos, siendo utilizadas en las industrias farmacéutica, cosmética y alimentaria en forma de diferentes formulaciones. Sin embargo, para poder cumplir con todos requisitos de seguridad e higiene exigidos por la industria, es necesario que sean sometidas a procesos de descontaminación eficaces y seguros. En este sentido, la irradiación es una tecnología aplicada en diversos países, que se considera segura no sólo para los consumidores, sino también para el medio ambiente. En este contexto, en el presente estudio se planteó como objetivo evaluar los efectos de la irradiación sobre distintas características físicas (color), nutricionales (proteínas, cenizas, grasa, hidratos de carbono y energía), químicas (azúcares, tocoferoles, ácidos grasos, ácidos orgánicos, compuestos fenólicos), toxicológicas (micotoxinas) y de bioactividad (actividad antioxidante y anti-proliferativa) en plantas usadas en medicina tradicional.

En particular, los efectos de la irradiación se evaluaron considerando:

i) diferentes especies de plantas: *Aloysia citrodora* P. (Verbenaceae), *Arenaria montana* L. (Caryophyllaceae), *Ginkgo biloba* L. (Ginkgoaceae), *Melissa officinalis* L. (Lamiaceae), *Melittis melissophyllum* L. (Lamiaceae), *Mentha x piperita* L. (Lamiaceae) y *Thymus vulgaris* L. (Lamiaceae);
ii) diferentes tipos de radiaciones ionizantes: radiación gamma y haces de electrones;
iii) diferentes dosis de radiación: 0, 1, 2, 5 y 10 kGy;
iv) diferentes períodos de almacenamiento: 0, 12 y 18 meses;

El tratamiento de los resultados se realizó mediante distintas herramientas estadísticas, como análisis de la varianza (ANOVA de una y de 2 vías), análisis de componentes principales (PCA) y análisis discriminante lineal.

1.3 Resultados y Discusión

1.3.1 Estudios con radiación gamma

1.3.1.1 Ginkgo biloba L.: Caracterización química y propiedades antioxidantes de infusiones, extractos acuosos y metanólicos.

Se evaluó la composición química y capacidad antioxidante en extractos obtenidos a partir de muestras de *G. biloba* irradiadas y no irradiadas.

Los extractos metanólicos obtenidos a partir de las muestras secas de *G. biloba* mostraron mayor capacidad de eliminación de radicales DPPH, poder reductor y capacidad para inhibir la peroxidación lipídica medida por el blanqueamiento de β-caroteno y la producción de sustancias reactivas al ácido tiobarbitúrico (TBARS), así como niveles más elevados de compuestos fenólicos totales y de flavonoides que las infusiones y extractos acuosos.

Se pudo comprobar que la irradiación no afectaba significativamente los contenidos de macronutrientes, ácidos grasos, γ- y δ-tocoferoles, fructosa, trehalosa, ácido quínico y ácido shikímico. La irradiación a dosis de 1 kGy tampoco afectaba a los contenidos de α-tocoferol, ácido oxálico y ácido málico, que, por el contrario, sí disminuían en el tratamiento con 10 kGy al igual que los niveles de glucosa y sacarosa. Por lo tanto, 1 kGy sería una dosis más recomendable desde el punto de vista de mantener el perfil nutricional de *G. biloba.* Curiosamente a esta dosis también se encontró cierto aumento en la actividad antioxidante tanto de las infusiones como de los extractos metanólicos obtenidos a partir de las hojas irradiadas. Asimismo, se observó que en las muestras irradiadas a 10 kGy se producía un aumento en los niveles de compuestos fenólicos en todos los tipos de extractos, lo que se podría explicar por una mejora en la capacidad de extracción de estos componentes favorecida por elde irradiación.

1.3.1.2 Aloysia citrodora Paláu, Melissa officinalis L., Melittis melissophyllum L. y Mentha x piperita L.: Efectos sobre nutrientes y propiedades antioxidantes. Evaluación en extractos metanólicos e infusiones.

