RICARDO JORGE DOS SANTOS NUNES

MICROMEDRONHO

DESIGN OF MICROENCAPSULATED *ARBUTUS UNEDO* LEAVES AND FRUITS BY SPRAY DRYING FOR SUPPLEMENTS AND FUNCTIONAL FOODS

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Thesis submitted for doctor degree in
Biotechnological sciences, specialty of food biotechnology

Supervised by:
Doctor professor Isabel Saraiva de Carvalho

UNIVERSIDADE DO ALGARVE

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Ricardo Jorge dos Santos Nunes

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Resumo

Arbutus unedo, conhecido em Portugal como medronheiro, é uma árvore comum por todo o mediterrâneo. Desempenha um papel importante na manutenção da economia de algumas regiões do país, nomeadamente Monchique no Algarve. Os seus frutos, vermelhos quando maduros, são colhidos e transformados em compota ou bebidas alcoólicas como licores e água-ardente. Desempenham também um importante papel ambiental, tanto na manutenção da população de abelhas como na recuperação após a ocorrência de um incêndio. Vários grupos de trabalho têm-se debruçado sobre o medronheiro, nomeadamente frutos e folhas, e têm investigado propriedades benéficas para a saúde, como a presença de antioxidantes, vitaminas e minerais, e medicinais como a atividade neuroprotetora e antiagregante. Quanto às flores, pouco se sabe sobre elas.

Este trabalho teve como objetivo aumentar o conhecimento sobre as folhas, frutos e flores de medronheiro, explorar os fatores que afetam a atividade antioxidante, determinar a sua atividade antibacteriana e antiproliferativa, o seu potencial no combate de doenças e desenvolver novos produtos tendo como base as diversas partes da árvore. No início apresentamos os resultados obtidos de uma pesquisa bibliográfica que incidiu sobre o tema do medronheiro em todas as suas vertentes. Esta pesquisa foi efetuada utilizando a *Web of Knowledge* para recolher os dados, o *endnote* para organizar e *o KH Coder* para conduzir o *text-mining*. Ao usar estes três programas foi possível recolher os dados e organizá-los de forma muito mais eficiente que seria possível fazer manualmente. Esta combinação pode ainda ser usada para explorar outros temas de forma rápida e sistemática.

De seguida exploramos a atividade antioxidante, nomeadamente a sua variabilidade entre 18 árvores diferentes, a diferença entre uma extração por soxhlet e por placa térmica com agitação, entre folhas e frutos, e entre diferentes solventes. Também investigamos o efeito da temperatura, tempo, rácio e agitação nos resultados obtidos e comparamos a atividade antioxidante do medronheiro com duas outras plantas da mesma família e concluímos que o medronheiro apresenta alta capacidade antioxidante. O próximo passo foi o estudo de potenciais propriedades medicinais dos estratos de 7 plantas colhidas no Algarve. Para tal avaliou-se a atividade antimicrobiana e antiproliferativa dos extratos. Foi ainda avaliado o perfil em compostos fenólicos e o conteúdo em zinco para o medronheiro e 5 outras plantas . Apesar do medronheiro não apresentar a maior diversidade ou quantidade de compostos fenólicos, verificou-se que ele apresenta altos conteúdos em ácido gálico e quercetina, dois potentes antioxidantes. Apresenta ainda um conteúdo apreciável de zinco e possui atividade

antimicrobiana especialmente contra *Staphylococcus aureus* e antiproliferativa contra a linha celular SW480.

O passo seguinte foi averiguar o uso potencial das diversas partes do medronheiro, analisando a sua composição proximal, conteúdo em vitamina E e perfil em ácidos gordos. Foi também investigado o seu potencial uso no tratamento de doenças crónicas, nomeadamente a diabetes, o Alzheimer e o Parkinson. Neste passo verificámos que as folhas e frutos eram maioritariamente constituídos por hidratos de carbono, possuindo as folhas uma maior quantidade de proteínas e gordura. Quanto ao conteúdo em vitamina E verificou-se que as folhas e frutos possuem este micronutriente em abundância, principalmente sob a forma de α-tocoferol, o vitamero com maior atividade e maioritariamente acumulado nos humanos. Em ambas as amostras a quantidade de ácidos gordos insaturados foi aproximadamente o triplo da quantidade de ácidos gordos saturados. Os principais ácidos gordos insaturados detetados foram o linoleico (ω6) e linolénico (ω3), sendo que ambas apresentaram uma quantidade maior de ω3 quando comparado com ω6 podendo potencialmente ajudar a combater os défices em ω3 constantes nas dietas comuns.

Quanto ao uso no tratamento de doenças crónicas verificou-se que tanto as folhas como os frutos inibem de maneira diferente α-amílases de diferentes origens. Enquanto os frutos mostram uma maior inibição contra α-amilase de origem fúngica, as folhas e frutos mostraram maior inibição contra α-amilase obtida de mamíferos, nomeadamente humanos (saliva) e suínos (pâncreas). Foi também verificado que todos extratos possuem uma atividade inibitória muito maior contra a α-glucosidase quando comparada com a α-amilase, que é o oposto do que acontece com a acarbose, um composto comummente usado no tratamento da diabetes tipo 2. As condições de extração também afetaram as inibições obtidas, mas com menor importância que a parte da planta ou origem da enzima. Quanto ao potencial uso no tratamento contra o Alzheimer verificou-se que todas as partes de quê ??estudadas possuem efeito inibitório contra a acetilcolinesterase e contra a butirilcolinesterase. A possibilidade de inibição destas duas enzimas é uma das terapêuticas utilizadas no tratamento do Alzheimer pelo que os extratos estudados possuem potencialmente o poder de ser utilizados para este mesmo fim. Apesar de ambas as enzimas mostrarem diferentes inibições, o fator *parte da planta* teve de maneira geral um papel mais importante, com os frutos a mostrarem muito menos inibição do que as folhas ou as flores. Verificou-se ainda que as flores inibem ambas as enzimas pelo método não competitivo, enquanto as folhas e os frutos inibem cada enzima por métodos diferentes. Quanto ao potencial uso no tratamento do Parkinson verificou-se que nenhum dos extratos obtidos com água possui capacidade de inibir mais de 50% da atividade

da enzima utilizada (tirosinase). Foi possível obter melhores resultados quando os extratos foram obtidos com 50% ou 100% de etanol, no entanto, o uso deste solvente iria impedir o consumo humano direto dos extratos obtidos. Apesar deste resultado os extratos podem ainda mostrar interesse potencial no tratamento do Parkinson uma vez que a tirosinase de origem fúngica utilizada e a tirosinase de mamíferos reagem de maneira diferente a certos inibidores.

A etapa final do trabalho passou por juntar todo o conhecimento adquirido e desenvolver três novos produtos através de folhas e frutos de medronheiro, que possam ser utilizados sozinhos ou no desenvolvimento de produtos de valor acrescentado. Neste passo foi desenvolvida uma bebida, cuja estabilidade foi testada utilizando diversas condições de armazenamento. Foi verificado que o armazenamento no frio é adequado e que mesmo após uma digestão *in vitro* a bebida ainda apresenta potencial anti-Alzheimer. Foram ainda desenvolvidas macrocápsulas, utilizando alginato e $CaCl_2$ com três formulações diferentes após a verificação prévia das melhores condições de encapsulação. Foi verificado que as cápsulas com extrato de folhas no seu interior apresentavam uma maior atividade antioxidante, apesar das cápsulas com extrato de fruto no seu interior serem mais estáveis ao armazenamento. Por fim os extratos foram colocados num equipamento de *spray-dryer* para a obtenção de micropartículas sob a forma de pó. Foram estudados o efeito da % de maltodextrina, da adição de gelatina e da temperatura de atomização. Foi verificado que todos os pós obtidos apresentam uma boa solubilidade em água e que mesmo após uma digestão *in vitro* o pó obtido a partir do extrato de folha possui uma alta atividade inibitória contra a acetilcolinesterase e contra a butirilcolinesterase. Este pó é mais fácil de armazenar e transportar que o extrato na forma liquida e apresenta uma maior estabilidade durante o armazenamento no que diz respeito à degradação das propriedades benéficas pelo que é uma boa alternativa.

Palavras-chave:

Arbutus unedo; *text-mining*; antiproliferativo; doenças crónicas; perfil fenólico e de ácidos gordos; novos produtos

Abstract

Arbutus unedo is a common tree in the Mediterranean area. Its fruits are consumed fresh or transformed, leaves are rich in antioxidants and not much is known about flowers. In this work we conduct a bibliographic survey utilizing text mining coupled with bibliography management software to expedite the process. We then study the best conditions to extract antioxidants from leaves, fruits and flowers of *A. unedo* and we compare their antioxidant activity with other plants from Algarve. In this step we found *A. unedo* to be the best plant. We then investigate the antiproliferative and antimicrobial activities of *A. unedo* and other plants from Algarve and also explore their phenolic profile and zinc contents, where we find *A. unedo* has antibacterial activity and retards sw480 cell line growth. Next we determine the vitamin E contents and fatty acid profile of the *A. unedo* extracts and explore their potential use in the treatment of chronic diseases, namely diabetes, Alzheimer and Parkinson, using enzymatic assays. At this step we find that leaves and fruits are rich in vitamin E, mostly α-tocopherol, and have a ω3:ω6 ratio above 1. Additionally, leaf and flower extracts have the best potential to be used in diabetes control, mostly via α-glucosidase inhibition. We also find the extracts show strong potential to be used in Alzheimer's control but not in Parkinson's and that they inhibit acetylcholinesterase and butyrilcholinesterase differently and via difference mechanisms. Finally we expose the development and stability to storage of three new products using *A. unedo* leaves and fruits as basis. These include a beverage with antioxidant and anti-Alzheimer activity, macrocapsules rich in antioxidants and microcapsules obtained using a spray dryer, which even after *in-vitro* digestion present the potential to be used in the treatment of Alzheimer's disease.

Keywords:

Arbutus unedo; text-mining; antiproliferative; chronic diseases; phenolic and fatty acid profiles; new products

List of publications

Published:

NUNES, R., ANASTÁCIO, A. & CARVALHO, I. S. 2012. Antioxidant and free radical scavenging activities of different plant parts from two Erica species. *Journal of Food Quality*, 35, 307-314.

NUNES, R., PASKO, P., TYSZKA-CZOCHARA, M., SZEWCZYK, A., SZLOSARCZYK, M. & CARVALHO, I. S. 2016. Antibacterial, antioxidant and anti-proliferative properties and zinc content of five south Portugal herbs. *Pharmaceutical Biology*, 1-10.

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Submitted:

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NUNES, R. & CARVALHO, I. S. Characterization of 18 *Arbutus unedo* trees from a demarked region and their leaf and fruit extracts antioxidant activity obtained using different conditions. *Journal of Agricultural Science and Technology*

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NUNES, R. & CARVALHO, I. S. Diabetes related enzymes inhibitory activity of *Arbutus unedo* L. extracts with focus on the influence of α-amylase origin. *Food Research International*

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Other work:

Publication:

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List of Abbreviations

A549 (Lung carcinoma)

Abs (Absorbance)

ABTS ($ABTS^{\cdot+}$ free radical)

ACHE (Acetylcholinesterase)

AD (Alzheimer's disease)

adjR^2 (Adjusted correlation coefficient)

ANOVA (Analysis of Variance)

ATCI (Acetylcholine iodide)

B% (Blue percentage)

°Brix (Degree Brix)

BCHE (Butyrilcholinesterase)

BJ (Human fibroblast)

°C (Degree Celsius)

CACO-2 (Human colon adenocarcinoma cell line)

CIb (Carr Index tapped on bottom)

Cis- (Carr Index tapped on side)

Cm (Centimetre)

DMSO (Dimethyl sulphoxide)

DPPH ($DDPH^{\cdot-}$ free radical)

DNS (Dinitrosalicylic acid)

DTNB (5,5'-dithiobis-(2-nitrobenzoic acid))

Dw (Dry weight)

Fb (Fibroblast)

FCT (Faculty of Science and Technology)

FRAP (Ferric reducing antioxidant power)

GAE (Gallic acid equivalent)

GC-MS (Gas chromatography-Mass spectrometry)

H (Hour)

HCA (Hierarchical cluster analysis)

HDL (High density lipoproteins)

HEK 293 (Transformed kidney cell line)

HEPG-2 (Human liver cancer cell line)

HR Tb- (Hausner ratio tapped on bottom)

HR Ts (Hausner ratio tapped on side)

HPLC (High performance liquid chromatography)

IC (Inhibition concentration)

IC_{50} (Extract concentration for inhibition of 50%)

LDL (Low density lipoprotein)

LSD (Least significant difference)

MIC (Minimum inhibitory concentration)

Min (Minute)

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)

ND (not detected)

NNw (Neural network methodology)

NQ (Not quantifiable)

PCA (Plate Count Agar)

pNPG (p-nitrophenyl α-D-glucopyranoside)

PUFA (Polyunsaturated fatty acids)

QE (Quercetin equivalents)

R% (Red percentage)

R^2 (Correlation coefficient)

ROS (Reactive oxygen species)

RP (Reducing Power)

RSM (Response surface methodology)

SD (Standard deviation)

SEM (Scanning electron microscopy)

SW 480 (Colon adenocarcinoma cell line)

SW 620 (Colon adenocarcinoma cell line)

TAA (Total antioxidant activity)

TE (Trolox equivalents)

TYR (Tyrosinase)

TFC (Total flavonoid content)

TPC (Total phenolic content)

UV-Vis (Ultraviolet-visible light)

Y% (Yellow percentage)

εTb (Porosity tapped on bottom)

εTs-(Porosity tapped on side)

μg (Microgram)

ρB (Bulk density)

ρP- (Particle density)

ρTb (Tapped density on the bottom)

ρTs (Tapped density on the side)

CHAPTER I - AIMS, OBJECTIVES AND WORK PLAN OF THE THESIS

In this chapter we expose the thesis motivation, the research aims and steps taken to achieve them, the outline of the thesis and the work plan followed.

Aims, objectives and work plan of the thesis

1 Thesis motivation

Human kind has for a long time used plants not only as a source of sustenance but also as a source of compounds with beneficial activities. While many times not knowing the reason of the benefits gained from their consumption, this knowledge has nonetheless been passed mainly by word of mouth through generations. Currently mankind is still interested in the use of natural products to attain beneficial effects that go well beyond the nutritional aspect, but now the focus is also on knowing the reason of those effects. Many plants are studied worldwide, from plants that are specific to a small region to plants that encompass a large are of the globe. One plant which is widely distributed and currently undervalued is the strawberry tree (*Arbutus unedo*), which can be found a little over all Europe, especially in the Mediterranean area. This tree belongs to the *Ericaceae* family, can reach several meters in height and produces dark red edible fruits, ripe in the autumn. In Portugal it is found mainly in the south, although it occurs also in some regions in the northern part of the country. In Monchique, a region in Algarve where this tree is abundant, fruits are used to produce alcoholic beverages and jams, having some impact in the economy of the region. Flowers are used by bees to produce honey, while wood is mainly used as fuel. In folk medicine, fruits are used to treat wounds due to their antiseptic power. They also have diuretic properties. Leaves are used as diuretics and to treat urinary disorders, diabetes and hypertension. They are also known to exhibit antimicrobial activity against some bacteria. Both leaves and fruits are indicated as being abundant in antioxidants, especially phenolic compounds, some minerals such as potassium, phosphorus and calcium as well as some vitamins which are important for the human being. Phenolic compounds encompass a vast group of phytoconstituents that possess antioxidant power. These compounds are important because the modern world abounds in oxidant sources, responsible for cancer, heart and degenerative diseases. Potassium, phosphorus and calcium are minerals important for the human being. Potassium is responsible for maintaining blood pH and for insulin secretion and muscle movement. The abundance in potassium is probably one of the reasons this fruit is regarded as antidiabetic agent. One of the reasons of hypertension is the lack of balance between potassium and sodium, the higher the sodium levels the higher the risk. This fruit is a very good source of potassium that has low sodium (proportion of 20 to 1), and as such can be used to fight this effect. Phosphorus is necessary for energy production, genetic material constitution and bone

formation. Calcium is also important in bone formation as well as nervous signals transmission, blood coagulation and muscular contraction.

While some of the properties mentioned before for the leaves and fruits have already been verified, the samples tested in this thesis are from a completely different environment and location which can affect their properties. Additionally most of the studies focus in one extraction following a predetermined condition, existing a great lack of information regarding the effect of extraction conditions on the aforementioned properties, despite knowing that both temperature and time can affect the properties of extracts. To verify these activities, the antimicrobial activity of extracts obtained with different solvents was tested on a panel of pathogenic bacteria. The enzymatic inhibitory effect of extracts obtained using different conditions was also tested on α-amylase, α-glucosidase (antidiabetic potential), acetylcholinesterase, butyrilcholinesterase (anti-Alzheimer's potential) and tyrosinase (anti-Parkinson's potential). The vitamin E content and fatty acid profile for leaves and fruits was also determined. Two drying methods for fruits were also evaluated. A more in depth study was conducted regarding the antioxidant activity, using a screening design to determine the factors having the most effect. The honey made from flowers is regarded as also being rich in antioxidants, which hints that the flowers themselves are also strong in antioxidants. Despite this, this part of the tree is rarely studied and not much is known about them. To determine if they are indeed rich in antioxidants and to fill the lack of information regarding flowers, the authors of this thesis have studied them not only for their antioxidant activity, but also for their antidiabetic, anti-Alzheimer and anti-Parkinson's potentials, and compared them with leaves and fruits. Since flowers fall from the tree after a certain time, the authors studied the activities described above both for flowers collected from the tree and from the ground.

As mentioned before, the *Arbutus unedo* tree has been the subject of some studies regarding its functional properties. In those studies it was found that leaves possess *in vitro* antiaggregant effects, reducing the risk of heart attacks (El Haouari et al., 2007), protective effect in induced lung inflammation in mice (Mariotto et al., 2008b), antihemolytic capacity (Mendes et al., 2011), and potential beneficial effects against breast cancer (Mariotto S et al., 2008). Fruits also possess antihemolytic capacity (Mendes, de Freitas 2011) and effect on neurodegenerative cells (Fortalezas et al., 2010). All these characteristics demonstrate that leaves, fruits and possibly flowers of this tree can benefit the consumer's health. There are however no new products being released to the consumer that capitalize on these properties and others the fruits, leaves and flowers may have. Additionally, the fruits of this tree get spoiled fast, which makes them hard to transport, store and unavailable throughout the year,

preventing the exploitation of their potential benefits. One way to surpass this problem is the production of microcapsules or microparticles using encapsulation technology. Encapsulation is defined as the process of confining active compounds inside a matrix or membrane to attain one or more desirable effects. From the point of view of plant origin products, encapsulation allows a group of desirable objectives to be reached, such as controlled supply and deliver, increased shelf life, separation of non-desirable compounds and improvement of the product final quality. Encapsulation also enables the isolation of sensitive compounds to prevent their degradation due to humidity, oxygen or other compounds that might react. Additionally, this treatment allows an easier transport and storage, and their inclusion in food matrixes. Thus encapsulation is a useful technological tool for the commercial sector in order to develop high value products and to allow a differentiation from competing companies.

In this work the authors create microcapsules in the form of powder using a spray-dryer. The effect of adding two different carrier agents, at two different concentrations and the use of two different temperatures was studied. The obtained powders from leaf and fruit extracts were then tested for their physical properties, antioxidant activity and anti-Alzheimer potential. The authors also create macrocapsules, which as the name indicates are larger, using a number of different conditions which are evaluated for their effect on the integrity and aspect of the obtained capsules. These capsules were then tested for their antioxidant activity and storage stability. Additionally a beverage using the obtained extracts was developed, initially using a mixture design to detect the best formulation and then evaluating it for the antioxidant activity, anti-Alzheimer potential and stability to storage and *in-vitro* digestion.

2 Research aims

In this thesis, the main goals were to complete the knowledge regarding *Arbutus unedo*, namely the properties of extracts obtained from different plant parts. This was especially important for flowers, whose activities are seldomly studied. The extracts were to be studied regarding their antioxidant activity and antibacterial activities and the effect of extraction conditions on these. The next goal was to study the potential application of the obtained extracts in the treatment of chronic diseases, namely diabetes, Alzheimer and Parkinson's. The final goal was to develop new products which could capitalize on the knowledge obtained before and could be included in other food matrixes as enrichment material for the creation of added-value products and functional foods.

To accomplish these objectives, different steps were taken throughout the study:

1. Determination of the best extraction methodology between soxhlet and hot plate for leaves. Evaluation of inter-tree sample variability. Evaluation of the extracts obtained at different conditions (temperature, time, ratio, stir) from leaves and fruits using different solvents and for flowers collected from the tree and from the ground using water as extraction solvent.
2. Evaluation of the antioxidant activity of leaves, fruits and flowers. Evaluation of the antibacterial activity of leaves and fruits. Evaluation of the antiproliferative activity and phenolic composition of leaves.
3. Evaluation of the potential health benefits using HPLC for vitamin E determination and GC-MS for fatty acid profile of leaves and fruits. Evaluation of the extracts in the treatment of chronic diseases, using enzymatic assays to determine potential applications in the treatment of type 2 diabetes, Alzheimer and Parkinson's diseases. Determination of the inhibition type. Study of the *in-vitro* simulated digestive process on the anti-Alzheimer's potential.
4. Development of new products, using a mixture design with leaves and fruits for the creation of a beverage. Evaluation of said beverage under adverse storage conditions and after exposition to the *in-vitro* simulated digestive process regarding its antioxidant and anti-Alzheimer potential. Development of macrocapsules using sodium alginate and $CaCl_2$ with varying concentrations and for varying time. Evaluation of capsules regarding their antioxidant activity and stability to storage. Development of leaf and fruit microcapsules obtained by spray-drying using different carrier agent %, type, and temperatures. Evaluation regarding their antioxidant and anti-Alzheimer potential before and after an *in-vitro* digestion.

3 Outline of the Thesis

In Chapter I we expose the motivation for conducting this work, the aims and steps taken to accomplish these aims.

In Chapter II we resort to a data analysis tool, called text mining. This is a useful tool that is seldomly used in the field of science due to the time it takes to prepare the data. However we describe how to use it and prepare the data using several programs that allow a more efficient use of the time spent and a degree of automatism in collecting and treating the data. The results are then analysed and conclusions are taken.

In Chapter III we conduct a characterization of antioxidant activity from plants belonging to the Ericaceae family, namely *Erica arborea, Erica australis* and *Arbutus unedo.* We investigate the influence of location and the inter-tree variation of the collected samples. We also investigate the best extraction conditions for *Arbutus unedo* regarding temperature, time, ratio, stir and solvent.

In Chapter IV we screen 7 different plants collected in Algarve for their medicinal properties. The plants studied are *Erica arborea, Erica australis, Arbutus unedo, Crataegus monogyna, Equisetum telmateia, Geranium purpureum, Mentha suaveolens and Lavandula stoechas spp. luisieri*. For all these plants we study the antimicrobial and antiproliferative activities. Additionally for *Arbutus unedo, Crataegus monogyna, Equisetum telmateia, Geranium purpureum, Mentha suaveolens and Lavandula stoechas spp. luisieri* we study the phenolic composition and zinc content.

In Chapter V we study the potential health benefits from the consumption of *Arbutus unedo* leaf, fruit and flower extracts. This is done by determining the vitamin E content and fatty acid profile of leaf and fruits using HPLC and GC-Ms respectively, as well as the antidiabetic, anti-Alzheimer and anti-Parkinson potentials of leaf, fruit and flowers using enzymatic assays.

Finally, in Chapter VI we outline the development of new products, namely a beverage, microcapsules and microparticles. For all these products the stability during storage and the anti-Alzheimer potential are investigated.

4 Work plan

To accomplish the objectives proposed in this thesis, the research was divided into four different elements including sampling (Figure I–1), extraction of bioactive compounds, testing of the extracts regarding their potential health benefits and development of new products (Figure I–2).

Figure I–1. The 18 trees selected for this study.

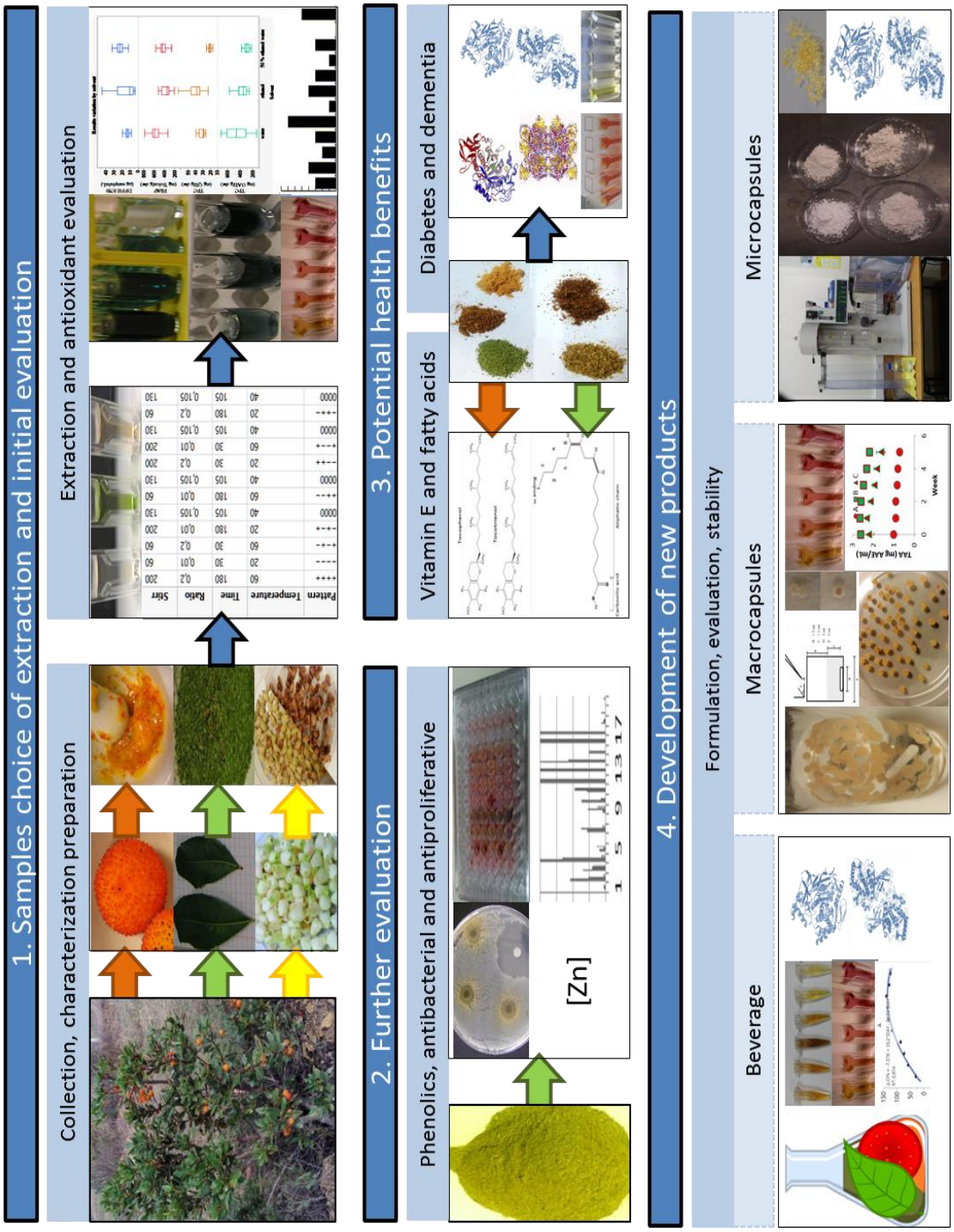

Figure I–2. Work plan.

The major parts of this study have been performed in:

-Food Science Lab from Universidade do Algarve, Portugal.

-The Drug Delivery Laboratory from Universidade do Algarve, Portugal.

-Laboratório de Bromatologia e Hidrologia from Universidade do Porto, faculdade de farmácia, Portugal.

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CHAPTER II - BIBLIOGRAPHIC REVIEW USING TEXT MINING TOOLS.

In this chapter we conduct a bibliographic review regarding the distribution of work and interest in the *Arbutus unedo* tree over the time. We also explore the main topics of research using a semi-automated data collection technique and text mining.

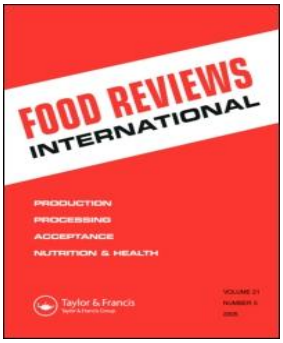
Arbutus unedo L. in literature, a bibliographic review and a case study for text mining using a combination of software programs to expedite the process

Running title: *Arbutus unedo* L. review using text mining

Ricardo Nunes[a], Isabel S. Carvalho[a]*

[a]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

Submitted to Food Reviews International:



1 ABSTRACT

Although text mining is quick, the construction of the required database is laborious. In this work we use different programs together to quickly build the database and make a revision concerning *Arbutus unedo*. The published work distribution showed an increased interest in this plant starting in the year 2008, with two main fields studied, the botanic/plant physiology and the medicinal properties. Abstracts analysis gave more information than titles. Text mining was an efficient tool to analyse the documents and the combination of software shown in this work could be used to quickly study other fields or areas of interest.

KEYWORDS

Arbutus unedo, text mining, Endnote, JMP, KH Coder, Web of Science, review

2 Introduction

Often the number of resources available concerning a theme can be overwhelming to study by conventional means, i.e. reading the documents (Kumar and Ravi, 2016). This is especially true in fields where there is an abundance of published work, as is the case in many subjects related to plant science such as photosynthesis, antioxidant activity or industrial production (Shan et al., 2016, You et al., 2013). In this work we intend to do an unconventional general

review using software that can be obtained at no cost, concerning the fruit tree *Arbutus unedo* L., commonly known as strawberry tree or "medronheiro" in Portuguese. Instead of reading the documents, the information present on them was inserted in a database and the database was analysed using text mining, a knowledge discovery tool (Ramageri, 2010). This technique has been used in areas as diverse as search algorithms for browsers, prebiotic surveys, identification of potential drug safety issues, food safety analysis, stock market predictions, enterprise evaluation and consumer research (Bolasco et al., 2004, Kumar and Ravi, 2016, Jing et al., 2016, Shan et al., 2016, Liu et al., 2017, Kate et al., 2014, Monakhova et al., 2011). It can also be used to identify potential working partners (Jonnalagadda et al., 2017). Although the creation of a database is quicker than reading all the documents, it is still a time consuming process if done manually (copy information from the document and paste it in the database), which we intended to expedite. To accomplish this goal, three tools were used to eliminate most of the processing necessary in the creation of the data base. They were the following: search engine Web of Knowledge© (https://apps.webofknowledge.com/), reference management software Endnote© and a free text mining software called KH Coder© (http://khc.sourceforge.net/en/), a versatile software widely used in Japan, but that has also been used by Povilanskas et al. (2016) to evaluate the tourism impact on the conservation of two world heritage islands. For this author two of the software key features were the treatment of all semantic parts equally, therefore allowing them to self-define their own associations and relations and the capacity it has to display complex information in a graphic outputs easily understandable. Excel® was used to collate all the gathered data from Endnote and JMP 12® (http://www.jmp.com/en_us/home.html) was used for the distribution analysis. Web of Knowledge is a search engine from the Web of Science™ which collects scientific articles, books, book sections, patents, and other forms of scientific knowledge dissemination. According to its website it has over 1 billion recorded citations from over 200 million source items and its coverage extends more than a century back, encompassing over 12 000 different scientific and scholarly journals. Other than its immense reach, it also has several interesting data concerning article statistics and functionalities. For this work the most important functionality was the ability to directly export bibliographic references into a reference manager software, which is easier to use and allows multiple exports at the same time, unlike Pubmed, which was used in a work by Markazi-Moghaddam (2016) to evaluate hospital performance, or ScienceDirect. This allows a massive amount of time saved when compared to manually imputing the information required for the process of text mining. The desktop version of Endnote was used for the purpose of this work, although Endnote web is free and

can be used instead (using tab delimited style). The final step required for the text mining was the introduction of the data into KH Coder, a text mining software available at no cost, which can be used in quantitative content analysis, also known as text mining, or in computational linguistics. The input can be in several languages other than English, although this work everything was in English. Several characters need to be removed from the text, together with some words, making this the highest time consumption part of the review, but data pre-processing is an integral step for the analysis to properly run and the results useable (Uysal and Gunal, 2014). KH Coder can analyse the relation of any word present in the text with other words, and represent the results using visualization aids such as co-occurrence networks of hierarchical cluster analysis. Since the final result consists in a list of grouped words which were already present on the document, it is an extractive technique and not an abstractive technique (Song et al., 2015). This technique is also often used in the economics area to classify documents into different subjects, a time consuming and subjective process previously done by researchers (Chakraborty et al., 2014, Fisher et al., 2010). It can be applied to a single document, or to a database of documents, as was the case in the present work (Xie et al., 2017, Wan and Xiao, 2010). Since the goal was not only to determine the interaction between words but also the distribution of works through time, a distribution analysis was performed using JMP. This software is focused on the statistical aspects of data and allows the creation of a quick data distribution, which can be further customized by showing counts and percentages for a large time period. It also allows a quick separation and analysis of a specific subset on time, journals or keywords. The conjunction of these software allowed a much quicker analysis of the documents, 190 with *Arbutus unedo* in the title, than would otherwise be possible, and still allowed the collection of important information regarding this tree fruits, leaves and flowers, their compounds and the fields of study.

3 Methodology

3.1 Web of Knowledge search

Initially a search was performed on the topic "*Arbutus unedo"*. This retrieved a total of 472 results (list 1). A search for the topic includes sources where a certain word is mentioned in places other than the title. It may be useful if the work includes several plants which due to scientific documents often having a character limit on the title may not be listed there. Of these 472, 2 of the sources were books, 23 were book sections and the remainder were articles. To search works that focus on *Arbutus unedo* in particular, instead of retrieving

results where it may have been only one of 25 plants studied, a new search was performed, this time with *"Arbutus unedo*" in the title. This search retrieved a total of 190 results, of which 5 were book sections and the remainder were articles (list 2). Using this base, a search for the three most common plant parts studied was performed using "leaf", "fruit" and/or "flower" as topics. Both lists were directly exported to endnote with information regarding year, title, keywords, abstract and journal.

3.2 Organizing references in endnote and exporting the results

Using the file from the Web of Knowledge export, the references were imported into two separate libraries on endnote. These libraries were then exported into excel using a custom output style which allowed the exportation of years, titles and journals all at once. Keywords and abstracts had to be exported individually to prevent formatting issues, which could only be done with custom output styles. These exports were then collated in excel.

3.3 JMP treatment

Two lists were used for the determination of the data distribution using JMP 12. The first was list 2 which consists in the year and journal publications, the second was list 3 which consists in the keywords. The data was directly imported from excel and the distribution was calculated, from the whole list 2 and then from subsets. The first subset was from the years 1924 until 1996. During this period there were years without publications containing *Arbutus unedo* in the title. The second subset was from 1997 to 2016, during which there were publications every year with *Arbutus unedo* on the title. Finally there was another subset created from 2010 to 2016, to have a better look at the current trend. For list 3, the data had to be previously treated to remove situations of words not being counted together due to uses of singular/plural forms, the treatment consisted in joining both words i.e. flavonoid and flavonoids into just flavonoids.

3.4 KH Coder

3.4.1 Data treatment

The data was treated previous to insertion in KH Coder due to limitations of the software in handling unknown symbols. The following symbols were removed "/", "<", ">"and "|". For the abstracts the code <h1> and </h1> was inserted before and after the abstract, i.e. <h1> "abstract text" </h1>. This step was necessary for the program to recognize where one abstract ends and the other begins. The data was then imported into KH Coder in the format of a ".txt" file. Previous to analysis the pre-processing was run on the program. This allowed

the correction of any errors or invalid symbols the file might still have. Only nouns, verbs and foreign words were analysed, this prevents words that are common in English language from being analysed i.e. the, in, on.

3.4.2 Word association

Using information obtained previously in JMP from the distribution of keywords, the most common were searched for their word associations in KH Coder, using "Jaccard" sorting method. Some keywords did not appear in the titles. For those that did, the 25 words most related to them were filtered and represented in a Co-occurrence network colour coded by "communities betweeness" parameter. The nodes size was chosen to be based on how many times a word is related to the keyword the search was based on.

3.4.3 Co-occurrence network

Two co-occurrence networks with all the data were constructed, one for the titles and one for the abstracts. The sorting method was "Jaccard" and the colour coding was "communities betweeness". Results were filtered to represent only the 60 most prominent words. The size of the nodes is related to the prominence of a word.

4 Results and discussion

4.1 Distribution of 190 works on Arbutus unedo according to the plant part

Of the 190 works that had "*Arbutus unedo"* on the title, 74 were about leaves, 58 about fruits and 10 about flowers (Figure II–2). There were 14 where leaves and fruits were studied and 3 which were about leaves, fruits and flowers. Of the 74 works related with leaves, 16 were related with antioxidants, 4 with antimicrobial activity and 3 with both. Fruits on the other hand were much more focused on the antioxidants, with 22 out of the 58 works focusing on this theme and 5 focusing on antimicrobial activity while 3 are focused on both. It should also be mentioned that most of the studies on flowers are concerning flower markers and honey quality, and only 1 work was about antioxidant activity, the same for antimicrobial activity. Of the 190 works, only 6 were pertaining the antioxidant activity of both leaves and fruits, 3 were pertaining their antimicrobial activities and only 2 are about their antioxidant and antimicrobial activities.

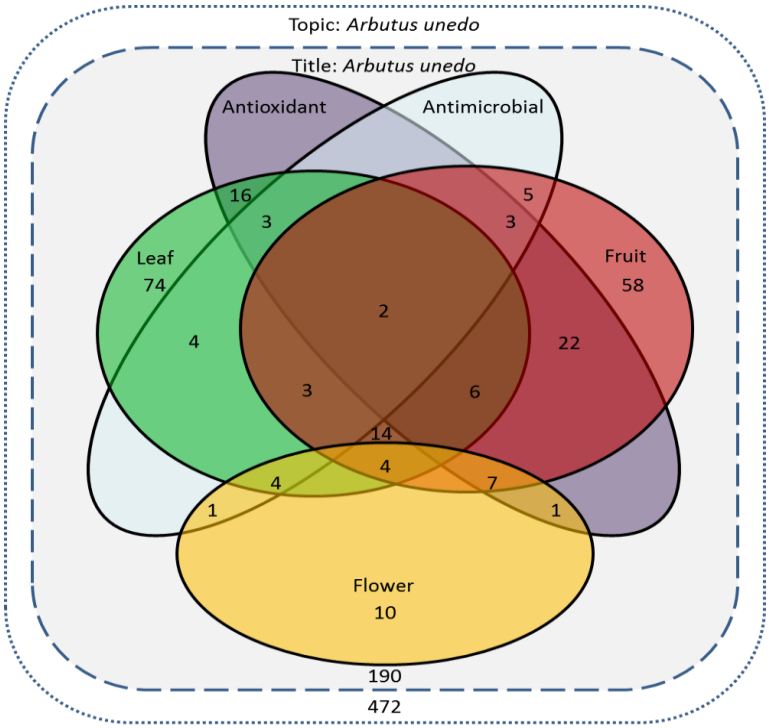


Figure II–1. Venn diagram of the results obtained on the Web of Knowledge search.

4.2 JMP

4.2.1 Distribution of year and journal in JMP

To understand the scattering of work published throughout the time, several distribution graphs with the list 2 were made in JMP. This can help determine the interest on the subject throughout a period of time, the journals that were used the most, shifts in trends and some current information. Regarding the distribution of published work throughout the years, the earliest report was in 1924, followed by a 25 year hiatus (Figure II–2). From the years 1968 to 1996 almost every year would have at least one work with *Arbutus unedo* on the title, with 1990 and 1995 being especially productive years during this period, with 6 articles each, accounting together for 26 % of a total of 47 works published during that period (Figure II–3). During this period, most works were on ecology/botany, as it can be seen by the distribution of places they were published. Oecologia journal was the place of publication most used, accounting for 21% of the total versus 9% on Flora journal, the second most used place of publication. Many journals only had one work published with *"Arbutus unedo"* in the title. Surprisingly during the years 1990 and 1995, only one work was published in Flora and none in Oecologia. From 1997 onwards, there was a work published every year. In total there were 143 works published in this period with a growing trend until 2011 after which it started to slightly dip, although there is still more work published than before 2007.

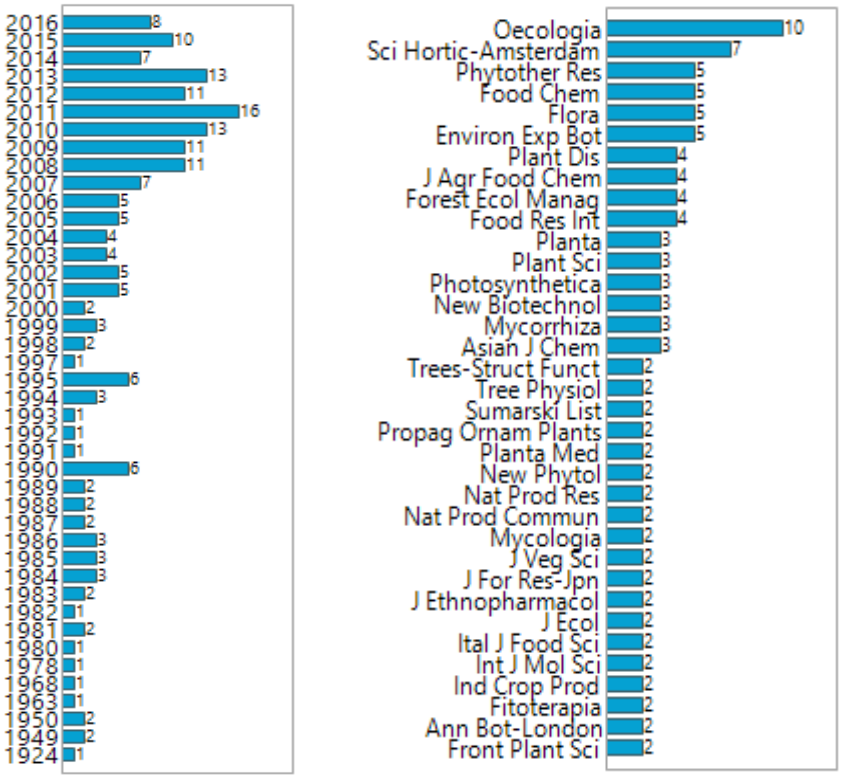


Figure II–2. Distribution of work over time and publication place (2 or more works).

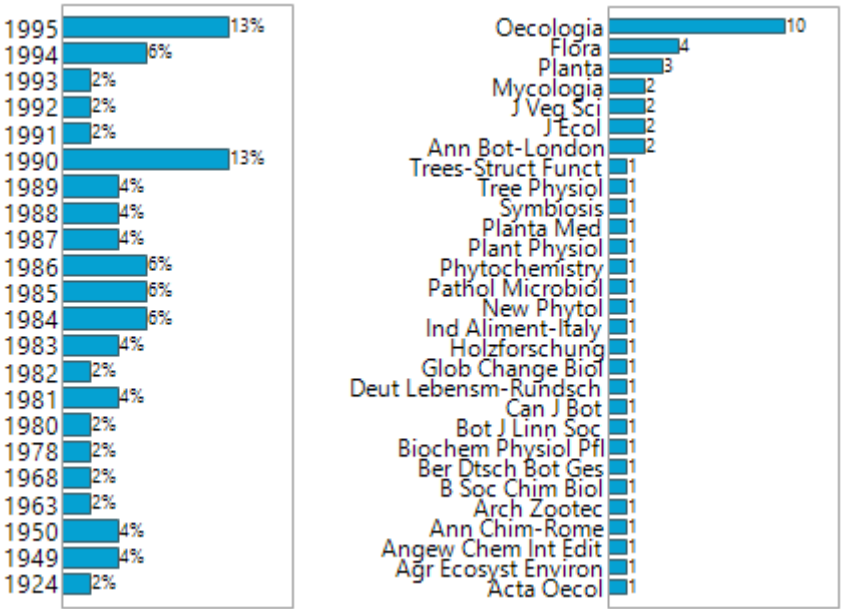


Figure II–3. Distribution of work over time (1924-195) and publication place.

Similarly to the period before, many journals only had one work published while others had clear interest on the subject. Scientia Horticulturae had the most work published with 7 in total, followed by Phytotherapy Research and Environmental and Experimental Botany with 5 each (Figure II–4A). The work published from 2010 onwards accounts for over 50% of the total work published during this period.

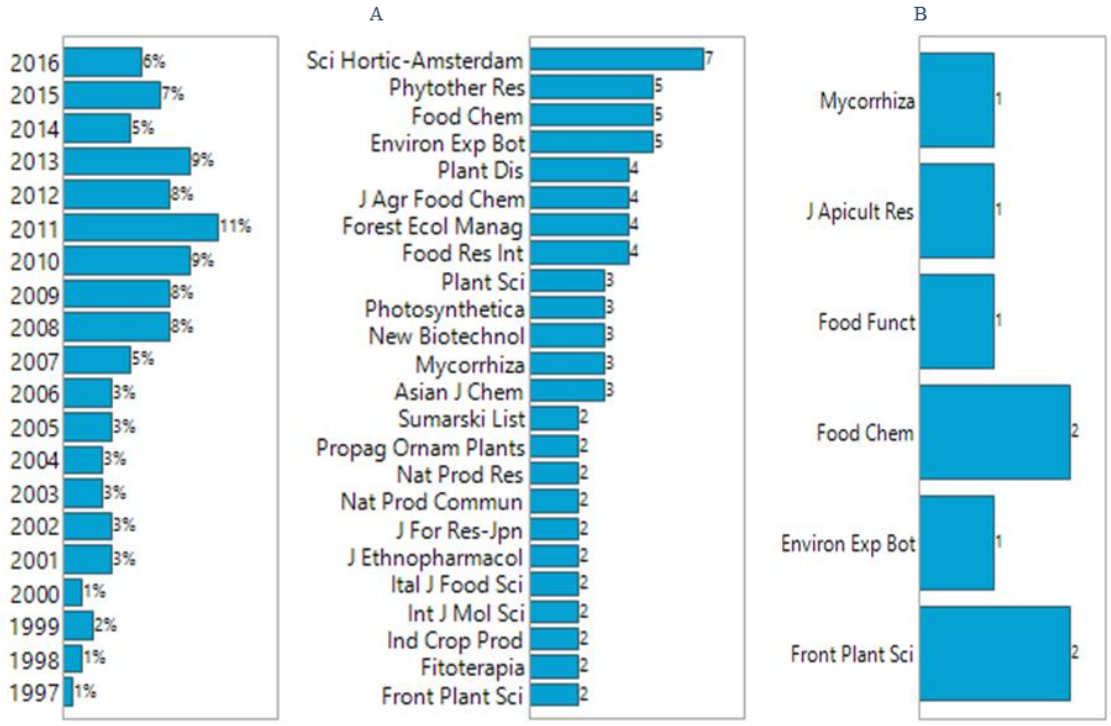


Figure II–4. Distribution of work over time and publication place (A-1997-2016; B- 2016)

Focusing on this period, the journals most used were Scientia Horticulturae and Food Research International with 4 each, followed by Mycorrhiza and Food Chemistry with 3 each. None of these journals however had any work published in 2015 when 10 total works were published. In the current year, Food Chemistry and Frontiers in Plant Science are the journals with most work published, with 2 each, from a total of 8 (Figure II–4B). The distribution information tells us that interest in the tree *Arbutus unedo* has grown and is still greatly present and that it has somewhat shifted from ecology/botany into plant science and chemistry. This knowledge could be used to decide which journal to submit to and which theme to focus on in order to have the best publishing success.

4.2.2 Keywords

The distribution of keyword usage can help understand which topics were the most studied, which complements the information of the journals most used (Figure II–5) (Salton and Buckley, 1988). From a total of 1727 keywords used, 1050 were different, of which 810 were used only once. From those that were used more than once, 40 were used 5 or more times and 14 over 10 times. The top three keywords were *Arbutus unedo* which unsurprisingly was at the top with 57 usages, followed by strawberry tree, and antioxidant activity with 33 and 20 each. The assays/properties most used in the keywords were in this order antioxidant activity > gas exchange > photosynthesis > water relations > water stress > antimicrobial activity >

stomatal conductance all with more than 5 uses and the goals were chemical composition, identification and selection, all with 7 or more usages.

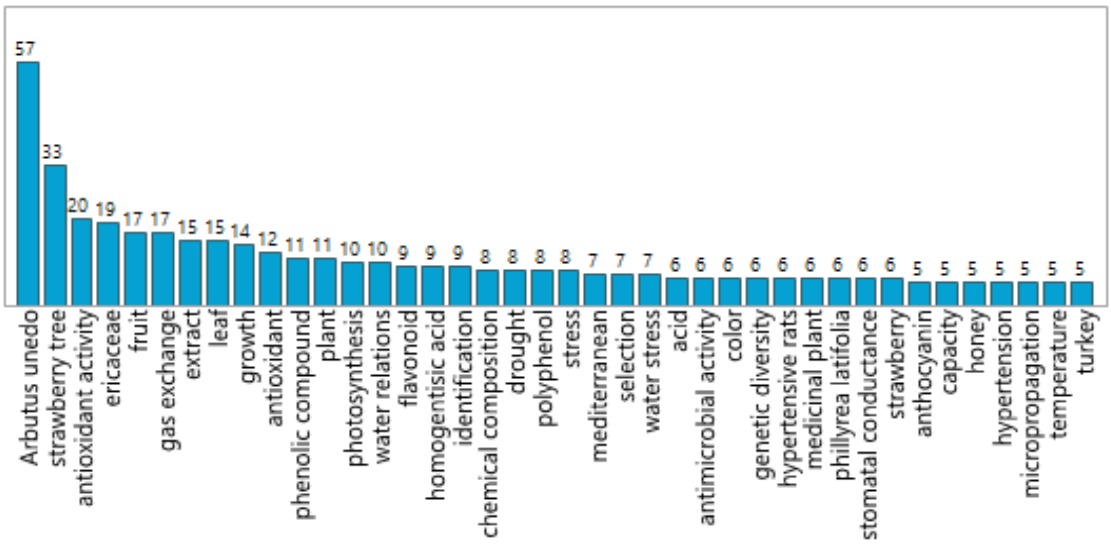


Figure II–5. Keyword distribution.

The plant parts most studied were fruits closely followed by leaves. On the other hand the compounds most present in the keywords were in this order phenolic compounds > flavonoids = homogentisic acid > polyphenols > anthocyanins, with at least 5 usages each. The keywords show there are two main themes, the ecology/plant physiology part with growth, water relations, photosynthesis, water stress, stomatal conductance and genetic diversity, and the medicinal one with antioxidant activity, phenolic compounds, flavonoids, and antimicrobial activity, which is in accordance with what was seen previously with the titles of the journals.

4.3 KH Coder

4.3.1 Word association and co-occurrence network for titles

One of the methods KH Coder has to examine data is word association. This means the program will display the words most often associated with any word typed into a search box. It can then construct co-occurrence networks which are easy to interpret. Other representations that can be used for representing data include bag of words or n-gram models (Le and Mikolov, 2014). For the easier interpretation of the networks, and to display only the most important words, a term frequency constraint was used, preventing words that appear less than 5 times from being represented (Salton and Buckley, 1988). This is especially important if different years are to be compared, otherwise a more prolific year would have a much more complex network, simply due to a higher word count, as shown by Li et al. (2016). If instead the goal is to find areas lacking research, the constraint can be changed so

that only those appearing fewer times are shown. The most common keywords, according to the distribution results from JMP, were searched for their association. Although some of them were not found in the titles, this analysis is considered a good indicator for document classification and information extraction (Periakaruppan and Nadarajan, 2015, Onan et al., 2016, Curci and Ospina, 2016) and their absence can be explained by the usual small size titles have, a constraint applied by the publishing journals. Out of the top keywords, those present in titles were "antioxidant"; "anthocyanins"; "antimicrobial activity"; "*Arbutus unedo"*; "flavonoid"; "fruit"; "gas exchange"; "homogentisic acid"; "leaf"; "phenolic"; "photosynthesis"; "stomatal conductance" and "water stress". Analysing the co-occurrence networks built from the word association, 5 different topics are visible, the influence of fruit ripening and flowering stage in antioxidant composition, the effect of phenolic content and its interaction in antioxidant activity, the effect of freezing, drying and colour in antioxidant activity and a final group which points towards being an underutilized source of valuable antioxidants. "Antimicrobial activity" was related to phytochemical screening, identification of phenolic compounds, and root, leaf and stems extracts. "Gas exchange" was mostly associated with transpiration, photosynthesis, sodium chloride and water. Also to note Portugal appears as a highly related word, meaning many of these studies were conducted either in Portugal or with plants collected in Portugal. "Photosynthesis" and "stomatal conductance" were mostly associated with transpiration, the variation of experimental conditions and once again Portugal. "Water stress" was mostly related to atmospheric control, stomata and growth on one side and xanthophyll, summer, photoprotection on the other. "*Arbutus unedo"* co-occurrence network had two major groups one mostly related to antioxidant composition where fruits and honey are present, and another which deals with plant physiology, where leafs, stomata, gas exchanges and factors influencing them are present. This separation is in accordance with the split also seen in the journals, some related to medicinal properties and others to botany. The word "fruits" was mostly related with optimization of phenolic extraction, effect of ripening on antioxidant composition, volatile components and nutritional assessment. "Leaf" was mostly associated with abscicic acid, antioxidant activity, and once again the plant physiologogy part with stomata, midday, photosynthesis and humidity. "Anthocyanins" were mostly associated with fruits and identification of phenolic derivatives. "Flavonoid" was mostly related with determination, chromatography, leaf and chlorogenic acid. "Homogentisic acid" was mostly associated with methods for rapid chromatographic determination and its use as a honey marker. "Phenolic" was mostly associated with antioxidant activity, metabolism and stability, identification of

homogentisic and gallic acid derivatives and anthocyanins, spasmoliytic properties and finally roots. Finally, analysing the global co-occurrence network, its visible the separation into several groups which delimit the topics of interest related to *Arbutus unedo*, such as antioxidant and antimicrobial activities, the use of bioactive compounds as genetic markers for honey, the interest in the in vitro micropropagation, study of temperature storage, and the plant physiology part (Figure II–6). It is also visible that fruit is the only plant part on the network, which is understandable given its use in several food products.

