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Effects of gamma radiation on chemical and antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity of borututu

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ABSTRACT

Borututu is a well-known medicinal plant in Angola for the treatment of liver diseases and for the prophylaxis of malaria. Our research group reported, in a recent study, that its infusion, pills and syrups display significant antioxidant and anti-hepatocellular carcinoma activities. However, during the processing and storage, it can be easily exposed to contamination that can lead to a microbial deterioration or insect infestation compromising its quality, shelf life, and efficiency. Herein, we investigated the effect of gamma irradiation, one of the most promising decontamination methods for many foodstuffs and plant materials, at different doses (1 and 10 kGy) on borututu regarding to its nutritional value, lipophilic (fatty acids and tocopherols) and hydrophilic (free sugars and organic acids) compounds, antioxidant and anti-hepatocellular carcinoma activities. In general, the irradiation treatment did not appreciably affect the nutritional value of the studied plant, but the highest energetic contribution (383.83 kcal/100 g), total sugars (8.63 g/100 g), organic acids (3.31 g/100 g dw), total tocopherols (336.72 mg/100 g dw), and PUFA (32.75%) contents were found in the sample irradiated at 10 kGy. Furthermore, this sample presented also the highest levels of total phenolics and flavonoids and, in general, the highest antioxidant activity (EC_{50} values of 0.04 to 0.24 mg/mL for the methanolic extract and 0.03 to 1.34 mg/mL for the infusion). Irradiated samples kept the anti-hepatocellular carcinoma activity, but a decrease was observed in the methanolic extract prepared from sample irradiated at 10 kGy (GI_{50}=188.97 µg/mL). Overall, gamma irradiation proved to be a suitable technique of preservation of dried herbs without affecting the bioactive compounds.

Keywords: Gamma irradiation; Borututu; Hydrophilic compounds; Lipophilic compounds; Antioxidant activity; HepG2.

1. Introduction

Cochlospermum angolensis Welw. (borututu) is a widespread medicinal plant in Angola, where the barks infusion has been traditionally used by healers for the treatment of many liver diseases and for the prophylaxis of malaria (Poppendieck, 1981; Presber, Herrman, & Hegenscheid, 1991; Silva et al., 2011). Recently, our research group reported that the infusion, pills (Pereira, Calhelha, Barros, & Ferreira, 2013) and syrups (Pereira, Calhelha, Barros, Queiroz, & Ferreira, 2014) of the dry barks display significant antioxidant and anti-hepatocellular carcinoma activities.

However, raw materials from medicinal plants are easily exposed (from the plants themselves, the soil, water, air and dust) to contamination with pathogens during the harvest or the air drying time, and the storage in dried form during long periods, which can lead to a microbial deterioration or insect infestation that decrease herbs quality and shelf life, compromising their efficiency and even posing a public health threat (Rosa, Medina, & Vivar, 1995; Pal et al., 2010). This kind of contamination, particularly with pathogenic non-spore forming, is one of the most significant causes of human suffering all over the world, and according to World Health Organisation (WHO), the infectious and parasitic diseases represented the most frequent cause of death (35%) worldwide (Loaharanu, 1994; Khan & Abrahem, 2010). Therefore, to improve the hygienic quality and guarantee the stability of active compounds of the plant materials during storage, making it suitable for human use and commercialization, an adequate technology for decontamination is required (Katusin-Razem, 2001; Thomas, Senthilkumar, Kumar, Mandal, & Muraleedharan 2008; Bhat, Sridhar, & Karim, 2010).

Food irradiation arises as one of the most promising decontamination methods for many foodstuffs and plant materials, reducing the reliance on chemical fumigants used by the food and pharmaceutical industries and chemical fumigants like gaseous ethylene oxide

or methyl bromide for decontamination or sterilization, that are carcinogenic and increasingly restricted in several countries, due to health, environmental or occupational safety reasons. Among the different types of radiation sources allowed for food processing (gamma, X-ray, UV, electron beam), gamma irradiation represents an effective and environment friendly technology to avoid the re-contamination and re-infestation of the product, since it can be done after packaging (Khattak, Simpson, & Ihasnullah, 2009; Aouidi, Samia, Hana, Sevastianos, & Moktar, 2011). This treatment has been carried out in several plant species and the doses used should guarantee consumer safety without compromising wholesomeness, structural integrity, functional properties, or sensory attributes, being often between 2 and 30 kGy (Olson, 1998; Khan & Abrahem, 2010).