Se estudió la composición química y capacidad antioxidante en extractos obtenidos a partir de muestras de *Aloysia citrodora* Paláu, *Melissa officinalis* L., *Melittis melissophyllum* L. y *Mentha x piperita* L. irradiadas y no irradiadas.

Se encontró que si bien la irradiación hasta dosis de 10 kGy producía cambios significativos en algunos componentes específicos, éstos no eran homogéneos sino que diferían según la especie considerada. El análisis de componentes principales (PCA) del conjunto de resultados obtenidos para todas las muestras de plantas no permitía diferenciar entre muestras irradiadas y no irradiadas, que se agrupaban de forma indiscriminada. Este resultado sugiere que el tratamiento de irradiación no da lugar a cambios consistentes en la composición química y nutricional suficientes para definir un perfil químico específico en las muestras irradiadas. El modo en que cada especie se ve afectada por la irradiación parece responder a patrones particulares dependientes de la planta. De una manera global, se puede considerar, en todo caso, que los cambios producidos no son demasiado extensos o relevantes como para desaconsejar el empleo de radiación gamma (hasta 10 kGy), sino que más bien ésta se postula como una técnica de conservación viable para las especies de Lamiáceas y Verbenáceas evaluadas. Ésta es una observación interesante por cuanto dosis de 10 kGy permitirían la desinfestación y decontaminación de las muestras.

1.3.1.3 Aloysia citrodora Paláu, Melissa officinalis L., Melittis melissophyllum L. y Mentha x piperita L.: Efectos sobre nutrientes y propiedades antioxidantes a lo largo de la vida útil.

El objetivo principal de este estudio era establecer si el tratamiento con radiaciones gamma podía prevenir o minimizar los potenciales cambios degradativos de las plantas durante el almacenamiento. Para ello, los resultados relativos a perfil nutricional, componentes individuales y propiedades antioxidantes obtenidos a los 12 y 18 meses se compararon con los obtenidos en el estudio anterior para muestras no irradiadas e irradiadas analizadas inmediatamente después del tratamiento de irradiación.

Se comprobó que los perfiles nutricionales después de 18 meses de almacenamiento eran similares en plantas irradiadas y no irradiadas, por lo que no parecía existir una influencia

significativa de este tratamiento sobre la capacidad de conservación de los nutrientes. En relación con otros componentes, la irradiación no mejoró los resultados con relación a las pérdidas de azúcares libres, ácidos orgánicos y tocoferoles producidas en las muestras no irradiadas, especialmente considerados al cabo de 18 meses. Sin embargo, si se encontró un efecto protector significativo en los niveles de ácido oleico, inhibición de blanqueo de β-caroteno (en infusiones), actividad captadora de radicales DPPH y el poder reductor (en extractos metanólicos) en las muestras irradiadas. Respecto al color, el análisis cromático en el espacio CIELAB indicaba la existencia de tonalidades más verdosas en las muestras irradiadas, lo que se podía percibir como un efecto favorable por parte del consumidor.

Por otra parte, y a pesar de la falta de similitud entre los efectos sufridos por cada especie de planta, el análisis de componentes principales permitió identificar los parámetros que más afectados por el tiempo de almacenamiento, así como sus correlaciones con períodos de 12 meses y 18 meses, sugiriendo que existen cambios específicos significativos en la composición a lo largo de almacenamiento que son independientes de la especie considerada.

1.3.1.4 Thymus vulgaris L. y Mentha x piperita L.: Influencia sobre citotoxicidad y compuestos fenólicos. Evaluación en extractos metanólicos.