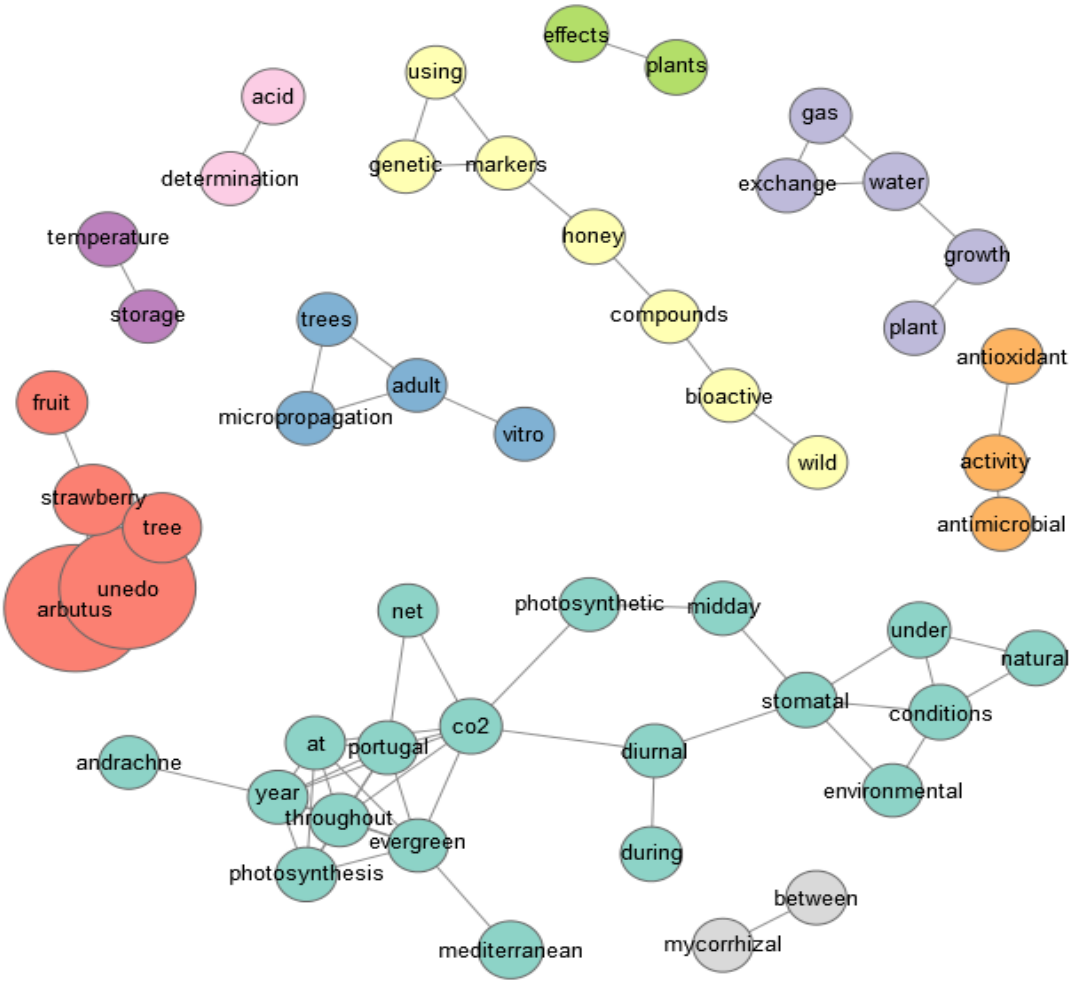


Figure II–6. Co-occurrence network for titles.

4.4 Word association and co-occurrence network for abstracts

Unlike what happened with titles, with abstracts all keywords appeared, however in the interest of comparing the information collected from titles to that collected from abstracts, only those present in titles will be studied. Analysing the co-occurrence network obtained from the word association for the expression “antioxidant activity” some differences from the network obtained in titles are visible, these differences from titles to abstracts is seen across all the keywords. In the abstracts, “antioxidant activity” is mostly associated with methods

used, such as DPPH radical and total phenolic content. The word ethanol is also highly associated, indicating many of the extracts studied are prepared in ethanol. “Antimicrobial activity” appears related with moderate antibacterial activity and the bacteria *Staphylococcus aureus* and *Escherichia coli* together with the word investigated indicating these are the bacteria most studied and the extract has inhibiting activity towards them. The focus on these two bacteria is no surprise, given they are both highly present in the environment, are pathogenic and are responsible for many food safety problems. “Gas exchange” was mostly associated with stress, transplanted plants and irrigation in greenhouses and as seen in titles with sodium chloride. “Photosynthesis” and “stomatal conductance” were mostly associated with the variation of experimental conditions, greenhouses, salinity and influence of transplantation. “Water stress” was mostly related to atmospheric control, stomata photosynthesis, carbon dioxide exchange mechanisms pigments, xanthophyll and zeaxanthin. “*Arbutus unedo”* co-occurrence network showed the most common associated words showed groups with leaf and fruit extracts phenolic content and antioxidant activity and the fact they are not currently used. To note that unlike with titles there was no separation with medicinal properties on one side and botany on the other. This is likely due to the fact that even on botany/plant physiology works the antioxidant potential is often mentioned but the physiology is not mentioned on works focusing on medicinal properties. This difference then causes the botany part to not appear due to the maximum 25 terms shown cut off. The word ”fruits” was mostly related with their production, their phenolic content and high antioxidant activity and natural characteristics. “Leaf” was mostly associated with stomatal conductance and higher antioxidant activity in water extracts. “Anthocyanins” were mostly associated with ellagic acid, antioxidant and vitamin sources, wide importance of gallic and ascorbic acids bioactive compounds, vitamin C and cyanindin quantification. “Flavonoid” was mostly related with calcium channel inhibition, colour, radical scavenging and MTT assay, dephinidin and carotenoids abundance. “Homogentisic acid” was mostly associated with honey of monofloral origin marker as a way to distinguish producers and with NMR which stands for Nuclear Magnetic Resonance spectroscopy. “Phenolic” was mostly associated with antioxidant activity and total content present in the extract, mainly of fruits and the scavenging activity of DPPH, indicating this is one of the most common methods. Finally, analysing the global co-occurrence network (Figure II–7), it appears water (WE-water extract) is associated with higher concentration in leafs but not fruits, which are themselves related with high antioxidant activity and total phenolic content. The effect of extract in the antioxidant activity is also studied and there is a separate group dealing only with different genetic populations.

Both titles and abstracts gave different valuable information. If the goal is to collect information regarding the works being mainly done in a certain subject, the text mining of titles is appropriate and at times preferable. This is due to limitations imposed by editors of scientific journals or books, which causes the title to be concise and display only essential information, allowing a more direct approach. However if more information is wanted, then the text mining should be conducted using abstracts, since the methodology used in the works and the main results and conclusions are usually present there.

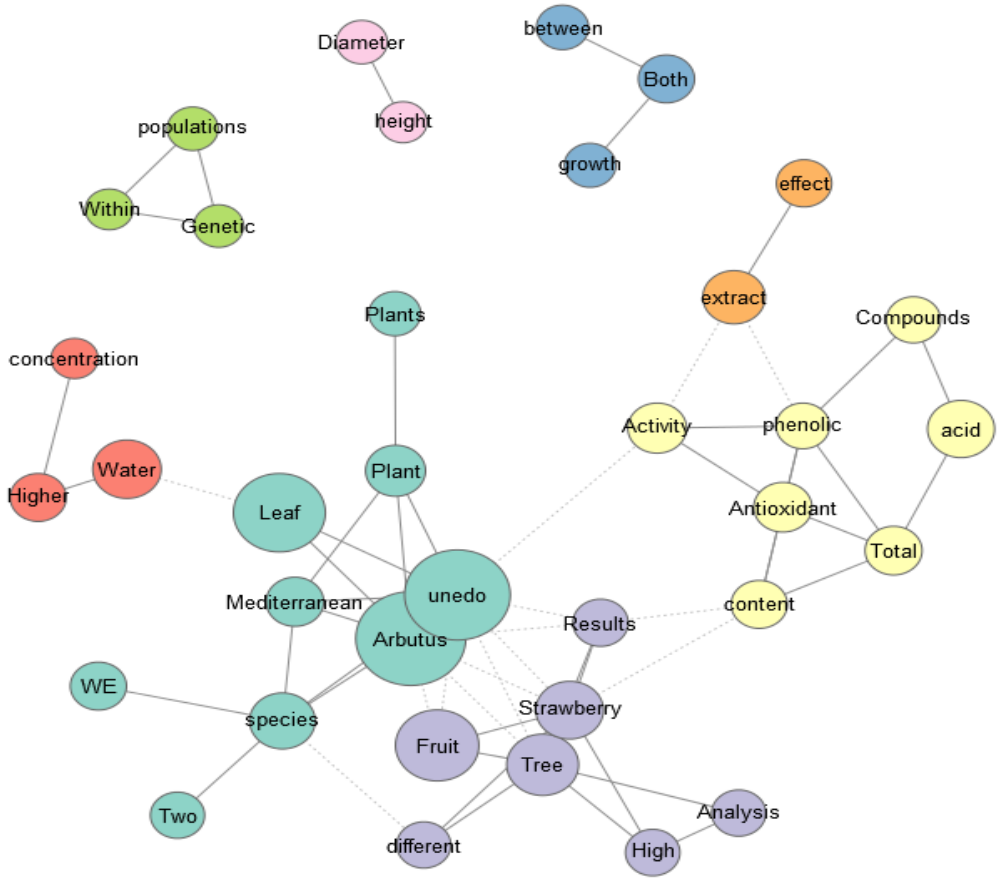


Figure II–7.Co-occurrence network for abstracts.

5 Conclusion

The preliminary analysis of keyword frequency and year distribution is a good way to not only verify if the field or subject is still of interest for the scientific community, but also to determine the most investigated parameters, either for the determination of a novel field of investigation or for focusing in a field of investigation that is still of interest. In conjunction with years and/or titles it also allows the selection of works with those keywords, which can be helpful when preparing a bibliographic review. By studying word association it's possible to check which words often appear related to a certain keyword, giving greater insight on the relations between different objects of study. The use of co-occurrence network to visualize the

information greatly helps in reading the groups and associations. Depending on the information that is desired, text mining should be conducted on titles, abstracts or both. Titles give more succinct information but their analysis might not be enough if the goal is to find the most commonly used methods, since that information is usually supressed from the title. Overall the use of the selected tools, web of knowledge, Endnote, JMP and KH Coder allowed the analysis of 190 documents much quicker than the time it would take to read all of them. Due to the interaction between web of knowledge and Endnote the time spent in the construction of the database is practically non-existent, meaning this methodology allows the analysis of a much greater number of documents if necessary and can be applied in a great number of fields. This approach can thus be extremely useful if the field of study encompasses a great number of documents, too great a number for all to be read.

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CHAPTER III - ANTIOXIDANT CHARACTERIZATION OF PLANTS FROM THE ERICACEAE FAMILY

In this chapter we investigate the inter-tree variability using a total of 18 different samples. We then evaluate the antioxidant activity of *Arbutus unedo* using different extraction conditions and different solvents and compare the findings with two other Ericaceae, *Erica australis* and *Erica arborea*.

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Antioxidant and free radical scavenging activities of different plant parts from two *Erica* species

Ricardo Nunes[1], Ana Anastácio[1] and Isabel S. Carvalho[2,3]

1IBB/CGB-Institute for Biotechnology and Bioengineering/Centre of Genomics and Biotechnology, Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas, Faro, Portugal

2Food Science Laboratory, Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas, Building 8, 8005-139 Faro, Portugal

1 ABSTRACT

In this work aqueous extractions from two plants consumed as infusions in several countries to heal ailments were investigated for their phenolic and flavonoid contents, along with antioxidant capacity and radical scavenging capacity using TAA, FRAP, RP and DPHH, ABTS radicals respectively. Antioxidant properties and total phenolic content differed significantly among selected plants. It was found that leaves aqueous extracts possessed in average the highest antioxidant capacities and phenolic contents (30.59 mg GAE/g dw) of all three plant parts. A significant correlation (r^2 = 0.952) between antioxidant capacity and total phenolic content was found, indicating that phenolic compounds are the major contributors to the antioxidant properties of these plants. Upon application of Hierarchical Cluster Analysis to the results from obtained from assays, leaves were grouped with flowers in one cluster and branches remained in another cluster, showing little interference from the collection site or specie factors.

Practical applications

Results obtained support that there may exist benefits for health from ingesting this plant infusions and that these plants have a great potential to serve as a cheap antioxidant source.

KEYWORDS

Erica; antioxidant capacity; cluster analysis

2 Introduction

Since ancient times, humans have used plants to treat many kinds of illnesses. This knowledge is passed down, from elders to younger people, passing it through generations

until our days. In most countries folk medicine was discarded by official medicine practice (Neves et al., 2009b). However the majority of approved drugs against cancer and infectious diseases are of natural origin meaning plants contain powerful compounds, capable of preventing or treating some illnesses (Newman et al., 2003, Akkol et al., 2008a). Over the time, with increasing pollution of modernized world and changes in life style, some illnesses became more common (Teixeira et al., 2010, Kan et al., 2008). Cancer, atherosclerosis, degenerative diseases such as Alzheimer as well as precocious aging, are caused or powered by free radicals, which can be neutralized by antioxidants, and some of the biggest natural sources are plants and fruits (Bondet et al., 1997). This relative abundance of antioxidants has led to an increasing interest in plants, which translated in numerous researches in the Mediterranean region, where Portugal is included. These investigations are important since not every plant has the same amount and type of antioxidants, making some plants more valuable. Studies usually focus on analyzing species that exist in their country, and some plants used in folklore medicine, usually served as infusions, are already proved to be beneficial. Some examples are *Melissa officinalis* and *Rosmarinus officilanili.* (Allahverdiyev et al., 2004, Benincá et al., 2011). Among the plants used as infusions, are bushes from the *Erica* genus. These bushes have woody branches, with small pine like leaves and white or pink flowers. They are commonly found in North Africa, Mediterranean region and Western Europe (Akkol et al., 2008a). *Erica spp.* is usually used in Morocco and Turkey's folk medicine as diuretic, antiseptic agent, and to treat urinary infections (Akkol et al., 2008a, Luís et al., 2009). In Portugal it's used to treat prostate, kidney, and bladder problems (Neves et al., 2009b). In addition *Erica* wood has economic value as pipe material. Ten species are reported in Portugal, but only *E. arborea* and *E. australis* were collected for this study, white and pink flower respectively. Some studies on *Erica* species, mainly *E. arborea*, exhibited compounds such as flavonoids, which are polyphenols that have been proven to help prevent heart diseases, anthocyanidins among others (Carvalho et al., 2011). Also a new phenylpropanoid glucoside, named ericarborin was identified in this specie (Nazemiyeh et al., 2008). Some medicinal properties have also already been studied, such as anti-inflammatory capacity of *E. arborea* extract (Akkol et al., 2008a). However most studies have lack of variability, few collection sites, and limited number of plant parts or low diversity of assays performed. Therefore, the present work was aimed to provide a broader analysis of these bushes regarding phenol content and antioxidant capacity of leafs, flowers and branches, in order to ascertain their potential value as sources of these compounds investigate the importance of collection site and differences between species.

3 Materials and methods

3.1 Reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, 2-2'-Azino-bis(3 ethylbenzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS), ammonium molybdate, sodium phosphate dibasic dehydrate, monosodium phosphate hydrous, gallic acid, quercetin and Trolox were purchased from Sigma-Aldrich Co. Ltd (Poole, UK). Iron (III) chloride, methanol, trichloroacetid acid, sodium acetate, sodium carbonate, sulfuric acid, glacial acetic acid, 2,4,6-tripyridyl-2-triazine (TPTZ), hydrochloridric acid and ascorbic acid were purchased from VWR (Pennsylvania, USA), Aluminium chloride and absolute ethanols were purchased from Merck (Nottingham, UK). All reagents were of analytical grade.

3.2 Plant materials

Samples from wild *Erica spp.* plants were randomly selected and collected during the blooming period in the ending of spring, beginning of summer of 2010. Five samples were collected, all from the Algarve region (Portugal): 1 (*Erica australis*, Ponte do Leitejo, -8.314858, 37.125644); 2 (*Erica australis*, Feiteira, -7.921556, 37.426508); 3 (*Erica australis*, Cotifo, 37.18757,-8.695348); 4 (*Erica arborea*, Barranco do Velho 1, -7.9382, 37.2319); 5 (*Erica arborea*, Barranco do Velho 2, -7.9353, 37.2304). All samples were collected on a dry day. After collection, plant material was stored on a dry place, protected from sunlight. Samples were naturally air dried for about one week. After drying, leaves, flowers and branches were manually separated. Leaves and flowers were stored on plastic vials at -4ºC while branches were stored at room temperature until extraction. On the day of extraction leaves were grinded on a mortar and pestle at room temperature, while flowers had to be frozen with liquid nitrogen prior to grinding, due to a hard core. Branches were grinded by kitchen grinding equipment. Plant materials (2 g) were packed in a cloth bag to reduce migration of powder, and inserted into 30 mL of distilled water (at initial temperature of 95ºC) for 15 minutes, with stirring. Infusions were filtered through Whatmman nº 4 paper, and their volume was made up to 25 mL. Aqueous infusions were finally transferred to Eppendorfs and stored at -4ºC. On the day of analysis, extracts were put on ice, protected from light, until unfrozen.

3.3 Total Phenol Content

Total Phenol content (TPC) of infusions was determined through a spectrophotometer (Huang et al., 2006). Briefly, 0.1 mL of properly diluted extract was mixed with 0.5 mL

Folin-Ciocalteau's reagent and 0.4 mL of a saturated sodium carbonate solution (7.5 %). After standing for 30 min in a dark room, absorbance was read at 765 nm against a blank in a spectrophotometer. Total Phenolic content was calculated by a calibration curve of gallic acid and was expressed as mg GAE/g dw (gallic acid equivalents per gram dry weight).

3.4 Total Flavonoid Content

Total Flavonoid Content (TFC) was analyzed using a spectrophotometer (Huang et al., 2006). Properly diluted extract (0.5 mL) was mixed with 2 % methanolic $AlCl_3.6H_2O$ (1.0 mL). The absorbance was measured at 430 nm after standing for 10 min in a dark room. Flavonoid content was calculated by a calibration curve of quercetin, and was expressed as mg QE (quercetin equivalents)/g dw.

3.5 Total Antioxidant Activity

Total Antioxidant Activity (TAA) of extracts was determined through a spectrophotometer (Prieto et al., 1998a). Briefly, 0.1 mL of properly diluted extract was mixed with 1.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Mixture was then incubated at 95ºC for 90 min in water-bath. Absorbance was measured at 695 nm and results calculated against an ascorbic acid calibration curve. Results are expressed as mg AAE (ascorbic acid equivalents)/g dw.

3.6 Reducing Power

Reducing Power (RP) was determined through a spectrophotometer (Yen et al., 2000). Briefly 0.2 mL of properly diluted extract in 0.5mL of sodium phosphate buffer (0.2 M, pH 6.6) was mixed with 0.5 mL potassium ferricyanide (1%) and mixture was incubated at 50ºC for 20 min. 0.5 mL of Trichloroacetic acid (10 %) was then added and mixture was centrifuged at 650 x g for 10 min. 0.5 mL of supernatant were then mixed with 0.5 mL of distilled water and 0.1 mL of ferric chloride (0.1 %). Absorbance was measured at 700 nm. Results were expressed as mg Trolox/g dw.

3.7 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined through a spectrophotometer (Benzie and Strain, 1996b). Three stock solutions were prepared, a 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$ for each liter of solution), pH=3.6, a 10 mM TPTZ solution in 40 mM HCl, and a 20 mM $FeCl_3.6H_2O$ solution. Working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3.6H_2O$ solution. This working solution was then heated to 37ºC. A 75 µL aliquot of

properly diluted extract was mixed with 1.425 mL of working solution. Absorbance was read at 593 nm 30 min after mixing. Results were calculated against a Trolox calibration curve and expressed as mg Trolox/g dw.

3.8 DPPH Radical Scavenging Capacity

DPPH Scavenging effects of extracts were determined using DPPH Radical Scavenging Capacity (DPPH) method with slight modifications (Luo et al., 2010). Briefly, 1.0 mL of a 0.16 mM DPPH solution was added to the test tube containing 1.0 mL of properly diluted extract in different concentrations. Mixture was then vortexed for 1 min at 800 rpm and kept in the dark for 30 min at room temperature. Absorbance of samples was measured at 517 nm. The % radical scavenging capacity (IC) was calculated using the following formula: (IC) = $((A_0-A_t)/A_0)$ x 100, where A_0 is absorbance of control at 30 min and A_t absorbance of sample at 30 min. Results were expressed as μg sample mL^{-1} in form of IC_{50}, determined by linear regression of IC and extract concentration at 50 % inhibition.

3.9 ABTS Radical Scavenging Capacity

Extract ABTS Radical Scavenging Capacity (ABTS) was determined through a spectrophotometer (Re et al., 1998). A stock solution of ABTS radical was prepared by reacting ABTS (7 mM in water) solution with potassium persulfate (2.45 mM final concentration) and allowing this mixture to stand in dark at room temperature for 12-16h before use. Stock solution of ABTS was diluted just before use to an absorbance of 0.70 (+0.02) at 734 nm. 2 mL of diluted ABTS was added to 100 μL of properly diluted extract, and mixed thoroughly. Absorbance was read 5 min after mixing. Results were expressed as μg sample mL^{-1} in form of IC_{50}.

3.10 Data Statistical analysis

Statistical analysis was performed using SPSS 18.0 (SPSS Inc, Chigaco). The α level was fixed at 0.05. All results are shown as mean ± S.D from three repetitions. ANOVA test was applied to data in order to verify if specie, plant part or location explained results variability. When homogeneity of variance was not verified by Levene's test, Welch statistics was used instead. Fisher's Least Significant Difference (FLSD) or Games–Howell (GH) post hoc tests were performed to determine statistically significant means. Finally, samples or assays were divided into similar groups through a Hierarquical Cluster Analysis using between groups linkage and Squared Euclidian distance.

4 Results and Discussion

4.1 Total phenol and flavonoid contents

In general both TPC and TFC results differ significantly ($p<0.05$) for specie, plant part and location (Table III–1), but it seems these factors have more influence in total phenolic contents than they have in flavonoid content. In both assays, *E. australis* performed generally better than *E. arborea* but plant part presented the same order for both methods, leaves>flowers>branches, with average results of 30.6; 24.4; 7.8 (mg GAE/g dw) and 4.0; 0.5; 0.21 (mg QE/g dw) respectively for both assays.

Table III–1. TPC and TFC results

Specie	plant part	location	Sample ID	TPC (mg GAE/g dw)	TFC (mg QE/g dw)
E. australis	leaves	1	1	21.90 ± 0.13^{a}	3.03 ± 0.18^{a}
		2	2	36.34 ± 0.36^{b}	5.59 ± 0.22^{b}
		3	3	40.28 ± 0.09^{c}	4.54 ± 0.03^{b}
			average	32.84 ± 8.38	4.39 ± 1.12
	flowers	2	4	32.43 ± 0.70^{d}	0.82 ± 0.04^{c}
		3	5	15.87 ± 0.82^{e}	0.23 ± 0.01^{de}
			average	24.15 ± 9.09	0.52 ± 0.33
	branches	1	6	10.58 ± 0.19^{f}	0.13 ± 0.02^{ef}
		2	7	12.20 ± 0.55^{f}	0.25 ± 0.01^{d}
		3	8	3.72 ± 0.08^{g}	0.16 ± 0.00^{f}
			average	8.83 ± 3..91	0.18 ± 0.05
E. arborea	leaves	4	9	17.45 ± 0.12^{e}	2.70 ± 0.06^{a}
		5	10	36.97 ± 0.75^{b}	4.12 ± 0.03^{g}
			average	27.21 ± 10.70	3.41 ± 0.78
	flowers	5	11	24.83 ± 0.26^{h}	0.44 ± 0.03^{h}
			average	24.83 ± 0.26	0.44 ± 0.03
	branches	4	12	7.37 ± 0.10^{i}	0.35 ± 0.00^{h}
		5	13	5.24 ± 0.36^{j}	0.16 ± 0.01^{f}
			average	6.30 ± 1.19	0.25 ± 0.10
average leaves				30.59 ± 10.19	4.00 ± 1.17
average flowers				24.38 ± 8.29	0.50 ± 0.30
average branches				7.82 ± 3.56	0.21 ± 0.09

Values are means ± standard deviation of three assays. Different letters in the same column mean statistically different results, by Games Howell test ($P < 0.05$). dw, dry weight; GAE, gallic acid equivalent; QE, quercetin equivalent; TFC, total flavonoid content; TPC, total phenolic content.

These results are different from other published for same genus. Both a Turkish and a Portuguese studies presented higher values than those obtained in the current study (Luís et al., 2009, Ay et al., 2007). These differences can be explained by several factors such as collection site, extraction procedures and quantification methodologies used.

4.2 Antioxidant capacity

For antioxidant capacity (Table III–2) results were in general statistically different ($p<0.05$). In average, leaves presented higher results followed by flowers, and branches.

Table III–2. TAA, RP and FRAP results

Specie	plant part	location	Sample ID	TAA	RP	FRAP
E. australis	leaves	1	1	26.94 ± 1.19^{ab}	34.33 ± 0.05^{a}	21.60 ± 0.12^{a}
		2	2	41.20 ± 3.29^{acd}	30.97 ± 0.11^{b}	52.43 ± 0.06^{b}
		3	3	47.06 ± 1.05^{c}	27.95 ± 0.08^{c}	52.57 ± 0.09^{b}
			average	38.40 ± 9.15	31.09 ± 2.76	42.20 ± 15.45
	flowers	2	4	36.37 ± 1.81^{d}	22.98 ± 0.11^{d}	25.96 ± 0.07^{c}
		3	5	16.27 ± 0.93^{e}	18.25 ± 0.09^{e}	18.91 ± 0.07^{d}
			average	26.32 ± 11.08	20.61 ± 2.59	22.44 ± 3.86
	branches	1	6	8.67 ± 0.13^{f}	8.11 ± 0.02^{f}	0.61 ± 0.00^{e}
		2	7	9.75 ± 0.33^{f}	8.85 ± 0.02^{g}	0.61 ± 0.02^{e}
		3	8	3.02 ± 0.32^{g}	4.75 ± 0.01^{h}	0.62 ± 0.01^{e}
			average	7.15 ± 3.14	7.24 ± 1.89	0.62 ± 0.01
E. arborea	leaves	4	9	20.08 ± 0.10^{be}	18.16 ± 0.08^{e}	20.82 ± 0.24^{a}
		5	10	35.18 ± 1.47^{d}	26.24 ± 0.16^{i}	46.40 ± 0.07^{f}
			average	27.63 ± 8.33	22.20± 4.43	33.61± 14.01
	flowers	5	11	35.89 ± 1.56^{d}	26.43 ± 0.24^{i}	26.69 ± 0.11^{g}
			average	35.89 ± 1.56	26.43 ± 0.24	26.69± 0.11
	branches	4	12	6.67 ± 0.13^{h}	9.50 ± 0.04^{j}	0.65 ± 0.02^{e}
		5	13	3.17 ± 0.21^{g}	5.16 ± 0.01^{k}	0.62 ± 0.00^{e}
			average	4.92 ± 1.93	7.33 ± 2.38	0.63 ± 0.02
average leaves				34.09 ± 10.81	27.53 ± 6.08	38.76 ± 16.22
average flowers				29.51 ± 11.47	22.55 ± 4.11	23.85 ± 4.30
average branches				6.26 ± 3.09	7.28 ± 2.18	0.62 ± 0.01

Values are means ± standard deviation of three assays. Different letters in the same column mean statistically different results, by Games Howell test ($P < 0.05$). AAE, ascorbic acid equivalent; dw, dry weight; FRAP, ferric-reducing antioxidant power; RP, reducing power; TAA, total antioxidant activity.

For TAA these were 34.1; 29.5 and 6.3 (mg AAE/g dw) respectively, with highest result being obtained for leaves from location 3, while lowest result, over 10 times less content than

the leaves, was obtained in the branches also from this location, 47.1 and 3.0 (mg AAE/g dw) respectively. This reflects a high disparity in antioxidant activity within the plant. In addition differences between species were also observed. Upon analysis of results from the RP assay, it appears this plant RP can be influenced by a diversity of factors. These results varied from 34.33 to 18.1; 26.4 to 18.3 and 9.5 to 4.75 (mg Trolox/g dw) for leaves, flowers and branches respectively. It appears neither collection site nor specie influence FRAP from this plant branches, as all results were very similar. Also this plant part showed a much lower FRAP, 0.6 (mg Trolox/ g dw) compared to the 38.8 (mg Trolox/g dw) of leaves and 23.9 (mg Trolox/g dw) of flowers. Results regarding DPPH and ABTS radical scavenging activities (Figure III–1) are presented as IC_{50}, which represent the concentration, needed to promote a 50% decrease in absorption. In average, and following the trend exhibited in the other assays, leaves infusions had higher radical scavenging capacity, followed by flowers and branches. However ABTS IC_{50} results were much higher comparing to DPPH, with results ranging from 66.6 to 537.6 and 296.3 to 4910.1 (µg/mL) respectively, meaning this plant has a higher affinity towards DPPH radical than towards ABTS.

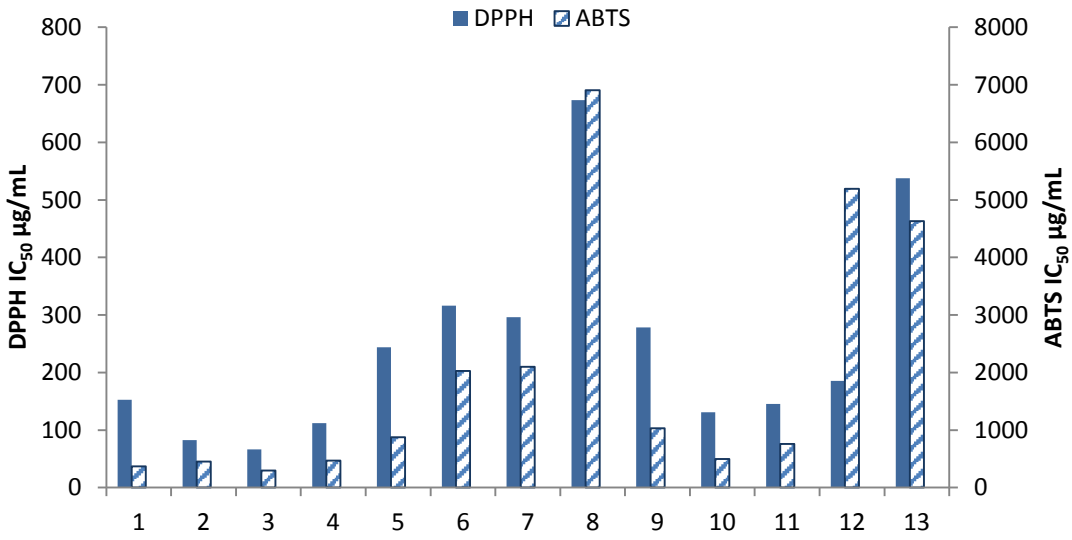


Figure III–1. DPPH (blue) and ABTS (light blue pattern) IC 50 values.

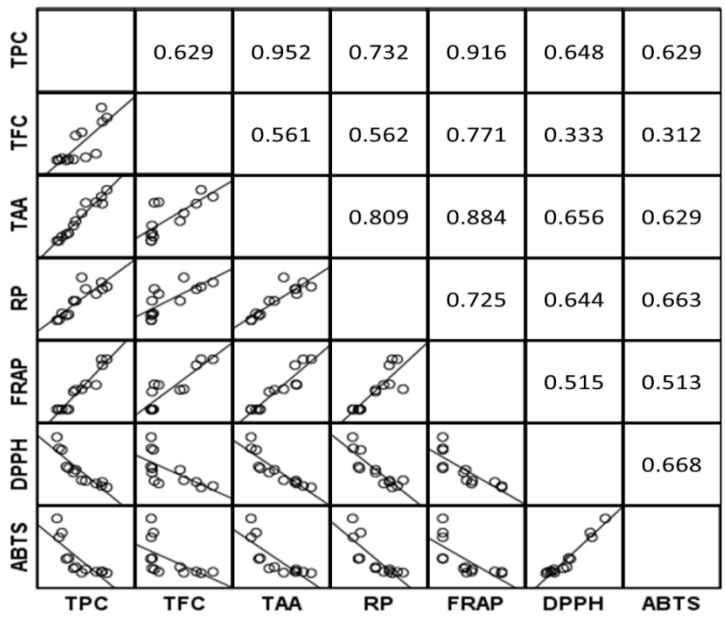
Also to be noticed that samples that had lower IC_{50} for DPPH didn't always had lower IC_{50} for ABTS, which seem to indicate some oxidant selectivity. Similar results were found with berry crops (Wang and Jiao, 2000). It also appears location plays a bigger role in ABTS scavenging capacity than it does in DPPH scavenging capacity, although both seem to be influenced by this factor. Regarding other studies, DPPH IC_{50} for *Erica spp.* were slightly lower than those obtained in the present study, therefore presented greater scavenging power,

which can be explained by different collection sites, extraction procedures and quantification methodologies (Luís et al., 2009, Ay et al., 2007). To the best of the author's knowledge, it is the first time this plants different parts are analysed, aswell as the first time TAA, RP, FRAP antioxidant activities and ABTS radical scavenging activity of this plants are studied, so this work greatly helps to widen the knowledge available about this plant and supports the view that this plant has a great potential to serve as a cheap antioxidant source. However since the studied plant parts responded differently in the several assays, and showed some oxidant selectivity, which means antioxidants present in them are probably different in structure and ammount, a deeper understanding of the plant is still required before commercial or fundamented health applications can be made.

4.3 Correlations and Hierarquical Cluster Analysis

Coefficients of determination (R^2) were computed to measure the strength of linear relationships between the different methods (Table III–3). TPC correlates strongly with all methods especially with TAA (0.952), and FRAP (0.916). The best correlations with TFC were obtained for FRAP (0.771), followed by TAA (0.561) and RP (0.562). FRAP and TAA assays present a strong linear relationship (0.884), while RP R^2 varied between 0.562 for TFC and 0.809 for TAA. Also there is a good linear relationship between DPPH and ABTS (0.668).

Table III–3. Bivariate coefficient of determination (R^2) matrix presented together with data scatterplots.



In order to group the different results by similarity, and see which factors influenced them the most, HCA was performed on samples and on assays (Figure III–2).

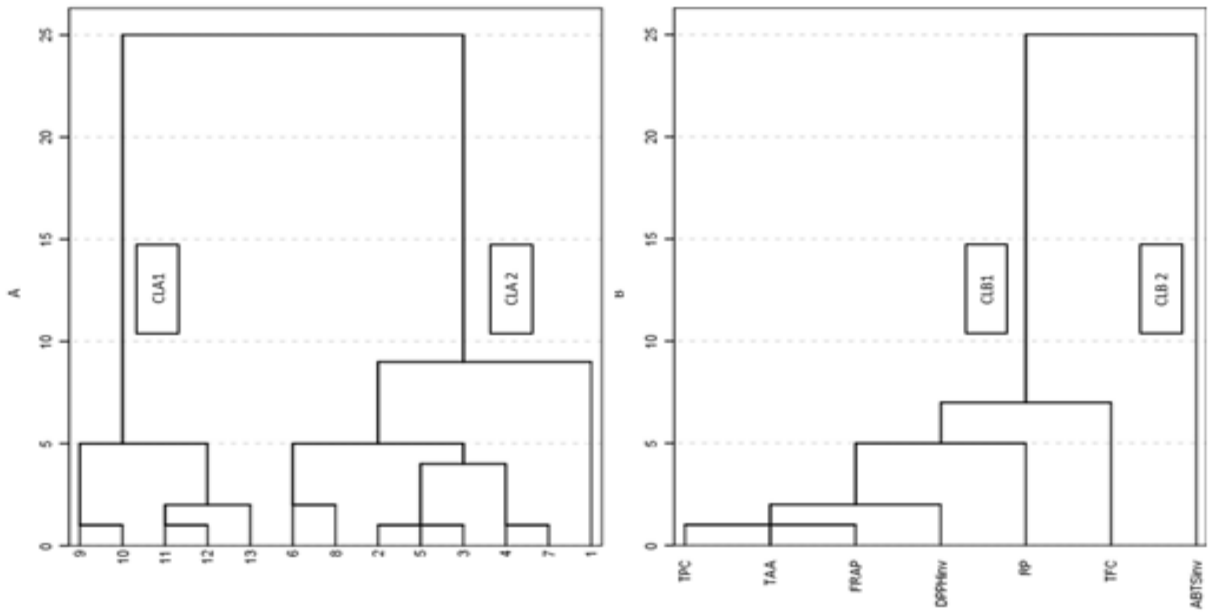


Figure III–2. Dendrogram built by Hierarchical Cluster Analysis with all samples (A) and parameters (B) using average link between groups method and Euclidean squared distance.

Upon application of this tool, samples (Figure III–2A) were grouped in two clusters, with all branches in one (CLA1) and leaves and flowers together in the second (CLA2). HCA confirmed that plant part factor was more responsible for the differences than specie or location, and also flowers are more similar to leaves than to branches.

Regarding methods (Figure III–2B) HCA supported the results obtained previously in the correlations, showing that TAA is mainly due to TPC, with TFC playing a lesser role, and the best method to measure it is FRAP.

5 Conclusions

In conclusion, the antioxidant capacity is mainly influenced by plant part studied, which means antioxidants present in them are probably different in structure and amount. Overall the findings of this study support the view that some plants, like *Erica* are promising potential sources of antioxidants, especially their leaves and flowers and may be efficient as preventive agents in the pathogenesis of some diseases. However, the strength of the existing data is not enough to suggest a reasonable mode of action for antioxidant effects. The data of this study may just enrich the existing comprehensive data of antioxidant capacity of Erica plant materials.

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Characterization of 18 *Arbutus unedo* trees from a demarked region in Portugal and their leaf and fruit extracts antioxidant activity obtained using different extraction conditions

Ricardo Nunes [1], Isabel S. Carvalho[1],*

[1]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

**Corresponding author: Isabel Carvalho, MeditBio, Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas 8005-139 Faro, Portugal.*
Email:icarva@ualg.pt

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1 Abstract

Background: *Arbutus unedo* is a tree common in the Mediterranean area, which plays a role in small economies and traditional medicine. While several studies regarding extractions have been conducted, most explore the effect of only one or two conditions on the results and none explored the high antioxidant variation that these trees are empirically known for.

Materials and Methods: In this work 18 healthy fruit bearing trees were selected from a demarked region in Algarve. Trees were characterized regarding their height, foliage, fruitification and flowering stages. Fruits were extracted using a hot plate while leaves were extracted using a hot plate and a soxhlet apparatus to determine the best method. Afterwards, a screening design to determine the influencing factors was performed, varying temperature, time, ratio and stir at the same time for three different solvents.

Results: Leaf extracts yielded a much higher concentration (mg/mL) of antioxidant compounds when using the soxhlet extraction but this advantage was diminished or even inverted when results were compared in mg/g of dry weight. No relation was found between physical measurements and antioxidant activity. Temperature and stir were the factors that influenced the most in the leaf and fruit extractions.

Conclusions: If only one solvent and one set of conditions were to be chosen for the highest antioxidant activity (mg/g dry weight), it would be water at 60°C for 30 minutes, 0.01 g/mL and 200 rpm for leaves and 50 % ethanol: water at 60°C, 180 minutes, 0.03 g/mL and 60 rpm for fruits.

KEYWORDS

Arbutus unedo, screening design, antioxidant activity, extraction methods

2 Background

Collection place affects the antioxidant activity of plants since plants produce antioxidants to protect themselves from environmental aggressions, and when under stress the amount produced is greater. Factors that can stress a plant and as such influence their antioxidant activity include exposure to sunlight, ultraviolet radiation and drought (Bautista et al., 2016, Giorgi et al., 2010, Liu et al., 2016). In a previous work where plants from the same family were studied, it was found that phenolic and flavonoid contents significantly changed between locations. However, in that work the locations were quite far from each other leading to the introduction of unknown variables which could account for at least some of the differences. In this work however we collected 18 samples from *Arbutus unedo* trees all from approximately the same area, which are exposed to approximately the same climate conditions, therefore the differences detected should be mostly due to the genetic makeup of the tree and how it copes with stress when compared to the surrounding trees.

Arbutus unedo L., known as strawberry tree, is a tree common in the Mediterranean area, including Portugal, although it exists on other parts of Europe and Africa (Celikel et al., 2008, Takrouni and Boussaid, 2010, Lopes et al., 2012). It belongs to the Ericaceae family and can grow up to 9 meters in high, however due to human intervention, mainly to facilitate collection of fruits and to increase the production, it doesn't usually go above 3 meters (Özcan and Hacıseferoğulları, 2007). These trees represent a source of income in small villages, mostly due to their fruits but they are also important to the honey production industry and

environment (Lopes et al., 2012). Despite all this *Arbutus unedo* L. started being neglected and is being replaced with other trees that are seen as more valuable on a commercial level, which led to a decline in the population. In an effort to counter this, Corte Velada Lda, a Portuguese company with Norwegian capital, acquired a terrain in the Algarve region and planted 72 000 trees. Their goal is not only an environmental one but also to monetize the tree using not only the fruits but also other plant parts, such as leaves.

The leaves of this tree are used in folk medicine as a diuretic, antiseptic and laxative, as well as to treat hypertension (El Haouari et al., 2007, Ziyyat et al., 2002, Afkir et al., 2008) and are known to possess high antioxidant activities, and to be rich in phenolic compounds (Andrade et al., 2009, Malheiro et al., 2012, Mendes et al., 2011, Oliveira et al., 2009). They have in their composition hydroxycinnamic and hydroxibenzoic acid derivatives, flavonols, flavanones and di-hydroflavonols (Boulanouar et al., 2013, Tavares et al., 2010) as well as tannins, α-tocopherol, lipids and vitamin E (Pabuçcuoğlu et al., 2003, Kıvc¸ak and Mert) which all have antioxidant properties. They also possess antimicrobial activity and neuroprotective effects amongst other properties (Dib et al., 2013, El Haouari et al., 2007, Fortalezas et al., 2010, Mendes et al., 2011, Tavares et al., 2010).

This tree produces fruits that are sweet in taste only when ripe, presenting before, astringent characteristics (Alarcão-E-Silva et al., 2001). They can easily be distinguished when ripe by the color, and for the possibility of squishing them, before which time it is nearly impossible. Besides their sweet taste and economic importance, these fruits also have a high antioxidant capacity and neuroprotective and antihemolytic properties (Mendes et al., 2011, Fortalezas et al., 2010).

To determine if within the small area there was significant variation in the samples, with the goal of later selecting the best samples for micropropagation, a total of 18 different fruit bearing trees were selected.

3 Objective

The goal of the present work was to conduct a botanical survey of the specimens present in the area, compare inside variation of leaves and fruits and compare both plant parts amongst them. Since extraction methods can also have a significant effect on the extraction of phytochemical compounds, it was decided that leaves would be extracted using a soxhlet apparatus and a hot plate, and both extraction results would be compared. The best method of extraction was then further analysed using a screening design to verify the influence of temperature, time, ratio and stir on the obtained results.

4 Materials and Methods

4.1 Plant material

All samples were collected on a dry day of November 2012, in a terrain belonging to the Corte Velada company (GPS: N 37 11.536; W 8 40.820). Trees were all planted 2 to 3 years before. Upon collection of leaves and fruits, they were put inside a plastic bag, tagged and stored inside a thermal contained which contained thermal blocks to maintain temperature. Upon reaching the FSLab in the university of Algarve, samples were characterized. Leaves were then dried in an oven at 40°C for 72 h, after which they were ground in a mortar and pestle and extracted. The remaining leaves were stored in falcons in a freezer at -20°C. For fruits no drying was performed.

4.1.1 Tree selection and characterization

Trees were selected from an area on a sunny hill around a damn facing both north and south. The two key features searched were healthiness and presence of fruits (samples 12 and 16 only had enough for the physical determinations). After tagging them they were photographed and characterized concerning their height, amount of fruits and flowers and the flowering stage. Notes were taken concerning the proportion of yellow versus red fruits, fruits on the ground, colour of fruits, their distribution around the tree and the strength of their connection with the stalk.

4.1.2 Samples characterization

Before drying, leaves were measured and weighted. After drying they were weighted once again and their humidity was calculated. For fruits the characterization was on fresh weight, their diameter and °Brix, which was measured after mashing a fruit sample in a mortar and pestle. For °Brix determination, measurements were made from 5 fruits each in triplicate and for each sample.

4.1.3 Soxhlet extraction

For leaves two extraction methods were used, soxhlet extraction and extraction in a hot plate with magnetic agitation. For fruits, due to being extracted in fresh state, which created a plug on the soxhlet extraction cartridge leading to deficient extraction, only the hot plate was used. For the soxhlet extraction 2 grams of ground dry leaves were inserted into an extraction cartridge which was covered with a cotton ball to prevent leaves from escaping during reflux. A volume of 60 mL of distilled water was used. The extraction lasted between 24 and 36 h, with the stoping point being the clarity of the solvent in contact with the leaves cartridge. For

the determination of the dry weight, an aliquot of 1 mL in triplicate was transferred into an Eppendorf previously weighted and put in an oven until dry. The remaining sample was transferred into Eppendorfs and stored in a freezer until use.

4.1.4 Plate extraction

For the extraction using the hot plate, the solvent was first heat to 95°C, after which the sample was inserted. For leaves 1 g was used in 30 mL volume, while for fruits 2 grams were used. The difference in weight was due to fruits still being fresh while leaves were dry. The extraction lasted for 15 minutes, after which the infusion obtained was passed through a Whatman n°4 filter paper. For the determination of the dry weight, an aliquot of 1 mL in triplicate was transferred into an Eppendorf previously weighted and put in an oven until dry. The remaining sample was transferred into Eppendorfs and stored in a freezer until use.

4.1.5 Screening design

After concluding on the best extraction technique a screening design with 4 factors on two levels and a slightly of center point made on JMP 10© was used to verify the influence of the temperature, time, ratio and stir (Table III–4). To assess the influence of solvent, the design was done with distilled water, absolute ethanol and 50% ethanol: water.

Table III–4. Leaves and fruits screening design.

	Condition	Temperature (°C)	Time (min)	Ratio (g/mL)	Stirr (rpm)
Leaves	1	60	180	0.035	200
	2	20	30	0.010	60
	3	60	30	0.035	60
	4	20	180	0.010	200
	5	40	105	0.020	130
	6	60	180	0.010	60
	7	40	105	0.020	130
	8	20	30	0.035	200
	9	60	30	0.010	200
	10	40	105	0.020	130
	11	20	180	0.035	60
	12	40	105	0.020	130
Fruits	1	20	180	0.030	200
	2	40	105	0.115	130
	3	20	30	0.030	60
	4	20	180	0.200	60
	5	60	30	0.030	200
	6	60	180	0.200	200
	7	20	30	0.200	200
	8	60	30	0.200	60
	9	40	105	0.115	130
	10	60	180	0.030	60

4.1.6 Total Phenol Content

Total Phenol content (TPC) of infusions was determined through a spectrophotometer (Huang et al., 2006). Briefly, 0.1 mL of properly diluted extract was mixed with 0.5 mL Folin-Ciocalteau's reagent and 0.4 mL of a saturated sodium carbonate solution (7.5 %). After standing for 30 min in a dark room, absorbance was read at 765 nm against a blank in a spectrophotometer. Total Phenolic content was calculated by a calibration curve of gallic acid and was expressed as mg GAE/g dw (gallic acid equivalents per gram dry weight).

4.1.7 Total Flavonoid Content

Total Flavonoid Content (TFC) was analyzed using a spectrophotometer (Lamaison and Carnat, 1990). Properly diluted extract (0.5 mL) was mixed with 2 % methanolic $AlCl_3.6H_2O$ (1.0 mL). The absorbance was measured at 430 nm after standing for 10 min in a dark room. Flavonoid content was calculated by a calibration curve of quercetin, and was expressed as mg QE (quercetin equivalents)/g dw.

4.1.8 Total Antioxidant Activity

Total Antioxidant Activity (TAA) of extracts was determined through a spectrophotometer (Prieto et al., 1998a). Briefly, 0.1 mL of properly diluted extract was mixed with 1.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Mixture was then incubated at 95°C for 90 min in water-bath. Absorbance was measured at 695 nm and results calculated against an ascorbic acid calibration curve. Results are expressed as mg AAE (ascorbic acid equivalents)/g dw.

4.1.9 Reducing Power

Reducing Power (RP) was determined through a spectrophotometer (Yen et al., 2000). Briefly 0.2 mL of properly diluted extract in 0.5mL of sodium phosphate buffer (0.2 M, pH 6.6) was mixed with 0.5 mL potassium ferricyanide (1%) and mixture was incubated at 50°C for 20 min. 0.5 mL of Trichloroacetic acid (10 %) was then added and mixture was centrifuged at 650 x g for 10 min. 0.5 mL of supernatant were then mixed with 0.5 mL of distilled water and 0.1 mL of ferric chloride (0.1 %). Absorbance was measured at 700 nm. Results were expressed as mg Trolox/g dw.

4.1.10 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined through a spectrophotometer (Benzie and Strain, 1996b). Three stock solutions were prepared, a 300 mM acetate buffer (3.1 g $C_2H_3NaO_2.3H_2O$ and 16 mL $C_2H_4O_2$ for each liter of solution), pH=3.6, a 10 mM

TPTZ solution in 40 mM HCl, and a 20 mM $FeCl_3.6H_2O$ solution. Working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of $FeCl_3.6H_2O$ solution. This working solution was then heated to 37°C. A 75 μL aliquot of properly diluted extract was mixed with 1.425 mL of working solution. Absorbance was read at 593 nm 30 min after mixing. Results were calculated against a Trolox calibration curve and expressed as mg Trolox/g dw.

4.1.11 Color analysis

Since three different solvents where used, which have different properties and can extract different pigments, color of leaf extracts was also analyzed(Kelebek et al., 2008). Direct absorbances at 420, 520 and 620 nm were taken. color intensity was determined by summing all the absorbances and % of each color was calculated. A spectrum of absorbance from 700 to 380 nm for all solvent extractions is also presented.

4.1.12 Statistical analysis

JMP 10© was used to plan and analyze the screening design. Relevant factors that influenced the extraction were detected using the screening modelling option of JMP 10©. This option analyses the influence of the main factors as well as main interactions. Statistical analysis was performed using SPSS 18.0 (SPSS Inc, Chigaco) with the α level fixed at 0.05. All results are shown as mean ± S.D from three repetitions. ANOVA test was applied to data after checking homogeneity of variance with Levene's test. Fisher's Least Significant Difference (FLSD) or Games–Howell (GH) post hoc tests were used to check for statistically differences. Correlations between physical parameters and antioxidant activity were explored.

Finally a Hierarchical Cluster Analysis was applied to the leaf results to group the different extractions by similarity. This analysis was successfully done in a previous work that grouped a different plant and its plant parts by similarity as well as the assays used (Nunes et al., 2012a). The relationships between the assays were also investigated after the results were standardized using z-score.

5 Results

5.1 Before screening design

As it can be seen on Table III–5 the trees greatly varied in height, with some being shorter than 60 cm while others were taller than 1.2 meters. The amount and stage of fruitification and flowering also varied greatly.

Table III–5. Leaves and fruits characterization.

	Tree					Leaves				Fruits		
	A	B	C	D	Notes	Length x width (cm)	Fw (g)	Dw (g)	% MC	Fw (g)	Θ (cm)	°Brix
1	vv	v	vv	v		7.5 ± 0.9 x 1.5 ± 0.2	0.542 ± 0.048	0.290 ± 0.034	53.5 ± 2.9	6.08 ± 0.43	2.3 ± 0.2	30.78 ± 0.15
2	vv	v	vvv	v		6.0 ± 0.3 x 2.5 ± 0.4	0.572 ± 0.052	0.291 ± 0.035	50.8 ± 2.9	8.27 ± 0.37	2.1 ± 0.1	26.05 ± 0.29
3	v	vvv	vvv	v		7.2 ± 0.5 x 2.1 ± 0.3	0.645 ± 0.063	0.324 ± 0.035	50.3 ± 3.0	4.56 ± 0.17	2.0 ± 0.2	20.60 ± 0.14
4	vv	vvv	v	v	(*)	6.5 ± 0.4 x 1.8 ± 0.4	0.558 ± 0.076	0.283 ± 0.045	50.7 ± 2.9	6.77 ± 0.33	2.3 ± 0.2	24.65 ± 0.48
5	vvv	v	v	vv		6.3 ± 0.3 x 1.7 ± 0.5	0.960 ± 0.065	0.615 ± 0.045	64.1 ± 2.0	9.39 ± 0.26	2.5 ± 0.2	25.15 ± 0.21
6	vv	vv	vv	v	(->)	6.2 ± 0.4 x 1.4 ± 0.3	0.531 ± 0.039	0.232 ± 0.016	43.7 ± 0.8	5.49 ± 0.18	2.1 ± 0.2	27.33 ± 0.40
7	v	vv	x	vv	(*)	4.1 ± 0.3 x 0.9 ± 0.3	0.373 ± 0.036	0.193 ± 0.024	51.8 ± 3.4	4.20 ± 0.09	1.7 ± 0.3	29.88 ± 0.17
8	vv	vv	v	vv	(Y>R) (f)	4.8 ± 0.3 x 1.2 ± 0.4	0.256 ± 0.037	0.146 ± 0.021	55.0 ± 5.7	5.18 ± 0.15	1.8 ± 0.2	26.25 ± 0.17
9	v	vv	vvv	v	(Y=R)	7.0 ± 0.2 x 1.5 ± 0.3	0.508 ± 0.043	0.261 ± 0.028	51.4 ± 3.6	5.12 ± 0.21	1.8 ± 0.2	21.88 ± 0.17
10	vvv	vvv	vvv	v	(Y=R)	5.6 ± 0.7 x 1.9 ± 0.5	0.372 ± 0.045	0.213 ± 0.035	57.1 ± 6.0	7.65 ± 0.22	2.1 ± 0.3	24.85 ± 0.21
11	v	vv	x	v	(->) (Y>>R)	5.1 ± 0.3 x 2.1 ± 0.2	0.662 ± 0.047	0.390 ± 0.038	58.8 ± 3.0	4.85 ± 0.15	1.9 ± 0.2	24.00 ± 0.41
12	vvv	vvv	vv	v	(*) (Y>>R)	5.5 ± 0.2 x 1.9 ± 0.3	0.631 ± 0.025	0.328 ± 0.027	52.0 ± 4.2	6.67 ± 0.22	2.1 ± 0.2	24.30 ± 0.18
13	vvv	vvv	vv	v	(R>>Y) (C)	3.8 ± 0.4 x 1.4 ± 0.3	0.397 ± 0.042	0.211 ± 0.026	53.1 ± 3.5	3.73 ± 0.35	1.8 ± 0.2	26.03 ± 0.22
14	v	vv	x	v	(Y)	4.6 ± 0.4 x 1.8 ± 0.3	0.320 ± 0.038	0.168 ± 0.021	52.6 ± 1.3	7.20 ± 0.53	2.2 ± 0.2	26.93 ± 0.43
15	vv	vvv	vvv	v	(*)(Y)	4.4 ± 0.3 x 1.7 ± 0.5	0.223 ± 0.052	0.343 ± 0.334	56.1 ± 8.5	7.49 ± 0.18	2.0 ± 0.2	26.53 ± 0.26
16	vv	vvv	v	vv	(Y>>R)	6.1 ± 0.3 x 1.6 ± 0.2	0.424 ± 0.027	0.288 ± 0.028	67.9 ± 3.4	7.87 ± 0.17	2.2 ± 0.3	22.90 ± 0.14
17	vv	vv	x	v	(->) (Y>>R)	5.5 ± 0.4 x 2.1 ± 0.4	0.354 ± 0.042	0.199 ± 0.036	56.0 ± 5.6	6.31 ± 0.27	2.0 ± 0.3	27.68 ± 0.33
18	vv	vv	o	o	(*) (Y>R) (C)	4.7 ± 0.2 x 1.4 ± 0.2	0.284 ± 0.017	0.157 ± 0.010	55.2 ± 0.9	7.58 ± 0.41	2.4 ± 0.3	22.13 0.10

A- Height; B- Fruit amount; C- Flower amount; D- Flowering stage; Fw- fresh weight; Dw- dry weight; MC- moisture content; Θ- diameter. Height [v, small (<60 cm); vv, medium (60<120 cm); vvv, tall (>120 cm)]; Fruit amount [v, low; vv, average; vvv, high]; Flower amount [x, very low v, low; vv, average; vvv, high]; Flowering stage [o, absent; v, very late; vv, late]; Notes [(*), some fruits fallen; (->), only fruits on one side; (Y), yellow fruits; (R), red fruits; (f), fruit firmly connected to the stalk; (C) fruits with a different color. Values are means ± standard deviation of three assays.