Nevertheless, as far as we know, no studies have been performed on borututu irradiated dry barks. The typical doses for insects disinfestation or parasite inactivation are up to 1 kGy and to reduce or eliminate spoilage or disease causing pathogenic microorganisms the common doses used are up to 10 kGy (Molins, 2001). Therefore, this study was undertaken to investigate the effect of different doses of gamma irradiation (1 and 10 kGy) on this plant regarding to its macronutrients composition, lipophilic (fatty acids and tocopherols) and hydrophilic compounds (free sugars and organic acids). Furthermore, due to its benefits in the prevention/treatment of liver diseases, the irradiated plant infusion and methanolic extract were also assessed to evaluate their antioxidant and anti-hepatocellular carcinoma activity depending on the different radiation doses.

2. Material and Methods

2.1. Samples and samples irradiation

Cochlospermum angolensis Welw. (borututu) was obtained from an herbalist shop, Américo Duarte Paixão Lda., in Alcanede (Portugal), imported from Angola, as dry barks material (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), sample irradiated at 1 kGy, and sample irradiated at 10 kGy, where 1 kGy and 10 kGy were the predicted doses.

The irradiation of the samples was performed in a Co-60 experimental chamber (Precisa 22, Graviner Manufacturing Company Ltd., UK) with four sources, 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, 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 irradiated at 1 kGy and 8.93 ± 0.14 kGy, 1.91 ± 0.03 kGy h^{-1}, 1.02 for sample irradiated at 10 kGy. For simplicity, in the text and tables we considered the values 0, 1 and 10 kGy, for the doses of non-irradiated and irradiated samples 1 and 2, respectively.

After irradiation, the samples were reduced to a fine dried powder (20 mesh) using a grinding mill, and mixed to obtain homogenized samples for subsequent analysis or preparation of infusions/extracts.

2.2. Standards and reagents

For 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, 1992) and Amber Perspex dosimeters (batch V, from Harwell Company, UK). 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, Billerica, MA, USA).

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, L-ascorbic acid, tocopherol, sugar and organic acid standards. Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA).

For 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 from Sigma (St. Louis, MO, USA).

For anti-hepatocellular carcinoma activity and hepatotoxicity evaluation: ellipticine, phosphate buffered saline (PBS), acetic acid, sulforhodamine B (SRB), trichloroacetic acid (TCA) and Tris were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum (FBS), L-glutamine, Hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively) and DMEM (Dulbecco's Modified Eagle Medium) were from Hyclone (Logan, Utah, USA).

Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3. Chemical composition

2.3.1. 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 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 equations: Energy (kcal)=4×(g protein+g carbohydrates)+9×(g fat).

2.3.2. Lipophilic compounds

Fatty acids. Fatty acids were determined after a lipid extraction of the sample (3 g) using a Soxhlet apparatus with petroleum ether; afterwards a transesterification procedure was applied to the lipid extract as described previously by the authors (Barros et al., 2013a), using a gas chromatography equipment (DANI 1000), 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). The results were expressed in relative percentage of each fatty acid.

Tocopherols. Tocopherols (α-, β-, γ- and δ-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 auto-sampler (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: α-tocopherol (y=1.295x; R^2=0.991); β-tocopherol (y=0.396x; R^2=0.992); γ-tocopherol (y=0.567x; R^2=0.991); δ-tocopherol (y=0.678x; R^2=0.992). The results were expressed in mg per 100 g of dry weight.