El tomillo y la menta son fuentes ricas de sustancias antioxidantes de naturaleza fenólica (especialmentes flavonas, flavonoles, flavanoneas y derivados hidroxicinámicos) que podrían contribuir a la prevención y control de enfermedades a través de su incorporación a una dieta normal o como suplementos. Los extractos metanólicos de estas plantas (irradiadas y no irradiadas) mostraron actividad anti-proliferativa en cuatro líneas de células tumorales humanas (MCF-7, NCI-H460, HeLa y HepG2), pero citotoxicidad en células no tumorales (línea PLP2). Los dosis de irradiación ensayadas (1 to 10 kGy) no influyeron sobre esta actividad ni sobre la composición fenólica en el caso de la menta, pero las muestras de tomillo irradiadas a 10 kGy aumentaron su capacidad anti-proliferativa en las líneas tumorales con relación a la observadas en muestras no irradiadas o tratadas con 1 kGy. En este sentido y en relación con las plantas consideradas, parece que 10 kGy es una dosis que podría ser utilizada para descontaminación de este tipo de muestras, ya que no influye negativamente sobre su potencial citotóxico.

1.3.1.5 Thymus vulgaris L.: Influencia sobre propiedades antioxidantes y compuestos fenólicos. Evaluación en infusiones

De acuerdo con los resultados obtenidos, el tratamiento a una dosis de 10 kGy aumenta la capacidad antioxidante de las infusiones preparadas a partir de las muestras irradiadas en los cuatro tipos de ensayos in vitro utilizados (captación de radicales DPPH, poder reductor, blanqueo β-caroteno e inhibición de laperoxidación lipídica). Esta dosis de radiación (10 kGy) también condujo a infusiones con mayor contenido de compuestos fenólicos totales y flavonoides. En cuanto a compuestos fenólicos individuales sólo tres (apigenina 6,8-di-*C*-glucósido, ácido cafeico and luteolina-7-*O*-glucurónido) de los 13 compuestos detectados no sufrían cambios significativos en sus concentraciones, mientras que se observaba un aumento en todos los restantes, que era especialmente relevante en los casos de metil-eriodictyol-*O*-pentosilhexósido, luteolina-7-*O*-glucósido y eriodictyol. Las observaciones realizadas sugieren que el empleo de radiación gamma a una dosis de 10 kGy (máxima permitida en la UE) no sólo no sería improcedente sino que podría incluso contribuir a mejorar el potencial antioxidante y contenido fenólico de las infusiones de *T. vulgaris* sin afectar significativamente a otros aspectos de composición química.

1.3.1.6 Aloysia citrodora L. y Mentha x piperita L.: Influencia sobre citotoxicidad y compuestos fenólicos. Evaluación en infusiones

Todas las muestras de hierba luisa y menta evaluadas mostraron actividad anti-proliferativa en cuatro líneas de células tumorales humanas (MCF-7, NCI-H460, HeLa y HepG2), sin que se observaran en general diferencias significativas entre muestras irradiadas y no irradiadas. Por otra parte, ninguna de las muestras estudiadas produjo efectos citotóxicos sobre una línea no tumoral (PLP2). Por otra parte, se observó un aumento estadísticamente significativo en los contenidos de compuestos fenólicos en el caso de las muestras de menta irradiadas, pero no entre las diferentes muestras de hierba luisa. La composición fenólica presentó mejores factores de correlación con la capacidad anti-proliferativa en las infusiones de menta que en las de hierba luisa.

1.3.1.7 Aloysia citrodora Paláu: Efectos sobre micotoxinas

La irradiación de las hojas secas de *A. citrodora* a dosis de 1, 5 y 10 kGy no produjo un descenso significativo en los niveles de aflatoxina B1 y ocratoxina A. Por lo tanto, en lo que los resultados aquí obtenidos se refiere, el tratamiento con radiaciones gamma incluso a las dosis máximas autorizadas en la UE (10 kGy) parece ser ineficaz para la descontaminación de micotoxinas, ello no significa que no pueda ser eficaz para ser utilizado con otros fines de seguridad alimentaria. En cuanto a composición fenólica, a pesar de que se observa una ligera disminución en los niveles de algunos compuestos, los tratamientos realizados no influyen de manera importante sobre el perfil fenólico de las muestras.