While some trees had no flowers (1 in this work), others had plenty, and while in some the flowering stage was still early, a few already had some fallen flowers. Leaves ranged greatly in length and width, from tree to tree and sometimes even in the same tree. The shortest were only 3.8 cm in length while the longest were 7.5 cm and the widest were 2.5 cm versus 1.2 cm for the thinnest. Their shape also changed, with some being rounder or with serrated edges, as did the average fresh weight. Humidity of leaves ranged from 43.7 to 67.9%, but most samples had humidity close to 53%. Fruits weight ranged from 3.73 to 9.39 g, and the diameter between 1.7 and 2.3 cm.

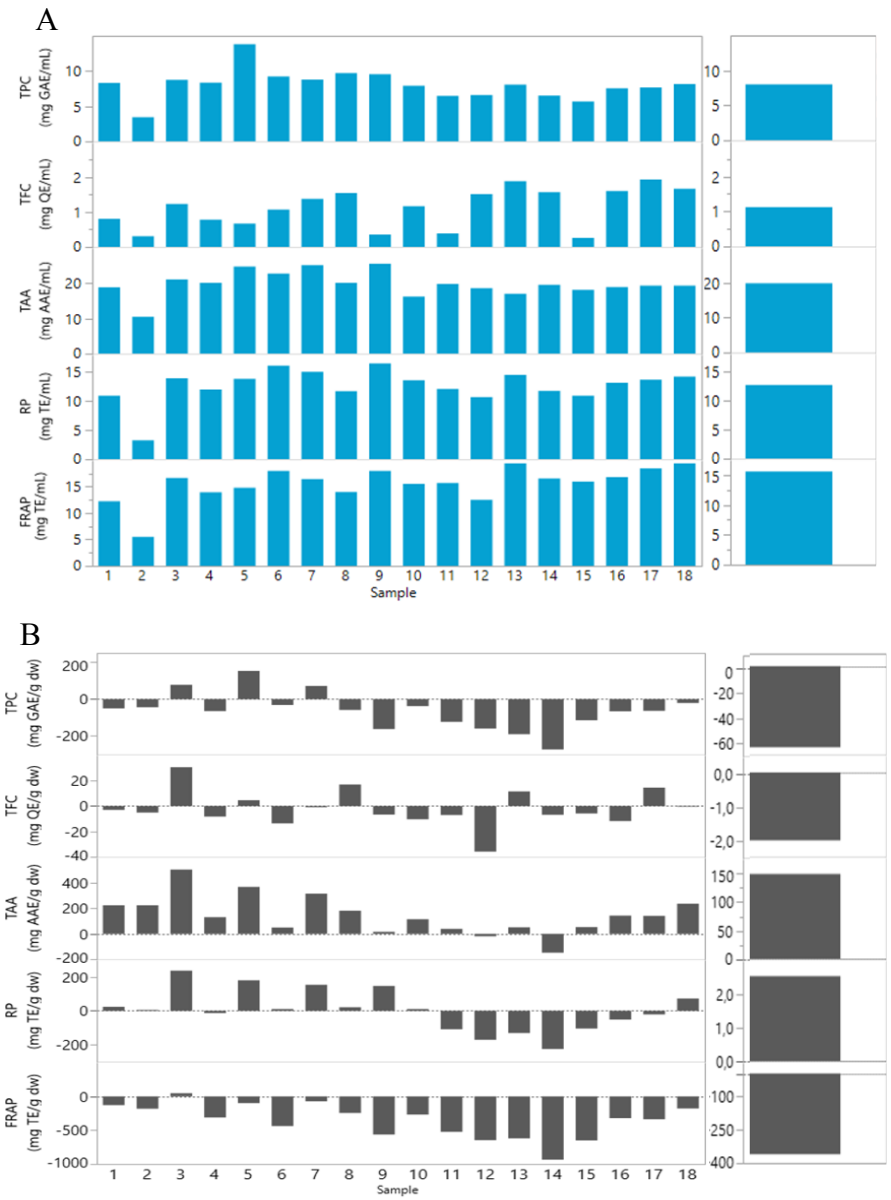

Figure III–3. Difference between the results of Soxhlet and plate extractions for each sample and on average in mg/mL (A) and in mg/g dw (B).

To note that the heaviest fruits were not always those with larger diameter. Finally the °Brix of samples from different trees ranged from 20.60 to 30.78, but due to the fruits collected for this work being all ripe, the intra-tree variation was reduced.

Regarding antioxidant activity the difference between soxhlet and hot plate results per mL of extract was always positive (Figure III–3A).

Comparing the results in mg/g dw reveals new information (Figure III–3B). While before the advantage was always in the side of using a soxhlet extraction, now for some samples the hot plate extraction was better. This was especially true for samples 12 and 14, which presented better results in all assays when extracted using a hot plate. Sample 11 also presented better results with the exception of TAA.

Comparing the results between fruits and leaves extracted in the hot plate, it's clear that per mg of dry weight extract, leaves are substantially superior in every aspect. This was especially true for RP, FRAP and TFC. Once again the values per mL were also compared (Figure III–4). While in mg/g leaves were always better, when it comes to mg/mL the same was not true, which is in part due to the higher concentration of fruit extracts when compared to leaves. Still, while TPC and FRAP were better on leaves on almost all samples, TAA was better on fruits. RP was better for some leaf samples while for others fruits were better, ending up showing on average no difference.

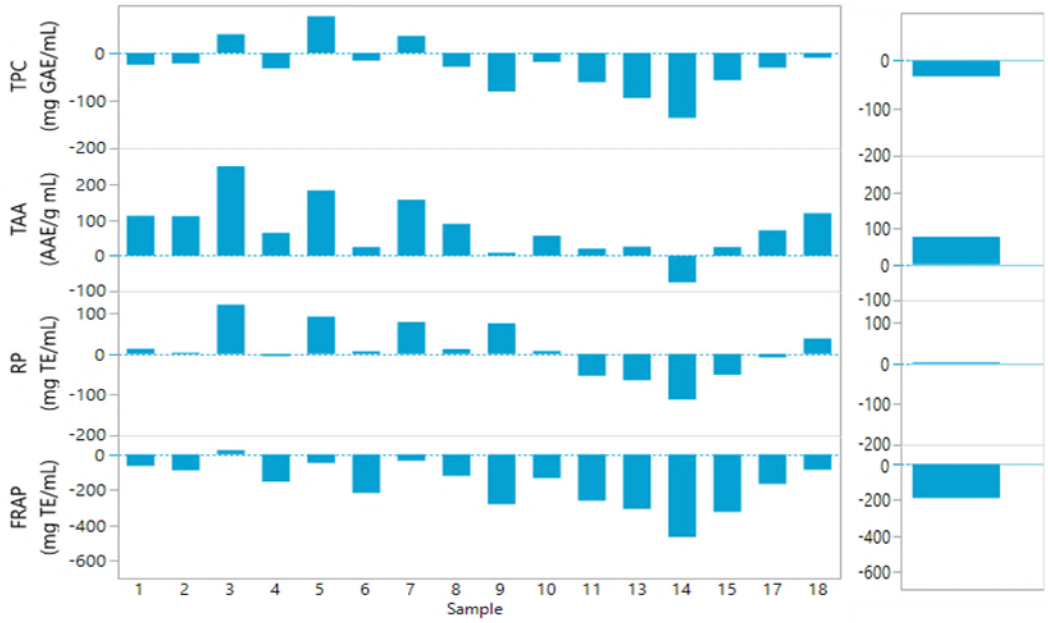

Figure III–4. Difference between plate extraction of leaves and fruits for each sample and in average in mg/mL.

Correlating the physical variables and antioxidant results, it was possible to find a positive significant correlation between average weight of the fruits and their RP per dry weight, but not with RP per mL. The °Brix was not correlated with any variable, while the average weight was not surprisingly correlated with the diameter, however the diameter was not correlated with RP. For antioxidant assays, correlations were found between TPC and RP. Contrary to expected, no significant correlation was found between FRAP and TPC for fruits, although there was one between FRAP and RP, both per dry weight. Also to note that correlations for results per mL were much stronger than those obtained when comparing results per gram of dry weight. Regarding leaves, correlations were similar for both extraction techniques, with the exception of the physical correlations. While for leaves extracted with hot plate no significant correlation was detected between the physical and the antioxidant parameters, there was a negative significant correlation between the weight of the leaves and the TFC values obtained using soxhlet extraction, both per g and per mL. The TPC per dry weight of leaves was well correlated with FRAP as expected, but also with TAA and RP. It was however not correlated with TPC per mL. On the other hand TFC per dry weight was only correlated with TFC per mL while the TFC per mL was also correlated with the RP per mL.

5.1.1 After screening design

For leaves, TPC results ranged from 643.44 to 141.22 mg GAE/g dw, both obtained with distilled water, which was overall the best solvent for TPC and FRAP and the worse for RP. On the other hand for flavonoid extraction and TAA the best solvent was absolute ethanol, while for DPPH absolute ethanol provided the best and worst results with IC_{50} ranging from 4.30 to 41.80. Fruit results were lower and different from those obtained for leaves. For TPC the highest result was obtained with 50% ethanol: water, but the best solvent was absolute ethanol. Regarding TAA, 50 % ethanol: water was once again the solvent with the highest and the best results overall, in stark contrast with water, whose highest result was three times lower than 50 % ethanol: water highest. A similar trend was observed with RP. For FRAP the trend was similar to what was seen with TPC, with absolute ethanol providing the best results. For DPPH, water provided for the first time the best results by far. Also different were the correlations between assays for the different solvents and plant parts (Table III–6).

Table III–6. Correlations for samples organized by plant part.

	Leaves						Fruit				
	Water										
	A	B	C	D	E	F	A	C	D	E	F
A	1	0.515	0.113	0.584*	0.565	-0.788**	1	0.269	0.665**	0.755**	-0.291
B		1	0.837**	0.974**	0.970**	-0.722**	x	x	x	x	x
C			1	0.847**	0.837**	-0.339		1	-0.125	0.310	-0.052
D				1	0.987**	-0.691*			1	0.681**	-0.526**
E					1	-0.692*				1	-0.487**
F						1					1
	Absolute ethanol										
A	1	0.649*	0.501	0.882**	0.841**	-0.206	1	0.348	0.521**	0.187	-0.575**
B		1	0.550	0.618*	0.416	-0.548	x	x	x	x	x
C			1	0.770**	0.706*	-0.630*		1	0.580**	0.628**	-0.534**
D				1	0.870**	-0.397			1	0.632**	-0.619**
E					1	-0.118				1	-0.258
F						1					1
	50% ethanol: water (v:v)										
A	1	0.781**	0.610*	0.879**	0.172	-0.297	1	0.596**	0.953**	0.893**	-0.917**
B		1	0.671*	0.532	0.133	0.015	x	x	x	x	x
C			1	0.504	0.180	-0.061		1	0.605**	0.506**	-0.531**
D				1	0.176	-0.424			1	0.968**	-0.909**
E					1	-0.100				1	-0.879**
F						1					1

A-TPC; B-TFC; C-TAA; D-RP; E-FRAP; F-DPPH; x- no correlation available. * Correlation significant at the $p<0.05$ level; ** Correlation significant at the $p<0.01$ level.

When studying the influence different factors have on the results, it was seen that the factors that influence the extraction depend on the method, the solvent used and the plant part (Table 4). In the case of leaves aqueous extractions for TPC ratio, stir and temperature had influence, while for TFC stir, temperature, and the interactions stir*time and temperature*time had influence in the results. When absolute ethanol was used, TPC, TAA and RP were only influenced by stir, while TFC was influenced only by temperature. Unlike what happened for extractions with distilled water and absolute ethanol, when 50% ethanol: water was used no factor had influence in TPC results. The interaction temperature*time had a negative influence on the TFC and RP results of aqueous extracts. On the other hand for absolute ethanol both stir and time had a negative influence in the results, while temperature had a positive influence. For 50% ethanol: water the main factors stir and time always had a positive influence while temperature had a significant negative influence in the TAA.

Table III–7. Influence the different factors have on leaf and fruit extracts organized by solvent. Only significant effects are shown.

Factor	**Water**						**Ethanol**						**50% Ethanol:water**					
	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
	Leaves																	
a	+	+	+	+	x	x	x	+	x	x	x	x	x	x	-	x	x	x
b	x	x	x	x	x	x	x	x	x	x	-	x	x	+	x	x	x	x
c	+	x	-	x	x	x	x	x	x	x	x	x	x	x	+	+	x	x
d	+	+	+	+	+	x	-	x	-	-	-	x	x	+	+	x	x	x
a*b	x	-	x	-	x	x	x	x	x	x	x	x	x	x	x	x	x	+
a*c	x	x	x	x	x	x	x	x	x	x	x	x	x	x	+	+	x	x
a*d	x	x	+	x	x	x	x	x	x	x	x	x	x	x	x	+	x	x
b*c	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	+	x
b*d	x	+	x	x	x	x	x	x	x	x	x	x	x	+	x	x	x	x
c*d	x	x	+	x	x	x	x	x	x	x	x	x	x	+	+	x	x	x
	Fruits																	
a	x		x	x	x	x	x		x	x	x	x	x		+	x	x	x
b	x		x	x	x	x	x		x	x	x	-	x		x	x	x	x
c	-		x	x	-	x	x		+	x	+	x	x		x	x	x	x
d	+		x	x	x	x	x		x	x	-	x	x		-	x	x	x
a*b	x		x	x	x	x	x		x	x	x	x	x		x	x	x	x
a*c	x		x	x	x	x	x		x	x	x	x	x		x	x	x	x
a*d	+		x	-	-	x	x		x	x	x	x	x		-	x	x	x
b*c	x		x	x	x	x	x		x	x	x	x	x		x	x	x	x
b*d	x		x	x	x	x	x		-	x	x	x	x		x	x	x	x
c*d	x		x	x	x	x	x		x	x	x	x	x		x	x	x	x
a	x		x	x	x	x	x		x	x	x	x	x		+	x	x	x

a, Temperature; b, Time; c, Ratio; d, Stir. "+", significant (P<0.05) positive interaction; "-" significant (P<0.05) negative interaction; "x" no significant interaction; A-TPC; B-TFC; C-TAA; D-RP; E-FRAP; F-DPPH

For fruits, temperature only had a significant global effect on 50 % ethanol: water TAA assay and it was a positive one. It was not possible however to see this effect on TPC although it was previously theorized that temperature of extraction was important in this assay. Time only had a significant global effect on absolute ethanol DPPH assay. Ratio influenced negatively TPC and FRAP from water extractions, and positively TAA and FRAP from absolute ethanol extractions.

From the samples HCA (Figure III–5A) it was possible to verify that for all solvents replicates of the center point (5, 7, 10 and 12) appear in the same cluster.

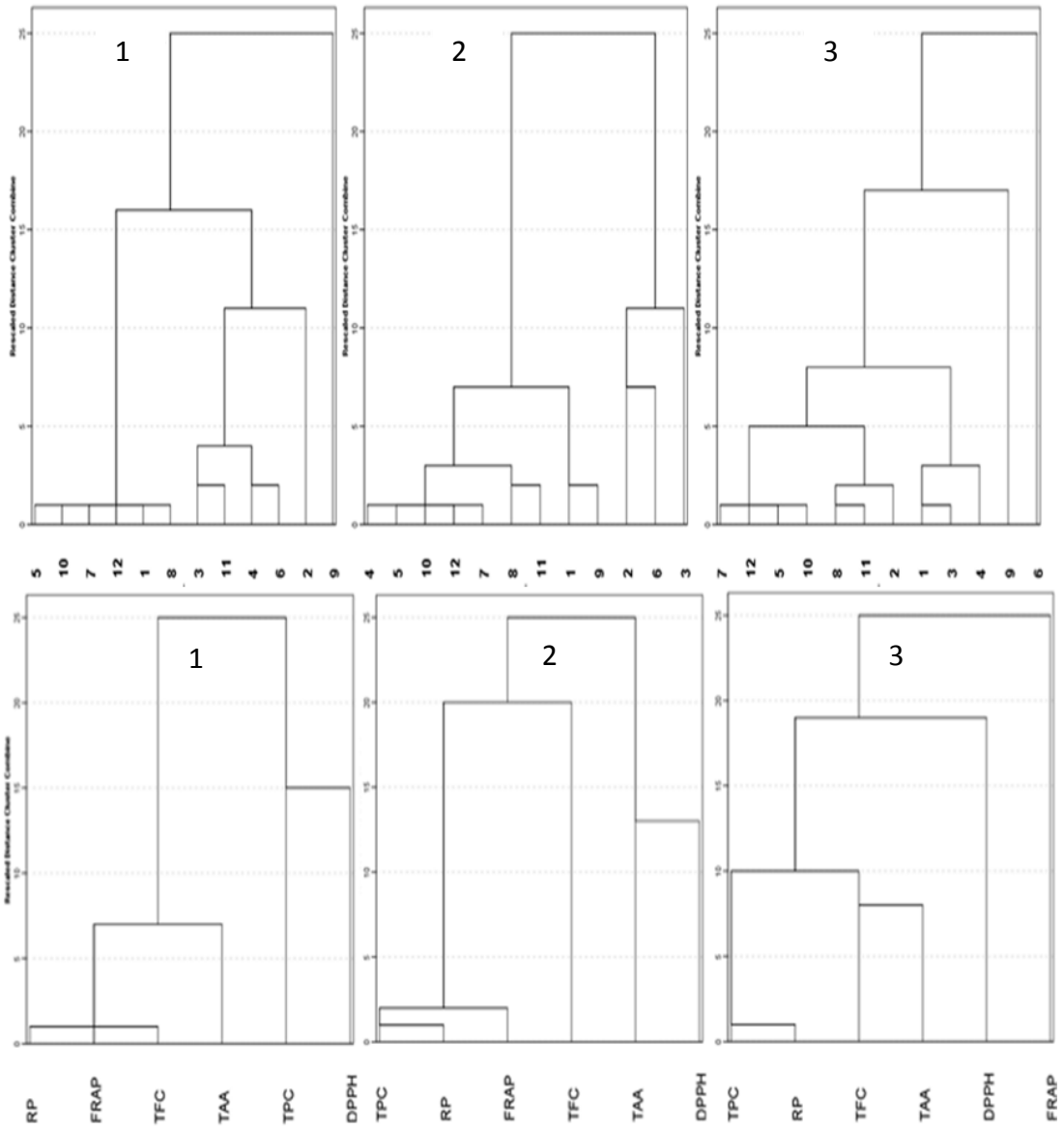

Figure III–5. Hierarchical cluster analysis for samples (A: 1A water; 2A absolute ethanol; 3A 50% ethanol: water) and assays (B: 1B water; 2B absolute ethanol; 3B 50% ethanol: water) obtained from leaf results.

We could also see that for water 2 main clusters were formed and extractions 2 and 9 are very different from the others. For absolute ethanol there wasn't such a distinct separation of clusters. Regarding 50% ethanol: water there were 3 distinct clusters. In the assays HCA (Figure III–5B) we can easily see that for water extractions (1B) the compounds mainly responsible for the RP, FRAP and TAA results are the flavonoids while for ethanolic (2B) extractions the main responsible are phenolics. Regarding 50% ethanol: water (3C) we can see that phenolics are the main responsible for the RP results but not for FRAP results which are not included in the cluster. This is in agreement with the conclusions taken before by analyzing the correlations but is presented much more clearly with this tool.

6 Discussion

6.1 Before screening design

Differences found on the tree characterization can be due to several factors such as age of the trees, different soil composition and different sun exposure among others. Additionally, *Arbutus unedo* L. trees are well known for their great variety of genetic makeups, which is most likely the reason for the differences found, with some trees being more resistant to adverse conditions than others, having a greater predisposition for vertical growth and producing fruits and flowers earlier and in different quantities.

A soxhlet apparatus recirculates the solvent so that the sample inside the extraction cartridge is always in contact with non-saturated solvent. This is important because when the solvent becomes filled with compounds its extraction power decreases. This is what happened with the extraction using a hot plate and helps to explain the results observed in mg/mL. However when comparing the antioxidant activity in mg/g of dry weight, the amount extracted is no longer important, and instead what is being measured is the potency of what has been extracted. In this case soxhlet extraction was no longer the best. This difference can likely be explained by the permanence time in contact with the boiling solvent on the case of soxhlet extraction, which is much higher than the 15 minutes in the hot plate. It is likely that the increased time leads to the destruction of phytochemicals with antioxidant activity, which increases the dry weight of the sample but does not contribute towards its antioxidant activity.

The fact that different extraction methodologies can give different results even when using the same solvent is well known. Time, temperature, presence or absence of stirring can all influence the extraction of phytochemical compounds. When (Luthria et al., 2007) compared different solvents and techniques used in the extraction of isoflavones from soybeans, they found that the method of extraction had a significant effect both on the levels and the compounds extracted. Similarly to what we found for our *A. unedo* samples, in that work they found some compounds were better extracted with soxhlet (daidzin, glycitin, genistin, acetyl daidzin and acetyl genistin), while others were better extracted using a hot plate with magnetic agitation (malonyl daidzin, acetyl glycitin and malonyl genistin). When summing all compound contents however they found no statistical difference between extraction methods. To note that in our work TPC and FRAP assays provided generally better results when extracts were obtained using the hot plate while TAA was markedly better when using soxhlet. For TFC and RP the difference was not as marked. This indicates that the compounds

responsible for FRAP results are most likely phenolics, while TAA is probably due to other compounds which are not losing their activity during the long exposure to high temperatures.

The fact that when comparing leaves and fruits antioxidant activity FRAP and TPC appear together but TAA is the opposite supports what was said before regarding the compounds being responsible for FRAP and TAA. Our results are generally in line with those described by other authors. (Orak et al., 2011) found the aqueous extract of leaves to have 197.16 mg GAE/g of extract, (Nenadis et al., 2015) found their content to be between 166.0 and 174.5 mg GAE/g and (Guendouze-Bouchefa et al., 2015) found them to be 179.6 mg GAE/g of dw for phenolics and 21.4 mg QE/g of dw for flavonoids in trees from Algeria. The differences could be due to different extraction methodologies, different locations or simply due to the differences from tree to tree. Regarding fruits, when (Oliveira et al., 2011) checked the influence of ripening on their ethanolic extract antioxidant activity, they found that yield, phenolics, reducing power and DPPH scavenging all changed from the intermediate to ripe stages. Yield and phenolics suffered a significant reduction while reducing power and DPPH scavenging increased. Given how the °Brix degree varies with fruit samples, and some trees were in a more advanced fruiting stage, it is possible that part of the differences in the results obtained between fruit samples in our work were due to this. However there are certainly other factors involved since no significant correlation, positive or negative, was found between °Brix and the phenolics or any antioxidant method tested. On the other hand (Alarcão-E-Silva et al., 2001) found that during the ripening stage tannins and ascorbic acid decrease, while anthocyanins, niacin and β-carotene increase. These are all compounds related with antioxidant activity, whose variation during ripening and from tree to tree could help to explain the differences observed in the present study. Despite differences between the samples, phenolic content extracted from fruits was overall in the range of those reported in the literature, even when using other solvents and different extraction methodologies. (Oliveira et al., 2011) reported the content of ripe fruits to be around 26.81 mg GAE/g of extract (Alarcão-E-Silva et al., 2001) reported values around 14.6 and (Fortalezas et al., 2010) values of 16.46 mg GAE/g of dw.

Following the lack of advantage in using soxhlet when comparing results per g of dry weight, and the difficulty such technique would pose in an industrial setting, the screening design was conducted using plate instead of soxhlet extraction.

6.1.1 After screening design

The type of design chosen is mainly used to determine factors that influence the results, using a lower amount of experiences than a full factorial design. Factors most commonly varied are temperature, time and ratio. It was also decided to vary stirring speed because it can influence the results, especially when the extraction time is short

The relation between the solvents and the results for the different assays is in agreement with other works (Deng et al., 2014, Fernández-Agulló et al., 2013, Kallel et al., 2014).This shows that studies like the one made here are important to know the product and use the appropriate extraction conditions according to what we want to obtain at the end. It also shows that when comparing different plants or solvents, more than one set of extraction conditions should be used, in order to achieve more trustworthy results.

The fact that the leaves phenolic content was higher when distilled water was used indicates that the majority of phenolics or other compounds which react with Folin reagent, present in the leaves of *Arbutus unedo* are hydrophilic. Some phenolic compounds that are highly soluble in water and are found in *Arbutus unedo* L. leaves are the substituted phenols such as tyrosol and flavanols such as catechin (Mariotto et al., 2008b). Some that are soluble in water and also ethanol include hydroxybenzoic acids, such as gallic and phenylpropenoic acids. Small tannins such as proanthocyanidins are also mildly soluble in water while large condensed tannins, such as galloylquinic acid, have a low solubility in water. Flavonoids such as myricetin, kaempferol and quercetin have a very low solubility in water but are highly soluble in ethanol (Mota et al., 2008, Queimada et al., 2009, Liu and White, 2012, Srinivas et al., 2010). Other compounds that can have antioxidant activity and have different solubilities are vitamins. Thiamine (vitamin B1) and riboflavin (vitamin B2) are soluble in water but not in ethanol. Niacin (vitamin B3) and pantothenic acid (vitamin B5) on the other hand are much more soluble in water than in ethanol. Both biotin and ascorbic acid are soluble in both solvents as is folic acid, although only slightly. Taking into consideration the solubility of the different compounds, it appears leaves are richer in substituted phenols and flavonols than in tannins and flavonoids. The chlorophylls and other pigments extracted are also different, with water extracting pigments that are brown in color, similar to 50 % ethanol: water extracts, while ethanol extracts green pigments. The extraction made with absolute ethanol was the only one with a peak at 660 nm, which according to (Moreau et al., 1998) may be due to porphyrins derived from chlorophyll. It is also possible to confirm that for 50% ethanol: water extraction conditions had a minimal effect, most likely because most compounds although being more soluble in one solvent, present some solubility to both solvents. By mixing the

solvent it is possible to extract most of these compounds, although there are some that due to a change in polarity of the solvent won't be extracted in such high amounts as with a pure solvent It is noteworthy that 50% ethanol: water never gave the highest results, which indicates that the polarity of many phenolic compounds present in this tree leaves is either close to that of the water or that of the ethanol, and not so much in between these two. It is also possible that by extracting different kinds of compounds we are getting antagonistic effects. The fact that in some extraction conditions the results for absolute ethanol and 50% ethanol: water are similar while the results obtained using distilled water are much higher (extractions 1, 5, 7 8, 10 and 12), reinforces the idea that most phenolics present are hydrophilic in nature which was mentioned earlier. In the case of flavonoid content, the fact that absolute ethanol gave the highest results was expected, since it is a known fact that absolute ethanol is a better solvent to extract flavonoids. Additionally, extracts obtained using this solvent showed the most variation with conditions, while those obtained with 50% ethanol: water showed the least. The fact that results obtained for TAA were better when absolute ethanol was used, like it happened for flavonoid content, indicates that the activity observed is related to flavonoids although there are other compounds that interfere resulting in no significant correlation for this solvent (table VII). For FRAP, which has a different reaction mechanism than RP, the results were best when leaves were extracted with distilled water, with absolute ethanol giving mostly the worst results.

For fruits, results show that the majority of phenolics present in the fruits of *Arbutus unedo* L. are more soluble in ethanol than in water. If we compare the results of 50 % ethanol water, the solvent with highest discrepancy, we can see that the extractions with the best results were the ones with low ratio and more time to extract. This seems to indicate that there are phenolic compounds inside some fruit matrix which can only be extracted if the extraction time is above 30 minutes and if the solvent is not saturated with other compounds. For condition 2, which had enough time and low ratio, the issue seems to have been the temperature being too low. Regarding TAA, the highest results all had a temperature of extraction of 60°C which once again is justified by the nature of the sample. For water however the best results were obtained at temperatures below 60°C. This indicates temperature may destroy some thermosensitive compounds that water can extract but 50 % ethanol: water can't. The time component however appears to be important since all the results obtained with only 30 minutes of extraction were below the average for water extractions. Extraction conditions for absolute ethanol don't seem to have much influence. For FRAP, absolute ethanol was better than either water or a mixture of both solvents. Since water and 50 % ethanol: water results

are similar, it seems the majority of compounds responsible for the FRAP activity have a polarity closer to ethanol than water. One type of compounds that fits in this category are fatty acids. Similar results were obtained by (Oliveira et al., 2011) which concluded that the best solvent to extract these compounds was absolute ethanol. In DPPH water extracts were the best. It would be expected that results obtained with a mixture of water and ethanol would follow and those obtained with only ethanol being the worse. That is however not true for all extraction conditions, although the average is indeed lower for 50 % ethanol: water. For extraction conditions 6, 7 and 8 results were better with absolute ethanol than with 50 % ethanol: water, likely due to the higher ratio used since it was previously seen for TPC that 50 % ethanol: water seems to give the best results when lower ratios are used. Overall these results show that it is important to use several assays to characterize the plant material, since different assays can give totally different results and subsequently different views of the sample analyzed. This was true for both leaves and fruits and should be taken in consideration especially when comparing them with other plant materials.

It is interesting to note that according to the correlations obtained, the compounds mainly responsible for the leaves antioxidant activity are different. When absolute ethanol is used, the phenolic compounds are the main responsible factor contributing to the results while for distilled water flavonoids are the main responsible factor. It would be interesting to determine which flavonoids are extracted with water, since these alone allowed higher results than a combination of flavonoids and other phenolic compounds extracted with absolute ethanol indicating they have a high ferric reducing antioxidant power. The negative influence temperature had in some assays, indicates the presence of thermosensitive compounds on leaves which are being degraded by the higher temperatures used. When it's the interaction of temperature* time that had the negative effect, this indicates that while there are thermosensitive compounds present, their degradation can be prevented by using a lower time at a high temperature or a lower temperature at a high time.

For fruits there were interactions that had significant effect while the individual factors did not. Since this design had wide ranges it is possible that one effect is not being considered significant because it is being masked by the results of some outliers. For this reason the screening analysis was also done without each of the extremes at a time (data not shown). When this happened several different conclusions could be taken. For example when the 60°C temperature is removed, temperature now has a positive influence on water extractions DPPH. This indicates there are compounds with DPPH scavenging activity that are being destroyed at 60°C but are not destroyed at 40°C, and this temperature is capable of extracting more

compounds than those extracted with 20°C. It also showed the effect of temperature and ratio on 50 % ethanol: water TPC what was theorized before. Additionally, for water extractions when the lowest temperature is removed, stir displays a positive effect in all assays except TAA. Also interesting is the fact that when the highest time was removed, this parameter had a negative effect on absolute ethanol TPC and RP which was not seen before and ratio had a negative effect, however when the lowest time was removed this effect disappeared and ratio now had a positive effect. One possible explanation for the dual time effect is the existence of two sets of compounds that are extracted at different times, both detected by TPC, RP and DPPH assays. The first set of compounds is extracted within a small time span, these compounds however appear to be destroyed as the extraction goes on, most likely due to temperature. This destruction leads to a lower result when the time increases from 30 to 105 minutes, thus the negative effect. However as time goes on, due to the degradation of the complex matrix which characterizes this fruit, new compounds that were bound to the matrix can be extracted which leads to a higher result and thus the negative effect introduced in the span 30 to 105 minutes disappears. This is supported by the effects presented when one ratio extreme is removed at a time. Taking into account these results it can be concluded that unlike what happens in most cases, for fruits the middle point condition is not the most appropriate one, at least regarding the time of the extraction. At a lower temperature, a lower ratio in conjunction with lower time and high stir speed should be used, while if we want to use a higher temperature a higher amount of time and a higher ratio should be used. This last alternative will result in a more concentrated extract or juice that retains most of the characteristics of the low ratio/low temperature, in a dry extract weight basis, but because it is more concentrated it can be more easily stored which may be advantageous from an industry point of view.

The HCA grouping of the center point (5, 7, 10 and 12) replicates for leaves in the same cluster indicates results are reproducible. Additionally, since some extractions are grouped together, it may be possible to obtain that same result using different sets of conditions, with one being perhaps quicker or more economical than the other. For example if we have plenty of fruit and little time, we can use 20°C instead of 40°C during 30 min instead of 105 min at a higher stirring speed and obtain the same result, which may be an important information in an industrial setting.

7 Conclusion

Arbutus unedo L. leaves and fruits possess a high antioxidant power, which may explain their known medicinal properties and use in folk medicine. The samples showed great variability, first on the physical attributes but also in a smaller scale on the antioxidant activity results. A soxhlet extraction yields a much more concentrated extract, which translates into higher antioxidant activity per mL of extract but has no advantage when comparing per gram of dry weight. In the case of FRAP and TPC it is even preferable to extract using a hot plate. Considering that per gram of dry weight the results of the hot plate extraction were comparable or superior to those of the soxhlet extraction, and in an industrial setting conducting a soxhlet extraction is unviable while an extraction with agitation is much easier to implement, it was decided to do the screening design using a hot plate extraction. After the screening design it was concluded that to compare different solvents several points should be used. The best solvent depends on the plant part, what we want to extract, the conditions used and the properties we want the extract to possess. Stir and temperature were the factors that had the most influence in the results and time the one that influenced the least. For fruits there are two extraction stages which may be explained by the complex matrix which characterizes it. If only one solvent and one set of conditions were to be chosen for the highest antioxidant activity per gram of dry weight for each plant part, it would be water at 60°C for 30 minutes, 0.01 g/mL and 200 rpm for leaves and 50 % ethanol: water at 60°C, 180 minutes, 0.03 g/mL and 60 rpm for fruits.

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Discussion of Chapter III

Analysing the results obtained in this chapter, we can see that *Arbutus unedo* leaves provided much higher results than those obtained for both *Erica australis* and *Erica arborea.* This was true for all assays performed on both species of *Erica* tested. *A. unedo* flowers also had higher results than either *Erica* flowers, at all tested conditions (Table III–8, Table III–9 and Table III–10). Additionally for both *Arbutus* and *Erica*, the flower results were similar to those displayed by the leaves. On the other hand when comparing plant parts of *A. unedo*, fruits had the lowest antioxidant activity of all. While flowers collected from the tree had flavonoids, similarly to leaves, those collected from the ground displayed no flavonoids, similarly to what was found on the fruits. Flowers collected from the ground however still displayed much stronger antioxidant activity than fruits, and even stronger activity per gram of extract obtained than leaves collected from the tree.

Table III–8. Extraction conditions for flower extracts.

Extraction	Temperature (°C)	Time (min)	Ratio (mg/mL)	Stirr (rpm)
1	65	10	75	600
2	65	90	50	400
3	25	10	100	400
4	25	50	50	600
5	65	50	100	200
6	45	10	50	200
7	45	50	75	400
8	45	90	100	600
9	25	90	75	200

Table III–9. Results obtained for extractions from flowers collected from the tree

Ext.	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg Trolox/g dw)	FRAP (mg Trolox/g dw)
1	184.83 ± 4.87	5.24 ± 0.59	295.77 ± 3.34	283.93 ± 2.74	413.61 ± 6.80
2	185.23 ± 3.79	7.48 ± 0.76	325.45 ± 11.23	287.44 ± 3.65	426.72 ± 8.98
3	119.82 ± 1.19	3.79 ± 0.49	226.19 ± 3.71	180.87 ± 0.81	277.90 ± 3.45
4	148.73 ± 0.52	4.25 ± 0.40	283.67 ± 15.4	225.90 ± 2.40	361.15 ± 9.00
5	160.18 ± 4.42	5.98 ± 0.07	298.52 ± 9.44	272.48 ± 4.67	370.81 ± 9.20
6	121.33 ± 0.88	3.83 ± 0.52	212.45 ± 10.89	188.12 ± 1.93	267.89 ± 8.35
7	180.24 ± 1.99	5.75 ± 0.22	305.27 ± 1.18	281.36 ± 8.66	399.89 ± 0.98
8	148.05 ± 1.35	4.86 ± 0.10	280.30 ± 5.05	237.58 ± 5.15	348.04 ± 5.25
9	134.88 ± 0.82	4.43 ± 0.39	234.00 ± 3.94	211.53 ± 4.21	317.64 ± 2.89

Results are presented as mean ± standard deviation of the replicates.

When comparing the same results per mL however leaves collected from the tree had a clear advantage (data not shown). A similar difference when comparing results per g and per mL was obtained for the leaves and fruits of *A. unedo*. This difference between results per mL and per gram of extract obtained is related to the efficiency of the extraction and the concentration of the extracts. These preliminary results indicate that flowers should be studied further, and flowers from the tree may possibly display medicinal properties which flowers from the ground don't display.

Table III–10.Results obtained for extractions from flowers collected from the ground

Ext.	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg Trolox/g dw)	FRAP (mg Trolox/g dw)
1	256.58 ± 4.09	n.d.	507.10 ± 17.26	445.31 ± 7.96	647.64 ± 7.34
2	285.49 ± 8.56	n.d.	359.26 ± 16.69	498.28 ± 4.00	440.86 ± 10.44
3	170.86 ± 18.11	n.d.	407.79 ± 22.60	293.33 ± 6.75	493.74 ± 12.73
4	159.84 ± 2.94	n.d.	240.84 ± 8.26	283.79 ± 9.73	280.15 ± 11.05
5	70.67 ± 2.99	n.d.	302.20 ± 15.07	131.96 ± 2.48	403.51 ± 8.73
6	214.61 ± 7.69	n.d.	184.52 ± 13.83	373.48 ± 8.80	221.74 ± 10.60
7	118.90 ± 1.12	n.d.	351.12 ± 4.83	214.99 ± 11.67	431.81 ± 6.17
8	111.84 ± 1.75	n.d.	314.60 ± 4.33	198.03 ± 3.81	452.58 ± 3.72
9	275.54 ± 2.08	n.d.	318.56 ± 5.98	470.49 ± 2.16	411.59 ± 16.61

Results are presented as mean ± standard deviation of the replicates.

When Guendouze-Bouchefa et al. (2015) tested the aerial parts of flowering *Erica arborea* and leaves of *Arbutus unedo*, they found similar results, with *Arbutus unedo* having higher TPC and TFC values even though the values they obtained for *Erica arborea* were higher than ours. In the work developed by Pavlovic et al. (2009) however the results were the opposite, with *Erica arborea* phenolic and flavonoid contents being higher than those of *Arbutus unedo*. The differences detected could be due to different locations, extraction methods and even collection time. It is known that plants antioxidant activity can vary throughout the year (Nenadis et al., 2015, Sivaci and Duman, 2014, Bujor et al., 2016) and both *Ericas* were collected in the end of spring, beginning of summer, while *Arbutus unedo* was collected during the month of November. Similarly to *Erica*, *Arbutus unedo* also presented different results in different locations. This was also seen by Pavlovic et al. (2011) which found the antioxidant activity of *Arbutus unedo* to vary significantly from Greece to Montenegro.

Table III–11. Leaf results for the individual samples after new extraction according to screening design

Leaves	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg Trolox/g dw)	FRAP (mg Trolox/g dw)	DPPH IC 50 (µg/mL)
1	278.00 ± 19.97	26.68 ± 2.01	414.28 ± 24.30	350.32 ± 17.15	634.41 ± 15.65	21.62 ± 2.09
2	366.62 ± 19.37	9.93 ± 0.71	501.89 ± 28.34	440.44 ± 22.32	795.84 ± 9.87	14.65 ± 1.00
3	308.76 ± 24.72	25.09 ± 3.34	462.26 ± 46.07	404.68 ± 7.55	706.88 ± 25.09	22.58 ± 2.46
4	357.44 ± 10.22	21.82 ± 1.59	518.35 ± 34.57	454.76 ± 27.67	789.43 ± 26.06	15.18 ± 1.08
5	293.20 ± 19.52	21.12 ± 1.45	332.92 ± 17.34	320.28 ± 29.75	551.55 ± 8.57	22.40 ± 1.02
6	363.65 ± 17.16	20.73 ± 1.59	458.23 ± 33.46	415.91 ± 25.35	770.48 ± 15.29	17.42 ± 0.82
7	305.37 ± 15.85	30.38 ± 0.59	432.23 ± 17.31	376.89 ± 10.22	647.66 ± 18.92	17.95 ± 0.85
8	321.06 ± 11.77	36.14 ± 2.49	447.66 ± 5.35	441.67 ± 21.86	671.92 ± 11.73	22.61 ± 2.08
9	444.97 ± 12.79	45.69 ± 2.36	558.41 ± 24.53	607.93 ± 28.90	982.00 ± 22.24	12.10 ± 0.60
10	292.53 ± 14.03	24.79 ± 0.48	441.08 ± 16.38	283.81 ± 9.04	676.71 ± 13.93	16.26 ± 0.75
11	409.01 ± 5.95	48.53 ± 2.35	591.80 ± 31.85	455.41 ± 52.82	892.86 ± 31.00	11.95 ± 0.86
12	495.79 ± 21.72	72.44 ± 2.69	800.04 ± 40.70	632.24 ± 19.83	1086.63 ± 18.45	10.14 ± 0.40
13	538.57 ± 9.30	67.30 ± 1.79	761.04 ± 22.41	597.62 ± 45.70	1217.94 ± 35.59	16.25 ± 1.09
14	440.45 ± 18.81	53.59 ± 1.52	698.16 ± 25.21	557.81 ± 18.09	985.62 ± 36.99	19.16 ± 10.00
15	450.91 ± 27.14	49.29 ± 1.58	572.67 ± 42.66	485.51 ± 22.32	954.82 ± 20.37	14.69 ± 2.67
16	275.07 ± 35.84	40.00 ± 1.07	430.92 ± 15.98	437.39 ± 46.55	661.72 ± 8.81	13.03 ± 0.94
17	370.20 ± 20.28	41.01 ± 1.89	506.33 ± 10.67	420.41 ± 16.11	739.22 ± 12.06	18.34 ± 2.13
18	325.48 ± 12.67	38.41 ± 0.73	461.92 ± 12.47	384.18 ± 22.95	737.93 ± 10.79	9.77 ± 1.18

Results are presented as mean ± standard deviation of the replicates.

Location can have a strong impact in the antioxidant activities. Since plants use phenolics as a way to defend themselves against aggression. The effect of UV-B exposure and rainfall was tested by Nenadis et al. (Nenadis et al., 2015) who found flavanol, quercitrin, theogallin and antioxidant activities of *Arbutus unedo* leaves were all affected by different UV radiation exposure.

Table III–12. Fruit results for the individual samples after new extraction according to screening design

Fruits	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg Trolox/g dw)	FRAP (mg Trolox/g dw)	DPPH IC_{50} (µg/mL)
1	18.67 ± 0.08	n.d.	258.58 ± 6.89	25.03 ± 0.37	31.19 ± 0.95	255.52 ± 16.60
2	16.16 ± 0.27	n.d.	238.96 ± 7.12	22.38 ± 0.29	29.25 ± 0.68	347.74 ± 24.61
3	23.92 ± 0.49	n.d.	228.45 ± 10.07	32.45 ± 1.58	48.07 ± 2.12	204.94 ± 19.71
4	30.19 ± 1.21	n.d.	226.52 ± 2.97	41.22 ± 0.97	65.16 ± 3.26	174.78 ± 6.20
5	17.47 ± 0.78	n.d.	203.45 ± 2.65	22.03 ± 0.75	28.75 ± 1.24	412.95 ± 18.00
6	22.14 ± 0.35	n.d.	196.00 ± 3.85	29.35 ± 1.41	45.87 ± 1.83	215.53 ± 9.62
7	28.03 ± 0.95	n.d.	267.23 ± 11.56	37.70 ± 0.51	59.82 ± 1.62	157.43 ± 9.77
8	42.67 ± 0.67	n.d.	226.79 ± 3.80	53.63 ± 0.12	94.35 ± 0.54	99.36 ± 4.81
9	26.53 ± 0.24	n.d.	197.13 ± 4.76	43.34 ± 1.53	57.32 ± 2.08	162.70 ± 9.45
10	22.99 ± 2.50	n.d.	228.98 ± 4.71	31.34 ± 0.22	40.73 ± 1.48	179.48 ± 26.78
11	34.31 ± 0.38	n.d.	230.55 ± 3.40	33.18 ± 0.33	62.08 ± 1.56	118.61 ± 14.15
12	27.04 ± 1.38	n.d.	245.13 ± 10.23	35.05 ± 0.38	48.43 ± 2.28	158.61 ± 6.60
13	24.09 ± 0.32	n.d.	200.60 ± 4.17	28.08 ± 0.14	45.20 ± 1.77	224.56 ± 23.75
14	28.36 ± 0.69	n.d.	205.35 ± 5.06	34.66 ± 0.31	50.82 ± 0.97	236.61 ± 17.61
15	21.37 ± 0.51	n.d.	221.31 ± 4.61	25.15 ± 0.28	37.27 ± 0.79	207.41 ± 87.03
16	18.39 ± 0.51	n.d.	214.90 ± 3.36	28.92 ± 0.59	26.75 ± 2.37	309.47 ± 15.60
17	26.47 ± 0.21	n.d.	216.51 ± 4.79	32.42 ± 0.58	50.45 ± 0.81	159.67 ± 22.34

Results are presented as mean ± standard deviation of the replicates.

After testing the effect of extraction technique using soxhlet and hot plate, it was concluded that the method of extraction had a significant impact on the results per mL, with clear advantage to the soxhlet extraction, but this advantage disappeared or was inverted when the results were compared in mg per gram of dry extract. For this reason it was decided that further extractions would be performed using a hot plate, which allows much quicker extractions and has conditions that are easier to replicate in an industrial setting when compared to a soxhlet extraction.

Comparing the results obtained before and after the application of the screening design on the 18 leaf samples and 16 fruit samples, it was possible to see that using the information gained from the screening design, the new extraction with water was more efficient when it comes to the amount of phytochemicals by mass of dry weight extracted, except for fruit samples FRAP. This was in part due to the lower sample/solvent ratio, meaning more compounds can be extracted until the solvent is saturated. The reduction of the extraction temperature was also important to prevent the destruction of compounds with antioxidant activity.

After finding the best conditions to extract the antioxidant compounds, the stability of the plant material itself was studied (Table III–13 and Table III–14). This was important especially for fruits, which are available for only 1 to 2 months every year, but it was decided to conduct the same study for the leaves, so that they can be used while their antioxidant activity is at its maximum.

Table III–13. TPC and TFC leaf variation over time.

Time (months)		0	3	6	9	12
		TPC (mg GAE/g dw)				
Sample	**1**	278.00 ± 19.97	280.58 ± 14.60	270.38 ± 16.57	257.79 ± 12.25	246.23 ± 6.69
	2	366.62 ± 19.37	373.16 ± 10.58	361.92 ± 17.85	358.55 ± 7.38	347.17 ± 19.01
	3	357.44 ± 10.22	369.59 ± 10.62	356.12 ± 9.56	351.16 ± 9.15	352.72 ± 8.92
	4	293.20 ± 19.52	303.40 ± 5.82	282.28 ± 15.79	262.89 ± 4.74	252.86 ± 8.21
	5	292.53 ± 14.03	293.19 ± 13.34	281.85 ± 13.53	272.30 ± 6.05	251.43 ± 13.27
	6	538.57 ± 9.30	536.83 ± 13.30	534.22 ± 4.36	541.19 ± 11.94	522.48 ± 8.48
	7	440.45 ± 18.81	446.68 ± 15.92	439.58 ± 3.37	434.34 ± 15.72	437.32 ± 41.96
	8	450.91 ± 27.14	437.07 ± 19.67	417.00 ± 20.38	365.82 ± 3.03	321.17 ± 1.97
	9	275.07 ± 35.84	286.84 ± 3.95	274.41 ± 26.87	286.89 ± 6.05	272.95 ± 2.44
	10	325.48 ± 12.67	318.51 ± 15.08	318.93 ± 10.08	322.35 ± 18.17	297.75 ± 8.67
		TFC (mg QE/g dw)				
Sample	**1**	26.68 ± 2.01	24.68 ± 0.82	25.25 ± 1.32	25.22 ± 2.18	19.35 ± 0.59
	2	9.93 ± 0.71	9.56 ± 0.89	9.91 ± 0.52	9.58 ± 0.36	7.88 ± 0.42
	3	21.82 ± 1.59	21.79 ± 0.80	21.16 ± 1.23	18.95 ± 1.57	27.83 ± 0.81
	4	21.12 ± 1.45	20.37 ± 0.45	20.32 ± 1.52	18.68 ± 1.06	29.22 ± 2.62
	5	24.79 ± 0.48	27.80 ± 1.06	26.61 ± 0.62	26.88 ± 0.53	31.68 ± 2.17
	6	67.30 ± 1.79	66.90 ± 0.74	66.51 ± 1.35	62.89 ± 2.92	28.51 ± 1.09
	7	53.59 ± 1.52	54.61 ± 0.97	52.90 ± 1.65	51.40 ± 1.49	28.26 ± 0.15
	8	49.29 ± 1.58	49.28 ± 2.12	46.49 ± 0.71	44.95 ± 3.77	27.35 ± 1.21
	9	40.00 ± 1.07	39.16 ± 0.45	37.93 ± 0.58	34.53 ± 2.46	16.22 ± 0.49
	10	38.41 ± 0.73	37.51 ± 0.40	37.31 ± 1.10	36.58 ± 2.80	34.64 ± 1.85

Results are presented as mean ± standard deviation of the replicates.

Table III–14. TAA, RP and FRAP leaf variation over time.

Time		0	3	6	9	12
		TAA (mg AAE/g dw)				
Sample	1	414.28 ± 24.3	412.83 ± 5.83	404.94 ± 11.76	393.20 ± 3.35	383.51 ± 16.40
	2	501.89 ± 28.34	508.37 ± 4.91	498.87 ± 13.98	492.74 ± 12.81	493.85 ± 26.02
	3	518.35 ± 34.57	495.33 ± 10.11	501.43 ± 18.49	496.08 ± 6.67	464.77 ± 17.03
	4	332.92 ± 17.34	344.52 ± 1.93	323.58 ± 9.36	310.86 ± 2.89	301.08 ± 11.13
	5	441.08 ± 16.38	412.53 ± 6.12	391.81 ± 4.17	375.01 ± 5.14	311.08 ± 16.69
	6	761.04 ± 22.41	733.15 ± 21.15	647.16 ± 23.65	473.89 ± 9.82	411.98 ± 46.93
	7	698.16 ± 25.21	618.88 ± 10.19	591.83 ± 29.53	420.45 ± 2.06	367.29 ± 43.42
	8	572.67 ± 42.66	509.94 ± 6.86	464.14 ± 23.54	348.27 ± 3.18	260.67 ± 29.88
	9	430.92 ± 15.98	414.95 ± 8.26	376.44 ± 8.98	314.2. ± 2.78	253.04 ± 9.00
	10	461.92 ± 12.47	437.59 ± 6.12	417.19 ± 7.07	372.38 ± 7.89	318.28 ± 9.04
		RP (mg TE/g dw)				
Sample	1	350.32 ± 17.15	347.86 ± 13.34	344.79 ± 15.71	327.16 ± 14.17	333.24 ± 17.82
	2	440.44 ± 22.32	422.74 ± 22.08	429.99 ± 19.59	406.28 ± 20.29	407.18 ± 23.42
	3	454.76 ± 27.67	456.86 ± 9.12	448.68 ± 18.64	426.25 ± 13.19	433.46 ± 17.39
	4	320.28 ± 29.75	319.95 ± 10.77	309.64 ± 16.96	298.42 ± 10.22	292.58 ± 5.45
	5	283.81 ± 9.04	293.28 ± 14.88	283.93 ± 14.22	288.71 ± 25.35	284.53 ± 30.72
	6	597.62 ± 45.70	565.21 ± 4.80	556.65 ± 27.27	499.16 ± 8.92	478.69 ± 38.78
	7	557.81 ± 18.09	560.39 ± 12.72	557.35 ± 24.45	551.97 ± 16.90	556.30 ± 39.35
	8	485.51 ± 22.32	474.54 ± 24.27	450.73 ± 15.17	414.33 ± 4.63	377.37 ± 6.88
	9	437.39 ± 46.55	426.31 ± 9.25	405.92 ± 33.41	379.89 ± 10.74	342.70 ± 7.58
	10	384.18 ± 22.95	377.87 ± 12.46	376.47 ± 16.03	367.67 ± 10.41	357.31 ± 6.66
		FRAP (mg TE/g dw)				
Sample	1	634.41 ± 15.65	633.61 ± 13.79	621.48 ± 4.61	622.73 ± 15.81	590.80 ± 21.20
	2	795.84 ± 9.87	752.33 ± 18.53	784.08 ± 12.93	767.39 ± 12.20	766.84 ± 17.81
	3	789.43 ± 26.06	776.88 ± 20.8	773.23 ± 10.08	772.05 ± 8.65	744.43 ± 24.54
	4	551.55 ± 8.57	538.68 ± 17.85	541.98 ± 0.45	522.37 ± 8.48	521.21 ± 16.49
	5	676.71 ± 13.93	663.02 ± 20.49	624.81 ± 8.38	553.74 ± 8.61	496.83 ± 7.01
	6	1217.94 ± 35.59	1071.56 ± 18.83	1076.05 ± 19.34	960.90 ± 25.15	741.22 ± 29.75
	7	985.62 ± 36.99	903.20 ± 5.02	809.60 ± 23.50	756.59 ± 16.12	524.09 ± 24.73
	8	954.82 ± 20.37	826.53 ± 14.35	788.11 ± 16.78	481.52 ± 18.00	401.98 ± 13.03
	9	661.72 ± 8.81	558.47 ± 9.01	543.02 ± 5.6	419.87 ± 10.99	333.51 ± 2.51
	10	737.93 ± 10.79	613.68 ± 24.41	601.40 ± 6.33	417.22 ± 8.96	388.23 ± 12.65

Results are presented as mean ± standard deviation of the replicates.