2.3.3. 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: fructose (y=0.864x; R^2=0.999); glucose (y=0.909x; R^2=0.999); sucrose (y=0.892x; R^2=0.999); trehalose (y=0.953x; R^2=0.999); raffinose (y=0.847x; R^2=0.999). 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, Pereira, & Ferreira, 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: citric (y=1E+06x+4170.6; R^2=1); fumaric acid (y=172760x+52193; R^2=0.999); malic acid (y=952269x+17803; R^2=1); oxalic acid (y=1E+07x+96178; R^2=0.999); shikimic acid (y=9E+07x-95244; R^2=1). The results were expressed in g per 100 g of dry weight.

2.4. Preparation of the extracts for the bioassays

Infusions (water extracts). A dry weight of each sample of borututu (2 g) was added to 200 mL of boiling distilled water, left to stand at room temperature for 5 min, and filtered through Whatman No. 4 paper. The following dilutions were used in the biological assays: 5 mg/mL to 19.53 µg/mL for antioxidant activity assays; 400 to 1.56 µg/mL for hepatotoxicity assays.

Methanolic extracts. The different samples of borututu (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), re-dissolved in methanol at 10 mg/mL (stock solution), and stored at 4 °C for further use. Successive dilutions were made from the stock solution at the same concentrations as described for the infusions.

2.5. Evaluation of bioactivity

2.5.1 General. The infusion (water extract) and methanolic extract were redissolved in *i)* water and methanol, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, or *ii)* water for antitumor activity evaluation (final concentration 8 mg/mL). The final solutions were further diluted to different concentrations to be submitted to 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 (sample concentration that inhibited 50% of the net cell growth) for antitumor activity. Trolox and ellipticine were used as standards in antioxidant and antitumor activity evaluation assays, respectively.

2.5.2. Quantification of total bioactive compounds. Total phenolics were estimated by Folin-Ciocalteu colorimetric assay according to procedures previously described (Sarmento et al., 2014); the results were expressed as mg of gallic acid equivalents (GAEs) per g of infusion and methanolic extract. Total flavonoids were determined by a colorimetric assay using aluminum trichloride, following procedures previously reported (Sarmento et al., 2014); the results were expressed as mg of catechin equivalents (CE) per g of infusion and methanolic extract.

2.5.3. 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 2h 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 (Fernandes et al., 2014).

2.5.4. Anti-hepatocellular carcinoma activity and hepatotoxicity

The anti-hepatocellular carcinoma activity was evaluated using HepG2, which is the most widely used tumor cell line and generally regarded as a good hepatocellular carcinoma model. HepG2 cells were routinely maintained as adherent cell cultures in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin, at 37 ºC, in a humidified air incubator containing 5% CO_2. The cell line was plated at 1.0×10^4 cells/well in 96-well plates. Sulforhodamine B assay was performed according to a procedure previously described by the authors (Barros et al., 2013a).

For hepatotoxicity evaluation, a cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, according to a procedure established by the authors (Barros et al., 2013a); it was designed as PLP2. 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/well, and commercial in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.6. Statistical analysis

Three samples were used for each preparation 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 SPSS v. 18.0 program (SPSS Inc.).

3. Results and Discussion

3.1. Chemical composition

Results regarding chemical composition of the control (non-irradiated) and irradiated samples are present in **Tables 1-3**. Nutritionally, all borututu samples revealed similar profiles (**Table 1**), with the exception of protein content; the remaining nutritional parameters did not reveal significant variation between irradiated and control samples. The protein content clearly decreased with the increasing irradiation dose, being the highest values presented by sample irradiated at 1 kGy (2.97 g/100 g), which is in agreement with a previous study that reported the effects of gamma irradiation in the disruption of the ordered structure and the degradation and aggregation of the polypeptide chains of proteins, due to oxygen radicals generated by irradiation (Moon & Song, 2001).