1.3.2 Estudios con radiación de haces de electrones

1.3.2.1 Aloysia citrodora Paláu, Melissa officinalis L., Melittis melissophyllum L. y Mentha x piperita L.: Efectos sobre nutrientes y propiedades antioxidantes.

Los efectos de rayos gamma y haces de electrones radiaciones sobre la composición química y características antioxidantes fueron altamente dependientes de la especie considerada. Sin embargo, cuando todos los resultados se trataron conjuntamente mediante análisis discriminante lineal, se pudo comprobar que los mayores cambios inducidos por los tratamientos de irradiación se producían en los perfiles de ácidos grasos y la actividad antioxidante, tanto cuando la discriminación se realizaba en función de las condiciones de irradiación como cuando se hacía de acuerdo a las especies de plantas.

Los resultados obtenidos sugieren que los haces de electrones podrían ser más adecuados para la irradiación de las plantas estudiadas, si se tiene en cuenta que sus extractos poseen porcentajes más elevados de ácidos grasos monoinsaturados (y también de algunos poliinsaturados) y mejores valores de actividad antioxidante que los de las muestras sometidas tratamiento con rayos gamma. En todo caso, como se ha señalado, los resultados obtenidos varían según la especie considerada, así como en función de la dosis aplicada, lo que aconseja realizar estudios específicos para el tipo de planta a irradiar.

1.3.2.2 Aloysia citrodora Paláu, Melissa officinalis L., Melittis melissophyllum L. y Mentha x piperita L.: Efectos sobre nutrientes y propiedades antioxidantes a lo largo de la vida útil.

En general, la composición proximal y los parámetros de color fueron los factores que experimentaron los cambios menos importantes durante el tiempo de almacenamiento de las muestras evaluadas, estuvieran o no irradiadas. Por el contrario, se observaron pérdidas importantes en los contenidos de azúcares libres, ácidos orgánicos, tocoferoles y ácidos grasos poliinsaturados durante el almacenamiento, aunque el tratamiento por haces de electrones, a pesar de no impedir totalmente estas pérdidas, tenía un significativo efecto atenuante. En todo caso, los factores considerados (tiempo de almacenamiento y proceso de irradiación) influían de forma diferente en las distintas especies de plantas, tanto en los cambios observados en la composición química (incluyendo compuestos fenólicos) como en los indicadores de actividad antioxidante.

1.3.3 Estudios comparativos con radiación gamma y haces de electrones

1.3.3.1 Arenaria montana L.: Caracterización nutricional y fitoquímica, propiedades antioxidantes y bioactividad.

Se determinó el perfil fitoquímico y la bioactividad en muestras de *A. montana* y se evaluaron los efectos de la radiación ionizante sobre los mismos. Entre los compuestos fitoquímicos, se identificaron y cuantificaron sustancias fenólicas individuales y tocoferoles, y se caracterizó la composición de ácidos grasos, con especial referencia a los de tipo omega-3 y omega-6, todos ellos asociados a efectos beneficiosos para la salud. Se encontró mayores valores de actividad antioxidante y anti-proliferativa en las infusiones que en los extractos metanólicos de la planta. Estos resultados apoyan el uso medicinal tradicional de *A. montana* y abren perspectivas a posibles aplicaciones en la industria alimentaria y farmacéutica, como ingrediente funcional o nutracéutico, teniendo en cuenta su destacada capacidad antioxidante y anti-proliferativa y contenido de compuestos bioactivos, como flavonas o tocoferoles.

La mayoría de los parámetros nutricionales y químicos estudiados (excepto azúcares y ácidos orgánicos) y también la actividad antioxidante mostraron variaciones estadísticamente significativas en la respuesta al tratamiento de irradiación. La aplicación de análisis discriminante lineal al conjunto de los datos permitió establecer los parámetros más afectados por la radiación gamma o haces de electrones, así como de acuerdo a la dosis de irradiación (1

ó 10 kGy). Así, la actividad antioxidante fue la característica que sufrió los cambios más significativos, especialmente a la dosis de 10 kGy y cuando se utilizaba radiación por haces de electrones.