The results show that although leaves demonstrate a high antioxidant activity when collected, with time this antioxidant activity will be reduced, although not equally for all samples. They also show that for the most part samples suffer no significant changes during the first 6 months of storage (Figure III–6). While TPC and RP remained for the most part

relatively the same, the other assays revealed the samples are susceptible to storage degradation. For TFC four samples had a decrease of 20% while there was no significant difference for three other samples ($p>0.05$).

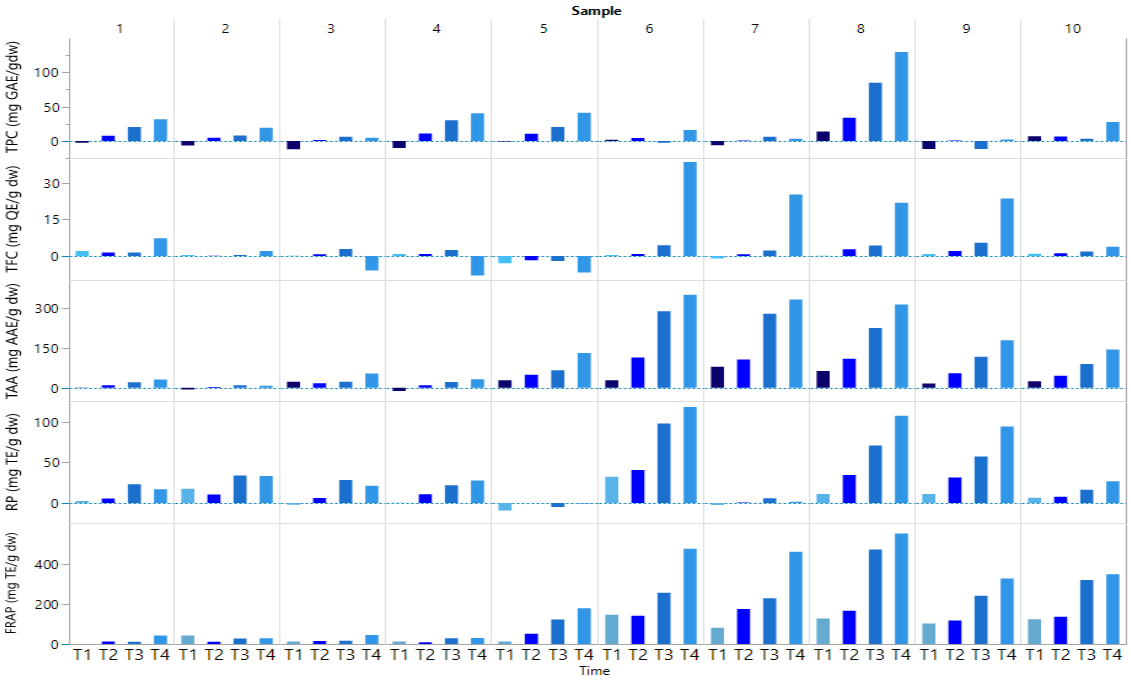

Figure III–6. Difference after 3 (T1), 6 (T2), 9 (T3) and 12 (T4) months for leaves.

This decrease and susceptibility of flavonoids is not surprising since many flavonoids have been demonstrated to be sensible to storage as well as oxidation (Mulinacci et al., 2011, Sokolová et al., 2012). TAA and FRAP revealed even more of the samples susceptibility to storage. The difference was specially marked for FRAP with six samples having a decrease of over 30 % of the initial value. The fact that TPC and RP remained relatively unaltered and TFC, TAA and FRAP mostly decreased indicates not only that after one year RP is mainly related to phenolic compounds that are not included on the flavonoid category, but also that both TAA and FRAP are likely due to flavonoids present in the samples, which is in accordance with what was seen previously. In addition, because these assays gave the most differences, these are the assays that should be used in the future to assess degradation of the antioxidant activity of *A. unedo* leaves while in storage.

Fruits (Table III–15) appeared more sensitive regarding TPC compared to leaves, with only 2 of the 6 samples being relatively unaffected by storage against 4 of 10 for the leaves.

Table III–15. Fruit TPC, TAA, RP and FRAP variation over time.

Assay	Time	Sample 1	2	3	4	5	6
TPC (mg GAE/g dw)	0	22.99 ± 2.50	24.09 ± 0.32	28.36 ± 0.69	21.37 ± 0.51	18.39 ± 0.51	22.08 ± 0.71
	3	20.28 ± 0.47	22.84 ± 0.67	23.67 ± 0.40	16.97 ± 1.41	19.18 ± 0.31	23.18 ± 0.26
	6	21.77 ± 0.91	23.83 ± 0.33	27.21 ± 0.58	20.42 ± 0.58	18.86 ± 0.30	22.47 ± 0.65
	9	22.03 ± 0.53	23.67 ± 0.56	26.55 ± 0.44	20.05 ± 1.33	19.42 ± 0.29	22.20 ± 1.10
	12	23.34 ± 1.25	23.83 ± 0.12	31.34 ± 1.03	19.54 ± 0.36	24.21 ± 1.32	19.13 ± 0.22
TAA (mg AAE/g dw)	0	228.98 ± 4.71	200.60 ± 4.17	205.35 ± 5.06	221.31 ± 4.61	214.90 ± 3.36	197.02 ± 3.08
	3	228.19 ± 1.23	216.79 ± 4.28	216.09 ± 2.43	221.20 ± 5.17	214.21 ± 1.19	195.45 ± 1.14
	6	227.19 ± 8.59	225.06 ± 2.80	221.31 ± 4.08	221.87 ± 2.22	210.78 ± 3.14	191.87 ± 4.31
	9	224.41 ± 14.83	268.09 ± 5.25	247.72 ± 8.42	222.80 ± 2.26	203.34 ± 11.09	183.67 ± 8.10
	12	239.74 ± 7.94	234.51 ± 9.12	220.55 ± 1.54	209.57 ± 1.05	208.45 ± 11.38	198.87 ± 12.54
RP (mg TE/g dw)	0	31.34 ± 0.22	28.08 ± 0.14	34.66 ± 0.31	25.15 ± 0.28	28.92 ± 0.59	27.78 ± 0.97
	3	30.21 ± 1.52	29.61 ± 0.72	35.19 ± 1.68	25.71 ± 0.65	31.06 ± 1.01	27.56 ± 0.48
	6	31.37 ± 0.31	30.04 ± 0.36	34.17 ± 0.40	25.59 ± 0.22	29.44 ± 0.38	27.85 ± 0.72
	9	31.40 ± 0.45	33.47 ± 1.02	33.09 ± 0.79	26.53 ± 0.50	30.41 ± 0.49	28.04 ± 0.37
	12	34.92 ± 0.12	32.45 ± 0.35	43.90 ± 0.37	29.39 ± 0.65	35.13 ± 0.92	28.54 ± 0.55
FRAP (mg TE/g dw)	0	40.73 ± 1.48	45.20 ± 1.77	50.82 ± 0.97	37.27 ± 0.79	26.75 ± 2.37	35.24 ± 1.06
	3	41.71 ± 0.68	46.87 ± 0.51	50.30 ± 1.09	36.82 ± 1.59	34.33 ± 1.08	36.57 ± 0.66
	6	43.94 ± 1.07	48.21 ± 0.57	49.60 ± 1.33	39.13 ± 0.97	36.00 ± 1.39	39.55 ± 0.64
	9	50.63 ± 2.41	52.88 ± 1.35	46.74 ± 2.77	42.98 ± 1.55	52.79 ± 0.75	48.57 ± 0.50
	12	56.20 ± 1.68	56.08 ± 1.53	63.24 ± 0.69	38.94 ± 2.34	51.12 ± 1.73	35.94 ± 1.48

Results are presented as mean ± standard deviation of the replicates

If results are seen on absolute basis however, fruits lost a lot less than leaves (Figure III–7). This is supported by the statistical analysis which showed that only one sample had significant differences due to storage for one year ($p<0.05$). On the other hand, regarding TAA, fruits showed a much smaller loss both in absolute and relative terms and in some cases the results after one year in storage were even higher than before. The difference between leaves and fruits loss with storage may be due to how the samples were stored. While leaves were stored in powder form and thus the inside was in contact with air, as little as it was, fruits were stored whole and thus the inside of the fruit was protected. This can lead to some enzymes, which need oxygen to work and degrade compounds with antioxidant activity into compounds without antioxidant activity, to be active on the leaves and not on the fruits.

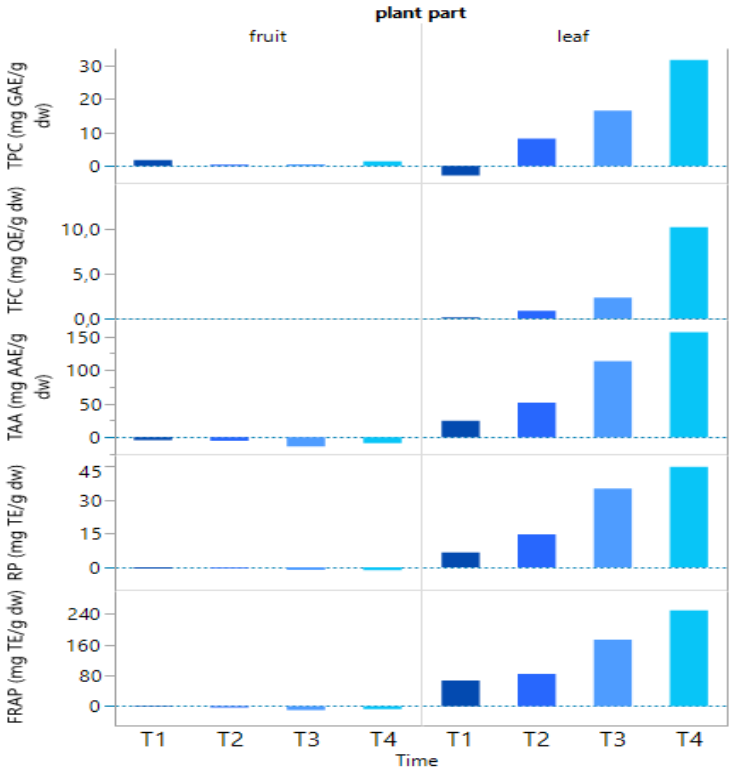

Figure III–7. Global variation of results over time by plant part

Regarding the increase of TAA in fruits, this behaviour was already detected in some other berries and even leafy vegetables, and was attributed to an increase in certain flavonoids and other compounds during storage, caused by the degradation of some matrices that previously prevented their action and detection (Mulinacci et al., 2011, Ahmad-Qasem et al., 2013).

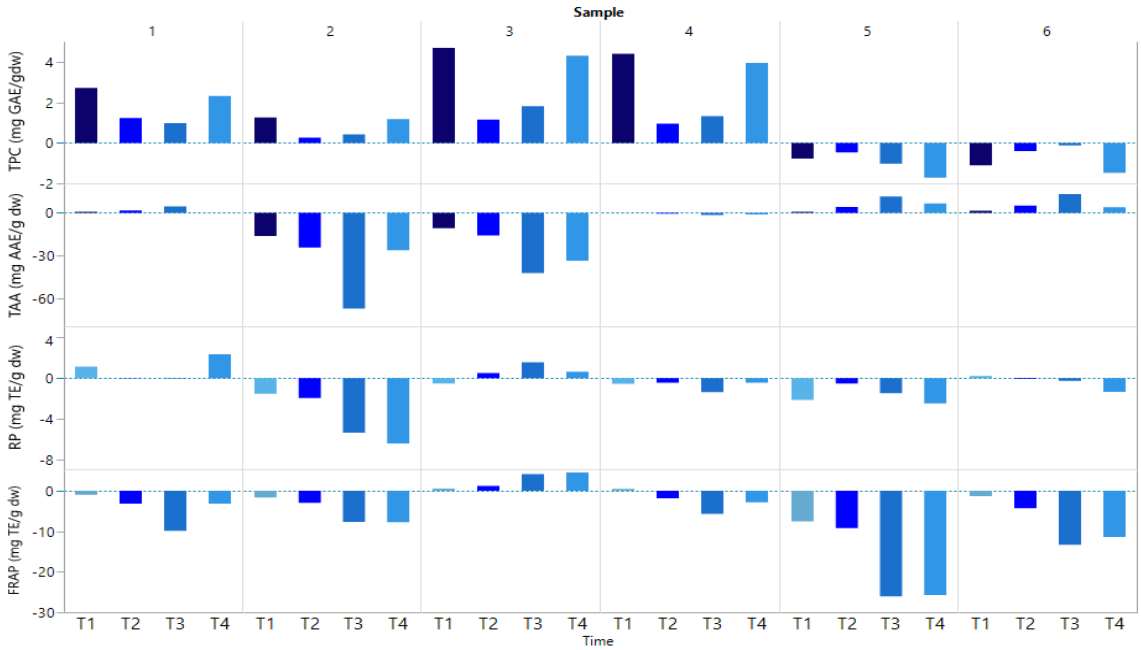

Figure III–8. Difference after 3 (T1), 6 (T2), 9 (T3) and 12 (T4) months for fruits.

While it was not possible to obtain a positive reaction from the fruit samples with $AlCl_3$, given how this reagent reacts mostly with flavonols and flavones luteolin, a negative reaction with $AlCl_3$ only means the sample does not have these compounds and not that it does not have other kinds of flavonoid compounds, which could increase during storage and alter the results (Pękal and Pyrzynska, 2014).

The same trend of increase during storage was observed for RP, although not significantly for most samples ($p>0.05$) while FRAP, which on leaves showed a high decrease, on fruits it showed a significant increase ($p<0.05$) of over 20% in 5 of the 6 samples. This indicates that after one year neither TAA nor RP are strongly connected to phenolic compounds and that most likely the same compounds that are responsible for TAA on fruits also affect their RP although with different magnitudes, which is further supported by the correlations obtained (Table III–16). While antioxidant results indicate that unlike leaves, fruits can be stored for one year on a freezer, it should be noted that during storage there is a degradation of their organoleptic properties, with the fruits after thawing being much softer and of lower consistency and thus not appetizing.

Table III–16. Correlations for leaves and fruits

Leaves					
	TPC	TFC	TAA	RP	FRAP
TPC	1	0.641**	0.739**	0.880**	0.792**
TFC	0.641**	1	0.523**	0.624**	0.516**
TAA	0.739**	0.523**	1	0.762**	0.942**
RP	0.880**	0.624**	0.762**	1	0.729**
FRAP	0.792**	0.516**	0.942**	0.729**	1
Fruits					
TPC	1	a	0.087	0.614**	0.524**
TAA	0.087	a	1	0.366*	0.194
RP	0.614**	a	0.366*	1	0.608**
FRAP	0.524**	a	0.194	0.608**	1

**, Correlations is significant at the 0.01 level; *, correlation is significant at the 0.05 level; a, not enough samples to calculate.

Overall this chapter shows that *Arbutus unedo* is a plant with high antioxidant activity, especially leaves, even when compared to other plants of the same family. For convenience, reproducibility in an industrial setting and greater antioxidant activity per gram of extract, the leaves and fruits should be extracted using a hot plate and not a soxhlet apparatus. There are significant differences in the extracts obtained with different solvents and using different conditions, and as such when starting a new work, the best conditions should be searched.

Finally, leaves can be stored in a freezer in the ground form for periods up to 6 months without losing antioxidant properties, while fruits can be stored for periods up to 12 months, enough for a new harvest.

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CHAPTER IV - SCREENING OF MEDICINAL PROPERTIES FROM SEVEN PLANTS COLLECTED IN ALGARVE

In this chapter we screen 7 different plants collected in Algarve for their medicinal properties. The plants studied are *Erica arborea, Erica australis, Arbutus unedo, Crataegus monogyna, Equisetum telmateia, Geranium purpureum, Mentha suaveolens and Lavandula stoechas spp. luisieri*. For all these plants we study the antimicrobial and antiproliferative activities. Additionally for *Arbutus unedo, Crataegus monogyna, Equisetum telmateia, Geranium purpureum, Mentha suaveolens and Lavandula stoechas spp. luisieri* we study the phenolic composition and zinc content.

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Effect of *Erica australis* extract on CACO-2 cells, fibroblasts and selected pathogenic bacteria responsible for wound infection

Ricardo Nunes[1], Susana Rodrigues[2], Pawel Pasko[3], Malgorzata Tyszka – Czochara[4], Ana Grenha[2], Isabel Saraiva de Carvalho[1,*]

[1]IBB/CGB-Institute for Biotechnology and Bioengineering/ Centre of Genomics and Biotechnology, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005 -139 Faro, Portugal

[2]CBME – Centre for Molecular and Structural Biomedicine / IBB – Institute for Biotechnology and Bioengineering, University of Algarve, Faculty of Sciences and Technology, Campus de Gambelas, 8005-139 Faro, Portugal

[3]Department of Food Chemistry and Nutrition, Medical College, Jagiellonian University, Krakow, Poland

[4]Department of Radioligands, Medical College, Jagiellonian University, Krakow, Poland

1 ABSTRACT

Plants from the genus *Erica* are used in many countries to treat several ailments. In this work we intend to evaluate the potential *in vivo* benefits of *Erica australis* L. by testing *in vitro* the effect induced by the plant extract when in contact with BJ fibroblasts (3 and 6 hours) and Caco-2 cells (3, 6 and 24 hours). Effects on five pathogenic microorganisms (*Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*) were also determined. It was found that the extracts enhanced fibroblast proliferation (maximum of 484% of control at 6 hour exposure) while Caco-2 cells viability was reduced in a concentration and time dependent manner (minimum of 22.3% of control at 24 hour exposure). Antimicrobial effects were also detected, with differences registered among the plant parts and solvent used, with the lowest minimum concentration for diffusion inhibition (MCDI) of 1 mg/mL. Results obtained with the fibroblasts and bacteria strongly show that this plant has potential to be used in wound healing as a stimulant of fibroblast growth and disinfection, as well as an antibiotic. Results obtained with Caco-2 cells indicate this plant also has some potential for and application as anticancer agent.

KEYWORDS

Erica australis L., Fibroblasts, Caco-2, antiproliferative effect, wound healing stimulant, antibacterial

2 Introduction

Plant extracts have been used for a long time by traditional healers and are referenced in folk medicine to treat several ailments. This knowledge was somewhat lost due to the rise of allopathic medicine which replaced natural treatments with chemicals and drugs (Neves et al., 2009a). This change, however, led to antibiotic resistant bacteria and a weakened immune system (Alanis, 2005). In addition, changes in lifestyle lead to a much larger exposure to oxidizing agents, which can cause problems that include degenerative diseases, heart problems and cancer (Jemal et al., 2008).

For this reason focus started to shift, turning the attention back to the study of plants and use of natural compounds for the treatment of diseases which led to extensive screenings for plants that could be used (Neves et al., 2009a). One of the most common aspects studied in plants is their content in antioxidants, which can help in the prevention of cancer and degenerative diseases (Ames et al., 1995). This antioxidant protection can be due to several actions, including the capture of Reactive Oxigen Species before they interact with lipids by direct scavenging of the radicals, the inhibition of enzimes such as cyclooxygenase and lipoxygenase or the stimulation of detoxifying enzymes such as indoles (Ames et al., 1995). Some plants however, in addition to free radical scavenging properties, also possess compounds that evidence cytotoxic properties. This may not only allow them to help preventing cancer occurrence, but potentially eliminating it (González-Sarrías et al., 2012). The plants here studied belong to the *Ericaceae* family and are used in many countries to treat several ailments, including digestive and urinary disorders, as well as wound healing and disinfection (Akkol et al., 2008b, Harnafi et al., 2007). Aqueous extracts from two species of this plant, *Erica australis* L. and *Erica arborea* L., were previously studied by our group and were found to possess relatively high activities, especially concerning the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical (IC_{50} of 60 µg/mL) (Nunes et al., 2012b). The sample with the best results was further studied and it was found that leaves contained almost 50 mg of amino acids per gram of dry weight, of which 20 mg are of essential amino acids They also possess a total of 16 phenolic compounds, including the strong antioxidant caffeic acid (500 µg/g) (Nunes and Carvalho). Now, continuing and extending the study of this plant, we intended to evaluate potential health benefits derived from the consumption/direct application of extracts from this plant, by analyzing the extract effect on fibroblasts (responsible for tissue regeneration), Caco-2 cells (intestinal cancer cells) and pathogenic bacteria commonly found in wounds. The selected bacteria were *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes*. *E. faecalis* is a pathogenic

microorganism responsible for 90% of the infections caused by the *Enterococcus* genus (F.D.A., 2012). These bacteria are amongst the most common bacteria isolated from infected surgical sites (Munaff, 2012). Pathogenic *E. coli* species can be fatal and also commonly found in infected wounds (F.D.A., 2012, Senthil Kumar et al., 2006). *B. cereus* can in some cases enable further contamination by other bacteria and is high prevalent in post-operative and post-traumatic wounds (F.D.A., 2012). *S. aureus* are also highly prevalent in infected wounds. Deaths are rare and only occur in people with compromised immune system (F.D.A., 2012). *L. monocytogenes* can provoke a severe form of infection with death rate between 15 and 30%, although if it triggers meningitis it can reach 70% (F.D.A., 2012).

3 Material and methods

3.1 Plant material

Samples were identified by a botanist and randomly selected and collected during the blooming period at the end of spring, beginning of summer of 2010 from the Algarve region (37.187596,-8.695455). A voucher specimen was deposited in the Herbarium from University of Algarve with the number 10945. After collection, plant materials were stored on a dry place, protected from sunlight and naturally air dried (ambient temperature of approximately 20ºC) for about one week. Leaves and flowers were manually separated and stored on plastic vials at -20ºC until extraction.

3.2 Biological material

Cell lines BJ (human fibroblasts) – ATCC CRL-2522(Rockville, USA) and Caco-2 (human colon adenocarcinoma) – ATCC HTB-37, bacteria strains: *Enterococcus faecalis* – DSMZ 20478, *Bacillus cereus* – ATCC 11778, *Escherichia coli* – ATCC 8739, *Staphylococcus aureus* – ATCC 6538, *Listeria monocytogenes* – DSMZ 7644. Bacteria strains were gently provided by Stress by Antibiotics and Virulence of Enterococci laboratory from IBET.

3.3 Chemicals

Phosphate buffer saline (PBS) tablets pH 7.4, , penicillin/streptomycin at +10,000 units/mL/+10,000µg/mL, nonessential amino acids (N.E.A.A.), L-glutamine 200 mM, trypsin–EDTA (ethylenediaminetetraacetic acid) solution (2.5 g/l trypsin, 0.5 g/l EDTA), Dulbecco's modified Eagle's medium (DMEM), trypan blue solution (0.4%), thiazolyl blue tetrazolium bromide (MTT) and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemicals, Germany. Dimethyl sulfoxide (DMSO), chloramphenicol

blue, Tris-acetate-EDTA (TAE) buffer ampicillin, plate count agar, RINGER tablets and cetrimide agar were purchased from Merk, Germany. Methanol was purchased from VWR, Pennsylvania, USA. Luria broth was purchased from Sigma-Aldrich Co. Ltd, Poole, UK. Ethanol was purchased from Merck, Nottingham, UK. Discs for minimum concentration for diffusion inhibition (MCDI) assay were purchased from Whatman, Maidstone, UK. FBS was purchased from Gibco, Invitrogen, USA. All reagents were of analytical grade.

3.4 Extraction procedure

The extraction was carried in a Soxhlet device with Electrothermal heating mantles (Electrothermal, Essex, UK), using 0.4 g of plant material and 60 mL of water or methanol. When the extraction was finished, the methanol was evaporated (Nahita serie 503, Navarra, Spain) and the material was re-suspended in water. Extracts were transferred to Eppendorfs and stored at -20ºC until next day. On the day of analysis, extracts were put on ice and in the dark until unfrozen.

3.5 Cell lines culture

The Caco-2 (HTB 37) and BJ (CRL-2522) line cells were used between passages 65-80 and 20-30, respectively. Both cells were grown in flasks in a humidified 5% CO_2/95% atmospheric air incubator at 37 °C. The cell culture medium for Caco-2 cells was DMEM supplemented with 10% FBS, 1% L-glutamine, 1% non-essential amino acids and 1% of penicillin/streptomycin. Medium was exchanged every 2-3 days and cells were sub cultured weekly. BJ cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and cells were sub cultured 3 times a week.

3.6 Bacterial strains culture

Prior to the assay, an overnight culture of each microorganism was initiated in 5 mL Luria Broth and incubated at 37ºC for 24 hours. After the 24 hours, a streak was made in a petri dish with 20 mL of dry plate count agar medium, and grown at 37ºC for 24 hours.

3.7 MTT assay

Taking into consideration the optimal confluence of culture and sufficient medium nutrients, cells were seeded in 96 well plates) at a density of 2.5×10^4 cells/well in 200 µl of medium for BJ cells and ii) at a density of 1×10^4 cells/well in 100 µl of medium for Caco-2 cells. Cells were then incubated overnight until the assay.

For the BJ cells, the medium was replaced by fresh medium after the overnight incubation, containing additionally MTT (5 mg/mL in PBS, pH 7.4). Dry methanol leaf extracts

(concentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 3 mg/mL) were previously added to the MTT to test the extract influence. Following incubation (3 and 6 hours), medium was removed and DMSO was added to each well to dissolve MTT formazan generated during incubation. The absorbance was measured at 540 nm (reference wavelength was 690 nm) using a microplate reader (Multiscan GO, Thermo Scientific) USA (Carmichael, 1987). Cells incubated in cell culture medium were considered as a control for 100% cell viability. The assay was performed on three occasions with six replicates at each concentration of test substance in each instance. All results are, therefore, means from triplicates and are presented as % of cell viability ± SD.

For the Caco-2 cells, the medium was also removed after incubation and replaced by 100 µL of aqueous leaf or flower extracts diluted in complete DMEM without FBS (concentrations of 0.1, 0.5, 1.0 and 2.0 mg/mL). After incubation with the extracts for 3, 6 or 24 hours, MTT was added and incubated for 3 hours. After that time, DMSO was used to dissolve formazan crystals and the absorbance was measured using a microplate reader (Tecan, Infinite M200, Austria) as referenced above. Due to the strong colour of the extracts, an intermediate step of washing with PBS prior to MTT addition was introduced. Cells incubated with cell culture medium were considered as the control for 100% cell viability. The assay was performed on three occasions with six replicates at each concentration of test substance in each instance. All results are means from triplicates and are presented as % of cell viability ± SD.

3.8 Disc diffusion assay

For the determination of minimum concentration for diffusion inhibition (MCDI) a method similar to that used in antibiotic susceptibility testing was used, but with plant extracts instead (Gaudreau and Gilbert, 1997a). Briefly, a grown microorganism colony was collected and suspended in a flask with Ringer solution. An aliquot of 50 µL was spread on a petri dish containing plate count agar. After dried discs were put in the petri dish and 10 µL of extract (10 mg/mL) were added to each one. Ampicillin (10 mg/mL) was used as control. This procedure was repeated for every strain. After 48 hour incubation, the inhibition diameter was measured. After assessing which strains were inhibited by the extract, several concentrations were tested to find the MCDI. All results are means from two measurements of each disc, three discs per plate triplicates ± SD.

3.9 Statistical methods

Statistical analysis of fibroblast cell and Caco-2 cells culture cytotoxicity experiments was performed by ANOVA followed by Duncan's post hoc test.

4 Results

4.1 Caco- 2 cells viability

The influence of aqueous extract exposure in Caco-2 cells was analyzed. Two different plant parts (leaves and flowers) and three different time points (3, 6 and 24 hours) were tested at 0.1, 0.5, 1.0 mg/mL for the 3 and 6 hour points and 0.5, 1.0 and 2.0 mg/mL for the 24 hour point (Figure IV–1).

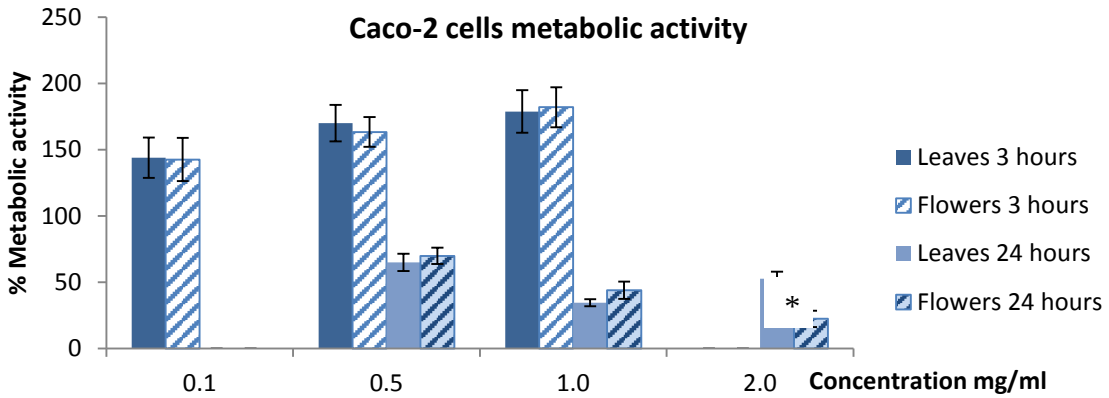

Figure IV–1. Metabolic activity (as % of control) of Caco-2 cells upon exposure to the samples for 3 h and 24 h exposure (mean ± SD, n = 3). Differences between plant parts ($p < 0.05$) are marked with *.

After incubating the cells with the extract, the amount of MTT formazan generated by the tricarboxylic acid cycle decarboxylases in mitochondria of viable, biochemically active cells, was analyzed. This reflects metabolic activity of the cells and, as such, their viability. As shown in Figure IV–1, at 3 hours there was an increase in the metabolic activity of cells upon contact with the extracts of both parts of the plant, an effect that increased in a concentration dependent manner. Upon 6 hours of continuous exposure there was still an increase for both plant parts, but of lower intensity (data not shown), suggesting that a more prolonged contact could result in a different effect. For this reason a time point of 24 hours was tested. This exposure induced a major decrease in metabolic activity, which resulted in a major decrease in cell viability, compared to that of the control. This decrease led to values 50% below that of the control, for a minimum concentration of 1 mg/mL and confirmed the previous suggestion of a time-dependent behavior. In general, the response between both parts of the plant was similar in all conditions ($p>0.05$) with a decrease in metabolic activity when the

concentration increased. There was however an exception, registered when the cells were exposed for 24 hours to the leaves aqueous extract at a concentration of 2 mg/mL. In that case, the metabolic activity of cells increased when compared to a concentration of 1.0 mg/mL ($p < 0.05$). Additionally, the result was statistically different comparing with the flower aqueous extract ($p < 0.05$), which continued to decrease. The highest cytotoxic effect (22% metabolic activity comparing with the control) was registered for the flower extract at a concentration of 2 mg/mL, followed by the leaves extract at 1 mg/mL (34% metabolic activity comparing with control), showing a very strong effect.

4.2 Fibroblasts proliferation

The influence of dry methanolic leaf extracts of *Erica australis* L. on fibroblasts proliferation was studied, testing different extract concentrations (Figure IV–2). The obtained results evidence, at first, that the tested extracts were not toxic to BJ fibroblasts, as cell viability was in all cases above 100%.

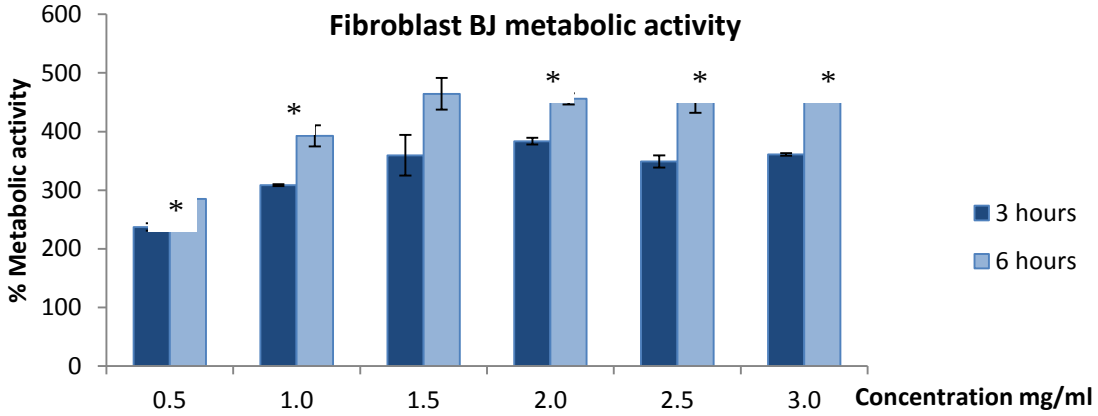

Figure IV–2. Fibroblast BJ cells metabolic activity (as % of control) with 3 h and 6 h exposure (mean ± SD, n = 3). Differences between times ($p < 0.05$) are marked with *.

In fact, upon both 3 hours and 6 hours incubation there was a significant increase in the metabolic activity, as evidenced by an increase in absorbance, which demonstrates the stimulation effect of the extract. This effect was observed for the range of concentrations between 0.5 and 2.0 mg/mL (3 hours exposure) ($p < 0.05$), and 0.5 and 1.5 mg/mL (6 hours exposure) ($p < 0.05$). At this time point (6 hours) the metabolic activity remained constant when a higher concentration of the extract (2 mg/mL) was used, suggesting that the stimulation reached the plateau at 1.5 mg/mL. The effect on the metabolic activity after 6 hours of incubation was significantly higher than after 3 hours ($p<0.05$), unlike the observed for Caco-2 cells, where the extract started showing a different trend after 6 hours of exposure.

4.3 Antibacterial effect

To ascertain if the extracts had antimicrobial properties the disc diffusion method was used, which is commonly applied to evaluate microorganism susceptibility to antibiotics. The extract at different concentrations is put in a porous paper disc. The exposure of the bacteria to the extract is accomplished via diffusion. It is verified if there is bacterial growth around the disc (Figure IV–3).

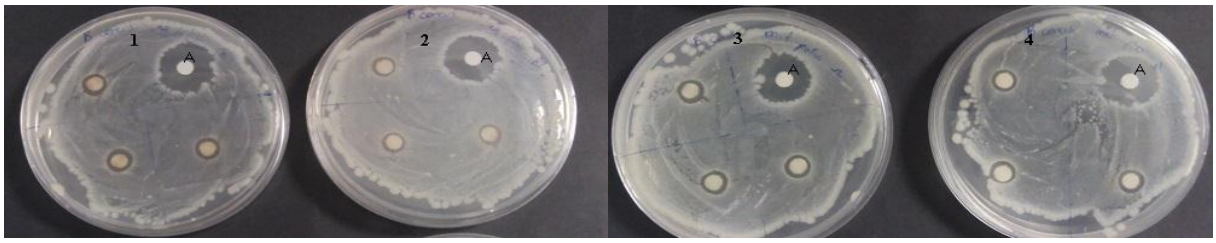

Figure IV–3.Inhibition of *B. cereus* by 4 plant extracts at 10 mg/mL (1- Aqueous leaf; 2- Aqueous flower; 3- Methanolic leaf and 4- Methanolic flower) and by A - Ampicilin.

The minimum concentration of extract needed to prevent the growth of the bacteria in these conditions can then be determined (Table IV–1).

Table IV–1. Microorganism inhibition by plant extract (mean ± SD, n = 3)

Extract	Parameter	*Microorganism*				
		E. faecalis	*B. cereus*	*E. coli*	*S. aureus*	*L. monocytogenes*
Aqueous leaf	Inhibition (mm) at 10 mg/mL	8.0 ± 0.0	9.0 ± 1.0	8.0 ± 0.0	8.0 ± 0.0	10.0 ± 0.0
	MCDI (mg/mL)	6.0	1.0	6.0	2.0	5.0
Aqueous flower	Inhibition (mm) at 10 mg/mL	n.i.	8.0 ± 0.0	n.i.	7.0 ± 0.0	9.3 ± 0.6
	MCDI (mg/mL)	n.i.	2.0	n.i.	2.0	8.0
Methanolic leaf	Inhibition (mm) at 10 mg/mL	n.i.	10.0 ± 0.0	n.i.	8.3 ± 0.6	n.i.
	MCDI (mg/mL)	n.i.	2.0	n.i.	4.0	n.i.
Methanolic flower	Inhibition (mm) at 10 mg/mL	n.i.	10.0 ± 0.0	n.i.	9.3 ± 0.6	n.i.
	MCDI (mg/mL)	n.i.	2.0	n.i.	4.0	n.i.
Ampicillin	Inhibition (mm) at 10 mg/mL	20.0 ± 0.0	18.0 ± 0.0	32.0 ± 0.0	50.0 ± 1.0	42.0 ± 1.0

n.i. – no inhibition

Two plant parts (leaves and flowers) and two extract types (aqueous and methanolic later evaporated and resuspended in water) were tested using a panel of foodborne 5 microorganisms, 3 of which are commonly found in wound infections, but all of them can contaminate wounds and cause an infection. It was found that the microorganisms responded differently, according to plant part and extract type used, which indicates the compounds with

antimicrobial activity differ from plant part to plant part. Aqueous leaves extract was the one showing better results, evidencing some activity against all the tested microorganisms. It was followed by the aqueous flower extract, which had effect in 3 out of 5 microorganisms and finally both methanolic extracts, which had effect in only 2 out of the 5 microorganisms. Not only the leaves extract was the one that had effect on more microorganisms, it also provided the strongest effects.

5 Discussion

One common *in vitro* assay to determine the cytotoxicity of plant extracts involves Caco-2 cells (Russo et al., 2005, Grey et al., 2010). These cells belong to a cell line derived from human colon tissue and were used in this study to evaluate the effects of the plant extract in human colon cancer cells (Russo et al., 2005, Grey et al., 2010, Ren et al., 2003). The observed effect evolved in a time dependent manner, mostly dependent on the concentration of the extract used. This is a common behavior that is observed for the majority of conditions. Except at the concentration of 2.0 mg/mL after 24 hours exposition, all results were similar in both leaves and flowers, which means both plant parts can be used when attempting to extract compounds with potential cytotoxic effect, with similar results up to a concentration of 1.0 mg/mL. At a concentration of 2 mg/mL, however, the flowers showed better cytotoxic ability than leaves, which can be due to some synergetic effect that manifests itself when the concentration of the extract is increased. Leaves on the other hand follow an opposite behavior, with a reduction of the cytotoxic effect at 2 mg/mL, perhaps due to an antagonistic effect caused by some compound present on the extract. The results obtained in this study for 24 hour exposure to the extract are higher than those obtained with wheat upon 24 and 96 hours exposure (Okarter, 2011) and with two types of cabbage at 24 and 48 hour exposure (Zarzour, 2011), but lower than those obtained with *T. gallica* upon 72 hour exposure (Boulaaba, et al., 2013). Although the obtained results show the potential of *Erica australis* as a cytotoxic agent, it has to be pointed out that in a normal ingestion of this plant infusion, the individual will not benefit from these properties, as according to the obtained results it takes over 6 hours of contact with the extract for the effect observation. After this time however, the effects can lead to a high reduction of the metabolic activity, so the use of this plant should not be discarded, but rather improved.

Quick wound healing depends on factors such as the proliferation of fibroblast cells and the prevention of an infection (Enoch and Leaper D., 2005); (Adetutu et al., 2011b, Shrivastava, 2011, Aslam et al., 2006). This has potentiated the research on the study of the

application of plant extracts as antimicrobial agents (Adetutu et al., 2011b, Steenkamp et al., 2004, Raja et al., 2011), an event of increased importance nowadays that bacteria resistance to antibiotics is a matter of concern. Finding suitable therapeutic alternatives has become a major priority. In this work, we evaluated the ability of plant extracts to enhance fibroblast activity in order to determine the *in vitro* wound healing potential. The extract effect on 5 microorganism strains commonly found in wound infections was further determined to assess the *in vitro* disinfection capacity and potential application in a wound treating product.

The results obtained in the study strongly indicate that *E. australis* might be a potential candidate for dermal wound healing, because of its proliferative effect on fibroblast cells and the antibacterial activity.

An explanation for such positive effect in fibroblasts can be the high and diverse composition of antioxidants evidenced by *E. australis* L., as preliminary studies have revealed (Nunes et al., 2012b). It is known that various plant extracts abounding in antioxidants are useful in prevention or treatment of skin disorders, especially those mediated by UV irradiation. Reactive oxygen can cause harmful effects in keratinocytes and fibroblasts if antioxidative defense mechanisms are exhausted (Adetutu et al., 2011a). Many different compounds have been tested alone or in combination (betacarotene, ascorbic acid, tocopherol, selenium and polyphenols) for prevention of sunburn, photodermatoses and photocarcinogenesis with divergent results (Tebbe, 2001). Different plant products are considered potential agents for wound healing and this kind of natural therapy is largely preferred because of the widespread availability, non-toxicity on skin cells, ease of administration and effectiveness even as crude preparations. In addition, because of the high concentration of caffeic acid in the investigated plant material, which is well known as a protector of human skin against UVB-induced erythema, *E. australis* has the potential to be used as a main compound on photoprotective cosmetics (Svobodová et al., 2003, Nunes et al., 2012b).

The only extract that had effect on *E. faecalis* and *E. coli* was the leaves aqueous extract, with a MCDI of 6 mg/mL in both cases. This relatively high MCDI and resistance to extracts was expected because these microorganisms are known to be resistant to a wide range of antibiotics (Munaff, 2012). The fact that only the aqueous extract had an inhibitory effect shows that the compound(s) responsible for the effect are only present in leaves and can be extracted with water but not with methanol. This hints at their polarity and is important information if compound extraction and isolation is to be pursued to achieve a more concentrated solution with higher activity. It is possible that the compounds responsible for

the activity against *E. faecalis* and *E. coli* are the same, which might be explained by the presence of both bacteria in the digestive system, as inhabitants, although one is Gram $^{-}$ while the other is Gram $^{+}$.

The growth of *B. cereus* and *S. aureus* was inhibited by all the tested extracts. Of these two, *B. cereus* was more susceptible than *S. aureus*, and more so to aqueous extract of leaves; the other extracts showing no difference among them. In the case of *S. aureus* a difference was seen according to the solvent used and not to the plant part in study. These results show that both plant parts being tested possess compounds responsible for preventing the growth of these bacteria and they can be extracted with water or methanol, meaning the extraction of compounds for a possible use does not need to be selective. This can be due to an intermediate polarity of the compounds or presence of some compounds that can be extracted with water and some with methanol. They also show that the compounds responsible for the inhibition are different or, at least, there are more compounds with that effect in the extract, when comparing to those that have antimicrobial activity against *E. faecalis* and *E. coli*.

L. monocytogenes growth was inhibited by both aqueous extracts but not by the methanolic extracts and more so by the leaves. These results show that while both leaves and flowers possess compounds capable of preventing this microorganism growth, it is not possible to extract them with methanol, indicating the polarity of the compounds. A similar trend was observed with *E. faecallis* and *E. coli*, where although leaves had compounds with antimicrobial activity, only water was able to extract them.

Overall, the results show that both plant parts and solvent used had influence on the antimicrobial activity. The combinations that yield a stronger antimicrobial activity are leaves extracted with water followed by flowers extracted with water. Methanolic extracts had a much lower activity, with the exception of when put into contact with *Bacillus cereus* where they performed similarly to flower aqueous extract. According to Fabry et al (1998), crude solvent extracts of plants are considered potentially useful in therapeutics if they have concentrations of inhibition values < 8mg/mL, which means all tested extracts are potentially useful. This is especially true for leaves extract and especially against *B. cereus* and *S. aureus*. Much lower MCDIs can be obtained if the particular compound that has the inhibitory effect can be isolated, which may be worthwhile to pursue considering the effects of some of the analyzed bacteria. Another important aspect relates to wounds. It was previously found that the extracts have potential to be used to treat wounds given the enhanced proliferation of fibroblasts. In addition to that, the fact that the extracts have some inhibitory effects against microorganisms found commonly in infected wounds, increases the potential use of this plant

in treating wounds and preventing their infection. It also supports the traditional use of this plant as a wound treating agent.

6 Conclusion

With this study we can conclude that the use of *Erica australis* in traditional medicine with the aim of wound healing is somewhat supported, since this plant extracts potentiated the proliferation of fibroblast cells. In addition, the plant shows antimicrobial activity against microorganisms commonly found in wound infections, and as such can serve a dual purpose in wound treatment. This activity was in some cases solvent and plant part dependent. While this plant displayed a cytotoxic effect in Caco-2 cells, this took over 6 hours to be evident, which makes challenging its use in the ambit of cancer therapy. However, the effects should not be underestimated since after 24 hours of exposition the cell metabolic activity was greatly diminished.

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Arbutus unedo L. leaves potential use in new products with antioxidant, antibacterial, antidiabetic and antiproliferative activities

Ricardo Nunes[a], Pawel Pasko[b,f], Malgorzata Tyszka-Czochara[c], Agnieszka Szewczyk[d], Marek Szlosarczyk[e], Isabel S. Carvalho[a]*

[a]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[b]Department of Food Chemistry and Nutrition, Medical College, Jagiellonian University, Krakow, Poland

[c]Radioligand Laboratory, Department of Pharmacobiology, Medical College, Jagiellonian University, Krakow, Poland

[d]Department of Pharmaceutical Botany, Medical College, Jagiellonian University, Krakow, Poland

[e]Department of Inorganic and Analytical Chemistry, Medical College, Jagiellonian University, Krakow, Poland

[f]Department of Clinical Science, Faculty of Health and Medical Science, Andrzej Frycz-Modrzewski Krakow University, Poland

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1 ABSTRACT

This study intended to evaluate the potential application of *Arbutus unedo* L. leaf extracts in commercial products, by measuring their antioxidant, antimicrobial, antidiabetic and antiproliferative activities Extracts displayed high antioxidant and radical scavenging activities (DPPH radical IC_{50} 12 μg/mL). All extracts inhibited *S. aureus* while only ethanolic extract inhibited *E. coli*, *E. faecalis* and *L. monocytogenes*. Leaves exhibited stronger α-glucosidase inhibition (IC_{50} of 9.6 μg/mL) than acarbose (standard) and specific inhibition activity against Hep G2 and SW 480 cell lines at the concentration of 1 mg/mL. Results are most likely due to the presence of gallic acid and quercetin, the two main compounds identified by HPLC and zinc. This work shows the potential leaves have to be used as antioxidant, antidiabetic and antibacterial agents in the formulation of new functional foods (for example to complement acarbose), in cosmetics (antioxidant activity), and creams (to prevent *S. aureus* infection of wounds).

HIGHLIGHTS

Absolute ethanol is the best solvent for antioxidant and antimicrobial activity.

Gallic, protocatechuic, p-hydroxybenzoic acids and quercetin were detected.

S. aureus was inhibited by all extracts, ethanol inhibited all except *B. cereus*.

Extracts exhibited greater inhibition power against α-glucosidase than acarbose.

Extracts exhibited antiproliferative activity against sw 480 and sw 620 cell lines.

KEYWORDS

Antioxidant, antidiabetic, antibacterial, antiproliferative, zinc, *Arbutus unedo*

2 Introduction

Arbutus unedo L. (Ericaceae), known as strawberry tree, is a tree common in the Mediterranean area, including Portugal, where its known as "medronheiro" (Celikel et al., 2008, Takrouni and Boussaid, 2010, Lopes et al., 2012). This tree produces fruits that are sweet in taste only when ripe, presenting before, astringent characteristics (Alarcão-E-Silva et al., 2001). These fruits represent a source of income to many small villages and companies on the inner part of the country, mainly in the form of distilled beverages and jams (Alarcão-E-Silva et al., 2001). These trees are also important to the honey production industry and environment (Lopes et al., 2012). Despite all its importance, these trees started being neglected and replaced with other trees, which led to a decline in its population. In an effort to counter this decline, Corte Velada Lda, a Portuguese company with Norwegian capital, acquired a terrain in the Algarve region and planted 72 000 trees. Their goal is to improve the production by selecting and cloning the best trees, and monetize its fruits and other parts, such as leaves, since they are rich in phytochemicals that are beneficial for health. The leaves of this tree are used in folk medicine as diuretics, antiseptics, laxatives and to treat hypertension (El Haouari et al., 2007, Ziyyat et al., 2002, Afkir et al., 2008). They exhibit high antioxidant activities, and are rich in phenolic compounds (Andrade et al., 2009, Malheiro et al., 2012, Mendes et al., 2011, Oliveira et al., 2009). They have in their composition hydroxycinnamic and hydroxibenzoic acid derivatives, flavonols, flavanones and di-hydroflavonols (Boulanouar et al., 2013, Tavares et al., 2010) as well as tannins, α-tocopherol, lipids and vitamin E (Pabuçcuoğlu et al., 2003, Kıvc¸ak and Mert) which all have antioxidant activity. They also possess neuroprotective and antiaggregant effects, amongst other properties (Dib et al., 2013, El Haouari et al., 2007, Fortalezas et al., 2010, Mendes et al., 2011, Tavares et al.,

2010). In this work, a composite sample obtained by mixing and gridding leaves from 18 different trees was studied for its antioxidant and antibacterial activities (against *B. cereus, E. coli, E. faecalis, L. monocytogenes, P. aeruginosa* and *S. aureus*) after extraction with water, absolute ethanol and two mixtures (50% and 80% v:v) of both. The antidiabetic activity was studied using two enzymes, α-glucosidase and α-amylase. The phenolic composition of its methanolic extract was determined with the help of a HPLC. Finally, its antiproliferative activity was tested against a panel of five different cell lines, Hep G2 (liver adenocarcinoma), HEK 293 (transformed kidney), A549 (lung carcinoma), sw 480 (colon adenocarcinoma) and sw 620 (colon adenocarcinoma), these results were compared with those obtained for Fb (fibroblasts) and the control.

3 Material and methods

3.1 Plant material

The leaves were collected from the "Herdade da Corte Velada" in Portugal, GPS: N 37 11.536; W 8 40.820. A total of 18 samples, each from a different 4 to 5 years old tree spread in an area of 180 hectare, were collected and put into a cooled isothermal container on November 2014, during the fruiting period. They were identified by naturalist José Manuel Rosa Pinto, of the University of Algarve's herbarium, where a voucher specimen (voucher 14796) was deposited. After identification the samples were dried in an incubator, at 40 °C for 72 h (Binder BD 53 incubator, Binder, Tuttilgen, Germany), and ground to powder in a kitchen mill. A composite sample, made with 2 grams of each powdered sample, was stored in a freezer until use.

3.2 Biological material

Bacteria strains: *Enterococcus faecalis* – DSMZ 20478, *Bacillus cereus* – ATCC 11778, *Escherichia coli* – ATCC 8739, *Staphylococcus aureus* – ATCC 6538, *Listeria monocytogenes* – DSMZ 7644. Bacteria strains were gently provided by Stress by Antibiotics and Virulence of Enterococci laboratory from IBET.

Human cell lines were obtained from the American Type Cell Culture collection, ATCC (LGC Standards-ATCC, -Teddington, Great Britain). ATCC designations were as follows: Fb, normal adherent human skin fibroblasts, HEK 297, human embryonic kidney cells, HEP G2, hepatocellular carcinoma., A549, lung carcinoma, SW 480 and SW 620 colorectal adrenocarcinomas.

3.3 Chemicals

Ethanol was purchased from Merck, Nottingham, UK. Luria broth 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, sodium phosphate dibasic dehydrate, monosodium phosphate hydrous, gallic acid, quercetin and Trolox were purchased from Sigma-Aldrich Co. Ltd, Poole, UK. Iron (III) chloride, methanol, trichloroacetic acid, sodium acetate, sodium carbonate, sulphuric acid, glacial acetic acid, 2,4,6-tripyridyl-2-triazine (TPTZ), hydrochloric acid and ascorbic acid were purchased from VWR (Pennsylvania, USA). Discs for minimum concentration for diffusion inhibition (MCDI) assay were purchased from Whatman, Maidstone, UK. Ampicillin, plate count agar, RINGER tablets, aaluminium chloride and absolute ethanol were purchased from Merk, Germany. All reagents were of analytical grade.

3.4 Extraction procedure

A 0.2 g mass of plant material (in powder form) was extracted with 30 mL of solvent for 30 min in an electro thermal heating mantle. Stirring speed was set to 200 rpm, solvent temperature was 75°C. For antibacterial and antidiabetic assays the solvent was evaporated (Nahita serie 503, Navarra, Spain) and the extracted material was re-suspended. The solvent chosen for the resuspension was distilled water. DMSO was not used to resuspend due its capability of inhibiting bacterial growth, even at relatively low concentrations (Wadhwani et al., 2009). Water was able to completely resuspend the plant extracts (after slightly heating it to 50°C). Extracts and resuspended extracts were transferred to Eppendorf tubes and stored at -20°C until analysis.

3.5 Cell lines culture

Cells were cultured according to ATCC's catalogue instructions. Briefly, cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 to confluence in Eagle's Minimum Essential Medium (EMEM, ATCC), supplemented with 10% v/v FBS (ATCC) and with antibiotic solution (100 IU/mL penicillin, 0.1 mg/mL streptomycin, Gibco Laboratories, NY, USA). For experiments, the cells were at an exponential phase of growth.

3.6 Bacterial strains culture

Prior to the assay a culture of each microorganism was initiated in 2 mL Luria Broth and incubated at 37°C for 24 h. After the 24 h the microorganism was cultured on solid Plate Count Agar medium using the streak method, and grown at 37°C for 24 h.

3.7 Qualitative and quantitative analysis

3.7.1 Total Phenolic Content

Total Phenolic Content (TPC) in samples was determined using a spectrophotometric procedure originally described by Singleton and Rossi (1965). Briefly, 0.1 mL of extract (diluted in the extraction solvent) was mixed with 0.5 mL of Folin-Ciocalteau's reagent, 0.4 mL of a saturated sodium carbonate solution (7.5 %) and incubated for 30 min in a dark room. Absorbance was read at 765 nm against a blank. Phenolic content was calculated using a gallic acid calibration curve and the results were expressed as mg GAE/g dw (gallic acid equivalents per gram of dry weight).

3.7.2 Total Flavonoid Content

Total Flavonoid Content (TFC) was determined according to the method of Lamaison and Carnat (1990). Briefly, 0.4 mL of diluted extract was mixed with 0.8 mL of a 2% methanolic $AlCl_3.6H_2O$ solution. After standing for 10 min in the dark the absorbance was measured at 430 nm. Flavonoid content was calculated using a quercetin calibration curve, and the results were expressed as mg QE (quercetin equivalents)/g dw.

3.7.3 Total Antioxidant Activity

Total Antioxidant Activity (TAA) of extracts was determined using a spectrophotometer and the method proposed by Prieto (1998b). Briefly, 0.1 mL of diluted extract was mixed with 1.0 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was then incubated in a water bath at 95°C for 90 min. Absorbance was read at 695 nm against a blank (negative control of water, ethanol or 80% ethanol: water according to the solvent used) and results were calculated from an ascorbic acid (positive control) calibration curve. Results were expressed as mg AAE (ascorbic acid equivalents)/g dw.