Regarding to fatty acids (**Table 2**), twenty-one different individual molecules were quantified in all the samples with prevalence of saturated fatty acids (SFA) and considerable percentages of polyunsaturated fatty acids (PUFA). The most abundant

fatty acids in all samples were linoleic (C18:2n6; 23.87-24.55%) and palmitic acid (C16:0; 21.47-21.71%), which is in agreement with a previous study performed by our research group (Pereira et al., 2014a). Among the three samples, sample irradiated at 1 kGy gave lower percentage of PUFA (32.75%) and SFA (43.32%), while control and sample irradiated at 10 kGy revealed quite similar amounts. Significant differences were not observed between the MUFA percentages of the studied sample. It was previously reported that unsaturated fatty acids, especially n-3 PUFAs, play an important role in the treatment or prevention of hepatic steatosis (Hanke, Zahradka, Mohankumar, Clark, & Taylor, 2013). Being borututu consumed essentially for hepatic purposes, it is important to keep those fatty acids after irradiation and, in fact, the sample irradiated with 10 kGy revealed the same levels in relation to control.

In agreement with Pereira et al. (2014a), in the present study the four vitamers of tocopherol were found in the borututu samples, with the prevalence of β-tocopherol (**Table 2**), but the highest total tocopherols content was obtained for the irradiated samples irradiated (both with 1 kGy and 10 kGy with 331.97 mg/100 g and 336.72 mg/100 g, respectively). With the exception of δ-tocopherol, the amount of the different isoforms was generally higher in the irradiated samples. Thus, irradiation seems to preserve these compounds with critical antioxidant importance in the prevention of PUFA oxidation, mostly α-tocopherol, which is the most effective chain-breaking lipid-soluble antioxidant in animal and human tissues (Valk & Hornstra, 2000; Fernandes et al., 2011; Fernandes et al., 2013). These observations can be related to the increase of atomic oxygen resulting from the dissociation of molecular oxygen present in the bag, which decrease the oxidation of tocopherols. Otherwise, control sample showed some degradation (**Table 2**), highlighting the efficiency of gamma irradiation in the preservation of these compounds.

Regarding free sugars, and also in concordance with Pereira et al. (2014a), fructose and sucrose were the most abundant molecules. As observed in **Table 3**, 1 kGy (sample 1) was not enough to preserve sugars content, where it was verified a decrease of fructose, glucose, sucrose, and trehalose. On the contrary, sample irradiated at 10 kGy showed the highest amount of these sugars that also contributed to the highest total sugars content (8.63 g/100 g). This is in agreement with previous works with irradiated plants that explained this increase through the depolymerization or degradation of polysaccharide molecules and glucoside group due to gamma irradiation in soybeans (Byun, Kang, & Mori, 1996) and ginseng products (Sung, Park, Lee, & Cho, 1982). Tissot, Grdanovska, Barkatt, Silverman, & Al-Sheikhly (2013) also reported the potential of ionizing radiation on facilitating the breakdown of cellulose into simple sugars.

With respect to organic acids (**Table 3**), it was possible to verify that the irradiation did not significantly affect these compounds in borututu samples, which showed the presence of oxalic, malic, shikimic, citric and fumaric acids in similar amounts, with higher oxalic, malic and citric acids content, similarly to what was observed in the previous work referred above (Pereira et al., 2014a). The preservation of organic acids is of great interest once they are related to plant foods organoleptic properties, namely their flavor, aromatic properties or consistence. The presence and ratio of organic acids in plants can affect their chemical and sensorial characteristics, such as pH, total acidity, microbial stability, sweetness and global acceptability; for instance malic acid possesses a smooth lingering taste and also a tart taste (not as sharp as that of citric acid, but longer lasting) (Tormo & Izco, 2004). Organic acids also play an important role on food technology, for example, citric and malic acids are used as acidulants and the latest is also used as flavor enhancer and as a potent growth inhibitor of yeasts and some

bacteria; on the other hand, fumaric acid is an important parameter to reveal microbial spoilage or processing of decayed food, and the naturally occurring concentration of some organic acids in plants can give information about the addition of synthetic preservatives to plant products, providing evidences of eventual authenticity issues (Gebre, Kuhns, & Brandle, 1994; Vaughan & Geissler, 1997).