1.3.4 *Melissa officinalis* L. y *Melittis melissoffillum* L. infusiones: Efectos en compuestos fenólicos de infusiones.

En estas especies se encontró un aumento en los niveles de compuestos fenólicos analizados individualmente entre muestras sometidas a distintos tratamientos de irradiación. En estudios previos sobre estas mismas plantas llevados a cabo por nuestro grupo (Koike et al., 2015b), apenas se habían observado diferencias en los valores de composición proximal, parámetros de color, azúcares, ácidos orgánicos, tocoferoles, ácidos grasos y actividad antioxidante. Los efectos favorables ahora observados sobre la composición fenólica al aplicar las mismas tecnologías se pueden considerar un argumento adicional para apoyar el empleo de las radiaciones ionizantes para la conservación de estas plantas desecadas. Otro aspecto que se pudo observar fue que diferentes compuestos se encontraban diferencialmente favorecidos según el tratamiento se realizara a 1 kGy o 10 kGy, así como dependiendo de si empleaban rayos gamma o haces de electrones para la irradiación, lo que podría ser evaluado como una posibilidad para maximizar específicamente la extracción de compuestos de mayor interés potencial.

1.4 Conclusión

El obejetivo de este estudio era no sólo el de analizar diversas especies de plantas usadas en medicina tradicional en términos de composición química y nutricional y propiedades bioactivas, sino también ensayar el potencial descontaminante y conservante de dos tipos de radiaciones ionizantes (rayos gamma y haces de electrones). De acuerdo con los resultados obtenidos, este tipo de procesado presenta ventajas destacadas, ya que permite preservar (e incluso aumentar) los contenidos de algunos componentes nutricionales y fitoquímicos, y en algunos casos también mejorar los indicadores de bioactividad, incluso irradiando a las máximas dosis autorizadas de10 kGy. No obstante, dependiendo del material vegetal irradiado y el tipo de proceso utilizado, también se observaron en ocasiones efectos negativos importantes sobre algunos moléculas de interés.

En general, la composición y propiedades bioactivas de las muestras irradiadas no se veían más afectadas a lo largo el posterior almacenamiento (hasta 18 meses), e incluso mejoraban algunas veces, con relación a las correspondientes no irradiadas. Aunque los cambios producidos durante el almacenamiento no eran muy diferentes entre muestras tratadas a 1 ó 10 kGy, para algunos compuestos se observaban mayores pérdidas a la dosis más elevada. Esta observación se hizo para los dos tipos de tratamientos de irradiación evaluados, aunque las pérdidas tendías a ser menos acentuadas con haces de electrones que con rayos gamma. En los casos en los que ambos tipos de tratamientos pudieron ser comparados en la misma especie vegetal, los haces de electrones se mostraban, en líneas generales, como una tecnología más adecuada para preservar las características de las plantas durante su procesado y almacenamiento.

Por otra parte, en los ensayos realizados, la irradiación no se mostró como una técnica eficaz para la descontaminación de micotoxinas, ya que fue capaz de reducir los niveles de ocratoxina A y aflatoxina B_1 en los materiales ensayados y a las dosis aplicadas (0, 5 y 10 kGy).

Por último, en lo que a resultados del presente estudio se refiere, resultaba evidente que el comportamiento de los distintos componentes evaluados era heterogéneo, dependiendo de la especie vegetal, fuente radiante y dosis aplicada.

Como conclusión general, se apunta a que la irradiación puede ser considerada como una tecnología prometedora para la conservación de plantas aromáticas y medicinales, aunque se requiere una evaluación previa para decidir sobre el tipo adecuado de tratamiento a aplicar en función de la material vegetal y del componente o características a preservar.