3.7.4 Reducing Power

Reducing Power (RP) was determined using the method previously described by Oyaizu (1986). Briefly, 0.2 mL of diluted extract were mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min. After incubation, 0.5 mL of trichloroacetic acid (10%) were added and the mixture was centrifuged at 650 x g for 10 min. 0.5 mL of the supernatant were mixed with 0.5 mL of distilled water and 0.1 mL of ferric chloride (0.1%). Absorbance was measured at 700 nm. Trolox was used as positive control and the negative control was water,

ethanol or 80% ethanol: water according to the solvent used in the extraction. Results were expressed as mg TE (Trolox equivalents)/g dw.

3.7.5 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined using the method previously described by Benzie and Strain (1996a). First, three solutions were prepared, a 300 mM acetate buffer, pH=3.6, a 10 mM TPTZ, 40 mM HCl solution, and a 20 mM $FeCl_3.6H_2O$ solution. Mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of FeCl3.6H2O solution and heating the mixture to 37°C prepared the working solution. After cooling down, 0.9 mL of the working solution were mixed with 0.1 mL of diluted extract. After staying 30 min in the dark the absorbance was read at 593 nm. Trolox was used as positive control and water, ethanol or 80% ethanol: water were used as negative control, according to the solvent used. Results were calculated from a Trolox calibration curve and expressed as mg Trolox/g dw.

3.7.6 DPPH Radical Scavenging Activity

Radical scavenging activity was determined using the DPPH radical (Blois, 1958) method (DPPH) with slight modifications. Briefly, 1.0 mL of a 0.16 mM DPPH solution was added to a test tube, which contained 1.0 mL of extract at different concentrations (two with more than 50% scavenging activity and two with less than 50% scavenging activity). This mixture was then vortexed and kept in the dark for 30 min, after which absorbance was measured at 517 nm. The radical scavenging percentage (IC) was calculated using the following formula: (IC) = $((A_0-A_t)/A_0)$ x 100, where A_0 is absorbance of control at 30 min and A_t absorbance of sample at 30 min. Trolox was used as positive control and the negative control was water, ethanol or 80% ethanol: water according to the solvent used. Results were expressed as μg sample/mL in form of IC_{50}, determined by linear regression of IC and extract concentration at 50% inhibition.

3.7.7 RP-HPLC analysis

Phenolic acids and flavonoids in dry biomass were quantified in methanol extracts (80°C, 2h) and after hydrolysis with 2 M aqueous HCl, 100°C, 1 h (Harborne, 1998). RP-HPLC analysis was conducted according to Ellnain-Wojtaszek and Zgorka (1999) but with modification on Merck-Hitachi liquid chromatograph (LaChrom Elite) equipped with a DAD detector L-2455 and Purospher ® RP-18e (250x4 mm/ 5μm) column. Analysis was carried out at 25°C, with a mobile phase consisting of A – methanol, B – methanol : 0.5% acetic acid

1 : 4 (v/v). The gradient was as follows: 100% B for 0–20 min; 100 – 80% B for 20 – 35 min; 80 – 60% B for 35 – 55 min; 60 – 0% B for 55 – 70 min; 0% B for 70-75 min; 0 – 100% B for 75 – 80 min; 100% B for 80–90 min at a flow rate 1 ml/min, λ = 254 nm (phenolic acids), λ = 370 nm (flavonoids). Identification was done by comparing retention times of peaks with standards and by adding the standards to the tested samples and verifying the increase in the peak area of the compound being identified (standard addition). Quantification was done by measurement of peak area with reference to the standard curve derived from five concentrations (0.03125 to 0.5 mg/ml). Caffeic, chlorogenic, cinnamic, gallic, gentizic, o-coumaric, protocatechuic, salicylic, sinapic, syringic acid, isorhamnetin, kaempferol, luteolin, quercetin, quercitrin, rutin, vitexin, *p*-coumaric, vanillic, ferulic and *p*-hydroxybenzoic acid diluted in HPLC grade methanol were used as standards.

3.7.8 Zinc

A Multipurpose Electrochemical Analyzer M161 with the M164 electrode stand (both MTM-ANKO, Poland) was used for all voltammetric measurements. The standard three-electrode cell consisted of a controlled growth mercury drop electrode (CGMDE) as a working electrode, Ag/AgCl in 3M KCl with a double junction filled with 3M KCl (Mineral, Poland) as reference and platinum wire as an auxiliary electrode. Voltammograms were recorded, interpreted and stored by EAGRAPH (MTM-ANKO, Poland) software. A standard stock solution of $Zn(NO_3)_2$ was prepared by proper dilution of solution both with concentration of 1 g/L (OUM, Łódź, Poland). The electrolyte used as ionic medium was prepared by dissolving KNO_3 (Merck, Suprapur®). For digestion procedures HNO_3 (Merck, Suprapur®) was used. All the solutions were prepared with double - distilled water from quartz distiller (SZ-97A, Chemland, Polska) and all reagents were of analytical grade. Samples were powdered in agate mortar and then dried at over 70°C for 4 hrs. Approximately 250 – 500 mg of sample material was weighed and inserted to high-pressure acid digestion vessel 4748 (Parr Instruments, USA) and treated with 5 mL of nitric acid. Next, the acid digestion bomb was closed and kept in the oven at 170°C for 24h. The digested sample was placed at the heated plate to let it evaporate and to remove the nitrate. The sampled solutions were cooled to room temperature, transferred quantitatively into volumetric flasks (10 ml) and filled up to the mark with double distilled water. All the procedures were repeated three times for each sample.

The zinc content of selected samples was determined using a differential pulse anodic stripping voltammetry (DP ASV) with a controlled growth mercury drop electrode (CGMDE)

under following parameters: pulse amplitude –20 mV; step height 2 mV; pulse width (waiting time – 20 ms; sampling time – 20 ms); resting time 5 s(Szlósarczyk et al., 2011). The voltammograms were recorded in the potential range from -1200 mV to 75 mV in diluted sample solution. Before each measurement, the solution in the voltammetric cell was de-aerated by high purity argon for 5 min. Voltammograms corresponding to individual additions were taken three times according to the standard addition method. All experiments were performed at room temperature.

3.8 Disc diffusion assay

For the determination of minimum concentration of diffusion inhibition (MCDI) a method similar to that used in antibiotic susceptibility testing was used (Bauer et al., 1966), but with plant extracts instead (Gaudreau and Gilbert, 1997b). Briefly, a colony was collected from the agar plate and suspended in a flask containing Ringer solution. An aliquot of 50 µL was spread on a petri dish containing solid plate count agar. After dried, discs were put in the petri dish and 10 µL of extract diluted in distilled water (10 mg/mL) were added to each disk. Ampicillin (10 mg/mL) and streptomycin (10 mg/mL), diluted in distilled water, were used as positive controls and distilled water was used as negative control. After 24 (not shown) and 48 h incubation, the inhibition diameter was checked. After assessing which strains were inhibited, several extract concentrations with 1 mg/mL of difference between them were tested to find the MCDI. All bioassays were repeated thrice in separated plates, and each plate had three discs per concentration. All results are means ± SD.

3.9 Antidiabetic activity

3.9.1 α-amylase

Inhibition was determined following the method of (Sancheti et al., 2013) with slight alterations. Briefly, 100 µL of 2% starch (prepared in pH 6.9 0.02 M and 0.0067 M NaCl phosphate buffer, and heated to 80°C for 10 min) were mixed with 50 µL of extract and incubated for 10 min at 20°C. After incubation, 100 µL of 2.4 U/mL of α-amylase enzyme were added and the mixture was incubated for 5 min at 20 °C. After adding 100 µL of colour reagent (sodium potassium tartarate and NaOH solution mixed with 96 mM 3,5-dinitrosalicylic acid), incubating for 15 min at 95 °C and adding 900 µL of distilled water, the absorbance was read at 540 nm. Acarbose was used as positive control and distilled water as negative control. Results are expressed as IC_{50} in µg sample/mL, determined by linear

regression of IC and extract concentration at 50% inhibition. Results are means ± SD of three repetitions.

3.9.2 α-glucosidase

Inhibition was determined following the method described by (Li et al., 2009) with slight alterations. Briefly, 150 µL of sample solution in pH 6.8 0.1 M phosphate buffer were mixed with 150 µL of 1 U/mL α-glucosidase enzyme. This mixture was incubated for 10 min at 37°C. After incubation 150 µL of 0.5 mM p-nitro-α-D-gluco-pyranoside (in 0.1 M phosphate buffer) were added to the mixture and incubated for 30 min at 37°C. After the addition of 600 µL of 0.2 M sodium carbonate solution the absorbance was read at 405 nm. Acarbose was used as positive control and distilled water as negative control. Results were expressed as IC_{50} in µg sample/mL, determined by linear regression of IC and extract concentration at 50% inhibition. Results are means ± SD of three repetitions.

3.10 Antiproliferative activity

Cell Viability Assay Microculture tetrazolium assay (MTT) was used as an indicator of cell viability. MTT assay determined by measuring the mitochondrial-dependent reduction of MTT to formazan was conducted as described previously (Tyszka-Czochara et al., 2014a, Tyszka-Czochara et al., 2016). Briefly, cells were seeded in 96-well microtiter plates (Sarstedt, Numbrecht, Germany). After optimum confluence, cells were treated with the extracts and incubated for 24 h at 37°C. Five different concentrations (0.01, 0.1, 0.5 1.0 and 5.0 mg/ml) for each extract were used. Cells treated with equivalents of vesicle were considered as controls. After incubation, cells were washed before adding medium containing MTT (5 mg/ml) and kept for 1 h at 37°C. Medium was then discarded and the formazan salt that had formed in cells was dissolved in DMSO. The optical density was measured at 570 nm (the reference wavelength was 630 nm) using a microplate reader (Tecan Austria GmbH, Austria). Results were reported using the following formula: (average OD value of three measurements for each experimental group/average OD value of control group)×100%.

3.11 Statistical methods

A one-way analysis of variance (ANOVA) and the Student's t test were used for the evaluation of MTT test. $P < 0.05$ was considered as the level of significance.

4 Results

4.1 Antioxidant activity, phenolic compounds and trace elements

Results for the antioxidant activity and phenolic composition can be seen on Table IV–2 and Table VI–3.

Table IV–2. Antioxidant activities of the Arbutus unedo extracts obtained with the different solvents

Extract	TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg TE/g dw)	FRAP (mg TE/g dw)	DPPH IC_{50} (µg sample/mL)
A	607.04 ± 6.51^{a}	29.33 ± 0.44^{a}	411.96 ± 14.54^{a}	362.51 ± 13.98^{a}	603.06 ± 27.78^{a}	12.00 ± 0.50^{a}
B	375.80 ± 4.33^{b}	23.58 ± 1.41^{b}	400.10 ± 25.97^{a}	830.92 ± 24.07^{b}	447.85 ± 6.46^{b}	21.34 ± 2.18^{b}
C	378.42 ± 16.27^{b}	62.63 ± 1.22^{c}	553.20 ± 27.56^{b}	544.71 ± 12.69^{c}	802.53 ± 20.11^{c}	17.85 ± 0.79^{b}
D	604.34 ± 27.75^{a}	61.24 ± 2.01^{c}	823.98 ± 118.32^{c}	917.93 ± 47.04^{d}	469.03 ± 9.17^{b}	12.14 ± 0.68^{a}

A – Water, B – 50% ethanol: water, C – 80 % ethanol: water, D – absolute ethanol; TPC – Total phenolic content, TFC – total flavonoid content, TAA – total antioxidant activity, RP – reducing power, FRAP – ferric reducing antioxidant power, DPPH –radical scavenging activity; GAE – gallic acid equivalents, QE – quercetin equivalents, AAE – ascorbic acid equivalents, TE – Trolox equivalents, dw – dry weight; Same letters in the same column indicate statistically similar results (p>0.05); Results are means ± standard deviation

Table IV–3. Detected compounds by HPLC and zinc content

Compound	mg/100g dw	Compound	mg/100g dw
Gallic acid*	38.21 ± 2.62	Kaempferol	n.d.
Protocatechuic acid*	5.51 ± 0.62	Chlorogenic acid	n.d.
p-hydroxybenzoic acid*	7.57 ± 0.32	Caffeic acid	n.d.
Vanillic acid*	n.d.	Ferulic acid	n.d.
Quercetin	87.08 ± 8.87	Vitexin	n.d.
Rosmarinic acid	n.d.	Coumaric acid*	n.d.
Rutin	n.d.	Syringic acid	n.d.
Neochlorogenic acid	n.d.	Zinc	1.91 ± 0.37

* – after HCl 2M digestion, n.d. – not detected; , dw – dry weight; Results are means ± standard deviation

The *Arbutus unedo* L. leaves extracts showed a high solvent-dependent antioxidant activity. Absolute ethanol and water provided the overall best results. In fact, with the exception of FRAP, where 50 % ethanol: water provided by far the best results ($p<0.05$), absolute ethanol was the best solvent in every antioxidant assay, with values of 604.34 mg

GAE/g dw for TPC, 61.24 mg QE/g dw for TFC, 823.98 mg AAE/g dw, 917.93 mg TE/g dw and 12.14 µg sample/mL for DPPH IC_{50}.

Statistical similarities ($p>0.05$) were found between absolute ethanol and water for TPC and DPPH, and between absolute ethanol and 80% ethanol: water for TFC. The only compound detected by HPLC without recurring to an acidic digestion was quercetin, with a concentration of 87.08 mg/100 g. After the acidic digestion three more compounds were detected, gallic, protocatechuic and p-hydroxybenzoic acids, with concentrations of 38.21, 5.51 and 7.57 mg/100 g respectively. None of the other 11 tested compounds was detected. The zinc content on the leaves was 1.91 mg/100 g.

4.2 Antibacterial activity

Four Gram positive (*B. cereus*, *E. faecalis*, *L. monocytogenes* and *S. aureus*) and two Gram negative bacteria (*E. coli* and *P. aeruginosa*) were tested (Table IV–4).

Table IV–4. Antimicrobial activity of the Arbutus unedo extracts obtained with the different solvents.

Extract	MIC (mg/mL) after 24h					
	B. cereus	*E. coli*	*E. faecalis*	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*
A	8.0 ± 0.0	n.i.	n.i.	n.i.	n.i.	6.3 ± 0.6
B	6.3 ± 0.6	n.i.	n.i.	n.i.	n.i.	5.0 ± 0.0
C	9.7 ± 0.6	n.i.	n.i.	n.i.	n.i.	10.0 ± 0.0
D	n.i.	6.0 ± 0.0	5.3 ± 0.6	5.7 ± 0.6	n.i.	4.7 ± 0.6

A – Water, B – 50% ethanol: water, C – 80 % ethanol: water, D – absolute ethanol; n.i. – no inhibition; Results are means ± standard deviation

The results show that *S. aureus* was the most susceptible of the tested bacteria, followed by *B. cereus*, *P. aeruginosa* was the most resilient. The antibacterial activity was solvent-dependent, similarly to the antioxidant activity. The positive control, ampicillin, inhibited all the microorganisms tested, with the exception of *P. aeruginosa*, for which streptomycin was used instead, on the other hand the negative control, water, inhibited none.

4.3 Antidiabetic activity

The antidiabetic activity of the extracts was highly solvent dependent, more so when the enzyme tested was α-glucosidase (Table IV–5). For both tested enzymes absolute ethanol was once again the best solvent, with an IC_{50} of 1.25 mg/mL for α-amylase and 9.61 µg/mL for α-glucosidase. The worse solvent was 50% ethanol: water for α-amylase (37.39 mg/mL) and water for α-glucosidase (72.90 µg/mL). Statistical similarities between absolute ethanol and

the other solvents were only found for α-amylase IC_{50}, where it was statistically similar to water and 80% ethanol: water ($p>0.05$).

Table IV–5. Antidiabetic activity of the Arbutus unedo extracts obtained with the different solvents

Extract	α-amylase (IC_{50} mg/mL)	α-glucosidase (IC50 µg/mL)
A	1.42 ± 0.11^{a}	72.90 ± 4.28^{a}
B	1.89 ± 0.08^{b}	32.38 ± 1.72^{b}
C	1.33 ± 0.10^{a}	25.73 ± 1.14^{c}
D	1.25 ± 0.32^{a}	9.61 ± 2.16^{d}

A – Water, B – 50% ethanol: water, C – 80 % ethanol: water, D – absolute ethanol; Same letters in the same column indicate statistically similar results ($p>0.05$); Results are means ± standard deviation

4.4 Antiproliferative activity

The direct effects of *A. unedo* leaf extract on human cells viability were evaluated using the MTT assay, which provides a sensitive measurement of the cell metabolic status and displays early cellular redox changes. To assess the anti-proliferative effect of extract towards cancer cells, a panel of human cancer cells (*hepatocellular carcinoma* Hep G2, *lung carcinoma* A549, *human embryonic kidney cells* HEK 297, *colorectal adenocarcinomas* SW 480 and SW 620) was chosen.

Our data indicates that concentrations of *A. unedo* leaf extract 0.5 mg/mL and higher effectively inhibited proliferation of exposed cells (Figure IV–4). At concentration of 0.1 mg/mL of extract, the growth of FB, HEK 293, Hep G2 and A549 cell lines was inhibited by extract, while cytotoxic effect measured towards both *colorectal adenocarcinomas*, SW 480 and SW 620 was lesser. Conversely, at 1 mg/mL of extract, the highest anti-proliferative effect was assessed for SW 480 cells, with approximately 60% of decrease of growth comparing to control cells. The addition to cell culture medium of 5 mg/mL of extract inhibited almost completely the proliferation of all tested cells.

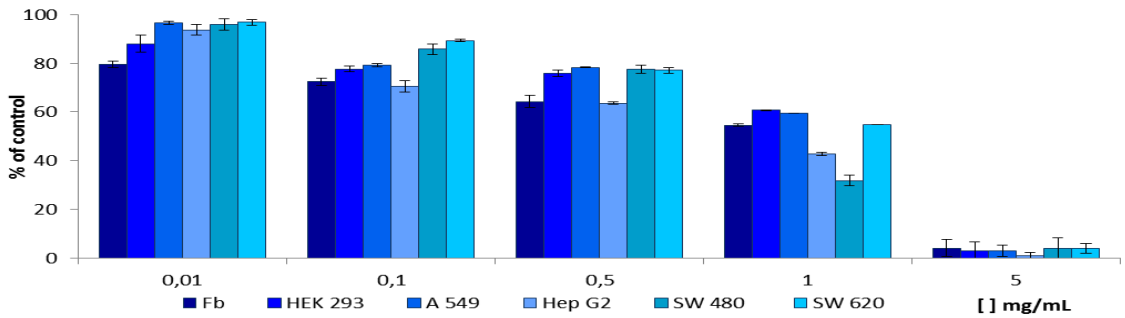

Figure IV–4. Antiproliferative activities of the *Arbutus unedo* L. extract.

5 Discussion

5.1 Antioxidant activity, phenolic compounds and trace elements

The antioxidant activity detected in the leaves was high with every solvent used, but it was specially so for absolute ethanol. This can most likely be attributed to quercetin, which was the compound detected in highest amount by the HPLC. Quercetin is a high antioxidant activity flavonoid which is also known for its antiproliferative and antibacterial activities. Since it is a flavonoid, this compound is best extracted with ethanol. This is supported by the increase in TFC value when the ethanol % also increases, with water being the solvent with lowest value. The second compound detected in high amount was gallic acid. Gallic acid is a phenolic compound which is best extracted with water. This compound is also known for its antioxidant and antimutagenic properties and its presence helps to explain the high results for TPC. The last two detected compounds were present in much lower amounts and were protocatechuic and p-hydroxybenzoic acids. These compounds also possess antioxidant and anti-inflammatory activities and protocatechuic has shown antiproliferative activity against certain cells such as those in leukemia (Tseng et al., 2000a). The comparison of detected compounds and their solubilities with the antioxidant activities of the extracts indicates the antioxidant activity detected in the pure ethanolic extract was mostly due to quercetin. The same TFC result for 80 % ethanol: water but lower results for TAA, RP and DPPH than the pure ethanol indicate that while quercetin is highly present on the 80% ethanol: water extract, there are some compounds that are having an antagonistic effect, lowering its antioxidant activity. The only assay where this was not true was for FRAP, where quercetin was actually aided by other compounds, allowing the 80 % ethanol: water mixture to have the highest activity of all four tested solvent combinations. The similar result between water and absolute ethanol for TPC, and DPPH and higher FRAP was most likely due to gallic acid, better extracted with water than with ethanol.

Zinc, despite being needed in very small quantities, plays an important role in human nutrition. It is necessary for normal growth and proper wound healing. It also boosts the immune system helping it fight against infections and parasites. It even helps against diabetes and has some connection with Alzheimer's disease. While zinc does not have an antioxidant activity on itself, it participates in the antioxidant processes of the body and helps to alleviate the production of free radicals and appearance of oxidant related injuries. It also induces the formation of metallothieonein in different organs. This metallothieonein has antioxidant activity under certain conditions and acts against mutagenesis (Szrok et al., 2016). The

amount detected in the *Arbutus unedo* L. leaves was 1.91 mg/100g. According to the United States Department of Agriculture; Agricultural Research Service (USDA) food composition database, this content is comparable to or above the content present in some cuts of pork, chicken, turkey, lamb and veal meat, some fishes, and vegetable products such as sun-dried tomatoes, tofu, whey, cowpeas, beans, cheese, corn flour, soybeans and eggs, among many other products (US Department of Agriculture, 2015). Also according to the USDA, a product which provides 20 % or more of the daily value is considered a high source of a nutrient, which in the case of zinc requires a value of 2.20 mg of zinc for an adult male with a daily dose of 11 mg of zinc. While the leaves cannot be considered a main source since their value is below the 20% and consuming 100 grams would be impractical, its importance and content should not be depreciated or undervalued since zinc is such an important micronutrient, especially during pregnancy and lactation (Chemek et al., 2016).

5.2 Antibacterial activity

Similarly to the antioxidant activity, ethanol was the best solvent when it came to the antibacterial activity. It inhibited the highest number of bacteria, 4 of the 6 (*E.coli*, *E. faecalis*, *L. monocytogenes* and *S. aureus*) while the other solvents only inhibited 2 (*B. cereus* and *S. aureus*). The ethanolic extract also had the lowest MIC, 4.7 mg/mL for *S. aureus*. While all extracts were able to inhibit some $Gram^+$ bacteria, only the ethanolic extract was able to inhibit $Gram^-$ bacteria. The added resilience of the $Gram^-$ bacteria can be attributed to the differences in their cell structure. While the $Gram^+$ bacteria peptidoglycan layer is a somewhat ineffective permeability barrier, the phospholipidic membrane of the $Gram^-$ bacteria makes these bacteria impermeable to lipophilic solutes, and porins present on the membrane act as a selective barrier to hydrophilic solutes. This difference in their structure makes these bacteria much more resistant to antibacterial substances (Nikaido, 2003). The results obtained with the ethanolic extract can most likely be attributed to quercetin, which has antibacterial activity against *E.coli*, *E. faecalis*, *L. monocytogenes* and *S. aureus* (Ozcelik et al., 2006, Rattanachaikunsopon and Phumkhachorn, 2010). Quercetin is also able to inhibit the growth of *P. aeruginosa*, however the concentration present in the extract was not sufficient (Ozcelik et al., 2006). While absolute ethanol was overall the best solvent, it was the only extract that showed no inhibitory activity towards *B. cereus*. Of those that did inhibit this bacterium, 50% ethanol: water was the best solvent, with a MIC of 6.3 mg/mL. Gallic acid, the second most abundant compound is also known to have antibacterial activity. It can inhibit *E. coli*, *L. monocytogenes*, *P. aeruginosa* and *S. aureus* (Borges et al., 2013a). However the aqueous

extract, in theory the richest in gallic acid, was only able to inhibit the growth of *B. cereus* and *S. aureus*, the same bacteria both ethanol: water mixtures inhibited. *S. aureus* was by far the most sensible microorganism. It not only had the lowest MIC of all (4.7 mg/mL), it was always lower or similar to the MIC of *B. cereus*, the second most sensible microorganism. Also, while the ethanolic extract was able to inhibit the growth of *S. aureus* it was unable to inhibit *B. cereus*. Given that *S. aureus* is associated with wound infection, mainly surgical wounds but can also occur in other wounds, if no other means of disinfection were available it is possible that washing a wound with an infusion of *Arbutus unedo* leaves could help against contamination with this microorganism, and therefore prevent the development of an infection (Cooper et al., 1999). Taking into account the fact that *S. aureus* forms biofilms when it contaminates a wound and these biofilms can envelop themselves in a fibril like layer that is able to prevent neutrophils from reaching them, a quick and thorough wound cleaning is important to prevent reaching that stage and facilitate treatment (Yamasaki et al., 2001). In addition it may have some compounds that are able to assist neutrophils in breaching through the fibril layer, as it is known that some 14-ring macrolides present in plants have that capability (Yamasaki et al., 2001). Since this is also one of the most common bacteria linked with food poisoning, one other use for the extracts could be the disinfection of food items prior to their consumption or preparation. When (Orak et al., 2011) tested the aqueous leaves extracts, they also found the leaves to inhibit *S. aureus.* While they tested a much higher concentration (250 mg/mL) they were still unable to see any *E. coli* inhibitory effect, which is in accordance with our aqueous extracts results. Also similarly to our work, their aqueous extract only inhibited $Gram^+$ bacteria. They however did not test the ethanolic extract. Their reasoning being that aqueous extract had the highest phenolic content, which also happened in our work when compared to the ethanol: water mixtures but not the pure ethanolic extract. Our results indicate the importance of using several solvents when studying the antibacterial activity of *Arbutus unedo* leaves, since different solvents can provide completely different results.

5.3 Antidiabetic activity

Centers for Disease control and Prevention (CDC) 2014 report estimates 29.1 million US citizens have diabetes, of which only 21 million have been diagnosed (2014). On the other hand World Health Organization (WHO) estimated the number of diabetic people worldwide in 2014 to be 422 million (2016). This condition affects both men and woman and is most prevalent on people aged 45 years or older, although it can occur on much younger people. In

fact WHO report indicates there has been a rise on the percentage of younger people with the condition, from 1980 till date. The same report estimates diabetes to have directly caused 1.5 million deaths in 2012 and a total of 2.2 million deaths due to high glucose levels. Unlike type 1 diabetes which is not preventable with current knowledge, type 2 diabetes, the most prevalent type, can be prevented with exercise and appropriate diet. While previously type 2 diabetes only seemed to occur in adults, the frequency of cases in children is rapidly increasing. This type of diabetes is characterized by the inefficient use of insulin the body produces. By reducing the fat and sugar intake, the insulin necessity is also reduced, helping to prevent or alleviate this condition. One treatment for type 2 diabetes is the intake of enzyme inhibitory substances, of which acarbose is an example (WHO, 2016). This drug is also sometimes used in prediabetes condition. While popular in China, its use in the USA is not as common due to the diarrhea and flatulence side effects. Its mechanism of action is the inhibition of α-glucosidase and α-amylase, preventing the degradation of complex carbohydrates into glucose. Because the glucose absorption is reduced, the blood glucose levels also decrease. The results from *Arbutus unedo* tree leaves indicate all solvents were able to extract compounds with inhibitory activities towards α-amylase and α-glucosidase. The IC_{50} for α-amylase varied between 1.25 mg/mL for ethanolic extracts and 1.89 mg/mL for 50% ethanol: water extracts. Comparatively, the IC_{50} for acarbose was around 261.88 μg/mL, when the enzyme concentration was 2.4 U/mL, indicating acarbose was a more powerful α-amylase inhibitor than any of the tested extracts. On the other hand, the concentration of acarbose needed to inhibit 50% of α-glucosidase was much higher than that of the extracts. While the IC_{50} of acarbose was over 30 mg/mL, the ethanolic extract only needed a concentration of 9.61 μg/mL to achieve the same result. The aqueous extract, which was the weakest of all extracts regarding α-glucosidase inhibition, only needed a concentration of 72.90 μg/mL to inhibit 50% of the activity of a 1 U/mL α-glucosidase enzyme solution. These results suggest the inhibition mechanism of acarbose and the extracts is different, since one appears to target α-amylase over α-glucosidase and the other is the opposite. Still, the results show that the extracts of *Arbutus unedo* leaves have the potential to be used in the prevention/treatment of type 2 diabetes. Since their mechanism to reduce blood glucose levels would pass by inhibiting the digestive enzymes, the extracts should be taken in the beginning of the meal. One option to use its inhibitory activities would be to accompany a meal with an infusion of the leaves. While the aqueous extract was not the best in either of the enzyme inhibition assays, the difference to the ethanolic extract regarding α-amylase inhibition while significant ($p<0.05$) was not marked, and it still proved to be a much stronger α-glucosidase

inhibitor than acarbose. The ingestion of an infusion would be much more feasible than doing an ethanolic extract and removing the ethanol by evaporation or other process. However if the aim was to extract compounds to be incorporated in pills or to make a soluble powder, the added efficacy of the ethanolic extract might prove advantageous.

5.4 Antiproliferative activity

Very intensive studies on *A. unedo* berries have been conducted (Fonseca et al., 2015), but recently the special attention has been paid to *A. unedo* leaves due to specific polyphenolic content discussed above. While fruits are seasonally available, *A. unedo* leaves may be a good source of polyphenolic compounds virtually through the whole year. (Nenadis et al., 2015) findings indicated that leaves of *A. unedo* can be a good source of antioxidants especially in autumn and winter, since their results showed a significant seasonal variation in the leaf content of phenols, with the lowest values found in spring and the highest in autumn and/or winter. Taking into consideration the circum-Mediterranean distribution of *A. unedo*, this widespread plant may be a precious source of available bioactive compounds (Miguel et al., 2014). What is more, in vivo human studies on bioavailability of phenolic compounds from *A. unedo* revealed a wide range of phenolic metabolites, showing the extensive metabolism that bioactive compounds underwent in the human body (Mosele et al., 2016). Anti-bacterial properties of those compounds are being intensively studied, but growing evidence suggest appreciable anti-tumour action of *A. unedo* leaf extracts as well as anti-cancer influence of specific compounds, as gallic acid and quercetin. (Mariotto et al., 2008a) reported that aqueous extract of *A. unedo* down-regulated of Signal Transducer and Activator of Transcription 1 (STAT1) protein expression elicited by interferon-γ (IFN-γ) in human breast cancer cell line MDA-MB-231 and in human fibroblasts. Moreover, anti-inflammatory activity of entire plant extracts of strawberry tree was also reported by (Carcache-Blanco et al., 2006). Polyphenolic compounds from *A. unedo* reinforced the anti-cancer effect through the anti-inflammatory activity, specifically through the inhibition of cyclooxygenase-2 (COX-2) (Carcache-Blanco et al., 2006). Our data revealed that *A. unedo* leaf extract was especially cytotoxic towards human *colorectal adenocarcinoma* SW 480 cells (even at concentration of 1 mg/mL). The successful inhibition of growth of tumorigenic SW 480 colorectal cancer cells is a relevant finding and anti-neoplastic mechanism of influence should be elucidated.

6 Conclusion

The extraction of compounds from *Arbutus unedo* leaves is highly solvent dependent. Overall the extract obtained with pure ethanol was better in antioxidant, antibacterial and

antidiabetic assays. Aqueous extract was second on the antioxidant activity. Quercetin and gallic acid were the compounds detected in highest amount. P-hydroxybenzoic and protocatechuic acids were also detected but in much lower amount. The high ethanolic extract activities are most likely due to the presence of quercetin, a flavonoid known for its antioxidant and antibacterial activities. This extract had the lowest MIC and was the only one capable of inhibiting Gram$^-$ bacteria. Of the tested microorganisms *S. aureus* was the most sensitive to the extracts. Compared to acarbose the tested extracts had a weaker α-amylase inhibitory activity. They were however much stronger α-glucosidase inhibitors. Of the tested cell lines, sw480 showed the highest sensitivity when exposed to an extract concentration of 1 mg/mL. Future uses for *Arbutus unedo* leaves extract could pass by wound disinfection since *S. aureus* is known to infect wounds. They could also be used to prevent high glucose levels in type 2 diabetes either by drinking an infusion or dissolving extracted powder into water. Given its difference in activity compared to acarbose, it could also be incorporated into acarbose pills to enable a wider inhibition and perhaps a better enzyme inhibitory and glucose levels control.

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Antibacterial, antioxidant and anti-proliferative properties and zinc content of five south Portugal herbs

Ricardo Nunes [a], Pawel Pasko [b, c], Malgorzata Tyszka-Czochara [d], Agnieszka Szewczyk [e], Marek Szlosarczyk [f] and Isabel S. Carvalho [a]

[a]MeditBio, Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas, Faro, Portugal; [b]Department of Food Chemistry and Nutrition, Medical College, Jagiellonian University, Krakow, Poland; [c]Department of Clinical Science, Faculty of Health and Medical Science, Andrzej Frycz-Modrzewski Krakow University, Poland; [d]Radioligand Laboratory, Department of Pharmacobiology, Medical College, Jagiellonian University, Krakow, Poland; [e]Department of Pharmaceutical Botany, Medical College, Jagiellonian University, Krakow, Poland; [f]Department of Inorganic and Analytical Chemistry, Medical College, Jagiellonian University, Krakow, Poland

1 ABSTRACT

Context: *Crataegus monogyna* L. (Rosaceae) (CM), *Equisetum telmateia* L. (Equisataceae) (ET), *Geranium purpureum* Vil. (Geraniaceae) (GP), *Mentha suaveolens* Ehrh. (Lamiaceae) (MS), and *Lavandula stoechas* L. spp. *luisieri* (Lamiaceae) (LS) are all medicinal.

Objective: To evaluate the antioxidant, antiproliferative and antimicrobial activities of plant extracts and quantify individual phenolics and zinc.

Material and methods: Aerial part extracts were prepared with water (W), ethanol (E) and an 80 % mixture (80EW). Antioxidant activity was measured with TAA, FRAP and RP methods. Phenolics were quantified with a HPLC. Zinc was quantified using voltammetry. Antibacterial activity (after 48 h) was tested using *Enterococcus faecalis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Listeria monocytogenes*. Antiproliferative activity (after 24 h) was tested using HEP G2 cells and fibroblasts.

Results: Solvents influenced results; the best were E and 80EW. GP had the highest antioxidant activity (TAA and FRAP of 536.90 mg AAE/g dw and 783.48 mg TE/g dw respectively). CM had the highest zinc concentration (37.21 mg/kg) and phenolic variety, with neochlorogenic acid as the most abundant (92.91 mg/100 g dw). LS was rich in rosmarinic acid (301.71 mg/100 g dw). GP and LS inhibited the most microorganisms: *B. cereus*, *E. coli* and *S. aureus*. GP also inhibited *E. faecalis*. CM had the lowest MIC: 5830 µg/mL. The antibacterial activity is explained by the phenolics present. LS and CM showed the most significant anti-proliferative activity, which is explained by their zinc content.

Conclusion: The most promising plants for further studies are CM, LS and GP.

KEYWORDS

HPLC; phenolics; flavonoids; Algarve; traditional medicine; solvent influence

2 Introduction

Since ancient times, the human race has been exploring the potential of plants to improve its health. From simple infusions to alleviate stomach problems to direct application on the skin to treat wounds, animal bites or other ailments, plants have had an important role in human history. When the modern times arrived however, the attention turned to the use of synthetic drugs or other chemical substances in high amounts which in part led to bacteria resistance and weak immune system (Fair and Tor, 2014, Fauci and Marston, 2014) Nowadays the focus is shifting again, this time from synthetic to the natural agents, with a great interest in antioxidant, antibacterial and anti-carcinogenic properties of plants and their extracts. Since some of plants' strongest antioxidants belong to the group of polyphenols, a preliminary analysis is often made in order to identify the most promising plants. This is important due to the difference in costs, with antioxidant assays being much cheaper and providing quicker results than antibacterial and anti-carcinogenic assays. Many aromatic plants have already been analyzed regarding their essential oil composition (hydrophobic compounds), due to its commercial value. On this work we analyzed hydrophilic extracts, which are less studied, and determined their zinc, phenolic and antioxidant contents as well as antibacterial and antiproliferative activities. The extracts were obtained from five plants endemic to the Algarve region in Portugal, all used in traditional medicine.

Crataegus monogyna L. (Rosaceae) is an endemic tree in Portugal, which produces edible berries appreciated by shepherds and hunters (Barros et al., 2011). They are considered nutritious due to their content in vitamins and micronutrients and are used to treat gastro-intestinal disorders, rheumatism, heart problems and respiratory infections (Jarzycka et al., 2013, Veveris et al., 2004). Ripened fruits are rich in carbohydrates while unripe ones are rich in polyunsaturated fatty acids. Leaves on the other hand are rich in phenolic compounds, tocopherols and ascorbic acid (Jarzycka et al., 2013).

Plants belonging to genus *Equisetum* (Equisetaceae) are distributed throughout the Mediterranean basin, from Spain to Turkey. In Portugal the most common ones are *Equisetum arvensis* L. and *Equisetum telmateia* L. They are used in the folk medicine of many countries to treat urinary, kidney, prostate and gastrointestinal problems (Gurbuz et al., 2009). They are also used to treat inflammations and skin disorders and are believed to possess antimicrobial properties (Milovanovic et al., 2007). Their phytochemical profile revealed the presence of silicic acid, flavonoids and manganese (Veit et al., 1995).

Lavandula stoechas L. ssp. *luisieri* belongs to the Lamiaceae family, is endemic in the Iberian Peninsula and common in Portugal. Lavandula oils, well known for their scent and

aroma, have been used in perfumery and food industry for many years (Da Porto et al., 2009). Its uses in traditional medicine include cold and headache therapy, an expectorant, stimulant and disinfectant. They are also believed to have antifungal and anti-depressive effects, and can be used to alleviate insect bites and burns (Cavanagh and Wilkinson, 2002).The oil is composed by irregular monoterpenoids such as necrodane derivatives (Baldovini et al., 2005) and is known to possess antifeedant and antibacterial activities (Baldovini et al., 2005, Gonzalez-Coloma et al., 2006). Although the oil is well studied, there are few researches on its hydrophilic extract composition. (Cavanagh and Wilkinson, 2002)

The genus *Geranium* (Geraniaceae) is distributed throughout the northern hemisphere and is constituted by approximately 250 different species including *Geranium purpureum* Vil. which can be found in Portugal. Its aromatic oils are used in perfumery and the plant itself is used in gardens (Camacho-Luis et al., 2008). Its uses in traditional medicine include the treatment of inflammatory diseases, gastric disorders, fever, gall bladder, gastritis and hemorrhages (Neagu et al., 2010). Both kaempferol and quercetin derivatives are reported to be present in the *Geranium* genus which may explain some of the uses in traditional medicine (Sohretoglu et al., 2011).

Mentha suaveolens Ehrh. belongs to the Lamiaceae family and is highly present in the Mediterranean area. Its leaves, flowers and stems are used frequently in herbal teas or as food additives in many countries for their aroma and flavor (Diaz-Maroto et al., 2003). Moreover the consumption of teas made from parts of Mentha spp. has many benefits on human health. In folk medicine the plant is used to treat nausea bronchitis, anorexia, ulcerative colitis and liver problems (El-Kashoury et al., 2013). This plant is known to possess anti-inflammatory, antiemetic, antispasmodic and analgesic properties, (Gulluce et al., 2007). It is also known that the essential oil from *Mentha suaveolens* has antimicrobial and antioxidant properties. According to its phytochemical analyses, the plant is rich in piperitenone, piperitone oxides, terpenic alcohol and other derivate compounds (Civitelli et al., 2014)

3 Material and methods

3.1 Plant material

Samples were randomly selected and collected during March 2013 in the Algarve region of Querença, Fonte Benémola, GPS coordinates 37.198946, -8.004309 and were identified by naturalist José Manuel Rosa Pinto, responsible for University of Algarve's herbarium, where a voucher specimen was deposited. The collected samples were *C. monogyna* (13501), *L. stoechas* spp. *luisieri* (13499), *G. purpureum* (13490), *E. telmateia* (13496) and *M.*

suaveolens (13489). Plants were stored on a dry place, protected from sunlight and naturally air dried (ambient temperature of approximately 20°C) for about one week, after which they were stored in plastic vials at -20°C until extraction.

3.2 Biological material

Bacteria strains were gently provided by "Stress by Antibiotics and Virulence of Enterococci" laboratory from IBET (Instituto de Biologia Experimental e Tecnológica) and consisted in: *Enterococcus faecalis* – DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) 20478, *Bacillus cereus* – ATCC (American Type Culture Collection) 11778, *Escherichia coli* – ATCC 8739, *Staphylococcus aureus* – ATCC 6538, *Listeria monocytogenes* – DSMZ 7644.

3.3 Extraction procedure

A 0.2 g mass of plant material (in powder form) was extracted with 20 mL of solvent for 30 min in an electro thermal heating mantle. Stirring speed was set to 200 rpm, solvent temperature was 90°C for water and 70°C for both ethanol and 80% ethanol: water 1:1(v:v). For antibacterial assays the solvent was evaporated (Nahita serie 503, Navarra, Spain) and the extracted material was re-suspended. The solvent chosen for the resuspension was distilled water (which was able to resuspend the extracts after slightly heating them to 50°C) instead of DMSO due to the latter capability of inhibiting bacterial growth, even at relatively low concentration (Wadhwani et al., 2009). Extracts and resuspended extracts were transferred to Eppendorf tubes and stored at -20°C until analysis.

3.4 Total Phenolic Content

Total Phenolic Content (TPC) in samples was determined using a spectrophotometric procedure originally described by Singleton and Rossi (1965). Briefly, 0.1 mL of extract (diluted in the extraction solvent) was mixed with 0.5 mL of Folin-Ciocalteau's reagent,0.4 mL of a saturated sodium carbonate solution (7.5 %) and incubated for 30 min in a dark room. Absorbance was read at 765 nm against a blank. Phenolic content was calculated using a gallic acid calibration curve and the results were expressed as mg GAE/g dw (gallic acid equivalents per gram of dry weight).

3.5 Total Flavonoid Content

Total Flavonoid Content (TFC) was determined according to the method of Lamaison and Carnat (1990). Briefly, 0.4 mL of diluted extract was mixed with 0.8 mL of a 2% methanolic $AlCl_3.6H_2O$ solution. After standing for 10 min in the dark the absorbance was measured at

430 nm. Flavonoid content was calculated using a quercetin calibration curve, and the results were expressed as mg QE (quercetin equivalents)/g dw.

3.6 Total Antioxidant activity

Total Antioxidant Activity (TAA) of extracts was determined using a spectrophotometer and the method proposed by Prieto (1998b). Briefly, 0.1 mL of diluted extract was mixed with 1.0 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was then incubated in a water bath at 95°C for 90 min. Absorbance was read at 695 nm against a blank (negative control of water, ethanol or 80% ethanol: water according to the solvent used) and results were calculated from an ascorbic acid (positive control) calibration curve. Results were expressed as mg AAE (ascorbic acid equivalents)/g dw.

3.7 Reducing Power

Reducing Power (RP) was determined using the method previously described by Oyaizu (1986). Briefly, 0.2 mL of diluted extract were mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min. After incubation, 0.5 mL of trichloroacetic acid (10%) were added and the mixture was centrifuged at 650 x g for 10 min. 0.5 mL of the supernatant were mixed with 0.5 mL of distilled water and 0.1 mL of ferric chloride (0.1%). Absorbance was measured at 700 nm. Trolox was used as positive control and the negative control was water, ethanol or 80% ethanol: water according to the solvent used in the extraction. Results were expressed as mg TE (Trolox equivalents)/g dw.

3.8 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined using the method previously described by Benzie and Strain (1996a). First, three solutions were prepared, a 300 mM acetate buffer, pH=3.6, a 10 mM TPTZ, 40 mM HCl solution, and a 20 mM $FeCl_3.6H_2O$ solution. Mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of FeCl3.6H2O solution and heating the mixture to 37°C prepared the working solution. After cooling down, 0.9 mL of the working solution were mixed with 0.1 mL of diluted extract. After staying 30 min in the dark the absorbance was read at 593 nm. Trolox was used as positive control and water, ethanol or 80% ethanol: water were used as negative control, according to the solvent used. Results were calculated from a Trolox calibration curve and expressed as mg Trolox/g dw.

3.9 DPPH Radical Scavenging Activity

Radical scavenging activity was determined using the DPPH radical (Blois, 1958) method (DPPH) with slight modifications. Briefly, 1.0 mL of a 0.16 mM DPPH solution was added to a test tube, which contained 1.0 mL of extract at different concentrations (two with more than 50% scavenging activity and two with less than 50% scavenging activity). This mixture was then vortexed and kept in the dark for 30 min, after which absorbance was measured at 517 nm. The radical scavenging percentage (IC) was calculated using the following formula: (IC) = $((A_0-A_t)/A_0)$ x 100, where A_0 is absorbance of control at 30 min and A_t absorbance of sample at 30 min. Trolox was used as positive control and the negative control was water, ethanol or 80% ethanol: water according to the solvent used. Results were expressed as μg sample/mL in form of IC_{50}, determined by linear regression of IC and extract concentration at 50% inhibition.

3.10 RP-HPLC analysis

Phenolic acids and flavonoids in dry biomass were quantified in methanol extracts (80°C, 2h) and after hydrolysis with 2 M aqueous HCl, 100°C, 1 h (Harborne, 1998). RP-HPLC analysis was conducted according to Ellnain-Wojtaszek and Zgorka (1999) but with modification on Merck-Hitachi liquid chromatograph (LaChrom Elite) equipped with a DAD detector L-2455 and Purospher ® RP-18e (250x4 mm/ 5μm) column. Analysis was carried out at 25°C, with a mobile phase consisting of A – methanol, B – methanol : 0.5% acetic acid 1 : 4 (v/v). The gradient was as follows: 100% B for 0–20 min; 100 – 80% B for 20 – 35 min; 80 – 60% B for 35 – 55 min; 60 – 0% B for 55 – 70 min; 0% B for 70-75 min; 0 – 100% B for 75 – 80 min; 100% B for 80–90 min at a flow rate 1 ml/min, λ = 254 nm (phenolic acids), λ = 370 nm (flavonoids). Identification was done by comparing retention times of peaks with standards and by adding the standards to the tested samples and verifying the increase in the peak area of the compound being identified (standard addition). Quantification was done by measurement of peak area with reference to the standard curve derived from five concentrations (0.03125 to 0.5 mg/ml). Standards were caffeic, chlorogenic, cinnamic, gallic, gentizic, o-coumaric, protocatechuic, salicylic, sinapic, syringic acid, isorhamnetin, kaempferol, luteolin, quercetin, quercitrin, rutin, vitexin, *p*-coumaric, vanillic, ferulic and *p*-hydroxybenzoic acid diluted in HPLC grade methanol.

3.11 Zinc

A Multipurpose Electrochemical Analyzer M161 with the M164 electrode stand (both MTM-ANKO, Poland) was used for all voltammetric measurements. The standard three-

electrode cell consisted of a controlled growth mercury drop electrode (CGMDE) as a working electrode, Ag/AgCl in 3M KCl with a double junction filled with 3M KCl (Mineral, Poland) as reference and platinum wire as an auxiliary electrode. Voltammograms were recorded, interpreted and stored by EAGRAPH (MTM-ANKO, Poland) software. A standard stock solution of $Zn(NO_3)_2$ was prepared by proper dilution of solution both with concentration of 1 g/L (OUM, Łódź, Poland). The electrolyte used as ionic medium was prepared by dissolving KNO_3 (Merck, Suprapur®). For digestion procedures HNO_3 (Merck, Suprapur®) was used. All the solutions were prepared with double - distilled water from quartz distiller (SZ-97A, Chemland, Polska) and all reagents were of analytical grade. Samples were powdered in agate mortar and then dried at over 70°C for 4 hrs. Approximately 250 – 500 mg of sample material was weighed and inserted to high-pressure acid digestion vessel 4748 (Parr Instruments, USA) and treated with 5 mL of nitric acid. Next, the acid digestion bomb was closed and kept in the oven at 170°C for 24h. The digested sample was placed at the heated plate to let it evaporate and to remove the nitrate. The sampled solutions were cooled to room temperature, transferred quantitatively into volumetric flasks (10 ml) and filled up to the mark with double distilled water. All the procedures were repeated three times for each sample.

The zinc content of selected samples was determined using a differential pulse anodic stripping voltammetry (DP ASV) with a controlled growth mercury drop electrode (CGMDE) under following parameters: pulse amplitude –20 mV; step height 2 mV; pulse width (waiting time – 20 ms; sampling time – 20 ms); resting time 5 s (Szlósarczyk et al., 2011). The voltammograms were recorded in the potential range from -1200 mV to 75 mV in diluted sample solution. Before each measurement, the solution in the voltammetric cell was de-aerated by high purity argon for 5 min. Voltammograms corresponding to individual additions were taken three times according to the standard addition method. All experiments were performed at room temperature.

3.12 Cell lines culture

Human cell lines were obtained from the American Type Cell Culture collection, ATCC (LGC Standards-ATCC, -Teddington, Great Britain). ATCC designations were as follows: BJ, normal adherent human skin fibroblasts, CRL-2522; HEP G2, *hepatocellular carcinoma.* Cells were cultured according to ATCC's catalogue instructions. Briefly, cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 to confluence in Eagle's Minimum Essential Medium (EMEM, ATCC), supplemented with 10% v/v FBS (ATCC) and

with antibiotic solution (100 IU/mL penicillin, 0.1 mg/mL streptomycin, Gibco Laboratories, NY, USA). For experiments, the cells were at an exponential phase of growth.

3.13 Bacterial strains culture

Prior to the assay a culture of each microorganism was initiated in 2 mL Luria Broth and incubated at 37°C for 24 h. After the 24 h the microorganism was cultured on solid Plate Count Agar medium using the streak method, and grown at 37°C for 24 h.

3.14 MTT assay

Cell Viability Assay Microculture tetrazolium assay (MTT) was used as an indicator of cell viability. MTT assay determined by measuring the mitochondrial-dependent reduction of MTT to formazan was conducted as described previously (Tyszka-Czochara et al., 2014b). Briefly, cells were seeded in 96-well microtiter plates (Sarstedt, Numbrecht, Germany). After optimum confluence, cells were treated with the extracts and incubated for 24 h at 37°C. Five different concentrations (0.01, 0.1, 0.5 1.0 and 5.0 mg/ml) for each extract (diluted in DMSO) were used. Cells treated with equivalents of vesicle were considered as controls. After incubation, cells were washed before adding medium containing MTT (5 mg/ml) and kept for 1 h at 37°C. Medium was then discarded and the formazan salt that had formed in cells was dissolved in DMSO. The optical density was measured at 570 nm (the reference wavelength was 630 nm) using a microplate reader (Tecan Austria GmbH, Austria). Results were reported using the following formula: (average OD value of three measurements for each experimental group/average OD value of control group)×100% (Nunes et al., 2014).

3.15 Disc diffusion assay

For the determination of minimum concentration of diffusion inhibition (MCDI) a method similar to that used in antibiotic susceptibility testing was used (Bauer et al., 1966), but with plant extracts instead (Gaudreau and Gilbert, 1997b). Briefly, a colony was collected from the agar plate and suspended in a flask containing Ringer solution. An aliquot of 50 µL was spread on a petri dish containing solid plate count agar. After dried, discs were put in the petri dish and 10 µL of extract diluted in distilled water (10 mg/mL) were added to each disk. Ampicillin (10 mg/mL) and streptomycin (10 mg/mL), diluted in distilled water, were used as positive controls and distilled water was used as negative control. After 24 (not shown) and 48 h incubation, the inhibition diameter was checked. After assessing which strains were inhibited, several extract concentrations with 1 mg/mL of difference between them were

tested to find the MCDI. All bioassays were repeated thrice in separated plates, and each plate had three discs per concentration. All results are means ± SD.

3.16 Statistical methods

A one way analysis of variance (ANOVA) and the Student's t test were used for the evaluation of MTT test. $p < 0.05$ was considered as the level of significance.

4 Results

4.1 Antioxidant activity, phenolic compounds and trace elements

Out of the 5 plants screened, those with highest content in phenolic compounds (Table IV–6) were *E. telmateia* (585.76 ± 8.83) and *L. luisieri* (482.59 ± 10.67) in absolute ethanol, followed by *C. monogyna* (475.12 ± 10.31 mg GAE/g dw) in 80% ethanol: water. Regarding flavonoids content *E. telmateia* provided the highest results (60.37 ± 1.28) when a mixture of both solvents was used, while *G. purpureum* was the best on water (24.73 ± 3.01) and absolute ethanol (59.82 ± 2.84 mg QE/g dw). Regarding radical scavenging activity, the plants with most potential (lower IC_{50}), were *L. stoechas spp. luisieri* for water (26.00 ± 1.59) and *E. telmateia* for absolute ethanol (22.60 ± 1.41) and 80% ethanol: water (32.52 ± 2.32 μg/mL).

From the examined plants *C. monogyna* was the richest in phenolic compounds, with 9 detected (Table IV–7) and had the highest zinc content (37.21 ± 4.03 mg/kg) (Table IV–8). The plant with the least phenolic diversity was *E. telmateia* in which only protocatechuic acid was detected (2.85 ± 0.41 mg/100 g dw). This compound was present on most analyzed samples, (was only absent in *M. suaveolens*) but always on small amount. On the other hand rosmarinic acid, was only present in *L. stoechas spp. luisieri* and *M. suaveolens*, but was the phenolic present in greatest amount (301.71 ± 10.15 and 161.40 ± 5.32 mg/100 g respectively).

Table IV–6. Antioxidant and radical scavenging activities of the five tested plants.

Extract		TPC (mg GAE/g dw)	TFC (mg QE/g dw)	TAA (mg AAE/g dw)	RP (mg TE/g dw)	FRAP (mg TE/g dw)	DPPH IC_{50} (µg sample/mL)
Crataegus monogyna L.	Water	55.02 ± 2.68	6.05 ± 1.32	173.13 ± 8.86	52.57 ± 2.51	74.54 ± 4.64	61.56 ± 4.00
	80 % ethanol: water	478.12 ± 8.45	10.70 ± 1.66	243.31 ± 9.61	177.86 ± 7.54	225.52 ± 10.91	37.64 ± 2.20
	absolute ethanol	241.87 ± 10.77	40.08 ± 1.74	137.79 ± 6.82	26.88 ± 3.69	168.56 ± 5.37	43.14 ± 2.02
Equisetum telmateia L.	Water	66.38 ± 3.05	8.68 ± 1.05	128.20 ± 7.27	8.67 ± 1.27	17.64 ± 1.96	79.53 ± 2.38
	80 % ethanol: water	371.82 ± 14.62	60.37 ± 1.28	370.05 ± 17.23	242.50 ± 2.66	353.42 ± 11.75	32.52 ± 2.32
	absolute ethanol	585.76 ± 8.83	16.50 ± 1.23	252.92 ± 9.94	9.17 ± 2.21	46.43 ± 5.47	22.60 ± 1.41
Geranium purpureum Vil.	Water	219.52 ± 9.35	24.73 ± 3.01	333.30 ± 15.01	169.07 ± 3.21	467.24 ± 7.85	211.57 ± 5.82
	80 % ethanol: water	293.22 ± 14.28	36.79 ± 1.19	472.04 ± 22.99	295.51 ± 9.53	705.91 ± 15.21	211.44 ± 10.33
	absolute ethanol	326.90 ± 7.82	59.82 ± 2.84	536.90 ± 21.67	681.58 ± 20.18	783.48 ± 20.50	197.16 ± 7.38
Lavandula stoechas L. *spp. luisieri*	Water	276.67 ± 12.77	13.26 ± 1.31	343.50 ± 7.61	35.81 ± 1.85	80.35 ± 2.80	26.00 ± 1.59
	80 % ethanol: water	99.21 ± 5.56	9.80 ± 1.94	173.84 ± 7.55	313.57 ± 6.61	447.40 ± 21.26	38.32 ± 1.54
	absolute ethanol	482.59 ± 10.67	7.18 ± 1.81	164.49 ± 8.56	51.98 ± 5.23	52.42 ± 8.12	123.02 ± 3.25
Mentha suaveolens Ehrh.	Water	172.97 ± 7.01	17.47 ± 1.23	289.00 ± 14.72	298.58 ± 14.34	396.60 ± 17.54	169.45 ± 5.54
	80 % ethanol: water	205.23 ± 5.40	39.74 ± 1.79	340.32 ± 12.74	333.67 ± 6.06	534.34 ± 21.12	150.11 ± 4.26
	absolute ethanol	101.66 ± 4.83	56.98 ± 2.73	229.88 ± 7.17	90.80 ± 5.81	218.03 ± 11.92	219.40 ± 6.00

The results are represented as mean ± standard deviation from three replicates.