3.2. Antioxidant properties, anti-hepatocellular carcinoma activity and hepatotoxicity

Four *in vitro* assays were used to evaluate antioxidant properties of infusions and methanolic extracts of the samples: scavenging effects on DPPH radicals, reducing power, inhibition of β-carotene bleaching and inhibition of lipid peroxidation in brain cell homogenates. As shown in Table 4, the infusion obtained from the sample irradiated at 10 kGy had a higher antioxidant activity in all the assays performed, with no statistical differences in the DPPH scavenging effect when compared to the control sample. On the other hand, the methanolic extract of this sample gave higher DPPH radicals scavenging activity and reducing power, and similar β-carotene bleaching and the lipid peroxidation inhibitions. Still, the methanolic extracts revealed higher scavenging activity, reducing power and β-carotene bleaching inhibition than the infusion. The latest can be compared with the infusion of borututu assessed in a previous study (Pereira et al., 2013) where it revealed antioxidant properties in lower concentrations (0.02-0.62 mg/mL). These data are also in accordance with a previous study where the ethanolic extracts revealed better results than the corresponding infusions prepared from irradiated Korean medicinal herbs (Byun et al., 1999).

Despite the results obtained in previous studies reporting that gamma irradiation did not influence the electron donating activity of Korean medicinal plants (Byun, Yook, Kim, & Chung, 1999) and did not influence the free radical scavenging effect of some Korean

soybean fermented foods (Byun, Son, Yook, Jo, & Kim, 2002), in the present work the results obtained are in agreement with Kim et al. (2009) that revealed that the DPPH radical scavenging activity of gamma irradiated *Hizikia fusiformis* extracts were increased with increasing irradiation dose, which was related to the increase of the total polyphenolic compounds content by the irradiation. Similarly to the referred observations, in the present study the total phenolics and flavonoids content in the 10 KGy irradiated sample was higher in both extracts, with respectively 26.85 mg GAE/g and 1.68 mg CE/g in the infusion, and 107.45 mg GAE/g and 33.77 mg CE/g in the methanolic extract, leading to a better understanding of the results discussed above.

The anti-hepatocellular carcinoma activity and hepatotoxicity of the infusions and methanolic extracts prepared from the three samples was also evaluated and the results are present in **Table 5**. All the methanolic extracts revealed anti-hepatocellular carcinoma activity that clearly decreased in the sample irradiated at 10 kGy (GI_{50}=188.97 μg/mL) relatively to the control sample (GI_{50}=160.02 μg/mL). Nevertheless, the infusions did not reveal anti-hepatocellular carcinoma activity in opposition to the results obtained in the mentioned previous work (Pereira et al., 2013), where the infusion revealed this capacity with a GI_{50} value of 146.06 μg/mL. This discrepancy, as those reported throughout the present discussion of results, can be explained by the kind of material analysed, once in the referred work a preparation for infusion was acquired while in the present study it was used bulk borututu, from another supplier. Moreover, the bioactivity of medicinal plants depends on numerous factors beyond the species, namely the origin of the sample, making it impossible to guarantee chemical homogeneity.

None of the infusions or methanolic extracts revealed toxicity in non-tumor porcine liver cells (GI_{50} > 400 μg/mL).

In conclusion, irradiation treatment up to 10 kGy does not appreciably affect the nutritional value of the studied plant, which can be explained by the low water content of dry herbs that limits the possibility of the formation of free radicals (Venskutonis, Poll, & Larsen, 1996; Murcia et al., 2004; Brandstetter, Berthold, Isnardy, Solar, & Elmadfa, 2009). In fact, sample irradiated at 10 kGy had the highest energetic contribution and the highest levels of total sugars, organic acids, total tocopherols, and PUFA. According to the results obtained for the antioxidant activity assays, the risk of limiting antioxidative properties of dried borututu due to the irradiation at the tested doses can be excluded, with sample irradiated at 10 kGy revealing, in general, the highest properties. Regarding to the anti-hepatocellular carcinoma activity, irradiated samples kept the activity, but a decrease was observed in the methanolic extracts prepared from sample irradiated at 10 kGy. Gamma irradiation proved to be a suitable technique to maintain bioactive compounds and effects of borututu (a widely used dried plant).