Table IV–7. Individual compounds of the five tested plants identified by HPLC.

	Crataegus monogyna L.	*Equisetum telmateia* L.	*Geranium purpureum* Vil.	*Lavandula stoechas* L. *spp. luisieri*	*Mentha suaveolens* Ehrh.
Caffeic acid	3.58 ± 0.92	n.d.	n.d.	3.94 ± 0.71	n.d.
Chlorogenic acid	37.05 ± 2.33	n.d.	n.d.	12.64 ± 1.32	n.d.
Coumaric acid*	4.26 ± 0.52	n.d.	n.d.	n.d.	n.d.
Ferulic acid	n.d.	n.d.	n.d.	17.30 ± 2.10	n.d.
Gallic acid*	n.d.	n.d.	162.38 ± 9.20	n.d.	n.d.
Kaempferol	n.d.	n.d.	24.14 ± 0.05	n.d.	n.d.
Neochlorogenic acid	92.91 ± 5.33	n.d.	115.35 ± 7.51	n.d.	n.d.
p-hydroxy b. acid*	1.79 ± 0.07	n.d.	n.d.	1.70 ± 0.04	0.19 ± 0.02
Protocatechuic acid*	17.46 ± 1.35	2.85 ± 0.41	2.55 ± 0.21	1.29 ± 0.29	n.d.
Quercetin	23.12 ± 2.15	n.d.	27.13 ± 2.76	n.d.	n.d.
Rosmarinic acid	n.d.	n.d.	n.d.	301.71 ± 10.15	161.40 ± 5.32
Rutin	n.d.	n.d.	53.87 ± 1.20	n.d.	n.d.
Syringic acid	14.60 ± 2.00	n.d.	n.d.	n.d.	n.d.
Vanillic acid*	n.d.	n.d.	n.d.	1.07 ± 0.07	n.d.
Vitexin	20.10 ± 0.07	n.d.	n.d.	n.d.	n.d.

Results are expressed as mg/100 g of dry weight. *after HCl 2M digestion

Table IV–8. Zinc content of the five tested plants.

Sample	Content of Zn(II) mg/kg ± SD
Crataegus monogyna L.	37.21 ± 4.03
Equisetum telmateia L.	28.30 ± 0.69
Geranium purpureum Vil.	11.63 ± 0.96
Lavendula stoechas L. *spp. luisieri*	23.55 ± 2.71
Mentha suaveolens Ehrh.	16.36 ± 1.58

The results are represented as mean ± standard deviation from three replicates.

4.2 Antibacterial activity

E. telmateia and *C. monogyna* were the plants that needed the lowest concentration to inhibit the growth of a microorganism, with a MIC around 6000 µg/mL for *P. aeruginosa* (*E. telmateia*) and *L. monocytogenes* and *S. aureus* (*C. monogyna*). On the other hand *G. purpureum* and *L. stoechas spp. luisieri* inhibited the highest number of microorganisms. *G. purpureum* inhibited *B. cereus*, *E .coli*, *S. aureus* and *E. faecalis*, while *L. stoechas spp. luisieri* inhibited the first three: All *L. stoechas spp. luisieri* extracts demonstrated some inhibitory activity, while only the ethanolic extract of *G. purpureum* had activity (Table IV–9). *C. monogyna* only inhibited two microorganisms, *L. monocytogenes* and *S. aureus,* but

this was the only plant with the ability to inhibit the growth of *L. monocytogenes* and had the highest activity towards *S. aureus*.

Table IV–9. Antibacterial activity of the five tested plants

Plant	Extraction solvent	MIC (µg/mL) after 48h					
		B. cereus	*E. coli*	*E. faecalis*	*L. monocytogenes*	*P. aeruginosa*	*S. aureus*
Crataegus monogyna L.	Water	x	x	x	x	x	9830 ± 410
	80 % ethanol:water	x	x	x	5830 ± 410	x	5830 ± 410
	Ethanol	x	x	x	x	x	7830 ± 410
Equisetum telmateia L.	Water	x	x	x	x	6000 ± 0	x
	80 % ethanol:water	x	x	x	x	x	x
	Ethanol	x	8000 ± 0	9670 ± 520	x	x	x
Geranium purpureum Vil.	Water	x	x	x	x	x	x
	80 % ethanol:water	x	x	x	x	x	x
	Ethanol	10000 ± 0	9670 ± 520	9830 ± 410	x	x	8000 ± 0
Lavandula stoechas L. spp. luisieri	Water	9830 ± 410	x	x	x	x	9830 ± 410
	80 % ethanol:water	x	7000 ± 0	x	x	x	x
	Ethanol	7670 ± 520	x	x	x	x	x
Mentha suaveolens Ehrh.	Water	x	x	x	x	x	x
	80 % ethanol:water	x	8000 ± 630	x	x	9670 ± 520	8330 ± 520
	Ethanol	x	x	x	x	x	x

The results are represented as mean ± standard deviation from nine replicates.x – no inhibition at maximum tested concentration (10000 µg/mL).

All microorganisms were inhibited by at least one plant. The most and least resistant microorganisms were *L. monocytogenes* and *S. aureus* respectively. The positive control, ampicillin, inhibited all the microorganisms tested, with the exception of *P. aeruginosa*, for which streptomycin was used instead, on the other hand the negative control, water, inhibited none.

4.3 Antiproliferative activity

The anticancer potential of herbal extracts was analyzed according to proliferation inhibition of cells using MTT assay. Cell lines were exposed to plant extracts for 24 h, at concentrations of 0.01, 0.1, 0.5 1.0 and 5.0 mg/ml. Studies were conducted on cancer and normal cell lines of human origin, *hepatocellular carcinoma* (HEP G2) and normal skin fibroblast (BJ) to address the specific anti-tumor activity of the herbal extracts toward cell types of different lineage. Dose response studies of the tested extracts are summarized in Figure IV–5.

Herbal extracts proliferation inhibition and cytotoxicity against cancer and normal cells varied in a dose-dependent manner. At a concentration of 0.01 mg/ml, only *L. stoechas spp. luisieri* had effect on the viability of Hep G2 cells. The incubation of both cell lines, tumor and normal ones, with addition of 5 mg/ml of any tested herbal extract, caused over 90% cell death. The highest anti-proliferative activity against Hep G2 cells was measured on *L. stoechas spp. luisieri* followed by *C. monogyna*. As shown below (Figure IV–5), *G. purpureum* and *E. telmateia* revealed mild anti-proliferative potential while the inhibitory effect of *M. suaveolens* extract was the lowest.

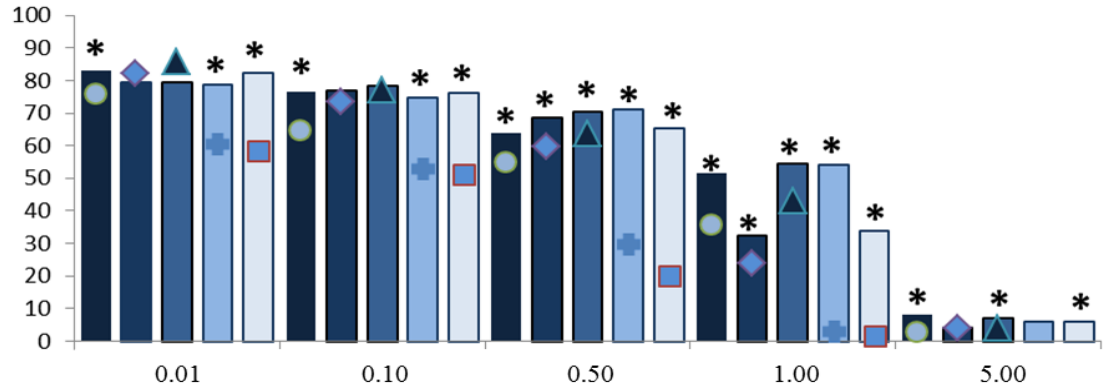

Figure IV–5. Effect of the five tested plants on the proliferation of human tumor (Hep G2) and non-tumor (fibroblasts) cell lines as determined by the MTT assay.

The bars represent the fibroblasts while the marks represent the Hep G2 cells. The mean values with asterisks were significantly different ($p<0.05$), n=3.

5 Discussion

5.1 Antioxidant activity, phenolic profile and trace elements

For the majority of the tested plants, the ethanol-water mixture was better at extracting phenolics while pure ethanol was better at extracting flavonoids. Exceptions were *L. stoechas spp. luisieri* and *E. telmateia.* On these two plants ethanol was much better than the mixture at extracting phenolics and the ethanol-water mixture was better at extracting flavonoids. Regarding antioxidant activity, the ethanol-water mixture was the overall best solvent for all plants except *G. purpureum* (for this plant pure ethanol was better). This solvent dependence is in accordance with the literature and shows the importance of using more than one solvent when studying the antioxidant activity of plants (Keser et al., 2014, Radojevic et al., 2012, Mimica-Dukic et al., 2008).

The individual phenolic compounds distribution was dependent on the plant being analyzed. The most common phenolics were protocatechuic and p-hydroxybenzoic acids, present in 4 and 3 of the 5 samples respectively. Despite being the most common, they were only found in low amounts. Both are strong antioxidants and protocatechuic acid has mild activity against leukemia cells, which is an early indicator that some of the plants may have antiproliferative activity (Tseng et al., 2000b). Those present in highest amounts were rosmarinic, neochlorogenic and gallic acids, rutin, quercetin and, chlorogenic acid in decreasing order s. Rosmarinic acid is a phenolic compound with antioxidant activity both in lipid protection and radical scavenging. It also has antimicrobial activity against several microorganisms including two tested in this work, *E. faecalis* and *P. aeruginosa* (Abedini et al., 2013). Chlorogenic acid and its isomer neochlorogenic acid are antioxidant and antibacterial compounds. Their antibacterial activity is due to the disruption of the bacterial membrane, which changes the intracellular potential and leads to the bacteria death (Lou et al., 2011). These are known to have activity against 4 of 6 tested bacteria, *B. cereus, E. coli, E. faecalis* and *S. aureus* (Fiamegos et al., 2011, Lou et al., 2011). Gallic acid is a trihydroxybenzoic acid with high antioxidant potential and anti-mutagenic potential that potentiates the expression of antioxidant related and DNA repairing enzymes (Abdelwahed et al., 2007). Its effect on protecting diabetic induced rats was also demonstrated (Punithavathi et al., 2011). According to Borges et al., (2013b) gallic acid inhibits the growth of *E. coli, E. faecalis, L. monocytogenes, S. aureus* and *P. aeruginosa*, with higher activity towards the latter. Rutin is a quercetin derivate with antibacterial activity against *S. aureus* . Quercetin is a flavonoid with high antioxidant and antibacterial activities against *S. aureus* and *E. coli*

(Rauha et al., 2000). The fact that gallic acid was present in only 1 of the 5 samples used in this study was somewhat surprising since its one of the most common phenolic compounds in terrestrial plants (Fernandes and Salgado, 2016). Kaempferol was only present in *G. purpureum*, ferulic acid was only present in *L. stoechas spp. luisieri*, and vitexin, coumaric acid and syringic acid were only present in *C. monogyna*.

According to the phenolic compound results, the high antioxidant activity detected in *G. purpureum* is due to gallic acid and quercetin, both strong antioxidants and best extracted with water and ethanol respectively. The antioxidant activity of *L. stoechas spp. luisieri* can be attributed to rosmarinic acid, commonly found in *Rosmarinus* and *Lavandula* genus (Erkan et al., 2008, Slobodnikova et al., 2013) . The higher ethanolic extract TPC results can be explained by its higher solubility in ethanol than in water (Wüst et al., 2016). Surprisingly, these results are not reflected in the antioxidant assays, since for RP and FRAP the best results were obtained with the mixture of both solvents. Pearson's correlation (data not shown) indicates that for *G. purpureum* and *L. stoechas spp. luisieri*, phenolics and flavonoids probably have a shared role on the antioxidant activity. However, while for *G. purpureum* both phenolics and flavonoids contribute positively for the antioxidant activity, in the case of *L. stoechas spp. luisieri*, and confirming the observation made before when comparing our results with other authors, TPC is negatively correlated with RP and FRAP and positively correlated with DPPH, meaning a higher TPC will lead to worse results. On the other hand, the antioxidant activity of *C. monogyna* and *M. suaveolens* is strongly correlated with phenolics, while *E. telmateia* antioxidant activity is probably mostly due to flavonoids. Regarding the trace elements analysis, only zinc was detected out of the tested elements. Zinc is an important mineral in human health, especially for pregnant woman and children on the developing stage (Favier and Hininger-Favier, 2005). It is also important to test for the presence of heavy metals such as (Cd, Pb, Cu) because they can interfere on the living cells (Chen et al., 2002, Hwang et al., 2013) leading to false positive results. Since none were detected it is safe to assume that the results obtained are in fact due to the compounds present in the tested samples.

Similarly to our work, Keser et al., (2014) found the FRAP and DPPH radical scavenging activity of *C. monogyna* to be better on ethanolic than on aqueous extracts. Also in agreement is the TPC (114.38 mg/GAE) of the aerial parts methanolic extract studied by (Simirgiotis, 2013) and the absence of gallic acid and kaempferol. That extract however had a higher TFC and DPPH radical scavenging activity (64.9 mg QE/g dw and IC of 3.34 μg/mL respectively) and no chlorogenic acid (Simirgiotis, 2013). In addition, when Urbonavičiūtėa et

al., (2006) studied this plant, they found the ethanolic % influences rutin and chlorogenic acid concentrations, with 60 to 80% ethanol providing the best results. Radojević et al., (2012) studied the methanol, acetone and ethyl acetate *E. telmateia* extracts while Mimica-Dukic et al., (2008) studied the aqueous and ethanolic extracts of *E. arvense* (a plant very similar to *E. telmateia*). Similarly to our work, they both found the TPC and TFC values to be solvent dependent while the RP value remained unaltered when extracted with water or ethanol. The radical scavenging activity obtained by Mimica-Dukic et al., (2008) was however higher than what we obtained, ranging from 2.37 to 37.20 μ/mL (versus 22.60 and 79.53 μg/mL). Despite the difference in values, which could be explained by the different plant and method of extraction used, in both cases ethanolic extracts were more powerful scavengers than aqueous extracts. When Milovanovic et al., (2007) studied *E. telmateia* they did not detect quercetin, similarly to us, but identified kaempferol which we did not. Jemia et al., (2013), Radulović et al., (2012) and Proestos et al., (2013) studied plants from the *Geranium* genus and obtained much lower TPC values than ours, 32.24 mg GAE/g dw for *G. robertianum* methanolic extract, 109.5 mg GAE/g dw for *G. macrohrryzym* ethanolic extract and 4.0 mg GAE/g dw for *G. purpureum* methanolic extract respectively. Our DPPH IC_{50} however was worse, an average of 205 μg/mL versus 19.98 μg/mL for *G. robertianum* methanolic extract and approximately 70 μg/mL for *G. purpureum* aqueous extract (Sohretoglu et al., 2011). Proestos et al., (2006) also studied the individual phenolics of *G. purpureum*, and found gallic acid to be present at a much lower concentration (14 mg/100 g). In addition, they identified caffeic, coumaric, vanillic, syringic and p-hydroxybenzoic acids, which we did not. The TPC and DPPH IC_{50} values of *L. stoechas spp. luisieri* studied by Baptista et al., (2015) and *L. stoechas* studied by Fouad & Mohamed (2014) are directly proportional while a higher TFC led to a lower DPPH IC_{50}, which is in agreement with our correlations. These results indicate that phenolics are not responsible for the DPPH scavenging activity and seem to in fact hamper it, since lower phenolic content extracts have a lower DPPH IC_{50}. Finally, the *L. stoechas* studied by Ceylan et al., (2015) had a higher caffeic and rosmarinic acids and lower ferulic acid concentrations (87.5 versus 3.94 mg/100 g; 834.7 versus 301.71 mg/100 g and 3.3 versus 17.30 mg/100 g, respectively). In addition they also identified quercetin, rutin and coumaric acid. Regarding the zinc content, *C. monogyna*, the plant with highest zinc content of our work was on par or above 19 out of 35 medicinal plants tested by Ansari et al., (2004).

5.2 Antibacterial properties

Strains from the microorganisms used, when detected from clinical samples, can cause

serious health problems and some can even lead to death if treatment is not administered. Due to the increasing antibiotic and synthetic drug resistances of microorganisms, it is increasingly important to search for sources of natural compounds capable of inhibiting their growth. Considering the antioxidant activity and individual phenolic results, the plant expected to exhibit the highest antibacterial activity was *G. purpureum* due to gallic acid and quercetin. Following *G. purpureum* would be *L. stoechas spp. luisieri* due to rosmarinic acid. This was in fact observed, with *G. purpureum* inhibiting the growth of *B. cereus, E. coli, E. faecalis* and *S. aureus* while *L. stoechas spp. luisieri* inhibited the same with the exception of *E. faecalis.* On the other hand, *C. monogyna* only inhibited *L. monocytogenes* and *S. aureus* but was highly active against the latter and was the only plant capable of inhibiting *L. monocytogenes*, while *E. telmateia* was the most effective plant against *P. aeruginosa*. The solvent used in the extraction influenced the antibacterial activities displayed by the plants, which is in accordance with the literature. When Şöhretoğlu et al., (2011) tested aqueous and ethyl acetate *G purpureum* extracts, their antibacterial activity was also dependent on the solvent, with the latter being much stronger. In that work the aqueous extract showed no inhibition towards *S. aureus* or *E. faecalis,* although that could be explained by the lower concentrations tested (1024 µg/mL), but *S. aureus* was, similarly to our work, more sensitive than *E faecalis*. When Ceyhan et al., (2012) studied the antibacterial activity of *L. stoechas* using four different solvents (water, ethanol, ethyl acetate and hexane), they found that only aqueous and ethanolic extracts had antibacterial activity, and the latter was more powerful against all microorganisms, including *S. aureus* and *B. cereus*. This is not in accordance with our results and could be due to the different variety of the plant, our plant belongs to the *luisieri* subspecies, or due to the extraction process. These variations could also explain why the MIC of their water extract for *S. aureus* is 20 times higher than ours and why there was no inhibition towards *B cereus* detected by them. Regarding *C. monogyna,* Ignat et al., (2013) found no inhibition towards *P. aeruginosa* and *E. coli*, but found, like us, this plant capable of inhibiting *S. aureus.* Out of the tested microorganisms the least sensitive to the tested plant extracts was *L. monocytogenes* which was only inhibited by *C. monogyna*. On the other hand *S. aureus* was the most sensitive microorganism. It was inhibited by at least one extract from each plant. In the case of *C. monogyna,* all extracts showed inhibition. This sensitivity of *S. aureus* and resilience of *L. monocytogenes* can be explained by the compounds present on the plants. *S. aureus* can be inhibited by quercetin, rutin, kaempferol, gallic, coumaric, caffeic, ferulic, neochlorogenic, chlorogenic, protocatechuic and p-hydroxybenzoic acids, a total of 11 different compounds. Among them the most powerful are kaempferol, chlorogenic,

neochlorogenic and protocatechuic acids (Fiamegos et al., 2011, Teffo et al., 2010, Pretto et al., 2004). From the tested plants *C. monogyna* was the plant with the highest number of them, 7, followed by *G. purpureum* with 6. Considering the compounds that were previously mentioned to be the most powerful against *S. aureus*, *C. monogyna* has the highest concentration of protocatechuic and chlorogenic acids, and a high concentration of neochlorogenic acid. *G. purpureum* also has some of the most powerful compounds, namely the highest concentration of neochlorogenic acid and was the only plant with kaempferol. It also has protocatechuic acid but at a much lower concentration. On the other hand, *L. monocytogenes* can only be inhibited by three of the detected compounds: gallic, ferulic and protocatechuic acids (Stojkovic et al., 2013, Takahashi et al., 2015, Borges et al., 2013b), with the latter being the strongest. Although *G. purpureum* and *L. stoechas spp. luisieri* had gallic acid and ferulic acid respectively, the concentration present was not enough since none of these plants inhibited its growth. *C. monogyna* did inhibit its growth, which can be explained by its high concentration of protocatechuic acid. These results show that conducting a screening of the plants regarding their phenolic and flavonoid total contents along with antioxidant activity or their individual compounds is a good approach to identify those with the highest potential to be explored for their antibacterial properties.

5.3 Antiproliferative properties

The identification of Portuguese local herbs with anticancer potential is important. This allows the establishment of their role as potential natural anticancer medicine and increases the attention given by the natural populace of the area and scientific community, perhaps helping even in their conservation. The plants screened in this this study (or plants of the same genus) have been previously reported to cause cytotoxic effects towards tumor cells and constitute therefore interesting candidates in a plant bioprospecting program for anticancer activity. The extract of *L. dentata* possess cytotoxic and pro-apoptotic properties assessed against human breast adenocarcinoma (MCF-7) cell lines using the MTT assay (Ali et al., 2014). Ku et al. (2014) evaluated antioxidant, anti-hyperglycemic, anti-cancer, anti-inflammatory, and anti-coagulant activities of hyperoside, an active compound from the genera of *Crataegus* and *Hypericum*. Venskutonis et al. (2010) reported that the extract of *G. macrorrhizum* possesses strong antioxidant properties. These are combined with cytotoxic and genotoxic activities on the bovine leukemia virus-transformed lamb kidney fibroblasts. *Mentha* leaf extracts were studied for pro-apoptotic properties and cell cycle arrest of tumor

cells (HeLa, MCF-7, Jurkat, T24, HT-29, MIAPaCa-2) (Jain et al., 2011, Elansary and Mahmoud, 2015).

By screening wild Portuguese plant extracts for their antiproliferative properties against human *hepatocellular carcinoma* (HEP G2) and normal skin fibroblast cell lines, we found that most of them exhibited promising cytotoxic activity towards tumor cell lines. This cytotoxic activity was dependent on the herbal extract. The obtained results also indicate this cytotoxic activity is selective as the anti-proliferative influence on tumor cells was more pronounced compared to the effects measured in normal human fibroblasts (Figure IV–5). Identifying novel bioactive compounds with anticancer properties from natural products is of great importance.

The selectivity in growth inhibition makes all tested herbal extracts interesting tools for further investigation of anticancer properties, although the most interesting for further studies regarding the identification of the mechanisms by which the bioactive compounds exerted the anticancer properties would be *L. stoechas spp. luisieri.*

The obtained results indicate that genus *Crataegus*, *Lavandula* and *Equisetum* contain not only polyphenolic compounds but also trace elements such as zinc. Zinc has been shown to exert beneficial effect on growth, proliferation and metabolism of normal human skin fibroblasts (Tyszka-Czochara et al., 2014b), protecting non-tumor human cells from damage, when used simultaneously with high content of polyphenols. It was found that the anti-proliferative effects of *E. telmateia*, *C. monogyna* and *L. stoechas spp. luisieri* towards tumor cells were more pronounced than towards normal fibroblasts, at the same extract concentration (Fig 1). This was especially true for the latter two. These data indicates the protecting properties of the herb compounds, specifically regarding non-tumor cells, and suggests the pleiotropic action of traditionally local used Portugal herbs.

6 Conclusion

There are significant differences between the results when different solvents are used. Water was generally the least effective solvent at extracting antioxidant compounds. Out of all the tested plants *G. purpureum* had average the one the highest antioxidant activity while *C. monogyna* had the biggest polyphenolic compound variety, with 9 different compounds identified. The compound present in highest amount was rosmarinic acid present in *L. stoechas spp. luisieri* and *M. suaveolens*. *G. purpureum* and *L. stoechas spp. luisieri* were the most promising in their composition with quercetin, caffeic and chlorogenic acids, all with known anti-cancer activity. The plants with the biggest antibacterial activity were *C.*

monogyna (lowest MIC) and *G. purpureum* followed by *L. stoechas spp. luisieri* (most microorganisms inhibited) which is attributed to the detected phenolic compounds. The highest concentrations of zinc, which has protective effect on normal fibroblasts, were detected in *C. monogyna, E. telmateia* and *L. stoechas spp. luisieri*. Overall the most promising plants for further studies were *C. monogyna* and *L. stoechas spp. luisieri* followed by *G. purpureum*.

Disclosure statement

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ORCID

Isabel S. Carvalho http://orcid.org/0000-0001-8057-3404

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Discussion of Chapter IV

In this chapter we study and discuss the medicinal properties of two Ericaceae, *E. australis*, the best of the two *Ericas* studied on chapter II and *A. unedo,* together with 5 other plants collected in Algarve, namely *C. monogyna*, *E. telmateia*, *G. purpureum*, *M. suaveolens* and *L. stoechas* spp. *luisieri*. The studied potential medicinal properties were the antimicrobial and antiproliferative activities. In addition, the antioxidant activity, zinc and phenolic contents were also determined for all plants except *E. australis*. Finally, the antidiabetic potential of *A. unedo* was also studied. The bacteria selected for this study were *B. cereus, E. coli, E. faecalis, L. monocytogenes, S. aureus* and *P. aeruginosa.* These are all bacteria which when in the nature can cause problems for human health and food safety. It is therefore important to search for plants which are able to inhibit the growth of these microorganisms and can be used in substitution or conjuction of antibiotics. The cell lines studied were Caco-2, a cell line isolated from a colorectal adenocarcinoma, for *E. australis,* HEP G2, a cell line isolated from a liver cancer, for *C, monogyna, E. telmateia, G. purpureum, M. suaveolens, L. stoechas* spp. *luisieri* and *A. unedo.* The antiproliferative properties of *A. unedo* towards the cell lines HEK 293, A549, SW 480 and SW 620 were also tested (human embryonic kidney cells, lung carcinoma, and colorectal adrenocarcinomas respectively).

In the previous chapter, *A. unedo* results regarding the antioxidant activity were better than those of both *Ericas*. However in this chapter *E. australis* showed a much more selective behaviour towards the tested cell lines. No only it inhibited the growth of Caco-2 cells, it even potentiated the proliferation of the fibroblast cells. This is in stark contrast with the results obtained for the antiproliferative activity of *A. unedo*, which showed only moderate selectivity towards Hep G2 and SW 480 cells. While the Caco-2 cells inhibition promoted by *E. australis* and the inhibition towards Hep G2 and SW 480 promoted by *A. unedo* cannot be directly compared, since it is possible that part of the difference in antiproliferative activity is due to being two different cell lines, the fact that *E. australis* promoted fibroblasts growth while *A. unedo* didn't cannot be denied. The inhibition towards bacteria was also lower on the *A. unedo* aqueous leaf extracts compared to *E. australis*, however when comparing the best extract of *A. unedo* (ethanolic) with the best of *E. australis* (aqueous) the inhibitory activities towards *E. coli, E. faecalis* and *L. monocytogenes* are quite close. On the other hand fruits inhibited none of the tested bacteria. Comparing with the other plants *A. unedo* always had the highest antioxidant activity, no matter the solvent used. On the other hand *C. monogyna* was the plant with the highest phenolic diversity. The inhibitory activities detected are most likely

linked to the phenolic compounds present in the plant extracts. It should be noted that extracts from every plant tested displayed different properties according to the solvent used. Just like *E. australis* and *A. unedo*, the other 5 plants tested displayed some selectivity on their antiproliferative activity. Out of them, *C. monogyna* and *L. stoechas* spp. *luisieri* were the most selective towards Hep G2 cells, even more than *A. unedo*. It is known that zinc can act as a protective agent towards fibroblast cells, and considering the results obtained for the zinc content, highest on *C. monogyna*, *E. telmateia* and *L. stoechas* spp. *luisieri*, it seems likely that the zinc present in *C. monogyna* and *L. stoechas* spp. *luisieri* is indeed helping in the protection of the fibroblast cells.

Diabetes mellitus is a condition that affects a large number of people throughout the world, and is a major cause of mortality, with projections indicating its impact will continue to increase. If diabetes arises, it is extremely important that blood glucose levels are monitored and treatment is performed adequately. For type 1 diabetes this invariably takes form of insulin injections while for type 2 diabetes insulin injections may be replaced with the intake of enzyme inhibitory substances, of which acarbose is an example (WHO, 2016). Its mechanism of action is the inhibition of α-glucosidase and α-amylase, preventing the degradation of complex carbohydrates into glucose. Because the glucose absorption is reduced, the blood glucose levels also decrease. Due to the side effects these inhibitors have, new sources of inhibition are constantly being sought. One of the sources which scientists usually look at is plants. With this in mind the potential antidiabetic use of *A. unedo* leaf extracts was studied using two different enzymes responsible for two steps of the digestive process. Results showed that *A. unedo* leaf extracts are a strong candidate to be studied in the incorporation into antidiabetic formulations or functional foods, due to their α-amylase and especially α-glucosidase inhibitory activities, which can help to control the amount of complex carbohydrates that is digested and in turn reduce the blood glycaemic level. They can also be incorporated into creams, due to their strong antioxidant and *S. aureus* inhibitory activities.

1 References

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CHAPTER V - ARBUTUS UNEDO POTENTIAL APPLICATIONS

In this chapter we study the potential health applications of *Arbutus unedo* leaves and fruits focusing initially on the nutritional properties via the analysis of the vitamin E and fatty acid contents of samples. We also investigate the potential use of leaf, fruit and flower extracts in the treatment of chronic diseases, namely type-2 diabetes, Alzheimer and Parkinson, using enzymatic assays.

Proximal analysis, fatty acid content and individual vitamin E vitamers of dried leaves and dried and lyophilized fruits from *Arbutus unedo* L.

Ricardo Nunes[a], Filipa Pimentel[b], M. Antónia Nunes[b], Anabela S.G. Costa[b], Maria Beatriz P.P. Oliveira[b] and Isabel Carvalho[a]

[a]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[b]REQUIMTE, LAQV/ Departamento de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, R. Jorge Viterbo Ferreira 228, 4050-313 Porto, Portugal

1 ABSTRACT

Two drying methods were used in fruits (oven at 70ºC and lyophilisation) and the effect of prolonged oven drying was evaluated regarding changes in nutritional composition, fatty acid profile and vitamin E content. Fruits were also compared with leaves.

Results showed that leaves had higher contents (%) of ashes, fat and protein while fruits had a higher amount of carbohydrates. Regarding vitamin E, α-tocopherol was, by far, the vitamer present in the highest amounts in all samples (89 to 95% of the total) with leaves presenting higher amounts (38.98 ± 2.40 mg/100 g) than fruits (16.79 ± 0.22 and 13.96 ± 0.99 mg/100 g for dried and lyophilized, respectively). The α-linolenic, linoleic, palmitic, stearic and oleic acids were common into leaves and fruits. The drying method only had a negative effect on the γ-tocopherol content, reducing it in half. Overall fruits are a good source of vitamin E and oven drying is a cheap method that can be used to preserve them or obtain powders that can be added to other products.

KEYWORDS

Fatty acid; vitamin E, drying effect; *Arbutus unedo*

2 Introduction

Edible plants and fruits have been, since ancient times, important sources of proteins, essential fatty acids and vitamins. In addition, many plants have in their composition compounds with potential for industrial, medicinal and/or pharmaceutical applications.

Fatty acids profile is important to maintain the cardiovascular, nervous, immune and reproductive systems and to produce prostaglandins, which regulate diverse body functions. Fatty acid deficiency is linked with serious health conditions, being of utmost importance, not only their intake, but also their balance (ω3:ω6) [1].

Related with the fat/fatty acids are Vitamin E contents. This fat-soluble vitamin main function is to prevent the oxidation of polyunsaturated fatty acids by breaking the oxidation chain. It is considered an essential micronutrient for the human body and is a powerful lipid-soluble antioxidant. It possesses cardio and neuroprotective properties, and helps to prevent cancer and hypercholesterolemia [2-4]. Vitamin E exists in eight different forms called vitamers, tocopherols (4) and tocotrienols (4). In humans, alpha tocopherol is the vitamer preferentially absorbed and stored [5]. α-tocopherol has an RDA (recommended daily allowance) of 15 mg for 14 years old and above, however this value can change if the amount of PUFA (polyunsaturated fatty acids) in diet is high [6].

Arbutus unedo L. (Ericaceae), known as strawberry tree (medronheiro) is common throughout the Mediterranean area, but can also be found on other parts of Europe and Africa [7-9]. Its fruits are red and sweet when ripe, presenting pre-ripening astringent characteristics and a yellow-green colour [10]. They represent a source of income to many small villages and companies in the country side, mainly as distilled beverages and jams [10]. The leaves are used in folk medicine as a diuretic, antiseptic and laxative, as well as to treat hypertension [11-13]. They are also described as possessing high antioxidant activities, due to the phenolic compounds present in its composition [14-17]. While their fruits have been previously characterized regarding vitamin E content and fatty acid profile, no work exists that studies the effect of oven drying on the fruit composition. This drying method may be a good option for preserving the fruits which are only available for 1 to 2 months every year. Additionally, no complete study of the leaves vitamin E contents has been performed previously.

The aim of this work was to characterize the products from the *Arbutus unedo* L. trees from the southern part of Portugal, regarding their vitamin E and fatty acids profiles for their possible inclusion on other food products. Thus two composite samples, one of leaves and one of fruits, collected from 18 different trees were tested. Leaves were dried at room temperature while fruits were dried in an oven at 70ºC, and also lyophilized to verify the effect of drying

on their fatty acid profile and vitamin E content and possible nutritional losses. Fresh fruits were also tested but only for their proximate composition, together with leaves and dried fruits.

3 Material and methods

3.1 Reagents and standards

Tocopherols (α, β, γ and δ) and tocotrienols (α, β, γ and δ) standards were purchased from Calbiochem (La Jolla, California, USA) and tocol (2-methyl-2-(4,8,12-trimethyltridecyl) was obtained from Matreya Inc. (Pennsylvania, USA). HPLC-grade *n*-hexane, anhydrous sodium sulfate (Na_2SO_4) and Kjeldahl tablets were purchased from Merck (Darmstadt, Germany); Absolute ethanol and sodium chloride (NaCl) from Panreac (Barcelona, Spain). Petroleum ether, sulphuric acid, 1,4-dioxane, Supelco 37 Component FAME Mix were purchased from Sigma Chemical Co. (St. Louis, EUA).

3.2 Equipment

The lyophilized fruit was obtained using a Cryodos lyophilizer from Telstar, Spain, while the heat drying process was carried in an oven from Binder, Germany. For moisture determination a moisture scale from Scaltec, Germany was used. For protein determination the digestion unit K-424 and distillation unit Kjelflex K-360 were both from Buchi, Switzerland. N_2 evaporation was carried in a sample concentrator model SBHCONC/1 from Stuart, UK. For ash determination the Muffle used was a Thermolyne 48000 purchased from Electrothermal Engineering Ltd, United Kingdom.

3.3 Plant material and sample preparation

Samples were collected on a dry day of November 2014 in Herdade da Corte Velada, Algarve, Portugal and transported to the lab in a thermic container. A total of 18 different trees, bearing fruits and with healthy leaves were selected. Leaves were air dried at ambient temperature while a portion of the fruits were dried at 60ºC during 72 hours. The samples were then ground to a powder with the aid of a kitchen mill and stored in a freezer at -20ºC. Another portion of the fruits was lyophilized and the remaining fruits were stored in a freezer at -20ºC without any treatment.

3.4 Proximate composition

Macronutrients were determined following the Association of Analytical Communities (AOAC) methods [18]. Results can be seen on table 1.

3.4.1 Moisture determination

Moisture content [18] was instrumentally determined using an infrared moisture analyzer (Model SMO 01, Scaltec Instruments, Germany). Briefly, approximately 1.5 grams of dried leaves and fruits and 4.0 grams of fresh fruit was weighted into an aluminium dish. The sample was spread with care to achieve an uniform coating on the dish and put under an infrared light at 100 ºC to achieve complete dryness. Results are presented as % of moisture and are presented as mean ± standard deviation, determinations were made in triplicate.

3.4.2 Ash determination

Ash content was determined according to the official method [18]. Briefly, for dried leaves and fruits, approximately 1 gram of each sample was weighted into porcelain crucibles. For the fresh fruit the weight was approximately 3 grams. The crucibles were then put into a muffle furnace and gradually heated up to 500 ºC. The sample was removed when the ashes became white and put into a desiccator to cool to ambient temperature after which it was weighted. Results are presented as % of dry sample as mean ± standard deviation of duplicate analysis.

3.4.3 Fat determination

Total fat was determined by Soxhlet method [18]. Briefly, approximately 5 grams of sample were weighted into a porcelain capsule, anhydrous sodium sulphate and treated sand were added, to remove moisture and ease sample dispersion. This mixture was then carefully transferred into a cellulose cartridge and extracted with petroleum ether for 8 hours. After extraction, the solvent was recovered by distillation and the extract put in an oven at 105 ºC to achieve full dryness. It was then transferred into a desiccator to cool to ambient temperature and weighted until constant mass. Results are presented as % of dry sample as mean ± standard deviation of triplicate analysis.

3.4.4 Protein determination

Protein content was determined according Kjeldahl procedure [18] (*N* x 6.25). A sample of approximately 1, 1.5 and 6 grams of dried leaves, fruits and fresh fruits, respectively, was weighted into a nitrogenless paper. This sample was transferred into a digestion tube and two Kjeldahl tablets were added together with 20 mL of H_2SO_4 and put into a digester at 420 ºC. After 90 minutes samples were removed from heating and put to cool down to ambient temperature. Distillation was done with NaOH and H_3BO_3 in an automatic distiller. After distillation was finished the sample was titrated with 0.2 M or 0.05 M H_2SO_4 depending on

the protein content, using metil red as indicator. Results are presented as % of dry sample as mean ± standard deviation of triplicate analysis.

3.4.5 Carbohydrate determination

Carbohydrates were determined by difference. It was considered that ash + fat + protein + carbohydrates = 100%. The results are presented as % of dry sample and are represented as mean ± standard deviation.

3.5 Fatty acid and vitamin E extraction

Fat extraction for the determination of fatty acids and vitamin E profiles was performed based on the method of Araujo et al. [19], with minor adjustments. Briefly, 1 g of each sample was weighed into amber Supelco® glassware vials, to which 20 µL of tocol (internal standard, IS, for vitamin E determination, 1 mg/mL,) were added. Extraction was performed (in duplicate) with 10 mL of *n*-hexane and 5 mL of absolute ethanol, under constant stirring, at room temperature for 30 minutes. Subsequently, 5 mL of NaCl (1%) were added and the final solution was centrifuged at 5000 rpm for 2 minutes. The left residue was twice re-extracted with 10 mL of *n*-hexane. Organic phases were combined and an adequate amount of anhydrous Na_2SO_4 was added. Extracts were taken to dryness under a N_2 stream, re-suspended with 1 and 3 mL of *n*-hexane and stored (-20ºC), for further fatty acids and vitamin E determination, by GC and normal phase HPLC, respectively.

3.5.1 Individual fatty acid methyl esters determination

Individual fatty acid methyl esters (FAME) determination was performed based on ISO 12966-2:2011[20]. Briefly, 2 drops of the extracted oil were mixed with 2 mL of n-Hexane, 200 µL of a 2 M methanolic KOH solution and vortexed for 1 minute. Na_2SO_4 was then added in enough quantity to remove any lingering humidity and the solution was vortexed for 1 minute. The solution was allowed to stand and 1 mL of the organic phase was transferred into injection vials.

A GC with a CP-Sil 88 silica capillary column for FAME (50 m x 0.25 mm i.d, 0.20 µm film thickness, purchased from Varian, Netherlands) coupled with a split/splitless Shimadzu AOC-20i auto-injector (Shimadzu, Japan) and with a flame ionization detector (FID) (Shimadzu, Japan) was used. The carrier gas used was helium, at a flow rate of 40 mL/min. The temperature program used was: 120 °C, for 5 min, with gradual increase to 220 °C at a rate of 3°C per min, and a constant temperature of 220 °C during 10 min. Injector and detector temperatures were 250 and 270 °C, respectively and the total run time was 48.33

min. A split ratio of 1:25 was used and the injection volume was 1.0 µL. Identification of the fatty acids was done based on comparison with a FAME standard that consisted in a mixture of 37 saturated and unsaturated fatty acids. Data were analysed using the Shimadzu software GC Solution (Shimadzu, Japan) based on the relative peak areas. Injections were made in duplicate. Results are presented on table 2 as % of total fatty acids.

3.5.2 Vitamin E vitamer determination

The previously obtained extracts were centrifuged and transferred into injection amber Supelco® vials. Chromatographic analysis was performed using an integrated HPLC system (Jasco, Japan) equipped with two PU-980 pumps, an AS-2057 automated injector, coupled with a MD-2018 multiwavelength DAD and a FP-2020 fluorescence detector. The separation of the compounds was carried out in a Supelcosil™ LC-SI normal phase column (3 µm; 75 mm × 3.0 mm; Supelco, USA), kept at room temperature. Mobile phase consisted of a mixture of *n*-hexane:dioxane (98.5: 1.5, *v:v*), which was pumped in isocratic mode at 0.8 mL/min. The sample injection volume was 20 µL. Data were analysed using JASCO-ChromNAV software (version 1.18.03; Jasco, Japan).

The quantification of compounds was based on the internal standard method, using the fluorescence signal response of each standard converted to concentration units through the calibration curves. These were obtained by preparing a standard stock solution containing individual compounds (α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol) in *n*-hexane, subsequently diluted (from 25.0 to 1.25 µg/mL). Each solution contained 20 µL of tocol (1 mg/mL). The compounds were identified based on their UV/vis spectra and by the comparison of their retention time with those of the standards. Injections were made in duplicate. Results are presented in table 3 as µg/g of extracted fat, mg/100 g of sample and % of total.

3.6 Statistical analysis

To determine the significant differences among the results, ANOVA with the post-hoc test LSD or Games-Howell was used, after checking the homogeneity of variances with Levene's test.

4 Results and discussion

4.1 Proximate composition

4.1.1 Moisture

Results for the proximate composition (Table V–1) show that fresh fruit was the sample with the highest amount of moisture, reaching almost 70%, followed by the lyophilized fruit, dry leaf and finally as expected, due to the higher temperatures used, the dry fruit which had only around 3% of moisture. The high moisture % of the fresh fruits is one of the reasons why these fruits get easily spoiled if no treatment is applied. To reduce the moisture % two different drying methods were tested, one involving high temperature but much simpler, cheaper and easier to accomplish, and the other involving more expensive equipment but low temperatures to prevent any temperature related degradation.

Table V–1. Proximal analysis of leaves and fruits.

		Dry leaf	Dry fruit	Lyophil. fruit	Fresh fruit
Moisture	(%)	6.76 ± 0.02	3.05 ± 0.01	7.55 ± 0.01	68.38 ± 0.08
Ashes	(g/100 g dw)	3.81 ± 0.04	2.09 ± 0.03	1.93 ± 0.04	1.91 ± 0.07
Fat	(g/100 g dw)	6.63 ± 0.36	2.12 ± 0.06	2.11 ± 0.07	2.10 ± 0.05
Protein	(g/100 g dw)	16.35 ± 0.24	1.88 ± 0.06	1.92 ± 0.01	1.95 ± 0.02
Carbohydrates	(g/100 g dw)	73.21 ± 0.16	93.87 ± 0.10	94.02 ± 0.03	94.04 ± 0.04

Results are presented as mean ± SD of three repetitions.

4.1.2 Ash

Ashes can be determined using either a dry or wet method, in this work the dry method was used, and refers to inorganic compounds [21]. According to Vidrih et al. [22] the major minerals present in the fruits are potassium, calcium, sodium and phosphorous. While ashes represent usually less than 5%, they have a high impact on nutrition and food technology [21]. Dry leaves had the highest amount of ashes, with 3.8%, almost double of the fruit content, which ranged from 1.9 to 2.1%, with no changed caused by the drying method. When compared to fruits present in the Agricultural Research Service National Nutrient Database from the United States Department of Agriculture [23] which contains information on a total of 73 fruits and fruit products, of which 26 are raw unprocessed fruits, the non-dry ash % of the fresh fruits studied in this work, 0.61%, ranks on the top 10 and is only lower than that of plantains, breadfruit, currants, passion fruit, ground cherry and figs, whose ash content varies between 1.17 and 0.66. When the value of the dry fruit is used to compare with other dry fruits, only dried longans and currants have a higher ash %. Commonly consumed fruits such

as orange (0.60 to 0.42), grape (0.57 to 0.50), papaya (0.39) tangerine (0.38) pear (0.38), mango (0.36) and apple (0.17) all have a lower ash % with the average of all the raw fruits on the database at 0.54%.

4.1.3 Fat content

Crude fat is a main dietary requirement which provides and stores energy along with many fatty acids that are essential for humans. Once again leaves had a higher amount than fruits, which were not affected by the drying method, with 6.63% versus 2.10%. While leaves had a higher crude fat content, since they cannot be directly eaten and water does not extract lipids, fruits are once again a better source than leaves. When compared to other fruits using the Agricultural Research Service National Nutrient Database and the fruit value 2.10%, the fruit ranks far below number 10, and has a much lower content than avocados (54.7%), horned-melon (11.4%), carissa (8.2%), rowal (7.0%), pomegranate (5.3%) guava (4.9%), groundcherries (4.8%) gooseberries (4.8%) , acerola (4.5%) or blackberries (4.1%), which are much better fat sources than *A. unedo* fruits [23].

4.1.4 Protein content

Proteins are the source of amino acids, essential and non-essential. They are also a source of energy, although not a preferred one, and are crucial in the diet. The protein content was the parameter measured in the proximal analysis where leaves and fruits showed the biggest difference. While ash and fat contents were only 2 to 3 times bigger on leaves than on fruits, on proteins it was 8 times, with leaves having a value of 16.35% and fruits having 1.92 (dried), 1.93 (fresh) and 1.95% (lyophilized). This higher difference is most likely due to the fruits preferential accumulation of sugars during ripening to prepare for the seeds future germination, which does not happen on leaves. Leaves on the other hand accumulate proteins which are used to facilitate membrane transport, energy generating reactions and control the production of other phytochemicals such as phenols which are highly present on leaves. In addition, since proteins life span is finite but their demand must always be met, plants must have a supply available of all 20 amino acids for plant growth and development to occur, mainly through foliage growth. All of this helps to explain the difference in protein content between fruits and leaves. Once again the drying method caused no change on the contents of the fruits. Compared with other fruits present on the Agricultural Research Service National Nutrient Database, *A. unedo* fruits are not a good protein source [23].

4.1.5 Carbohydrate content

Carbohydrate content was determined by subtracting the other main compounds, ash, water, fat and protein from the total. While disregarding humidity, all fruits presented a similar amount of carbohydrates which was higher than that of leaves. This difference was to be expected since fruits normally demand a high amount of carbohydrates in order to supply the seeds with the needed sustenance for them to germinate as mentioned before. According to Barros et al. [24] the majority of the sugars present in *A. unedo* fruits are fructose and glucose, although sucrose is also present. On leaves they are most likely composed of large amounts of cellulose which is a structural carbohydrate and a main compound present on leaves. When humidity is taken into account, all samples show differences in their carbohydrate amounts. Dry fruit is the sample with the highest amount (91.00%), followed by lyophilized fruit (86.92%), dried leaf (68.26%) and finally fresh fruit (29.74%) (data not shown). When compared with fresh fruits present in the USDA database, only plantains have a higher carbohydrate %, with 31.89. Commonly consumed fruits such as orange (15.50 to 11.89), grape (17.50 to 13.93), papaya (10.82) tangerine (13.34) pear (15.23), mango (14.98) and apple (12.76) all have a lower content, with the average of all the raw fruits on the database at 15.63%. When compared with the fresh fruits but considering humidity at 0% (with the ash, fat and protein values properly transformed) apples (95.72), followed closely by pears (94.88) and persimmons (94.41) are the only fruits with higher carbohydrate amount [23].

Overall the results for the proximate composition of the leaves and fruits of *A. unedo* were similar to those obtained by Barros et al. [24], Morales et al. [25] and Vidrih et al. [22] although the fruits used in this work had slightly higher fat content (2.10 vs 1.37 g/100 g dw) but lower protein (1.95 vs 3.09 g/100 g dw) [24] and higher moisture [22,26], but not as high as the moisture content of the fruits analysed by Fonseca et al. [27]. The fruits are a good carbohydrate source, but only an average source of proteins and fat. Additionally the drying method caused no appreciable change on the nutritional composition of the fruits.

4.2 Fatty acids determination

4.2.1 Leaf and fruit quantification

The structure of the detected fatty acids can be seen on Figure V–1. A total of 5 fatty acids on fruit samples and 8 on leaves were detected (Figure V–2). These include the essential α-linolenic and linoleic acids.

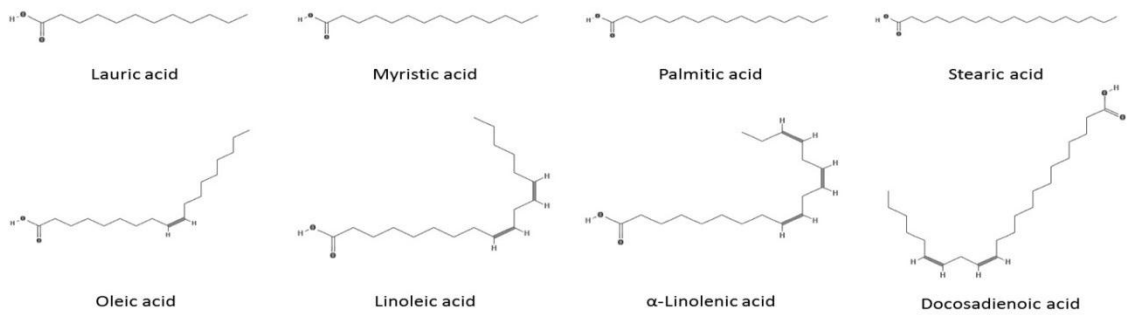

Figure V–1. Structures of the detected fatty acids.

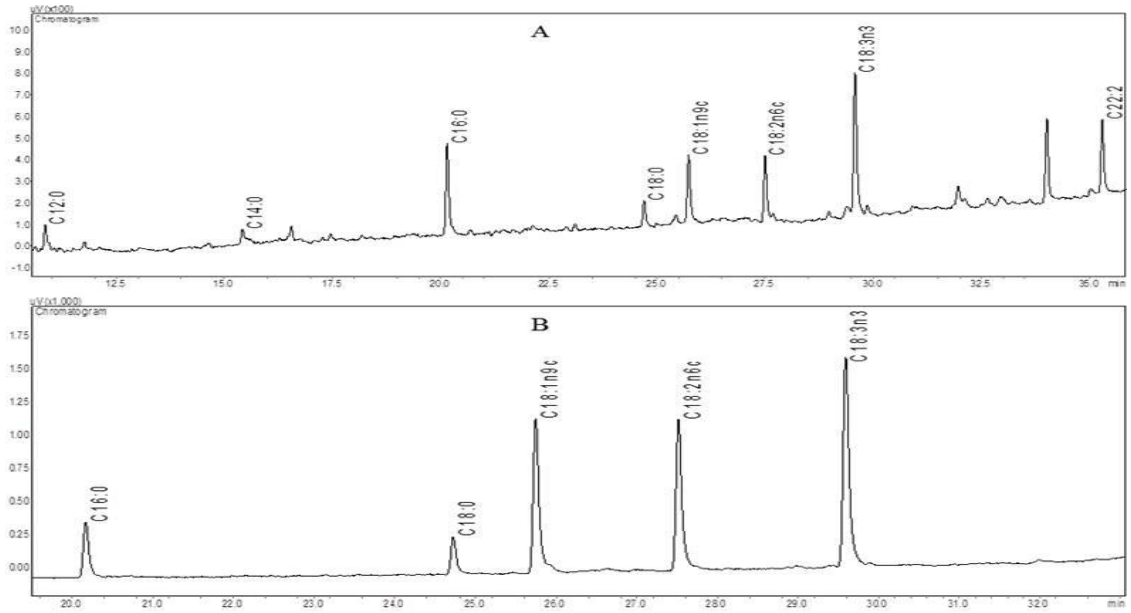

Figure V–2. GC-MS chromatogram. A is leaf, B is fruit.

Palmitic (C16:0), stearic (C18:0), oleic (C18:1ω9c), linoleic (C18:2ω6c) and α-linolenic (C18:3ω3) acids were present on both fruits and leaves while lauric (C12:0), myristic (C14:0) and docosadienoic (C22:2ω6) acids were only detected on the later (table 2). α-linolenic acid was the most abundant fatty acid on all samples, accounting for over 30% of the total (Table V–2). α-linolenic (36.94%), linoleic (25.38%) and oleic acids (24.43%) accounted for over 75% of the total fatty acids on fruits. On the other hand, α-linolenic (30.26%), palmitic (18.19%) and docosadienoic (14.13%) acids were the most abundant fatty acids on leaves and accounted for 62% of the total. Similarly to what Delgado et al. [28] found for chestnut drying, oven drying had no effect on the fatty acid composition of fruits. Of the detected fatty acids the most important are α-linolenic and α.linoleic, both essential fatty acids. The first is an ω3 and precursor to eicosapentaenoic and docosahexaenoic fatty acids, both important in maintaining health [29].

Table V–2. Fatty acid content of leaves and fruits.

Fatty acid	Dry leaf	Dry fruit	Lyophil. fruit
		Fatty acid %	
C12:0	3.35 ± 0.15	n.d.	n.d.
C14:0	2.05 ± 0.03	n.d.	n.d.
C16:0	18.19 ± 0.42	8.46 ± 0.22	9.66 ± 0.69
C18:0	4.95 ± 0.18	4.79 ± 0.33	5.91 ± 1.24
C18:1ω9c	13.49 ± 0.06	24.43 ± 0.21	23.37 ± 0.21
ΣMUFA	13.49 ± 0.06	24.43 ± 0.21	23.37 ± 0.21
C18:2ω6c	13.56 ± 0.16	25.38 ± 0.14	24.68 ± 0.40
C18:3ω3	30.26 ± 0.46	36.94 ± 0.29	36.37 ± 1.35
C22:2ω6	14.13 ± 0.10	n.d.	n.d.
ΣPUFA	57.96 ± 0.46	62.31 ± 0.31	61.06 ± 1.70
Σsaturated	28.55 ± 0.51	13.25 ± 0.47	15.57 ± 1.84
Σunsaturated	71.45 ± 0.51	86.75 ± 0.47	84.43 ± 1.84
ω3/ω6	1.09 ± 0.02	1.48 ± 0.04	1.45 ± 0.02

Results are presented as mean ± SD of two repetitions from two replicates.

The second is an ω6, precursor to arachidonic acid and important in maintaining the skin water permeability barrier [30]. As the precursor of the arachidonic acid it has importance in the production of many regulatory factors and in controlling inflammation and the immune system, however when in excess problems may arise [31]. Oleic acid is also important since it is the main fatty acid stored in human fatty tissue [32]. Its consumption is believed to lower cholesterol levels [33] and while some defend its benefits against cancer [34] others have studied its effect on breast cancer development [35]. On both dried and lyophilized fruits the unsaturated fatty acids accounted for around 85% of the total, of which 70% were polyunsaturated, which is in agreement with the results obtained by Morales et al. [25]. On the other hand leaves had a lower total percentage of unsaturated fatty acids, around 70%, but over 80% of these were polyunsaturated. Saturated fatty acids are usually considered less desirable from a health point of view than unsaturated ones, since it is believed they promote the formation of LDL which can lead to clogged arteries and heart problems, although some studies defend it is not so linear [36-38]. They defend that not all of the saturated fatty acids promote heart problems, with the main difference being in the size of the LDL formed [39,40].

One of the main sources of PUFAs is the consumption of fish or fish oil. However there are several limitations to using fish oil such as the presence of contaminants, antibiotics, very strong odour, flavour and taste which are considered by many to be undesirable. Finally, stability problems, high cost and decrease of fish stocks mean alternative PUFA sources are

needed [30]. Other sources include microorganism synthesized PUFAs, microalgae, animals (mainly herbivores and their products), and plants, which are the best source of α-linolenic acid, the essential ω3 fatty acid [30].

When Oliveira et al. [41] studied the fruits fatty acid composition at different ripening stages they also detected C16:0 (5.92 to 6.73%), C18:0 (3.72 to 4.67%), C18:1ω9 (26.75 to 29.38%), C18:2ω6 (18.84 to 20.14%) and C18:3ω3 (36.90 to 43.07%) in similar amounts to those detected on the present work. Additionally, they detected C14:0, C15:0, C16:1n7, C16:1ω9, C20:0, C18:3ω6, C20:1ω9, C20:2ω6, C22:1ω9 and C24:0, each accounting for less than 1% of the total. Also similar were the results of Morales et al. [25], which detected mostly C18:1ω9 (24.82%), C18:2ω6 (24.22%) and C18:3ω3 (31.26%), with C16:0 accounting for 10.54% and C18 for 4.32%. Additionally they also detected C6:0, C8:0, C10:0, C11:0, C12:0, C14:0, C16:1, C17:0, C18:3ω6, C22:0 and C24:0, each accounting for less than 2% of the total. Barros et al. [24] identified C16:0 (8.20%), C18:0 (4.00%), C18:1ω9 (21.01), C18:2ω6 (21.50%) and C18:3ω3 (36.51%) as the main fatty acids, which were present in similar amounts to those detected in the present work. Also similar were the results of Delgado-Pelayo et al. [26] when they investigated the fatty acid composition of lyophilized fruits collected in Spain. They detected palmitic (10.71%), stearic (5.31%), oleic (24.57%), linoleic (24.41%) and linolenic acids (30.94%) in similar amounts. Additionally, they detected lauric, myristic, arachidic and gadoleic acids, which were not detected on the present work. Additionally, they also detected C6:0, C8:0, C10:0, C12:0, C15:0, C16:1, C17:0, C20:0, C20:1, C20:2, C20:3ω3 + C21:0, C22:0, C23:0 and C24:0 in low quantity. On the other hand, Fonseca et al. [27] results were quite different. They had a much higher proportion between C16:0 and C18:2+C18:3 than we did, with their C16:0 varying between 3 to 23 mg/100 g fresh fruit while the C18:2+C18:3 varied between 1 and 4 mg/100 g fresh fruit. Additionally, they found traces of C12:0, C18:0 C22:0, C24:0 and C16:1 before alkaline hydrolysis and traces of C6:0, C8:0, C9:0, C10:0, C13:0, C15:0, C17:0 C20:0 and C23:0 after hydrolysis. The location played a role on the main fatty acids detected, with different contents for C16:0 (3 to 23 mg/100 g fresh fruit), C18:0 (traces to 5 mg/100g fresh fruit), C18:2+C18:3 (1 to 4 mg/100 g fresh fruit) and in particular C18:1ω9 which was only detected on two samples after alkaline hydrolysis. They explain these differences with both intrinsic and extrinsic factors, such as genetic makeup of the trees or climate which could also explain the differences between our results and theirs.

Fruits of *A. unedo* have some similarities with other fruits but also some differences. When compared with blackthorn and rose fruits, the main differences were the higher C18:2ω9 of

blackthorn (57.58%), higher C18:2ω6 of rose (39.51%) and lower C18:3ω3 of both (2.79 and 26.33% respectively) [24]. When Morales et al. [25] studied other fruits (*Crategus monogyna, Prunus spinose* and *Rubus ulmifolius*) the main differences were the higher SFA due to C16:0 on *C. monogyna* (30.56%) and *P. spinosa* (23.42%), the lower C18:2ω6 on the same fruits (10.52 and 14.18%) but higher on *R. ulmifolius* (48.56%) and lower C:18:3ω3 in all of them (16.10, 10.58 and 13.28% respectively). *A. unedo* fruits have half of the C16:0 (14 to 18%) and C18:1ω9 (48 to 64%), but have a much higher content in C18:3ω3 (2 to 7%) and slightly higher C18:2ω6 (14 to 26%) when compared to *Quercus* fruits. According to Vinha et al. [42] they also possess a much higher PUFA/MUFA ratio.

Regarding leaves, the fatty acid composition is not as studied, but Dib et al. [43] determined leaves to possess C12:0, C14:0, C16:0, C18:2ω6, C18:2ω3 and C18:1ω9, which is in agreement with the present work. Also similar was the C18:3ω3 and C18:2ω6 proportions. Unlike us however, they also detected C13:0, C15:0, C17:0, C20:0 and C22:0, in quantities similar to that of C14:0. In that work C16:0 and C18:1ω9 accounted for over 50% of total fatty acids detected. Koukos et al. [44] also studied *A. unedo* leaves and obtained slightly different results. Similarly to us they detected C14:0 (5.3%), C16:0 (25.5%), C16:1 (0.8%), C18:0 (2.8%), C18:1 (6.8%), C18:2 (7.9%), C18:3 (44.2%) and C20 (1.3%) but there was a big difference regarding the proportion between C18:2/C18:3 and the C16:0 amount (much higher on their work). These differences are likely due to the origin, location, extraction and drying methods (they collected leaves from Athens 370 m above sea level while we collected between 110 and 130 m above sea level, dried at 60°C while we used ambient temperature and extracted with chloroform-methanol while we used *n*-Hexane and ethanol).

4.2.2 ω3:ω6 balance

Another difference between leaves and fruits was the ω3:ω6 balance. While fruits proportion was close to 1.5, leaves proportion was close to 1, due to the presence of docosadienoic acid. These ratios are similar to what other authors detected [41,26,24,43,25]. The ω3:ω6 ratio is important because ω6 fatty acids have regulatory effect in low amounts with studies even suggesting that a slightly higher intake on ω6 leads to lower heart disease risk, but when high amounts are consumed its effects become harmful [45] due to their function as prothrombotic and proaggregatory stimulants. An increase in the intake of ω3 fatty acids leads to a partial replacement of ω6 fatty acids (especially arachidonic acid) on cell membranes, causing a decreased production of prostaglandin E2 metabolites. It also decreases the production of thromboxane A2, a potent platelet aggregator and vasoconstrictor, replacing

it with thromboxane A3, which is weaker, and replaces leukotriene B4 which is a strong inflammation inducer with B5, a weak inducer of inflammation and chemotactic agent [45,46]. It should be noted that many vegetable sources including most nuts, seeds and vegetable oils have ω3:ω6 ratio lower than 1. Soybeans and tofu (two very popular products) ω3:ω6 proportion is also much lower than 1 [23], and . The fact that ω6 tends to be present in higher amounts than ω3 in so many widely consumed products, results the importance of finding sources like the fruits tested on this work, since their consumption can help to offset all the rich sources of ω6 in the common diet, allowing the balance to move towards the ω3 end.

Overall the results show that drying method had no effect on the fatty acid profile of fruits. Both leaves and fruits have very high unsaturated fatty acid content with the majority of them being polyunsaturated and with both essential fatty acids representing over 60% of the total fatty acids of fruits and 40% of leaves. Other than these two, they also possess oleic acid. In addition, they have a high ω3:ω6 balance, and can therefore be used to offset the effects of the rich ω6 modern diet.

4.3 Vitamin E profile

The results obtained for the individual vitamin E vitamers are presented on Table V–3 as μg per gram of extracted fat, as mg per 100 grams of used sample and as % of the total vitamin E detected. The chromatograms obtained can be seen on Figure V–3. A total of 5 vitamers were detected on leaves versus 4 on fruits. α-tocopherol, β-tocopherol, γ-tocopherol, and γ-tocotrienol were detected on both of them while β-tocotrienol was only detected on leaves. While several vitamers were detected, α-tocopherol relative amount (between 89 and 96%) was higher than all the other vitamers combined, with the next most abundant vitamer, γ-tocotrienol, accounting for less than 6%. α-tocopherol is the vitamer with the highest biological activity and the highest half life time (73 to 81 hours), especially if compared with tocotrienols, which when exogenously administered have a half-life of less than 5 hours [5]. This means that for people lacking vitamin E supplementation should be done mostly with α-tocopherol [5]. For the first time the fruit drying method made a significant difference, with the air drying method causing a 40% decrease of γ-tocopherol levels. This sensitivity of γ-tocopherol to drying procedure was also seen on chestnuts, indicating the higher sensitivity of this vitamer [28] However, due to this vitamer low amount, the vitamin E levels remained mostly unaffected. When the results are analysed using the mg/100 g of sample instead of μg/g extracted fat it is possible to see differences between the dry and lyophilised fruits.

Table V–3. Vitamin E profile of leaves and fruits.

Vit E vitamer	µg/g of extracted fat			mg/100g of sample used			%		
	Dry leaf	Dry fruit	Liofilized fruit	Dry leaf	Dry fruit	Liofilized fruit	Dry leaf	Dry fruit	Liofilized fruit
A	5879.43 ± 361.7	7918.2 ± 98.78	6615.16 ± 467.36	38.98 ± 2.4	16.79 ± 0.22	13.96 ± 0.99	95.79 ± 2.1	92.13 ± 0.03	89.92 ± 0.16
B	47.55 ± 1.38	49.67 ± 3.90	47.50 ± 4.04	0.28 ± 0.02	0.10 ± 0.01	0.08 ± 0.00	0.55 ± 0.27	0.57 ± 0.02	0.54 ± 0.03
C	9.59 ± 0.57	147.18 ± 7.95	360.21 ± 9.15	0.06 ± 0.00	0.31 ± 0.01	0.64 ± 0.05	0.11 ± 0.06	1.68 ± 0.03	4.13 ± 0.04
D	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
E	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
F	105.39 ± 4.85	n.d.	n.d.	0.63 ± 0.06	n.d.	n.d.	1.22 ± 0.62	0.00 ± 0.00	0.00 ± 0.00
G	200.38 ± 11.06	492.60 ± 20.56	470.90 ± 25.91	1.20 ± 0.06	1.02 ± 0.01	0.84 ± 0.04	2.33 ± 1.15	5.62 ± 0.05	5.40 ± 0.14
H	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total	6206.87 ± 381.39	8594.22 ± 139.25	7356.2 ± 478.99	41.15 ± 2.53	18.22 ± 0.24	15.52 ± 1.07			

Results are presented as mean ± SD of two repetitions from two replicates

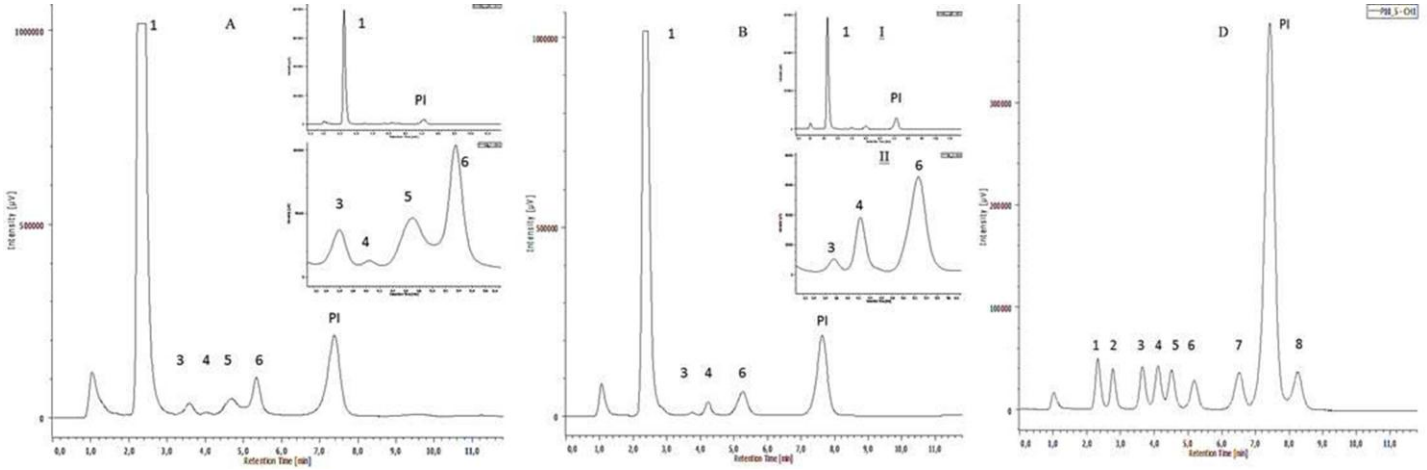

Figure V–3. HPLC chromatogram. A is leaf, B is fruit, PI is internal standard. (α-tocopherol (1); β-tocopherol (3); γ-tocopherol (4); δ-

This difference was due to the characteristics of the lyophilized fruit which tended to form small clumps that made the extraction slightly less efficient. Several authors have previously quantified tocopherols and tocotrienols on the fruits of *A. unedo*, with the results ranging greatly. However when comparing with authors that used similar extraction methods, the results obtained in this work are in the same range. Barros et al. [24] found the fruits α-tocopherol content to be similar to ours, at 21.98 mg/100 g dw. Also similar were the much lower β-tocopherol (0.44 mg/100 g dw) and γ-tocopherol (1.03 mg/100 g dw) contents and the absence of δ-tocopherol. Their total content was 23.46 mg/100 g dw while ours was 18.22 mg/100 g dw. Fonseca et al. [27] also had similar results regarding α-tocopherol content (4 mg/100 g fw) which when removing the 69% moisture detected on that work translates into 13 mg/100 g dw. However in their case the fruits had 50% more γ-tocopherol than α-tocopherol, which is in stark contrast with what happened in the current work. While this proportion was different from location to location, all fruits collected in that work had a higher or equal amount of γ-tocopherol than they did of α-tocopherol. Morales et al. [25] also studied fruits and detected all 4 tocopherol vitamers. Similarly, to us however the vitamer in highest amount was α-tocopherol with 3.49 mg/100 g fw, which at a 57.56% moisture translates into 8.22 mg/100 g dw, about half of what we detected in the present work and accounting for the majority of the content detected (9.14 mg/100 g dw). On the other hand, *Crataegus monogyna*, *Prunus spinose* and *Rubus ulmifolius* fruits had a total of 10.89, 16.94 and 54.16 mg/100 g dw, respectively. It should however be noted that for *R. ulmifolius* α-tocopherol only accounts for 13.58 mg/100 g dw, with the rest being γ and δ-tocopherols, which have a lower half-life time and lower activity. When Oliveira et al. [41] extracted oil from the fruit seeds they detected a higher proportion of γ-tocopherol than we did, with its content ranging between 12.09 and 68.89 and α-tocopherol ranging between 21.16 and 271.47 μg/g of extracted fat. These contents were both lower than what we detected on our fruits. On the other hand γ-tocotrienol content, the main vitamer in their work, was much higher than what we detected, ranging from 556.60 to 1368.58 μg/g of extracted fat. Pallauf et al. [47] also studied the fruits and found their vitamin E content, measured as α-tocopherol, to be much lower, 0.023 mg/100 g of fresh fruit. Leaves vitamin E contents are not as well studied as fruits, but Munné-Bosch, Peñuelas [48] found their α-tocopherol to range between 1.2 and 2 μmol/g dw which translates into 51.7 to 86.1 mg/100 g dw, about twice the content we detected on the current study.

The differences detected were most likely due to extraction procedures, some authors re-extracted two times such as Barros et al. [24] while others like Pallauf et al. [47] only

extracted once and with different solvents. Additionally, tocopherol content can vary from location to location [27], likely due to the different stresses plants are subjected to, since as demonstrated by Munné-Bosch, Peñuelas [48] water stress has a significant effect on tocopherol content. It is also possible that different plants cope with that stress differently. One other factor that can influence the tocopherol content is the ripening of the fruits, with contents being higher on unripe fruits [41].

Considering that the vitamer most abundantly present on the tested samples is α-tocopherol which is the one primarily accumulated by the human body, and the levels in which it was detected, the fruits can be an exceptional source of vitamin E, especially for people that may need vitamin E supplements, but also to the population in general.

5 Conclusion

Leaves and fruits were both rich in vitamin E, mainly α-tocopherol, and had similar α-linolenic acid contents, with fruits being richer in polyunsaturated fatty acids. The use of oven drying at 70ºC only affected significantly ($p<0.05$) the content of γ-tocopherol, but its amount is so low compared to the most abundant vitamer that this drying method can still be considered adequate, and has the advantage of being much cheaper than lyophilisation. The most remarkable characteristic of the fruits was their vitamin E content, mainly in the form of α-tocopherol. They also possessed a better ω3:ω6 ratio and were richer in carbohydrates. Overall *A. unedo* fruits can be considered an excellent source of vitamin E and can be used in dry form to enrich other food products.

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Diabetes related enzymes inhibitory activity of *Arbutus unedo* L. extracts with focus on the influence of α-amylase origin

Ricardo Nunes[1], and Isabel S. Carvalho[2,3]

[1]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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1 ABSTRACT

One of the treatments for type 2 diabetes, a condition affecting millions of people, is the intake of substances that inhibit complex carbohydrate digestion, such as acarbose. In this work we study the antidiabetic potential, measured as *in-vitro* inhibition of three different α-amylases and α-glucosidase caused by *Arbutus unedo* leaf, fruit and flower extracts obtained with different conditions, and their inhibition mechanism. Overall, extraction conditions had mild influence in the results while the α-amylase source and plant part had a high impact. The most powerful plant part (leaves) inhibited α-amylase (IC_{50} of 173.80 ± 34.33 μg/mL) via a competitive mechanism and inhibited α-glucosidase (IC_{50} of 4.39 ± 0.34 μg/mL) by uncompetitive and non-competitive mechanisms. Overall, *A. unedo* leaf and flower extracts have a high potential to be used as antidiabetic agents due to their inhibition of mammalian α-amylases and extremely low concentration required to inhibit α-glucosidase.

KEYWORDS

Antidiabetic; α-amylase; α-glucosidase; inhibition type; *Arbutus unedo*

2 Introduction

Diabetes mellitus is a condition affecting men and woman throughout the world. While it is more common on older people, it can manifest itself at any age. This condition is characterized by lack or ineffective insulin which causes abnormal glucose levels. While glucose is necessary as an energy source and for proper functioning of the human body, with the brain alone is consuming approximately 20% of the total glucose-derived energy

(Mergenthaler et al., 2013), its accumulation in the blood can brink severe consequences. These consequences are related to the change in the blood pH towards more acidic levels, known as ketoacidosis, which can lead to kidney failure, heart attack, vision loss, nerve damage and slow wound healing (Forbes and Cooper, 2013). Glucose is obtained from the diet, either as a simple sugar of after digestion. This digestion begins in the mouth with starch being digested by amylase and continues throughout the body, especially in the intestine, where α-amylase from pancreatic origin will come in contact with the chyme, together with α-glucosidase, another enzyme related with carbohydrate digestion. After the digestion, glucose passes into the blood and is delivered to all the cells of the body with the help of insulin, produced in the pancreas. Insulin also induces the absorption of glucose present in the blood into the skeletal muscle, liver and adipocyte cells, for energy storage as glycogen or fat. In addition, when present at high concentrations it acts as an inhibitor towards the production and excretion of glucose by the liver cells (Girard, 2006). When this hormone is not present in enough quantity, or its efficiency has been reduced, the levels of blood sugar cannot be regulated and diabetes arises. Depending on the reason for the lack of hormone in enough quantity, diabetes can be divided into either type 1 or type 2. There is another type which can only occur in pregnant woman called gestational diabetes (Rother, 2007).

Type 1 diabetes, is characterized by insufficient or lack of insulin production and therefore requires daily administration of insulin. Its causes are not totally known, it can be congenital, meaning its symptoms can appear on early childhood, or it can trigger after an infection by a virus or bacteria. The infection of genetically predisposed individuals can cause the white cells, one of the body defences against pathogenic microorganisms, to attack the insulin producing beta cells in the pancreas, leading to diminished or absence of insulin production (Rother, 2007).

Type 2 diabetes, which was previously called adult diabetes to distinguish the fact that symptoms would appear at a latter age, can affect people at any age, however it is more prevalent in middle-aged or older individuals. The first step is usually the development of insulin resistance, meaning while there is production of insulin, cells don't properly use it to help in transporting glucose to their interior. This lack of efficiency leads to an increased production of insulin by the pancreas which in the early stages can keep up with the added demand. However if this overstress in insulin production continues, the pancreas will reach a point where it can no longer keep up with the demand, leading to higher blood glucose levels. Unlike type 1 diabetes which is not preventable with current knowledge, type 2 diabetes, the most prevalent type, can be prevented with exercise and appropriate diet. This is

accomplished via the reduction of fat and sugar intake, therefore also reducing the insulin levels required, which helps to prevent or alleviate this condition (Rother, 2007).

There is another type of diabetes which can only occur on pregnant woman. This appearance is related to certain hormones produced. At the late pregnancy stage, women may start to be insulin resistant and if the pancreas cannot keep up with the demand then it is said the woman has developed gestational diabetes. This is more common in overweight woman or those that gain a lot of weight during pregnancy. Often this type of diabetes disappears when the baby is born but it increases the chance of developing diabetes type 2 at a later stage in life. Their babies are also more likely to develop type 2 diabetes (Banerjee et al., 2016).

2.1 Detecting diabetes

The detection of diabetes is paramount to the health of the individual, giving the risk it poses if left untreated. Individuals with diabetes can exhibit several symptoms that can arise suspicion of being diabetic. Such symptoms include abnormal thirst, hunger and tiredness, blurry vision, dry skin and blurry eyesight. However it is also possible that no symptoms are present. The CDC (Centers for Disease Control and Prevention) lists the following as risk factors: overweight, having a direct relative with diabetes, a history of gestational diabetes, high blood pressure, low HDL and high triglyceride levels and lacking physical activity (Stanifer et al., 2016). Diabetes type 2 also affects people of different ethnicity differently, with people of south Asian and African origins being at a higher risk (Zaini and Abdulsatar, 2016). If the blood test accuses the individual of having prediabetes (high glucose blood level but not yet high enough to be considered diabetic) it is possible to act and prevent the development of diabetes with controlled diet and exercise (Mayor, 2016).

2.2 Prevalence of diabetes

A report published by CDC regarding 2014 estimates that a total of 29.1 million US citizens have diabetes. Of these, 21 million have been diagnosed (2014). This number is equivalent to almost 10% of the entire US population of which over 25% are unaware. The same report estimates over 30% of the US population has prediabetes. Another report published by WHO (World Health Organization) regarding diabetes in Europe shows that approximately 10% of the entire European population, which translates into 60 million people, has diabetes (WHO, 2016). Worldwide, the WHO report estimates a total of 422 million people are afflicted by this condition, mostly in developing countries, with a rise on the percentage of younger people with the condition, from 1980 till date. The same report estimates diabetes to have directly caused 1.5 million deaths in 2012 and a total of 2.2 to 3.4

million deaths due to high glucose levels. These deaths are concentrated in low and middle-income countries, whose deaths account for 80% of the total. Not only are the numbers distressing, WHO, based on and article by (Lopez and Mathers, 2006) estimates the deaths caused by diabetes to double between the years 2005 and 2030.

2.3 Preventing and treating diabetes

Type 1 cannot be prevented, due to the lack of knowledge regarding what the genetic markers and triggers actually are. Type 2 diabetes however can be prevented. Several studies have shown there is a relation between obesity and type 2 diabetes, and therefore regular physical activity helps in its prevention (Mayor, 2016). For the same reason weight loss, if necessary, and healthier eating can also decrease the risk of developing type 2 diabetes. Due to the increasing number of individuals afflicted with diabetes, the extra cost it has both for the families and the health services and the increasing lack of exercise practice in many communities of the modern world, several institutions are involved in spreading the information about the problems and how to prevent them (Zaini and Abdulsatar, 2016).

If diabetes arises, it is extremely important that blood glucose levels are monitored and treatment is performed adequately. For type 1 diabetes this invariably takes form of insulin injections, which should be coupled with healthier eating and exercise. For type 2 diabetes insulin injections may not necessary and other treatments can be applied. One such treatment for type 2 diabetes is the intake of enzyme inhibitory substances, of which acarbose is an example (WHO, 2016). This drug is also sometimes used in prediabetes condition. While popular in China, its use in the USA is not as common due to the diarrhoea and flatulence side effects (Hollander, 2007). Its mechanism of action is the inhibition of α-glucosidase and α-amylase, preventing the degradation of complex carbohydrates into glucose. Because the glucose absorption is reduced, the blood glucose levels also decrease. Similarly to type 1 diabetes, for type 2 diabetes the treatment should be coupled with exercise and healthier eating choices.

2.4 Curing diabetes

Despite several teams actively researching a cure for diabetes, there still exists no cure. There are however several different approaches being studied, which could open the path for curing diabetes. One example is organ transplantation, namely the pancreas, since this is the organ that produces insulin and the kidney (Chan et al., 2016, Watson, 2015). However it is unknown how the new organ would cope with the insulin resistance of the body. Related to the pancreas, the transplantation of islet cells and creation of an artificial pancreas that could

produce insulin are also being studied (Pellegrini et al., 2016, Rekittke et al., 2016). A more controversial cure being researched is the use of genetic manipulation, to transform non-insulin producing cells into cells capable of producing insulin (Basta et al., 2015). However, all these still have a long way to go and many problems to solve, including society acceptance.

2.5 Interaction of diabetes with other diseases

Not only is diabetes a complicated disease to deal with, it can bring other problems with it. As mentioned before due to the drop in blood pH several problems that could be life threatening, including heart attacks, have an increased chance of occurring. However this disease can also potentiate other diseases, such as tuberculosis. It has been known for centuries that there is a link between diabetes mellitus and tuberculosis. Now there is increasingly more evidence that both diseases are fuelled by one another (Lo et al., 2016, Dooley and Chaisson, 2009). Diabetes is a risk factor for the development of tuberculosis, and an individual with both diseases can respond differently to the treatment for tuberculosis (Sola et al., 2016, Magee et al., 2016, Gomez-Gomez et al., 2015). On the other hand tuberculosis also makes it more likely for someone to develop glucose intolerance, leading to a worse glycemic control (Singh et al., 2015). On one hand this combination is important in lower income regions, since proper nutrition, sanitation and administration of antibiotics, all important in tuberculosis control, are many times lacking. On the other hand, urbanization, changes in lifestyle, improper diet, lack of exercise, stress and increased life expectancy are all factors present in many industrialized countries which can lead to the appearance of type 2 diabetes and are responsible for the increasing number of diabetes cases (Lopez and Mathers, 2006).

3 Methodology

3.1 Biologic material

Three α-amylases were used. *Aspergillus oryzae*, porcine pancreas (TYPE VI-B) and human saliva (TYPE XI) α-amylases were purchased from Sigma Aldrich, UK. The α-glucosidase used was obtained from yeast and purchased from Megazyme, Ireland. All α-amylases were in lyophilised powder form while α-glucosidase was in ammonium sulphate buffer suspension.

3.2 Plant material collection

All plant part samples were collected during the fruiting period of November 2014 from "Herdade da Corte Velada" in Algarve, Portugal, GPS N 37 11.536; W 8 40.820. Flowers were collected both from the tree and from the ground, with special care to leave the ovary connected to the tree. Upon collection samples were stored in tagged bags and kept in an isothermal container until arrival at the lab in the University of Algarve. Leaves and flowers were dried in an oven at 45°C while fruits were dried in an oven at 60°C and in a lyophilizer. All samples were ground to a powder after drying and then stored in a freezer until extraction.

3.3 Extraction conditions

Extracts were obtained using the ground powder of each plant part. For all extractions water and a hot plate were used, with a stir set to 200 rpm. Details of the conditions used can be seen on Table V–4.

Table V–4. Extraction conditions for antidiabetes potential determination

Code	Temperature (°C)	Time (min)	Ratio (g/mL)		
			Leaf	Fruit	Flower
1	40	105	0.035	0.035	0.01
2	60	30	0.035	0.035	0.01
3	20	180	0.035	0.035	0.01
4	60	180	0.035	0.035	0.01
5	95	15	0.035	0.035	0.01
6	95	5	0.035	0.035	0.01

3.4 α-amylase inhibition

Three α-amylases from different origins were tested, *Aspergillus oryzae*, porcine pancreas and human saliva. Inhibition was determined following the method described by (Sancheti et al., 2013) with slight modifications. Briefly, 100 µL of a 2% starch solution were mixed with 50 µL of extract and incubated for 10 min at 20°C. After incubation, 100 µL of 2 U/mL of α-amylase enzyme (prepared in pH 6.9 0.02 M and 0.0067 M NaCl phosphate buffer) were added and the obtained mixture was left to react for 5 min at 20 °C. After adding 100 µL of colour reagent (sodium potassium tartarate and NaOH solution mixed with 96 mM 3,5-dinitrosalicylic acid) the mixture was incubated for 15 min at 95 °C. After cooling down, 900 µL of distilled water were added and the absorbance was read at 540 nm. For removal of the samples absorbance, buffer solution was used instead of enzyme. Acarbose was used as positive control and distilled water as negative control. Results are expressed as IC_{50} in µg

sample/mL, determined by regression of inhibition and extract concentration. Results are means ± SD of three repetitions.

3.5 α-glucosidase inhibition

Inhibition was determined following the method described by (Li et al., 2009) with som modifications. Briefly, 150 μL of sample solution were mixed with 150 uL of 0.5 U/mL α-glucosidase enzyme in pH 6.8 phosphate buffer (0.1 M). The obtained mixture was incubated for 10 min at 37°C, after which 150 μL of 0.5 mM p-nitro-α-D-gluco-pyranoside (in 0.1 M phosphate buffer) were added and a subsequent incubation for 30 min at 37°C took place. After adding 600 μL of sodium carbonate solution (0.2 M) absorbance was read at 405 nm. Acarbose was used as positive control and distilled water as negative control. Results are expressed as IC_{50} in μg sample/mL, determined by regression of inhibition and extract concentration. Results are means ± SD of three repetitions.

3.6 Determination of inhibition type

The inhibition type was determined for the extracts which showed the highest inhibitions. This was done using a Lineweaver-Burk plot (Rafiei et al., 2016). In this plot the x axis corresponds to 1/[s] and the y axis to the 1/v of the reaction. By comparing the places were the y and x axis are crossed between sample and blank it is possible to determine the type of inhibition that is happening. For α-amylase, measurements were taken after 2 minutes of incubation using a starch concentration of 1.6, 1.4, 1.2, 1.0, 0.8 and 0.4%. For α-glucosidase the measurements were taken after 2 minutes of incubation and the PNPG concentrations were 0.5, 0.4, 0.3, 0.25, 0.15, 0.1 and 0.08 mM.

3.7 Statistical analysis

Results were analysed for statistical differences using ANOVA test with LSD or Games-Howel *post-hoc* tests after checking for the homogeneity of variances using Levene's test in SPSS 22. Results regarding the IC_{50} were also analysed using principal components analysis (PCA) in JMP Pro 12. Data was automatically scaled by the software and the estimation was made using the default method of the software.

4 Results and discussion

4.1 Enzyme and plant part comparison

The number of cases in the USA, Europe and the rest of the world justify the classification given to diabetes as the pandemic of the 21st century. In addition, the high prevalence of this

condition in developing countries stresses the importance of finding new ways to control blood glycaemia that are more affordable to the population in general. The α-amylase inhibition of extracts from different *A. unedo* parts extracted with different conditions was determined using three different α-amylases, one from filamentous fungi and two from mammals (Table V–5).

The extracts with the highest α-amylase$_{A.\ oryzae}$ inhibitory activity were obtained using conditions 1 for leaves and 1 through 4 for lyophilised fruits. For flowers and dry fruits the extraction conditions did not have as big an influence. Regarding α-amylase$_{pancreatic}$ inhibition, the best results were obtained with condition 6 for leaves, 1 and 5 for dry fruits, 3 and 4 for lyophilized fruits, 1,4 and 5 for flowers collected from the tree and 5 and 6 for flowers collected from the ground. This comparison is regarding the IC_{50} of the extracts in μg/mL and not considering every condition provided the same concentration. While the conditions used in the extraction caused significant differences on the results obtained, the relationship between the obtained extracts and the most inhibited enzymes was the same. There is not much information regarding the effect of temperature and time on the α-amylase inhibitory activity of extracts, but the results obtained are in agreement with the work published by (Chen et al., 2016) on the *Pleurotus citrinopileatus* extract, an edible mushroom, in which they found cold extracts (4°C, 24 hours) to possess higher inhibitory activity than hot extracts (boiling for 1 or 2 hours). Additionally, α-amylase origin also influenced significantly the results obtained regarding the inhibitory activity of the tested extracts. For instance, the same concentration of extract 1 from leaves inhibited 40% of α-amylase$_{A.\ oryzae}$, versus 80% inhibition against α-amylase$_{pancreatic}$. Comparing the two amylases from mammal origin, the same conclusion is reached, although the difference is less perceptible, a 75% α-amylase$_{pancreatic}$ inhibition versus 83% α-amylase$_{saliva}$ inhibition. Regarding dry fruits, the same comparison between α-amylases is not so linear. While at a 2x dilution the inhibition of α-amylase$_{A.\ oryzae}$ is around 55% and of α-amylase$_{pancreatic}$ is 50%, when using a 3x dilluted extract, the inhibitions are 33% and 0% for the same enzymes and 33% for α-amylase$_{saliva}$. This different relation depending on the dilution was also found for lyophilized fruits, although it not as evident. Regarding flowers, the enzyme most easily inhibited was α-amylase$_{pancreatic}$, followed by α-amylase$_{saliva}$ with α-amylase$_{A.\ oryzae}$ being the enzyme against which the extracts showed the least inhibitory activity. This was true for flowers collected from the tree and from the ground, although for those collected from the ground there was less difference between α-amylase$_{pancreatic}$ and the other amylases inhibition.

Table V–5. α-amylase and α-glucosidase IC_{50} for the different extracts and extraction conditions.

		α-amylase IC_{50} (μg/mL)			α-glucosidase IC_{50} (μg/mL)
		A. Oryzae	Porcine pancreas	human saliva	
Leaf	1	6574.84 ± 311.57	339.49 ± 64.57	313.37 ± 14.00	4.49 ± 0.37
	2	9525.73 ± 1572.94	444.65 ± 92.84	323.20 ± 111.99	5.40 ± 0.37
	3	16737.02 ± 1558.48	459.81 ± 32.15	425.45 ± 78.01	7.28 ± 0.36
	4	16446.56 ± 1897.55	569.14 ± 507.35	480.79 ± 33.20	4.39 ± 0.34
	5	13903.70 ± 825.03	337.90 ± 30.66	504.46 ± 18.40	5.56 ± 0.56
	6	10795.61 ± 916.62	173.80 ± 34.33	585.78 ± 51.28	10.01 ± 0.62
Fruit (dry)	1	10409.79 ± 864.78	11127.11 ± 1085.18	12941.90 ± 2953.57	148.54 ± 8.54
	2	10233.53 ± 1744.24	14212.87 ± 2067.59	12889.31 ± 2349.97	73.01 ± 9.35
	3	11224.73 ± 1122.91	14724.86 ± 1605.31	13705.92 ± 1979.79	34.78 ± 13.91
	4	9943.58 ± 1334.84	13165.92 ± 1860.70	11062.67 ± 2306.89	88.43 ± 1.73
	5	9391.50 ± 495.11	12184.39 ± 549.24	10016.21 ± 1136.33	54.81 ± 10.2
	6	12273.21 ± 1008.05	13806.01 ± 1754.58	12278.36 ± 2139.98	51.95 ± 14.65
Fruit (lyophilized)	1	4438.64 ± 395.86	7157.27 ± 1363.35	6700.34 ± 554.98	21.64 ± 0.81
	2	5850.69 ± 403.60	5739.16 ± 1430.86	6280.12 ± 346.91	24.21 ± 0.86
	3	5761.61 ± 484.96	4148.83 ± 1045.82	5829.62 ± 290.25	24.17 ± 1.98
	4	5752.24 ± 2514.05	4709.95 ± 274.00	4757.58 ± 592.67	17.83 ± 1.75
	5	7592.17 ± 600.98	5936.67 ± 2427.04	6809.25 ± 319.02	22.98 ± 0.87
	6	9058.06 ± 1797.53	5730.22 ± 1201.48	6194.44 ± 127.91	22.84 ± 1.71
Flower (tree)	1	4865.29 ± 654.94	665.05 ± 213.18	1383.88 ± 150.28	1.52 ± 0.18
	2	3374.62 ± 280.65	793.79 ± 38.38	1323.40 ± 65.07	1.37 ± 0.08
	3	3356.68 ± 423.80	774.45 ± 16.24	1380.17 ± 57.79	1.32 ± 0.06
	4	3403.79 ± 455.56	612.54 ± 22.40	1058.01 ± 106.81	0.96 ± 0.05
	5	3672.66 ± 423.10	646.33 ± 17.25	1054.52 ± 85.63	1.23 ± 0.13
	6	3867.86 ± 619.99	779.19 ± 44.58	1393.98 ± 356.47	1.37 ± 0.08
Flower (ground)	1	3710.84 ± 401.71	990.77 ± 16.67	2675.15 ± 258.13	1.36 ± 0.11
	2	3138.13 ± 282.47	760.79 ± 27.43	1782.38 ± 127.58	1.34 ± 0.19
	3	4160.68 ± 422.02	981.49 ± 59.25	2517.27 ± 291.50	4.64 ± 0.32
	4	3258.05 ± 353.68	888.82 ± 54.21	2553.21 ± 194.42	3.13 ± 0.24
	5	3036.02 ± 222.26	690.32 ± 65.14	2038.22 ± 336.34	1.58 ± 0.56
	6	3372.43 ± 367.11	732.86 ± 52.76	2443.17 ± 347.61	1.21 ± 0.05

Results are presented as mean ± SD and were obtained from a linear regression with $R^2 \geq 0.900$.

This difference in inhibitions towards α-amylases from different origins is in accordance with other works published and is related to their structure. α-amylase$_{A.\ oryzae}$ is a very different enzyme from α-amylase$_{pancreatic}$ and α-amylase$_{saliva}$. α-amylase$_{A.\ oryzae}$ is composed by 478 amino acid residues folded into two domains, versus the three domains of the 496 amino acid α-amylase$_{pancreatic}$. On α-amylase$_{A.\ oryzae}$ domain A has 380 amino acid residues, contains the N-terminal and consists of a β/α_8 barrel with eight β strands alternating with eight helices joined by loops while domain B is an 8 stranded antiparallel β sheet linked to domain A. On

the other hand on α-amylase$_{pancreatic}$ domain B is inserted in domain A, which stretches from amino-acids 1 to 99 and 170 to 404. The active site for both enzymes is located in a cleft at the C-terminal of domain A and in both enzymes the catalytic amino acid residues are glutamic and aspartic acids, although in different positions. On the other hand, both the bond cleavage location and the amino acids surrounding the active site are different. For α-amylase$_{A.\ oryzae}$ cleavage occurs between subsites 4 and 5 while for α-amylase$_{pancreatic}$ it occurs between subsites 3 and 4 (Wong, 1995). These differences in structure justify the different inhibition of molecules towards α-amylase, such as acarbose, which strongly inhibits α-amylase$_{pancreatic}$ via a hydrogen bond with the glycosidic NH, but is much weaker in its α-amylase$_{A.\ oryzae}$ inhibition (Robyt, 2005). Similarly to our work, when Kim, Wang & Rhee (2004) studied the inhibition of pine leaves towards 5 α-amylases from different origins, they also found α-amylase$_{A.\ oryzae}$ to be differently inhibited from the amylases of mammalian origin. Extracts didn't inhibit α-amylase$_{A.\ oryzae}$ but inhibited both mammalian α-amylases with an IC_{50} of 1.7 μg/mL. Similarly, when Robyt (2005) tested the inhibition of α-amylase from 4 different origins (*A. oryzae*, *B. amyloliquefaciens*, porcine pancreas and human saliva) he found that acarbose had a much lower Ki towards mammalian α-amylases (approximately 1 μM), than towards *B amyloliquefaciens* or *A. oryzae* α-amylases 13 and 270 μM respectively). They found this difference in inhibitions to be related to the different bonding between acarbose and the α-amylases. While on the mammalian α-amylases acarbose binds from the -1 to the +2 subsites of the enzyme, the binding between acarbose and α-amylase$_{A.\ oryzae}$ occurs between subsites -1 and +3. They also found that the addition of maltodextrins to its non-reducing translated into a much higher affinity of the acarbose molecule to the active site, therefore increasing the inhibition displayed.

While fruit extracts were better at inhibiting α-amylase$_{A.\ oryzae}$, they were the worst extracts for inhibiting the mammalian α-amylases. There were differences detected between the lyophilized fruit samples and those dried in an oven, with lyophilized fruits displaying a higher α-amylase inhibition, no matter the source of the enzyme. This indicates that the oven drying at 65°C destroys inhibitory compounds. While there are differences regarding the activities of flowers collected from the ground and from the tree, on average the two samples are almost equivalent regarding their α-amylase inhibition. When there are differences however, flowers collected from the tree display a much inhibition towards the enzymes. In a previous work we found that while the flowers collected from the ground retain for the most part their antioxidant activity, they completely lacked flavonoids which were present on the flowers collected from the tree. This indicates flavonoids are partially responsible for the

higher inhibitory activity detected on extracts obtained using flowers collected from the tree. The importance of flavonoids is further supported when considering the higher inhibition caused by leaves when compared to fruits, whose extract possesses no flavonoids according to an earlier work. Additionally, in a previous work leaf extracts obtained using ethanol, a better solvent to extract flavonoids than water, also showed a higher inhibitory activity towards α-glucosidase when compared to aqueous extracts. This observation regarding the importance of flavonoids is in agreement with published works regarding their antidiabetic activity. Li et al. (2015) tested the effect of a concentrated flavonoid extract obtained from *Ipomea batatas* on the glycaemia and lipid level of diabetic rats and found the extract caused a great reduction on both levels at a concentration of 100 mg/kg of body weight, when compared to untreated diabetic rats and similar levels compared to rats treated with glibenclamide, a drug used in type 2 diabetes treatment. Vinayagam and Xu (2015) conducted a review on the antidiabetic potential of dietary flavonoids. In that work they classified diosmin, fisetin, morin, eriodictyol, hesperidin, naringenin, apigenin, baicalein, chrysin, luteolin, tangeretin, wogonin, isorhamnetin, kaempferol, rutin, quercetin, genistein and daidzein as having positive effects on diabetes control, by either decreasing glucose formation, increasing its absorption or protecting cells responsible for insulin production. Of these flavonoids, quercetin was previously detected on the leaves used in this work, which explains in part the results obtained with this plant part. This flavonoid not only shows inhibitory activity towards the enzymes responsible for the carbohydrates digestion, it also acts on the intestinal cells, decreasing the absorption of glucose via the inhibition of GLUT2 (Kwon et al., 2007). In an assay conducted in diabetic mice, a daily quercetin dose of 15 mg/kg injected in rats protected β-cells from oxidative stress caused by streptomycin injected at 50 mg/kg, caused a lower serum glucose level when compared with untreated diabetic rats and increased insulin sensitivity (Coskun et al., 2005). These properties displayed by quercetin support that extracts rich in flavonoids and specifically quercetin, as is the case of *A. unedo* leaf extract, may help in diabetes control not only via the inhibition of the enzymes responsible for carbohydrate digestion, but also via the direct control of blood glycaemia and insulin producing cells protection.

While differences regarding the sensitivity of α-glucosidase towards N-ethylmaleimide and diethyl pyrocarbonate have been noted, overall the enzymes are considered similar regarding substrate specificity and inhibitor sensitivity, therefore the results using α-glucosidase from yeast origin should accurately translate into the α-glucosidase of mammalian origin (Suresh Babu et al., 2004, Dhanawansa et al., 2002). For α-glucosidase, the inhibitory activity of the extracts was once again influenced by extraction conditions. For leaves the best condition was

5 and the worse was 6, with the remaining conditions having no difference among them. On the other hand for dry fruits the results were obtained with condition 3 while the worse were obtained with condition 1. For fruits both extracts obtained at 95°C showed the same inhibitory activity. All extracts showed a much higher inhibitory activity towards α-glucosidase than towards any of the α-amylases. The most active extracts were leaves and flowers, with fruits once again displaying the worse inhibitory activity, although with a lesser difference for α-glucosidase. However the IC_{50} displayed by these fruits is quite low when compared with other fruits. For instance, when (Park et al., 2012) tested 50 different fruits and vegetables at a 50 mg/mL only 5 water extracts and 17 ethanolic extracts showed α-glucosidase inhibition. Of these the best results were 30.4% inhibition for lotus root and 20.0% for Japanese apricot (aqueous extracts), 73.9 and 28.3 for potato and pear respectively. Comparatively *A. unedo* fruit extract after a 1000x dilution, which corresponds to a concentration of 1μg/mL was still able to inhibit 60% of the enzymatic activity. The difference between leaves and fruits is in agreement with a previous work where leaves were tested using different solvents, and with published work from other authors. When (Shai et al., 2010) tested the inhibitory activity of six medicinal plants towards α-amylase and α-glucosidase, they found that while all extracts were capable of inhibiting α-glucosidase, with IC_{50} between 0.6 and 3.0 mg/mL, but could only inhibit less than 20% of α-amylase activity. Other researchers have noticed the same tendency, with plant phytochemicals more strongly inhibiting α-glucosidase than α-amylase, a direct opposite of acarbose. This difference means herbal extracts could be used in conjunction with acarbose to achieve a better glycaemic control, since the enzymes targeted by each one are different. The preferred inhibition of α-glucosidase also has the advantage of possibly preventing the drawback of acarbose, which has the side effects of abdominal distention, flatulence and diarrhoea caused by the high amounts of undigested starch, which can be fermented by the bacteria present in the colon (Hoffmann, 1990). From the tested extracts, fruits followed by leaves were the least effective with flower extracts being the most active against α-glucosidase. This order is different from what was seen for α-amylase, indicating the compounds responsible for inhibiting α-amylase and α-glucosidase are different. Several classes of compounds, all present in plant products, have been shown to inhibit α-glucosidase. These include mostly terpenes, flavonoids and phenylpropanoids but phenols and alkaloids have also demonstrated inhibitory activity (Yin et al., 2014). In addition to quercetin, which was also able to inhibit α-amylase, gallic acid, a phenolic highly present in the leaves of *A. unedo* studied in this work and p-hydroxybenzoic acid, also present in the leaves but in lower amount, also showed strong α-glucosidase

inhibitory activity (Lam et al., 2008, Li et al., 2015, Oboh et al., 2016, Ma et al., 2015). The IC_{50} of gallic acid was even lower than acarbose, which supports the claim that this compound is one of those responsible for the inhibitory activity detected in this work.

(Bnouham et al., 2007) tested the antihyperglycemic activity of *A. unedo in vivo*. They verified that administering *A. unedo* water extract, at non-toxic concentrations, to rats prior to glucose loading led to a significant reduction of the glucose levels, when compared to the control. This reduction was caused by the inhibition of jejunal glucose absorption (31.6%). Therefore *A. unedo* extracts consumption may be an important way of reducing postprandial glucose levels, not only due to enzymatic inhibition but also due to the reduction of glucose absorption, helping to control the glucose levels in the blood and therefore ameliorate the problems of type 2 diabetes and reduce its risks.

4.2 PCA

To further compare the enzymes, scaled results for IC_{50} for all samples and treatments were analysed via PCA on JMP Pro 12. PCA graph showed that over 90% of the results can be explained with 2 components (72.8% for CP1 and 20.8% for CP2) (Figure V–4).

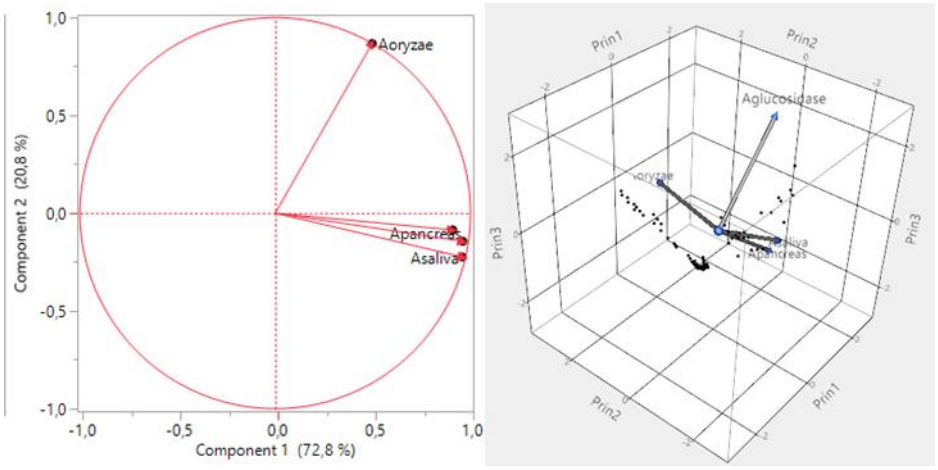

Figure V–4. PCA results (2 components on the left, 3 components on the right).

It also showed that mammalian enzymes were grouped together with α-glucosidase and separately from *A. oryzae* α-amylase. It was expected that both mammalian amylases would be grouped together and *A. oryzae* amylase would be separate due to the differences observed previously, but it was not clear that α-glucosidase would be grouped with the mammalian enzymes. It should however be mentioned that this grouping disappears when using three components, as it can be seen on the 3D scatterplot, with α-glucosidase pointing in a different direction of all α-amylases.

4.3 Type of inhibition

Following the results obtained above for the α-amylase and α-glucosidase inhibitions it was deemed important to determine the type of inhibition to better understand the process that is happening, since no information regarding these extracts mode of inhibition is currently available. The samples obtained with the extraction condition 2 were used for this part of the work. From the α-amylases only the pancreatic was used. The determination was done using a Lineweaver-Burk plot. From this plot it was possible to verify that both lyophilised and dry fruit extracts inhibited α-amylase via a non-competitive mechanism. On the other hand leaves displayed competitive inhibition and flowers a mix of competitive and uncompetitive inhibition. For α-glucosidase, leaf and fruit inhibitions were a mix of non-competitive and uncompetitive mechanisms. This indicates not only that there are at least two different compounds being responsible for the inhibition, but also that some compounds can bind to the free enzyme or to the enzyme-substrate complex, while others can only bind to the enzyme-substrate complex. On the other hand flower inhibition was uncompetitive, meaning the bond is formed between the enzyme-substrate complex and the inhibitor present in solution.

5 Conclusion

Overall fruit extracts were better at inhibiting α-amylase$_{A.\ oryzae}$ while leaf and flower extracts were better at inhibiting mammalian origin α-amylases. Flower, followed by leaf extracts were the best at inhibiting α-glucosidase. The drying process of the fruits significantly influenced the inhibitory activity of their extracts, as did the flower origin, although on a much smaller scale and only partially. While the extraction temperature and time caused some changes in the inhibitory activity of the extracts, the enzyme and plant part factors had a much higher influence on the IC_{50}. The stronger inhibitory activities of leaf and flowers towards mammalian α-amylases are likely related to their flavonoid content, while α-glucosidase inhibition is likely linked to flavonoids and phenolics. Both leaf and flower extracts displayed interesting activity and have the potential to be used as antidiabetic agents for a better glycaemic control. This is especially interesting for flowers from the ground, which exist every year and are currently being wasted.

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Potential application of *Arbutus unedo* L. leaf, fruit and flower extracts in the treatment of Alzheimer's and Parkinson's diseases

Ricardo Nunes[1] and Isabel S. Carvalho[1]

[1]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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1 ABSTRACT

Alzheimer and Parkinson's diseases are responsible for many deaths. Since there is a link between increased radical formation and both diseases, and increased neurotransmitters availability is an accepted therapeutic, in this work we study the inhibitory activity of *Arbutus unedo* leaf, fruit and for the first time flower extracts (from the tree and from the ground), which previously showed strong antioxidant activity, on enzymes related with both diseases. All extracts showed extraction dependent and enzyme specific inhibition towards acetylcholinesterase (ACHE) and butyrilcholinesterase (BCHE), especially leaves and flowers. Leaf extract showed non-competitive and uncompetitive inhibitions for ACHE and BCHE (Alzheimer's), while fruit was the opposite. Flowers inhibition method was non-competitive for both enzymes. *In-vitro* digestion caused changes in inhibitory activities. No aqueous extract was able to inhibit over 50% of tyrosinase activity (Parkinson's). This work shows that leaf and flower aqueous extracts show promising anti-Alzheimer potential with different inhibitory mechanisms.

HIGHLIGHTS

Alzheimer and Parkinson related enzymes inhibition was tested.

Effect of different extraction conditions was evaluated.

Leaf and flower showed strong Alzheimer enzyme inhibition via different mechanisms.

Extracts lost some inhibition power after *in-vitro* digestion.

Extract tyrosinase inhibition was improved when extracting with ethanol.

KEYWORDS

Arbutus unedo; acetylcholinesterase; butyrilcholinesterase; tyrosinase; inhibition type; dementia.

2 Introduction

Chronic diseases or conditions are understood as long lasting or permanent conditions. They include diabetes, Alzheimer, Parkinson, obesity, heart problems, cancer and arthritis, and are responsible for a great burden both on the health system, society and personal life of the affected people. They also usually do not occur alone, and bring with them more health problems. For example diabetes is highly related with blindness and kidney failure, while obesity increases the risk of heart problems [1].