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Table 1. Composition in macronutrients and energetic value of borututu samples submitted to different gamma irradiation doses (mean ± SD).

	0 kGy	1 kGy	10 kGy
Ash (g/100 g dw)	7.87 ± 0.62a	7.48 ± 0.74a	6.91 ± 0.23a
Proteins (g/100 g dw)	2.83 ± 0.00b	2.97 ± 0.05a	2.76 ± 0.06b
Fat (g/100 g dw)	2.24 ± 0.15a	2.31 ± 0.04a	2.29 ± 0.07a
Carbohydrates (g/100 g dw)	84.06 ± 0.55a	84.24 ± 0.52a	85.04 ± 0.15a
Energy (kcal/100 g dw)	379.71 ± 1.22b	381.64 ± 2.24ab	383.83 ± 0.89a

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

Table 2. Lipophilic compounds (fatty acids and tocopherols) of borututu samples submitted to different gamma irradiation doses (mean ± SD).

	0 kGy	1 kGy	10 kGy
C6:0	0.13 ± 0.04	0.18 ± 0.02	0.16 ± 0.02
C8:0	0.25 ± 0.04	0.15 ± 0.04	0.34 ± 0.00
C10:0	0.15 ± 0.02	0.09 ± 0.01	0.20 ± 0.02
C11:0	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.01
C12:0	1.09 ± 0.03	1.27 ± 0.00	1.09 ± 0.02
C13:0	0.09 ± 0.01	0.08 ± 0.00	0.05 ± 0.00
C14:0	2.77 ± 0.08	2.81 ± 0.04	2.67 ± 0.06
C14:1	0.09 ± 0.00	0.10 ± 0.00	0.05 ± 0.00
C15:0	0.68 ± 0.04	0.63 ± 0.02	0.63 ± 0.01
C15:1	0.10 ± 0.03	0.06 ± 0.00	0.08 ± 0.00
C16:0	21.70 ± 0.15	21.47 ± 0.23	21.71 ± 0.09
C16:1	0.41 ± 0.02	0.49 ± 0.00	0.37 ± 0.05
C17:0	0.97 ± 0.00	1.00 ± 0.00	0.95 ± 0.00
C18:0	13.89 ± 0.72	15.09 ± 0.36	13.48 ± 0.25
C18:1n9	16.35 ± 0.13	16.07 ± 0.08	16.97 ± 0.14
C18:2n6	24.44 ± 0.05	23.87 ± 0.09	24.55 ± 0.02
C18:3n3	7.89 ± 0.05	7.81 ± 0.07	8.21 ± 0.04
C20:1	6.71 ± 0.26	6.67 ± 0.05	6.46 ± 0.12
C22:0	0.93 ± 0.15	0.85 ± 0.03	0.98 ± 0.06
C23:0	0.39 ± 0.04	0.38 ± 0.04	0.35 ± 0.03
C24:0	0.93 ± 0.12	0.89 ± 0.05	0.66 ± 0.04
Total SFA (relative %)	44.01 ± 0.33ab	44.94 ± 0.26a	43.32 ± 0.28b
Total MUFA (relative %)	23.65 ± 0.33a	23.38 ± 0.06a	23.03 ± 0.25a
Total PUFA (relative %)	32.34 ± 0.00a	31.68 ± 0.21b	32.75 ± 0.03a
α-tocopherol (mg/100 g dw)	2.49 ± 0.05b	2.69 ± 0.02a	2.47 ± 0.04b
β-tocopherol (mg/100 g dw)	280.32 ± 1.26b	306.59 ± 4.42a	313.10 ± 3.14a
γ-tocopherol (mg/100 g dw)	0.61 ± 0.01b	2.03 ± 1.37a	0.59 ± 0.01b
δ-tocopherol (mg/100 g dw)	22.66 ± 1.92a	20.66 ± 1.36a	20.56 ± 1.06a
Total (mg/100 g dw)	306.08 ± 3.12b	331.97 ± 7.13a	336.72 ± 4.24a

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecanoic acid (C11:0); Lauric 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 aci d (C18:1n9); Linoleic acid (C18:2n6); γ-Linolenic acid (C18:3n6); α-Linolenic acid (C18:3n3); Eicosenoic acid (C20:1); Behenic acid (C22:0); Tricosanoic acid (C23:0); Lignoceric acid (C24:0). In each row different letters mean significant differences (p<0.05).

Table 3. Hydrophilic compounds (sugars and organic acids) of borututu samples submitted to different gamma irradiation doses (mean ± SD).

	0 kGy	1 kGy	10 kGy
Fructose (g/100 g dw)	2.01 ± 0.08b	1.79 ± 0.01c	2.14 ± 0.04a
Glucose (g/100 g dw)	1.31 ± 0.00b	1.17 ± 0.08c	1.47 ± 0.02a
Sucrose (g/100 g dw)	3.04 ± 0.06b	2.87 ± 0.02c	3.39 ± 0.05a
Trehalose (g/100 g dw)	0.96 ± 0.02a	0.83 ± 0.02b	0.83 ± 0.02b
Raffinose (g/100 g dw)	0.60 ± 0.07b	0.68 ± 0.03b	0.80 ± 0.00a
Total (g/100 g dw)	7.92 ± 0.12b	7.34 ± 0.11b	8.63 ± 0.04a
Oxalic acid (g/100 g dw)	0.24 ± 0.00a	0.23 ± 0.00a	0.29 ± 0.08a
Malic acid (g/100 g dw)	1.30 ± 0.02a	1.26 ± 0.02a	1.63 ± 0.48a
Shikimic acid (g/100 g dw)	0.01 ± 0.00a	0.01 ± 0.00a	0.01 ± 0.00a
Citric acid (g/100 g dw)	1.22 ± 0.04a	1.13 ± 0.03a	1.35 ± 0.41a
Fumaric acid (g/100 g dw)	0.01 ± 0.01a	0.02 ± 0.01a	0.02 ± 0.01a
Total (g/100 g dw)	2.78 ± 0.04a	2.65 ± 0.06a	3.31 ± 0.98a

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

Table 4. Antioxidant activity (EC_{50} values, mg/mL) of infusions and methanolic extracts obtained from borututu samples submitted to different gamma irradiation doses (mean ± SD).

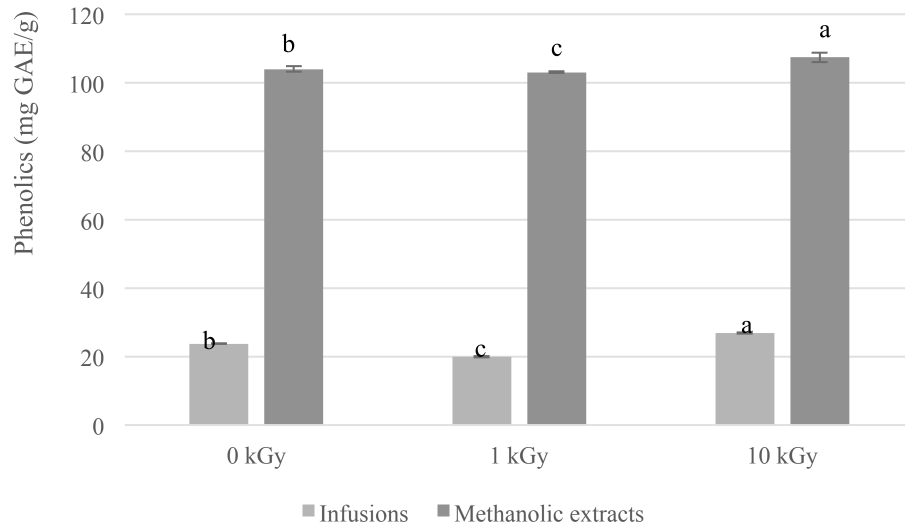
	0 kGy	1 kGy	10 kGy	Trolox
Infusions				
DPPH scavenging activity	0.93 ± 0.05b	1.27 ± 0.09a	0.86 ± 0.05b	0.04 ± 0.(
Reducing power	0.74 ± 0.01b	0.90 ± 0.01a	0.65 ± 0.01c	0.03 ± 0.(
β-carotene bleaching inhibition	2.95 ± 0.13b	3.12 ± 0.03a	1.34 ± 0.14c	0.003 ± 0.
TBARS inhibition	0.04 ± 0.00a	0.04 ± 0.00a	0.03 ± 0.00c	0.004 ± 0.
Methanolic extracts				
DPPH scavenging activity	0.24 ± 0.01a	0.25 ± 0.01a	0.21 ± 0.00b	0.04 ± 0.(
Reducing power	0.16 ± 0.01a	0.16 ± 0.01a	0.12 ± 0.00b	0.03 ± 0.(
β-carotene bleaching inhibition	0.25 ± 0.04a	0.25 ± 0.04a	0.24 ± 0.06a	0.003 ± 0.
TBARS inhibition	0.04 ± 0.00b	0.06 ± 0.00a	0.04 ± 0.00b	0.004 ± 0.

nd- not detected . In each row different letters mean significant differences (p<0.05).

Table 5. Anti-hepatocellular carcinoma activity and hepatotoxicity (GI_{50} values, μg/mL) of infusions and methanolic extracts obtained from borututu samples submitted to different gamma irradiation doses (mean ± SD).

	0 kGy	1 kGy	10 kGy	Ellipticine
Infusions				
HepG2 (hepatocellular carcinoma)	>400	>400	>400	3.22 ± 0.67
PLP2 (non-tumor liver primary culture)	>400	>400	>400	2.06 ± 0.03
Methanolic extracts				
HepG2 (hepatocellular carcinoma)	160.02 ± 13.34b	158.65 ± 7.52b	188.97 ± 5.65a	3.22 ± 0.67
PLP2 (non-tumor liver primary culture)	>400	>400	>400	2.06 ± 0.03

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



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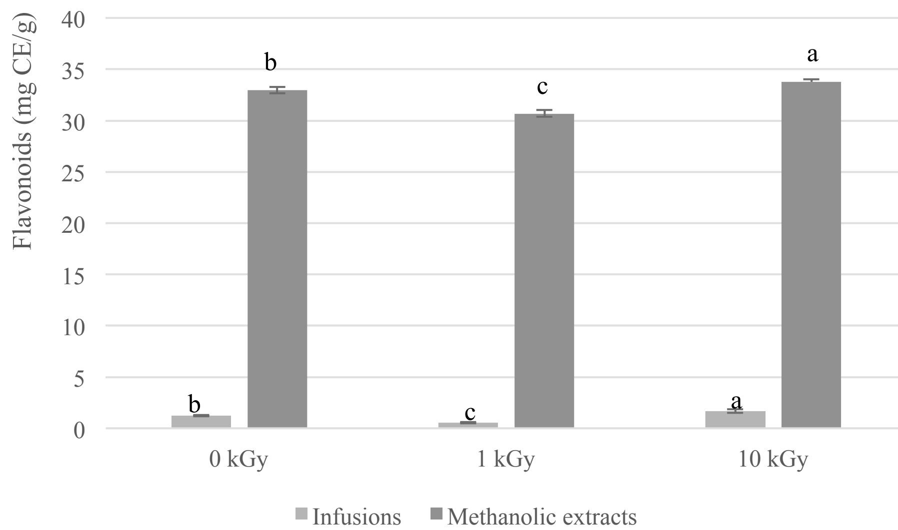

B

Figure 1. Phenolics and flavonoids content in infusions and methanolic extracts obtained from borututu samples submitted to different gamma irradiation doses (mean ± SD). Different letters mean significant differences (p<0.05).