Alzheimer's is the most prevalent non-transmissible chronic neurodegenerative disorder. It is responsible for a great percentage of elderly people deaths, who often don't even know are afflicted by it. It is a type of dementia that affects the brain neurons causing problems with memory, behaviour and thinking processes [2]. This is due to changes which include the formation of plaques or deposits of β-amyloid, the presence of microscopic strands of τ protein which became twisted and tangled, inflammation which is caused by the body's own immune system, loss of connection between the brain cells, which is the main responsible, for the memory and cognitive losses, and finally at a an advanced stage, shrinkage of the brain tissue, caused by the death of brain cells [3, 4]. Given the importance of early diagnosis to try and mitigate the damage, several diagnosis processes to detect the disease even before the first symptoms are being tested. These include brain imaging, searching for specific proteins in the blood, urine and cerebrospinal fluid (τ and β-amyloid proteins) or searching for mutations in the DNA [5-8]. However they are still in experimental stage meaning the earlies detection is usually by the family of the affected person or in routine checks. The earliest symptom of the Alzheimer's disease is usually taking more time to conclude usually performed tasks. This is caused by disturbances in the brain part responsible for learning and memory. As the disease progresses and different parts of the brain are affected, the symptoms worsen and evolve into disorientation and mood and behaviour changes [9]. Together with the brain tissue damage that occurs in affected people, there is a loss of the cholinergic neurons, located mainly in the basal part of the forebrain. This loss is accompanied by a diminishment of the neurotransmitter acetylcholine, responsible for passing information from one neuron to the next in the synapses. Since there is a positive correlation between acetylcholine levels decreasing and the decrease in the cognitive functions displayed by Alzheimer's patients, the

control of acetylcholine levels could reveal advantageous in alleviating Alzheimer' symptoms [3, 4, 10]. In order to accomplish this goal two paths can be pursued, either increase acetylcholine levels or decrease acetylcholinesterase (ACHE) activity. The first option is not easily feasible since the ACHE enzyme is highly efficient, degrading over 2000 acetylcholine molecules of acetylcholine every second [11]. In order to artificially raise the levels, an extremely high dose of acetylcholine would need to be administrated, which since acetylcholine is one of the main neurotransmitters responsible for peristaltic movements could cause discomfort [12]. Therefore most studies focus on the inhibition of the ACHE enzyme. So far several drugs based on this principle have been approved, with more being developed. The administration of these drugs has led to an improvement of the symptoms, but these are often coupled with unwanted side-effects, one of the reasons search for new treatments continues [3]. Given how some studies tend to point towards a link between oxidative stress and cell death and in the case of Alzheimer's disease cell death is already occurring together with increased radical production caused by the β-amyloid proteins present in the plaques, it is important that whenever possible, the ACHE inhibitor also displays strong antioxidant activity, as it could further help to control the spread of the disease [2, 13].

Following Alzheimer's, Parkinson is the next most common neurodegenerative disease [14]. In this disease there is once again the death of cells, but this time it is of the basal ganglia and substantia nigra cells [14-16]. Since these cells are responsible for secreting dopamine, an amine synthesized from the L-DOPA molecule, their destruction causes the levels of dopamine to decrease. Similarly to acetycholine, dopamine is also a neurotransmitter, and as such its destruction or low levels lead to impairment in the transmission of information between neurons. One of the functions of dopamine is controlling the motor system, whose impairment is one of the symptoms displayed by Parkinson's patients [14, 15]. Since dopamine is so important in the control of the motor system, the most widely used treatment for Parkinson is the administration of its precursor, which is commercialized as Levedopa [17]. However, since dopamine is also involved in the reward-motivated behaviour, is responsible for the addiction of many psychoactive drugs, and is related to schizophrenia, the administration of Levedopa needs to be well established and monitored [18-20]. This is usually done by increasing the administrated levels until no more beneficial effects appear and before serious side effects start to emerge. Therefore other ways to treat or ameliorate the symptoms of Parkinson's disease need to be found and studied. Since the tyrosinase (TYR) enzyme can catalyse the oxidation of L-Dopa into the precursor of melanin, reducing the available L-Dopa to be converted into dopamine, the inhibition of this

enzyme could prove important in the reduction of Parkinson's symptoms [21, 22]. Furthermore, the intermediate product of that oxidation, dopaminequinone, leads to the production of radical species known to promote cytotoxicity and can also react with α-synuclein forming toxic intermediate products in the nigral cells, which can lead to their death [21, 23]. For this reason the administration of compounds with high antioxidant activity, capable of scavenging the radicals produced could also help ameliorate the symptoms [24, 25].

Therefore, and following a previous study where *Arbutus unedo* leaf and flower extracts displayed a high α-glucosidase inhibitory activity, which translates into antidiabetic potential via the control of blood glucose levels, and more importantly for this work, a high antioxidant activity, we decided to continue to study the potential of this tree extracts in the treatment of chronic diseases by evaluating their anti-Parkinson's and anti-Alzheimer's potentials using enzymatic assays.

3 Material and methods

3.1 Chemicals

All chemicals used were of analytical grade. The enzymes used were electric eel ACHE, equine BCHE and mushroom TYR, all purchased from Sigma-aldrich (UK).

3.2 Plant material and sample preparation

Leaves and fruits were extracted according to conditions present on Table V–6.

Table V–6. Extraction conditions for leaves, fruits and flowers

		code	T (°C)	t (min)	ratio (mg/mL)
Leaf	1	A1	40	105	20.0
	2	A2	60	30	10.0
	3	A3	20	180	35.0
Fruit	1	B1	40	105	11.5
	2	B2	60	180	30.0
	3	B3	20	30	30.0
Flower tree	1	C1	45	50	7.5
	2	C2	65	50	10.0
	3	C3	25	50	5.0
Flower ground	1	D1	45	50	7.5
	2	D2	65	50	10.0
	3	D3	25	50	5.0

To begin the material was collected directly from the trees present in the "Herdade da Corte Velada", in Algarve, Portugal, on a dry day of November 2014. Flowers were collected both from the tree and from the ground, with special care to leave the ovary connected to the tree.

Samples were then tagged, put into separate bags inside a thermal container and transported to the FSLab in the University of Algarve. Leaves and flowers were dried in an oven at 45°C while fruits were stored in a freezer until extraction on the following days. Before extraction, leaf and flower samples were ground to a powder while fruits were macerated using a mortar and pestle. The selected extraction conditions can be seen on.

3.3 Acetylcholinesterase (ACHE) and butyrilcholinesterase (BCHE) inhibition assays

The inhibitory activity of the enzymes were evaluated following the method firstly reported by Ellman, Courtney, Andres and Featherstone [26] with modifications by Sancheti, Sancheti and Seo [27], but with volumes adjusted for not using 96 well plates. Brielfy, 100 µL of a prepared ACHE solution (0.03 U/mL in 7.2 mM with 0.1 mM EDTA 7.2 pH phosphate buffer) were mixed with 50 µL of test sample and 900 µL of Tris-Hcl buffer (50 mM, pH 8). After incubation for 30 min at 4°C, 100 µL of a 0.3 mM 5,5-dithio-bis(2-nitrobenzoic acid) and 100 µL of a 1.8 mM acetyltiocholine iodide solution were added and incubated for 20 min at 37°C. For BCHE the method was very similar but the enzyme concentration used was 0.1 U/mL and the acetyltiocholine concentration 6.8 mM. The absorbance was then read at 412 nm in a T70 + UV-VIS spectrophotometer from PG instruments (UK). Inhibition was calculated using the following equation:

$$Inhibition\ (\%) = \frac{Acontrol - Asample}{Acontrol} \times 100$$

The concentration of extract correspondent to 50% inhibition (IC_{50}) was determined, when possible, from concentration versus inhibition curves.

3.4 Tyrosinase (TYR) inhibition assay

The inhibitory activity of the TYR enzyme was determined following the method by Lin, Hu, Lin, Liu, Chen, Zhang and Chen [28]. Briefly, 900 mL of a 0.5 mM L-DOPA solution in 50 mM NaH_2PO_4–Na2HPO4 buffer (pH 6.8), were pre-incubated at 30 °C with 50 µL of extract. After incubation, 50 µL of a TYR aqueous solution (250 U/mL) were added and the solution was mixed. At a constant temperature of 30 °C, the measurement was performed at 475 nm after 1 minute, with a UV spectrophotometer. The inhibition was calculated as shown above.

3.5 Determination of inhibition type

The inhibition type was determined for the extracts that showed an inhibition above 50%. This was done using a Lineweaver-Burk plot, where the x axis corresponds to 1/[s] and the y

axis to the 1/v (measured in variation of absorbance) of the reaction. The type of inhibition is determined by comparing where the x and y axis are crossed between sample and blank.

3.6 In-vitro digestion

The effect of the digestive process on the samples was simulated using an in-vitro digestion described by Liu, Glahn and Liu [29]. Briefly, 0.9 mL of the extract sample (diluted two times) were mixed with the saline solution of NaCL (140 mM), KCl (5 mM) and BHT (150 μM), to give a final volume of 3.6 mL. This solution was then acidified to pH 2 with the addition of 1M HCL and 0.1 mL of pepsin solution in HCL (0.4 g of pepsin in 10 mL HCL) were added and mixed. The vials were then immersed in water and incubated for 2 hours in an orbital shaker. The shaker was set for 100 rpm and 37 °C. After digestion, the pH of obtained digestate was increased to 6.9 using a solution of $NaHCO_3$ at 1 M. Further digestion in the orbital shaker was carried out for 3 more hours after the addition of a solution (0.5 mL) containing pancreatin and bile extract (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M NaHCO3). The final volume of the digested sample was adjusted to 4.5 mL and stored until analysis in the following day.

4 Discussion

4.1 Alzheimer's

4.1.1 Anti-Alzheimer's potential of extracts measured by IC_{50}

The anti-Alzheimer's potential was tested using two different enzymes which can degrade acetylthiocoline, a neurotransmitter whose diminished concentration is responsible for lack of proper neuron communication. The inhibition of these enzymes is an acceptable therapeutic in the treatment of Alzheimer's symptoms, with several drugs already approved by USA and EU health authorities [30]. In this work, all tested plant parts showed inhibitory activity towards acetylcholinesterase (ACHE) and butyrilcholinesterase (BCHE), in a dose dependent manner. The activity displayed was also affected by the extraction conditions (Table IV–4). Concerning ACHE inhibition, for leaves the best results were achieved with extraction A2 which had an IC_{50} of 118 μg/mL. Both the other extraction conditions yielded extracts with an IC_{50} of 173 μg/mL. This indicates there are compounds with inhibitory activity present in leaves which can only be extracted using temperatures above 40°C, although even when using lower temperatures it is possible to obtain an extract with considerable inhibitory activity towards ACHE.

Table V–7. IC_{50} results for leaf, fruit and flower extracts using acetylcholinesterase (ACHE) and butyrilcholinesterase (BCHE)

Plant part	Sample	ACHE (IC_{50})	BCHE (IC_{50})
Leaf	A1	173.20 ± 33.48^{ab}	169.40 ± 11.78^{a}
	A2	118.60 ± 32.80^{a}	54.31 ± 5.58^{b}
	A3	173.86 ± 13.12^{b}	518.43 ± 22.67^{c}
Fruit	B1	2345.89 ± 476.05^{c}	10256.90 ± 2067.52^{d}
	B2	n.d.	n.d.
	B3	28444.73 ± 762.29^{d}	31399.02 ± 584.43^{e}
Flower	C1	205.94 ± 76.09^{ab}	3078.96 ± 182.04^{f}
	C2	181.90 ± 58.71^{ab}	328.87 ± 42.29^{g}
	C3	590.17 ± 76.39^{e}	384.23 ± 29.23^{g}
	D1	1429.83 ± 320.25^{f}	n.d.
	D2	n.d.	n.d.
	D3	n.d.	n.d.

Results are presented as average ± SD, of replicates from three repetitions. n.d., not determinable. Different letters in the same column mean statistically different results ($p<0.05$)

For the fruits however, using high temperature yielded the extract with the worst performance (B2). Iin fact, it was so low that even the crude undiluted extract could not inhibit more than 50% of the acetylcholinesterase enzyme activity. This indicates that the compounds responsible for ACHE inhibition, that are being extracted from fruits using lower temperatures, are thermosensitive and are being destroyed by the higher temperatures used in extraction B2. Overall, fruit extracts were also much weaker than leaf extracts, with the best extraction, B1, having an IC_{50} of 2345 µg/mL, more than ten times higher that of the leaves. The range of IC_{50}'s was also much broader for fruits, since the next extract for which IC_{50} could be calculated, B3, had an IC_{50} of 28 mg/mL. Within flowers collected from the tree, the best results were obtained using extractions C1 and C2, with IC_{50} values of 205 and 181 µg/mL, versus 590 µg/mL of the extraction C3. Therefore we verify that similarly to leaves, higher temperatures yielded more powerful extracts, although in this case there was no statistical difference ($p > 0.05$) between extractions C1 and C2. These results were significantly different from those obtained using flowers collected from the ground, in which only extraction D1 showed an inhibition above 50%, with an IC_{50} of 1429 µg/mL. Results obtained using BCHE were for the most part different than those obtained with the previous enzyme. Using BCHE, the best result was obtained with extraction A2, with an IC_{50} of 54 µg/mL, significantly better than the result obtained with ACHE. This extraction also showed the highest inhibitory capability with ACHE. In addition, whereas before the extractions A1 and A3 showed the same inhibitory capability, A3 is now significantly weaker, although A1

remains the same. This indicates that a temperature higher than 20°C is necessary to obtain a much more powerful leaf extract, and temperature keeps improving the result even at 65°C, therefore the compounds must be at least partially resistant to temperature degradation. Similarly to what happened previously, for fruits only extractions B1 and B3 showed inhibitory activities above 50%, they also followed the same order as before, B1 stronger than B3. The concentration of B1 extract needed for inhibiting 50% of BCHE activity was however much higher than for ACHE. For B2 extract was the opposite, while before it inhibited only 18% of ACHE activity, the same extract inhibited 44% of BCHE activity. For flowers collected from the tree, all extracts showed inhibitory activity above 50%, similarly to what happened for ACHE, although while previously extracts C1 and C2 were the best, with no statistical difference, now C2 and C3 are the best, and the C1 extract requires a much higher concentration to inhibit BCHE, meaning temperature promoted the destruction of phytochemicals responsible for the inhibitory activity displayed on the other extracts. Once again, flowers collected from the ground yielded extracts with much lower inhibitory activity, so low that contrary to what happened with ACHE, extract D1 was unable to inhibit more than 50% of the enzymatic activity, inhibiting only 46%. Nanasombat, Thonglong and Jitlakha [31] tested several mixtures of medicinal plants and found the highest ACHE inhibitions of non-alchoholic beverages to be 22.78%. These were even higher than the alcoholic beverages tested in that work, which showed a maximum of 21.35% inhibitory activity. They also tested a pure grape extract, which showed an inhibition of only 0.71% at a concentration of 1 mg/mL. On the other hand α-tocopherol showed an inhibition of 18.62% at a concentration of 1mg/mL and their positive control had an inhibition of 35.82 at 0.1 mg/mL. When Nanasombat, Bubpasawana, Tamaputa and Srimakhan [32] tested Thai medicinal plants they achieved better inhibition percentages, with the crude methanolic extracts of black galingale rhizomes, sacred lotus petals, serpentine roots and Asiatic pennywort leaves showing inhibitions of over 70% at a concentration of 1 mg/mL while the extracts of gingko leaves, saffron pollen and blue pea glowers only inhibited between 32.73 and 25.55% at the same concentration. These inhibitions are much lower than those obtained in the present work for both leaves and flowers, which had IC_{50}'s as low as 118 μg/mL and 181 μg/mL respectively. Dzoyem and Eloff [33] also tested medicinal plants, in their case 12 different plants used traditionally in South Africa. In their work the IC_{50} results ranged from 118 μg/mL for *Leucaena leucocephala* to 487 μg/mL for *Ehretia rigida*. Their average was close to 350 μg/mL, which puts the best results obtained with leaves and flowers of *A. unedo* well within the range obtained for medicinal plants. Girones-Vilaplana, Valentao, Moreno,

Ferreres, Garcia-Viguera and Andrade [34] tested lemon juice enriched with exotic berries. They found all extracts inhibited similarly ACHE and BCHE, with their IC_{50} ranging between 8.83 and 21.76 mg/mL for 5% açaí in citric acid+ lemon juice and for 5% maqui in citric acid respectively. They also found a relationship between the antioxidant activity and the inhibitory properties of the prepared blends, which is in accordance with the present work. These inhibitions are lower than those detected in this work, where we tested the inhibitory activity of aqueous *Arbutus unedo* fruit extracts, with the best extract having an IC_{50} of bellow 3 mg/mL. This indicates that the extracts obtained in this work have potential to be used as ACHE and BCHE inhibitors consumed in the form of beverages, especially those from leaves and flowers. Additionally, while Fortalezas, Tavares, Pimpao, Tyagi, Pontes, Alves, McDougall, Stewart, Ferreira and Santos [35] found no effect caused by fruits on neuroblasma viability, when Tavares, Fortalezas, Carrilho, McDougall, Stewart2, Ferreira and Santos [36] tested leaf and fruit extracts, they found both crude and enriched extracts displayed neuroprotective properties via the inhibition of MMP-2 and MMP-9 activities. They also found that the inhibition of the enriched extract was higher than the crude extract and not significantly different from that displayed by blackberry and green tea, two highly documented health-promoting products.

4.1.2 Inhibition type

Considering the results obtained, the best extracts were further analyzed regarding their inhibition type. To do this, a Lineweaver-Burk plot using 2.16, 1.8, 1.44, 0.72, 0.6, 0.45 and 0.36 mM of acetylcholine concentration for ACHE and 9.54, 8.48, 6.36, 5.3, 4.24 and 3.18 and 2.12 mM for BCHE was created, for the blank and the extracts A2, B1 and C2. The linear regression equations were determined and the resulting y and x axis crossing was extrapolated. Lineweaver-Burk plot showed that not only were the IC_{50} of leaves and flowers similar, their method of inhibiting ACHE was also the same, with both extracts showing non-competitive inhibition (Figure V–5). This means that they inhibit by binding either to the free enzyme or to the enzyme-substrate complex, therefore forming either enzyme-inhibitor or enzyme-substrate-inhibitor complexes. This has the effect of lowering Vmax of the reaction, since the number of available enzymes decreases, however because the substrate can connect to the enzyme even if the inhibitor is already connected, the affinity of the enzyme towards the substrate is unaltered.

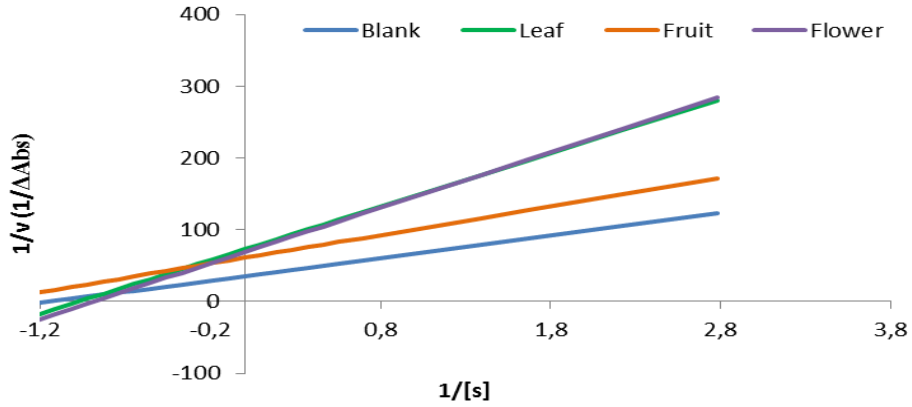

Figure V–5. Lineweaver Burk plot for acetylcholinesterase inhibition.

On the other hand, fruit extracts, which displayed the least powerful inhibitory potential, inhibit the reaction mostly via the uncompetitive method. This means they can only connect to the enzyme if the enzyme-substrate complex is already formed, therefore forming enzyme-substrate-inhibitor complexes. This inhibition type has the characteristic of lowering the Vmax of the reaction and increasing the affinity of the enzyme towards the substrate, since when the inhibitor connects with the enzyme-substrate complex, the substrate can no longer leave the active centre of the enzyme.

For BCHE, not only the inhibitory activities changed, the type of inhibition was also different for some extracts (Figure V–6).

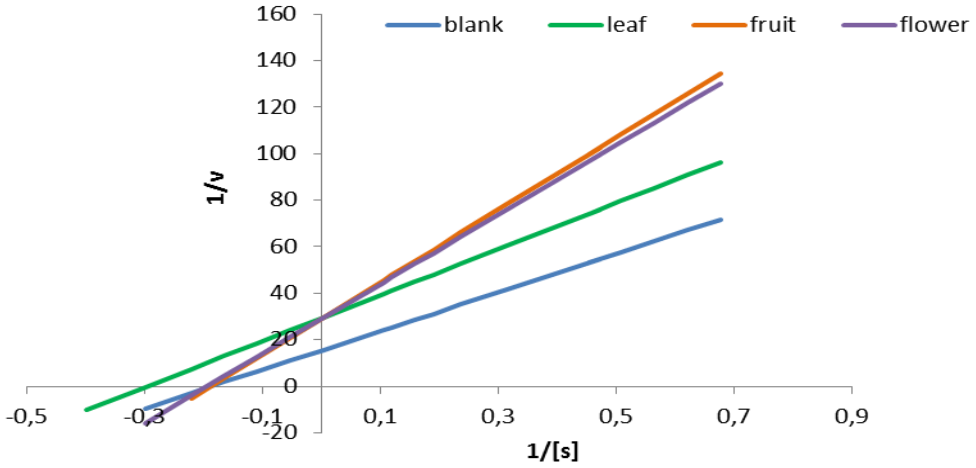

Figure V–6. Lineweaver Burk plot for butyrilcholinesterase inhibition.

While before leaves would bind to the free enzyme or to the enzyme-substrate complex, with BCHE they can only bind to the enzyme-substrate complex, meaning it is an uncompetitive inhibition. On the other hand, fruits which inhibited ACHE via the uncompetitive method, with BCHE they can not only bind to the enzyme-substrate complex,

but also to the free enzyme, since they display a non-competitive inhibition. Flowers were the only extract for which the inhibition type is the same in both enzymatic assays, non-competitive. Overall the results show that the anti-Alzheimer activities vary from plant part to plant part and are dependent on the extraction conditions. Leaf and flower extracts show elevated ACHE inhibitory activity, higher than other plant extracts, including those obtained from medicinal plants. Fruit extract showed a much lower activity than leaves or flowers, but in values close to those expected.

When comparing the anti-Alzheimer activity of different plants (measured as IC_{50}) it is important to use the same enzyme, since testing with a different enzyme, also related with acetylcholine degradation, caused the results to change considerably. Fruits and flowers showed lower inhibitory activity towards BCHE than towards ACHE, while leaves inhibitory activity remained somewhat similar. The inhibitory mechanism also changed for leaves and fruits, from non-competitive to uncompetitive and from uncompetitive to non-competitive respectively, while for flowers the mechanism was always non-competitive. This indicates that while previously it was hypothesized that the compounds responsible for the ACHE inhibition on leaves and flowers were the same, this is clearly not true for the inhibition displayed towards BCHE.

4.1.3 In-vitro digestion effect on anti-Alzheimer potential

The simulated in-vitro digestion can be used to determine the bioavailability of certain phytochemicals of interest and their release or degradation. In this work, the goal was to determine the effect of the simulated digestion process in the activity displayed by the extracts by comparing the final activity with the activity of the original extract. This process had different effects for the different plant parts (Table V–8). All leaf extracts lost some of the inhibitory activity towards both enzymes, especially extract A3 for ACHE and extract A1 for BCHE. This loss in activity is likely related to the degradation of the phytochemicals responsible for inhibiting the enzymes, caused by the low pH of the simulated gastric juice, the higher pH of the simulated pancreatic and intestinal juices or the enzymes contained in them. Epigallocatechin gallate, the ester of epigallocatechin and gallic acid, is an example of a compound which loses its activity due to digestion. While this catechin displayed a high ACHE inhibitory activity, its breakdown product γ-valerolactone showed almost no inhibitory activity [37]. Flowers collected from the tree, also show a reduced activity after being subjected to the digestive process, although in this case the highest difference was for samples C1 for ACHE and C2 for both enzymes.

Table V–8. Enzymatic activity before and after simulated *in-vitro* digestion

Plant part	Sample	[μg/mL]	ACHE activity (%)		BCHE activity (%)	
			Before	After	Before	After
Leaf	A1	140	50.63 ± 0.31	79.48 ± 2.41	56.26 ± 0.83	73.55 ± 1.31
	A2	300	23.07 ± 0.45	62.78 ± 3.17	50.89 ± 0.40	65.64 ± 0.80
	A3	220	27.76 ± 0.45	86.72 ± 1.88	84.44 ± 1.21	91.07 ± 1.21
Fruit	B1	1600	50.94 ± 0.27	86.82 ± 0.92	51.56 ± 0.86	72.52 ± 0.99
	B2	900	92.66 ± 0.81	76.96 ± 1.2	98.02 ± 1.26	76.17 ± 0.86
	B3	3500	80.57 ± 2.66	80.1 ± 1.64	64.88 ± 1.30	75.92 ± 0.86
Flower	C1	600	44.84 ± 0.41	73.54 ± 1.19	57.51 ± 0.69	75.06 ± 1.72
	C2	400	45.36 ± 0.39	77.95 ± 0.79	49.02 ± 0.36	78.30 ± 1.38
	C3	300	59.84 ± 1.08	76.69 ± 1.57	52.10 ± 0.29	76.90 ± 0.86
	D1	315	97.66 ± 1.77	63.83 ± 2.02	88.39 ± 0.82	85.42 ± 1.13
	D2	240	81.93 ± 2.13	74.12 ± 1.2	97.67 ± 2.31	93.28 ± 1.04
	D3	250	96.82 ± 0.59	67.45 ± 0.73	90.47 ± 1.78	73.59 ± 1.72

Results are presented as average ± SD, of replicates from three repetitions. Different letters in the same column mean statistically different results ($p<0.05$)

On the other hand, for extracts obtained using fruits changes were not so linear. Extract B1 showed a decrease in inhibition towards both enzymes while for extract B3 the decrease was only towards BCHE, with the inhibitory activity towards ACHE showing no difference caused by the digestive process. The change caused by the digestive process on extract B3 indicates that while the compounds responsible for inhibiting ACHE are resilient to the digestive process, the same is not true regarding the compounds responsible for inhibiting BCHE. This also means that the compounds responsible for both inhibitions are different. Extract B2 showed an increase in inhibition towards both enzymes after the digestive process. This increased inhibition was unexpected considering the results obtained thus far. Usually extracts inhibition is either maintained or reduced [38-40]. An increase in inhibition could happen if the initial matrix is complex, in which case a digestion would lead to an increased bioavailability of the compounds when compared to the pre-digested matrix. However in this work the increased inhibition was detected in an aqueous extract of the food product, meaning the compounds with inhibitory activity should already be in solution. These results indicate that either the extract obtained was still a complex matrix, with the inhibitory compounds bound to other molecules thus preventing their action on the enzyme, and the digestive process caused their release, or the digestive process transforms the structure of some compounds in a way that enables them to inhibit the enzymes when previously they could not.

Pyrogallol is a benzene triol obtained through the decarboxylation of gallic acid, which displays a higher inhibitory activity towards ACHE than gallic acid [37]. While this decarboxylation normally occurs via heating, it might be possible that the digestive process could in some way contribute to the decarboxylation of gallic acid for the formation of pyrogallol or the transformation of other compounds which also possess higher inhibitory activity. Regarding flowers collected from the ground, for sample D2 there was an increase in inhibition, similarly to the extract B2 for fruits, but unlike fruits this was not the only sample that showed an increase in inhibition after the digestive process. Also unlike fruits, the inhibition increase was higher towards ACHE than towards BCHE. Despite leaf extracts inhibition decrease after the simulated digestive process, it should be noted that leaf extract still displayed a higher inhibitory activity than fruits. While digestate A2 inhibits 38% of the ACHE activity at a concentration of 300 μg/mL, the B2 digestate only inhibits 23% at a concentration of 900 μg/mL. Regarding flowers, while the original extract inhibitory activity towards ACHE is much stronger when using flowers collected from the tree, the same is not true when comparing both flowers after the digestion. Therefore depending on whether or not the extract can be protected from degradation during digestion, a different flower origin should be used.

4.2 Parkinson's

The antiParkinson's potential was determined using the tyrosinase (TYR) enzyme. This enzyme is able to catalyze a reaction which reduces L-Dopa concentration. Since L-Dopa administration is the most common way of ameliorating Parkinson's symptoms, the reduction of L-Dopa concentration is undesirable. In this work, all tested plant parts showed weak inhibitory activity towards TYR. The maximum inhibitions were obtained with A1, B1 and C1 extracts, which after 1 minute showed an inhibition of only 24, 21 and 20% (Table V–9). This was unexpected since *A. unedo* is known to possess arbutin, a glycosylated hydroquinone that is used as standard in this assay [41]. This hydroquinone derivative is also found in some other plants such as bearberry, branberry and blueberry, and is used as a skin-lightning agent due to its melanin formation inhibition [42]. This inhibition occurs at non-cytotoxic concentrations in melanocytes via a reversible competitive process [43]. These unexpected results can likely be explained by the concentration and type of arbutin present, since Pavlovic, Lakusic, Doslov-Kokorus and Kovacevic [41] extracted this compound using a 70% ethanol: water mixture instead of just water.

Table V–9. Maximum TYR inhibition obtained with the pure crude extracts.

Extract	Solvent	Inhibition
A1	H_2O	23.72 ± 0.37^a
	Et100	53.86 ± 2.35^b
	Et50	46.73 ± 2.99^b
B1	H_2O	22.20 ± 2.40^a
	Et100	54.67 ± 2.92^b
	Et50	18.15 ± 0.74^a
C1	H_2O	18.04 ± 1.51^a
	Et100	45.92 ± 1.47^b
	Et50	49.97 ± 3.20^b
D1	H_2O	21.66 ± 4.12^a
	Et100	49.97 ± 2.00^b
	Et50	29.01 ± 3.08^a

Results are presented as average ± SD, of replicates from three repetitions. Different letters in the same column mean statistically different results ($p<0.05$)

In addition, when Funayama, Arakawa, Yamamoto, Nishino, Shin and Murao [42] tested the inhibitory effects of two kinds of arbutin towards tyrosinase from different origins, they found that while β-arbutin is capable of inhibiting tyrosinase from mushroom and mouse melanoma, α-arbutin is only capable of inhibiting tyrosinase from mouse melanoma. This arbutin however is 10 times stronger in its inhibition of the melanoma than β-arbutin. Since the tyrosinase used was obtained from mushrooms, it is possible that even though the results obtained were not promising, *A. unedo* extracts can still display antiparkinsonian effects when administered *in-vivo* or when tested against a tyrosinase of different origin. It is also possible, although unlikely that the arbutin concentration present in the extracts was exceedingly high, since paradoxically, this compound can also promote darkening if a concentration threshold is passed [44] and can increase the tyrosinase activity [45]. In this assay there was no difference between flowers collected from the tree and from the ground, indicating that flavonoids are unlikely to be responsible for the inhibition detected, since flowers collected from the ground are lacking in these compounds. Since none of the pure crude extracts showed considerable inhibitory activity, no Lineweaver-Burk plot was constructed. Following the low inhibition results obtained with just water, it was decided to use the same conditions but with two other solvents, pure ethanol, and a 50 % ethanol: water (v:v) mixture. The results improved considerably for all plant parts, especially when using absolute ethanol. The new results were 54, 55 for leaves and flowers, and 50% inhibition for both flowers when using absolute ethanol. These were different from those obtained when using the 50% ethanol: water (v:v)

mixture, 47, 18, 51 and 29 for leaves, fruits, flower from the tree and flower from ground respectively.

Using different solvents to extract inhibitory compounds, Neagu, Radu, Albu and Paun [22] also obtained better results with a mixture of ethanol: water than with just water, when they tested the TYR inhibition of *P. officinalis* and *C. umbellatum* extracts. In addition, and similarly to the present work, they also obtained extracts with a lower inhibiton towars TYR than towards ACHE. While some of the undiluted extracts obtained in the present work were able to inhibit over 50% of the TYR enzyme activity when compared to the blank, it should be noted that the concentrations necessary were quite high. It should also be noticed that to achieve those inhibition values, ethanol had to be used. Therefore the use of this plant extracts as a drinkable beverage with potential antiParkison's effect is not realizable without previous processing to remove the ethanol from the extract.

5 Conclusion

The extracts showed considerable inhibitory activity towards both enzymes related to Alzheimer's disease. Leaf and flower extracts had especially strong inhibitory activity. This activity was influenced by extraction temperature and was different for both enzymes. The inhibition method for leaves and fruits was also different. Leaf extract showed non-competitive and uncompetitive inhibitions for ACHE and BCHE respectively, while fruit extract was the opposite. Flowers inhibition method was non-competitive for both enzymes. The simulated digestive process influenced the activity of the obtained extracts. It decreased the inhibitions displayed by leaf and flower C extracts, but increased those of extracts B2 and flower D extracts. Leaf extracts still displayed a higher inhibition than either fruits or flower D extracts however. The inhibitory activity towards tyrosinase (related with Parkinson's) displayed by the extracts was much lower than that displayed towards ACHE and BCHE. So low in fact that using only water no extract was able to inhibit more than 50% of this enzyme activity. It was possible to obtain extracts with inhibition above 50%, but only using ethanol, which needs to be removed from the extract prior to consumption. This work shows that leaf and flower water extracts show promising anti-Alzheimer potential and when evaluating the potential inhibitory activity towards ACHE and BCHE enzymes of extracts, care should be taken to subject the obtained extracts to a simulated digestive process since it can significantly change the conclusions regarding the extracts with the highest potential.

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Discussion of Chapter V

Chronic diseases or conditions are responsible for a great number of obits worldwide. According to a WHO report, in 2005 over 60% of the deaths occurred due to chronic diseases or related problems. These include obesity, heart, kidney, liver or other organ problems, diabetes, Alzheimer and Parkinson among many others.

Many of these diseases can be prevented or have their symptoms ameliorated by consuming antioxidant compounds. Previously we studied the antioxidant activity of water extracts from leaves and fruits. This chapter deals with fatty acids and vitamin E, two groups of compounds with antioxidant activity usually not extracted with water. They are however fundamental for human health and must be ingested. Additionally there is mounting evidence that fatty acids may help retard Alzheimer's progress during its earlier stages (Freund-Levi et al., 2006), help reduce β-amyloid (Quinn, 2016) and decrease cognitive loss (Wu et al., 2016).

Both leaves and fruits revealed to be poor in fat content, but rich in vitamin E. The main fatty acids present were polyunsaturated, ω3 and ω6, with a ratio between 1 and 1.5. Given how an imbalance between ω3 and ω6 currently exists in many modern diets and this imbalance can lead to health problems, finding sources that have more ω3 than ω6 is important. Out of the vitamers detected, α-atocopherol was the most abundant, accounting for over 90% of the vitamin E content. This is the vitamer with the most antioxidant activity and the highest half-life time in the human body. This makes the *A. unedo* fruits a prime source for vitamin E in a diet which is lacking this essential micronutrient.

Following the high antioxidant activity and the presence of quercetin on leaves, knowing that this compound can exert antidiabetic effect, and the preliminary antidiabetic assay performed on leaves, the aqueous extracts of leaves, fruits and flowers were tested for their potential antidiabetic effects. Finding plant sources which exert antidiabetic activity is important due to the high prevalence of this condition and the strain it puts in people's lives and the national healthcare budget. According to a national health inquiry the number of diabetes cases self-reported in Portugal rose from 2005 to 2014 on the population 15 years old or older, from 7.7% to 9.3%, with no distinction between men and woman (INSA, 2015). This increase however may be simply due to improvements in diagnostics and not due to increased cases. Given these numbers, it is very likely the number of people affected with diabetes in Portugal reaches an alarming number of 1 million. This number is all the more daunting considering that according to the INE (Instituto Nacional de Estatística – Statistics of Portugal) the normalized mortality rate per 100 000 citizens during the year 2014 was on average 19.8. Regarding intra-hospitalar mortality, diabetes related deaths accounted for

almost 25% of the occurrences, 11736 of a total of 47245, despite a constant reduction of diabetes related deaths per hospitalar case from 2005 (5.5%) to 2014 (1.8%) (WHO, 2016). In our work we found that all extracts displayed some inhibitory activity against α-amylase and a high inhibitory activity against α-glucosidase. The results concerning the α-amylase were different based on the extraction conditions but were mostly influenced by plant part and enzyme origin. The drying of the fruits and in smaller part the origin of the flowers had an impact on the results, with extracts from lyophilized fruits and flowers collected from the tree displaying a higher antidiabetic activity than their counterparts. Fruit extracts were the best at inhibiting α-amylase from the *Aspergillus orizae* mould while leaf and fruit extracts were best at inhibiting mammalian α-amylase, and therefore present the highest potential to be used as antidiabetic agents for α-amylase inhibition. All extracts displayed a much greater inhibition towards α-glucosidase, with leaf and flowers being the most potent extracts. This difference in inhibition has been seen in other works with plants and it is the opposite of the inhibition displayed by acarbose, a drug used in type 2 diabetes, which inhibits α-amylase better than α-glucosidase. This difference makes the use of herbal extracts or compounds extracted from them an attractive perspective in better glycemic control. Additionally it is possible the side effects caused by acarbose, which include diarrhea and flatulence won't occur with herbal extracts.

Dementia related diseases are another cause of mortality in Portugal and in the world. Of these, Alzheimer and Parkinson are the most common. Not only are they deadly, they potentiate the occurrence of other problems and cause a huge strain on the quality of life of the people affected and those around them. Given how important these diseases are and knowing arbutin, a compound with antiparkinsonian effect is present in leaves from the *A. unedo* tree it was decided to study the effect of leaf, fruit and flower extracts on enzymes related with Alzheimer and Parkinson's diseases. While leaf and flowers collected from the tree extracts displayed excellent inhibitory activity towards acetylcholinesterase (ACHE) and butyrilcholinesterase (ACHE) (related with Alzheimer), fruits did not fare as well, but were still better than extracts obtained using flowers collected from the ground. The inhibitory activity displayed towards ACHE and BCHE was different for most of the leaf extracts and all flower extracts. This was also true regarding the method of inhibition for leafs, which inhibited ACHE non-competitively but inhibited BCHE uncompetitively. Inhibition displayed by flowers was always non-competitive. While it was expected for at least leaf extracts to display high inhibitory activity towards tyrosinase that was not the case. This is likely due to a lower than expected content of arbutin. Additionally the extracts lost most of activity after

just 24 hours on the fridge and showed no activity after 48 hours. The same behaviour was found when testing pure arbutin, therefore it is likely that arbutin is indeed being responsible for the observed effect on tyrosinase, but is not in enough quality to inhibit 50% of this enzyme. This is all the more important given the high enzymatic unit count used in this assay, which might mean there is just too much enzyme for the extract to inhibit the reaction. Additionally, while the extracts showed low inhibition, it should be noted that the tyrosinase used was not of mammalian origin, and other authors have noted differences between inhibition displayed towards tyrosinase from mammals or other sources.

Overall fruits and leaves possess a high vitamin E content and good fatty acid quality. Fruits could be used to supplement a diet lacking in ω3. The leaves and flower extracts also showed good inhibition towards diabetes and Alzheimer related enzymes, which could be further explored in new-products obtained from these *A. unedo* parts, to be consumed alone or as an additive for functional foods.

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CHAPTER VI - DEVELOPMENT OF NEW PRODUCTS

In this final chapter we develop three new products using *Arbutus unedo* extracts. Initially we develop a beverage, with anti-Alzheimer potential. Secondly we develop macrocapsules with alginate and calcium chloride, rich in antioxidant activity. Finally we use a spray-dryer to obtain microcapsules with anti-Alzheimer potential to be added to other products. All the new products were tested regarding their stability during storage.

Development of a functional drink from A*rbutus unedo* L. leaves

Ricardo Nunes[1], and Isabel S. Carvalho[2,3]

[1]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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1 ABSTRACT

The goal of the present work was the development of a functional drink with *Arbutus unedo*. Leaf and fruit extract mixtures showed none or antagonistic interactions, therefore it was decided to use only leaves, which had shown the best antioxidant and enzymatic inhibition activities previously. The degradation of the obtained drink antioxidant activity was studied at different storage temperatures and light intensity levels, for a period of 10 days and after *in-vitro* digestion. In the end the acetylcholinesterase inhibition was determined for each condition. Fridge and freezer maintained the acetylcholinesterase inhibitory activity and for the most part the antioxidant activity. Oven storage and direct intense light caused not only a decrease in antioxidant and inhibitory activities but also a change in colour. The obtained beverage has the potential to be used as an anti-Alzheimer functional drink, even after the digestive process, but should be conveniently stored to maintain its properties.

HIGHLIGHTS

A beverage was prepared with *Arbutus unedo* leaf extract from several formulations.
Several storage conditions were used, *in-vitro* digestion effect was evaluated.
Colour, antioxidant activity and Alzheimer enzyme inhibition were studied.
Refrigerated storage was adequate, light caused colour and antioxidant change.
Beverage showed strong enzymatic inhibition, even after *in-vitro* digestion.

KEYWORDS

Beverage development, stability, *in-vitro* digestion, anti-Alzheimer

2 Introduction

Several studies have indicated a positive effect from the consumption of compounds with high antioxidant power. Some of the best sources of antioxidants are vegetables and fruits, however many times these are not freshly available and their consumption in the modern society has decreased. An alternative would be the production of beverages from sources rich in antioxidants. Not only are beverages easier to store, they are also often more resistant to spoilage. *Arbutus unedo* fruits are commonly used in the preparation of alcoholic beverages and jams, however leaves are currently underutilized, despite possessing a much stronger antioxidant activity and high antidiabetic potential via α-glucosidase inhibition as seen in a previous work. In a previous work the acetylcholinesterase (ACHE), butyrilcholinesterase (BCHE) and tyrosinase (TYR) inhibitions were studied, since these enzymes are related with the neurodegenerative diseases Alzheimer (ACHE and BCHE) and Parkinson's (TYR). It was found that leaves and flowers yielded extracts with a high inhibitory activity towards ACHE and BCHE but low inhibitory activity towards TYR. These were also the extracts with the highest antioxidant activity. However, since the flowers collected from the ground lost most of their inhibitory activity and collecting flowers from the tree would be a time-consuming process, in this work only leaf and fruit extracts are studied. It is known that many phenolic compounds can exhibit synergetic interactions when combined (Pereira et al., 2015). It is also known that phytochemicals can react differently to adverse external conditions such as light and heat, and bioactive compounds such as vitamins, carotenoids and anthocyanins, all with antioxidant activity, can suffer a reduction in their contents during storage (Chung, Rojanasasithara, Mutilangi, & McClements, 2016; Sinela et al., 2017; Touati, Barba, Louaileche, Frigola, & Esteve, 2016). Therefore it is important to monitor the changes in antioxidant activity during storage, whose variation can be used as valid criteria for the determination of quality deterioration (Miller, Diplock, & Rice-Evans, 1995; Touati et al., 2016). In this work we first measure antioxidant activity and phenolic and flavonoid contents in pure leaf and fruit extracts and mixtures of them. We then pick the best combination and study the variation of the antioxidant activity measured with TAA, FRAP and RP assays, and the total phenolic and flavonoid contents along a period of 10 days, when stored in a freezer, fridge, ambient temperature in the dark, ambient temperature and light, ambient temperature and high light intensity and oven at 35°C. The colour intensity and yellow, red and blue % were also monitored. Since bioactive compounds can also be degraded during digestion, in this work we also test for the degradation in antioxidant activity and enzymatic inhibition caused by in-vitro digestion.

3 Materials and methods

3.1 Plant material and sample preparation

Leaves and fruits were collected directly from the trees present in the "Herdade da Corte Velada", in Algarve, Portugal, on a dry day of November 2014. Samples were then tagged, put into separate bags inside a thermal container and transported to the FSLab in the University of Algarve. Leaves were air dried at ambient temperature while fruits were stored in a freezer until extraction on the following days. Before the extraction, leaf samples were ground to a powder while fruits were macerated using a mortar and pestle. The selected extraction conditions can be seen on Table VI–1.

Extracts were transferred to Eppendorf tubes and the mixtures were prepared to determine the synergistic effects, with the concentration of each extract being between 0 and 100% at 20% increments.

Table VI–1. Extraction conditions for leaves and fruits.

		code	T (°C)	t (min)	ratio (g/mL)
Leaf	1	A1	40	105	0.02
	2	A2	60	30	0.01
	3	A3	20	180	0.035
Fruit	1	B1	40	105	0.115
	2	B2	60	180	0.03
	3	B3	20	30	0.03

3.2 Storage conditions for degradation determination

For the determination of storage conditions effect on the antioxidant activity, a freshly obtained extract obtained replicating the conditions that yielded the best results was transferred into separate Eppendorf vials which were stored at different environmental conditions (Oven at 35ºC [A]; ambient temperature and light [B]; ambient temperature and intense ligh [C]; ambient temperature and in the dark [D]; fridge [E] and freezer [F]).

3.3 Total Phenolic Content

Total Phenolic Content (TPC) in samples was determined using a spectrophotometric procedure originally described by Singleton and Rossi (1965). Briefly, 0.1 mL of extract (diluted in the extraction solvent) was mixed with 0.5 mL of Folin-Ciocalteau's reagent,0.4 mL of a saturated sodium carbonate solution (7.5 %) and incubated for 30 min in a dark room. Absorbance was read at 765 nm against a blank. Phenolic content was calculated using a

gallic acid calibration curve and the results were expressed as mg GAE/g dw (gallic acid equivalents per gram of dry weight).

3.4 Total Antioxidant activity

Total Antioxidant Activity (TAA) of extracts was determined using a spectrophotometer and the method proposed by Prieto (1998). Briefly, 0.1 mL of diluted extract was mixed with 1.0 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). This mixture was then incubated in a water bath at 95°C for 90 min. Absorbance was read at 695 nm against a blank (negative control of water, ethanol or 80% ethanol: water according to the solvent used) and results were calculated from an ascorbic acid (positive control) calibration curve. Results were expressed as mg AAE (ascorbic acid equivalents)/g dw.

3.5 Reducing Power

Reducing Power (RP) was determined using the method previously described by Oyaizu (1986). Briefly, 0.2 mL of diluted extract were mixed with 0.5 mL of sodium phosphate buffer (0.2 M, pH 6.6) and 0.5 mL potassium ferricyanide (1%). The mixture was then incubated at 50°C for 20 min. After incubation, 0.5 mL of trichloroacetic acid (10%) were added and the mixture was centrifuged at 650 x g for 10 min. 0.5 mL of the supernatant were mixed with 0.5 mL of distilled water and 0.1 mL of ferric chloride (0.1%). Absorbance was measured at 700 nm. Trolox was used as positive control and the negative control was water, ethanol or 80% ethanol: water according to the solvent used in the extraction. Results were expressed as mg TE (Trolox equivalents)/g dw.

3.6 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined using the method previously described by Benzie and Strain (1996). First, three solutions were prepared, a 300 mM acetate buffer, pH=3.6, a 10 mM TPTZ, 40 mM HCl solution, and a 20 mM $FeCl_3.6H_2O$ solution. Mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution and 2.5 mL of FeCl3.6H2O solution and heating the mixture to 37°C prepared the working solution. After cooling down, 0.9 mL of the working solution were mixed with 0.1 mL of diluted extract. After staying 30 min in the dark the absorbance was read at 593 nm. Trolox was used as positive control and water, ethanol or 80% ethanol: water were used as negative control, according to the solvent used. Results were calculated from a Trolox calibration curve and expressed as mg Trolox/g dw.

3.7 Acetylcholinesterase (ACHE) and butyrilcholinesterase (BCHE) inhibition assays

The inhibitory activity of the enzymes were evaluated following the method firstly reported by Ellman et al. (1961) with modifications by Sancheti et al. (2013), but with volumes adjusted for not using 96 well plates. Brielfy, 100 μL of a prepared ACHE solution (0.03 U/mL in 7.2 mM with 0.1 mM EDTA 7.2 pH phosphate buffer) were mixed with 50 μL of test sample and 900 μL of Tris-Hcl buffer (50 mM, pH 8). After incubation for 30 min at 4°C, 100 μL of a 0.3 mM 5,5-dithio-bis(2-nitrobenzoic acid) and 100 μL of a 1.8 mM acetyltiocholine iodide solution were added and incubated for 20 min at 37°C. For BCHE the method was very similar but the enzyme concentration used was 0.1 U/mL and the acetyltiocholine concentration 6.8 mM. The absorbance was then read at 412 nm in a T70 + UV-VIS spectrophotometer from PG instruments (UK). Inhibition was calculated using the following equation:

$$Inhibition\ (\%) = \frac{Acontrol - Asample}{Acontrol} \times 100$$

The concentration of extract correspondent to 50% inhibition (IC_{50}) was determined, when possible, from concentration versus inhibition curves.

3.8 In vitro digestion

The in vitro digestion of the samples was performed following the method described by Liu et al. (2004). Briefly, 0.9 mL of extract sample were mixed with saline solution containing NaCL (140 mM), KCl (5 mM) and BHT (150 μM), for a final volume of 3.6 mL. This solution was acidified to pH 2.0 by adding HCL (1 M). After acidification 0.1 mL of pepsin solution in HCL (0.4 g of pepsin in 10 mL HCL) were added, mixed and incubated in an orbital shaker immersed in water. The shaker was set for 100 rpm and 37 °C. After digestion, the pH of the mixture was increased to 6.9 adding solution of 1 M NaHCO3. Further digestion in the orbital shaker was carried out with the addition of 0.5 mL of a solution containing pancreatin and bile extract (0.45 g of bile extract and 0.075 g of pancreatin in 37.5 mL of 0.1 M NaHCO3). The final volume of the digested sample was adjusted to 4.5 mL and stored until analysis in the following day.

3.9 Statistical analysis

After verifying the homogeneity of variance, a one way analysis of variance (ANOVA) and the LSD or Games-Howel post-hot tests were used for the evaluation of antioxidant

degradation due to environmental conditions and in-vitro digestion. Graphs, linear and quadratic fits and prediction equations were done on JMP 12 Pro ®.

4 Results and discussion

4.1 Choice of beverage composition

Many juices and beverages are prepared using more than one fruit or plant, not only due to the improvement in sensorial quality, but also due to improvements in the benefits gained from ingesting it (Pereira et al., 2015). These improvements can be additive, if the final value is similar to the simple addition of both ingredients, or synergetic, when the improvement is significantly higher than the simple addition. The mixture of phenolic compounds can also have an antagonistic effect, when the value obtained is significantly lower than the average of both components. Given that leaves exist all year round in the *Arbutus unedo* trees, but fruits only exist during 1 or 2 months, it was considered important to include leaves in the beverage. It was also a point to consider, the much higher antioxidant contents displayed by leaf extracts in previous works. On the other hand the sweetness of the fruit could help to balance and improve the taste of the final beverage. For this reason we prepared several beverage compositions in varying percentages of leaf and fruit extracts. These beverages were then evaluated regarding their antioxidant activity, to determine the best one composition and to scan for possible synergetic interactions. As expected the pure leaf extract was always the best beverage considering the antioxidant activity and the results of the fruit extract antioxidant activity improved considerably upon its addition. However while the addition of the leaf extract raised the antioxidant activity of the mixture, the expected vs obtained results for the mixtures prepared showed no synergism. In fact the obtained results were often lower than expected. This lack of synergism was unexpected and happened at all temperatures of extraction. Since not all phenolics show positive interactions between themselves and the phenolic profile of both extracts tested is likely different, it is possible that some compounds which display antagonistic effects when together were present in the mixture. It is also possible that the addition of both extracts increased the concentration of certain compounds turning them from antioxidants into prooxidants. An example of a compound which can have dual function is catechin aglycate. The effect of this compound in the oxidation of LDL protein was studied by Cirico and Omaye (2006) who found that from 2 to 4.3 μMol it has an antioxidant effect lowering the oxidation of LDL protein, at 9.3 the antioxidant effect is reduced and at 20 μMol the compound actually acts as a prooxidant. Similar results were obtained for hesperedin which started showing prooxidant effects at 20 μMol. On the other

hand ferulic acid showed the maximum prooxidant effect at a concentration of 2 μMol, while at the concentration 20 μMol there was no prooxidant effect. Finally, quercetin showed a strong prooxidant effect at all concentrations, but lower at higher concentrations (Cirico & Omaye, 2006). This effect seems to be related to the number of –OH substitutions on the flavonoid structure when in the presence of Cu^{2+} and it is likely that the same compounds can exhibit different activities under different conditions. In that same work however, the mixture of all compounds showed antioxidant activity towards the protection of LDL protein at high concentrations. Girones-Vilaplana et al. (2012) also tested several beverage formulations, in their case lemon juice enriched with exotic berries. In that work they found all extracts had considerable antioxidant properties, with some of the mixtures showing synergetic effects while others didn't.

4.2 Changes in antioxidant activity and colour

4.2.1 During storage

Following the lack of synergism in this work, and in some cases the presence of antagonistic effects between leaf and fruit extracts, it was decided to continue the study using the pure leaf extract. To this end a new extraction using leafs was performed following the conditions set on code A2, which yielded the highest antioxidant activity, but with a higher solid/solvent ratio to obtain an extract with higher concentration. The obtained extract was then submitted to different storage conditions.

A linear fit seemed to be the best option for the determination of the degradation equations for every sample and antioxidant assay (Table VI–2). Storage temperature had a significant effect on the extract degradation. Only the extracts in the freezer (F) suffered no degradation in their antioxidant activity or contents (except flavonoid) ($p>0.05$) throughout the whole storage duration. This was significantly better than those at ambient temperature, which showed significant phenolic differences after 5 days in storage, with no difference between refrigerated (E) and ambient temperatures. On the other hand, extracts in the oven showed degradation after only 2 days. Regarding flavonoid content, oven storage was once again the worse, showing the degradation effect of the temperature, with statistical differences from the original extraction after only 3 days. Light also had caused a faster degradation of flavonoids. While extracts stored at ambient temperature but exposed to light showed degradation after 7 (B) and 8 (C) days, the sample in the dark (D) only showed degradation in the flavonoid content after 10 days. This was the only assay where even the sample stored in the freezer showed degradation when compared to original values. This was unexpected, but can likely

be explained by the formation of ice-crystals due to the below zero temperatures which caused some loss in the flavonoid content.

Table VI–2. Equations for the change in antioxidant activity during storage.

Assay	Treatment	m	b	R^2	Obs
TPC	A	4.094	-0.133	0.924	linear reduction
	B	4.501	-0.139	0.764	linear reduction
	C	5.271	-0.123	0.888	linear reduction
	D	4.303	-0.087	0.854	linear reduction
	E	4.215	-0.066	0.788	linear reduction
	F	4.263	-0.004	0.023	no change
TFC	A	0.272	-0.005	0.837	linear reduction
	B	0.296	-0.008	0.736	linear reduction
	C	0.278	-0.006	0.670	linear reduction
	D	0.259	-0.001	0.013	no change
	E	0.280	-0.007	0.812	linear reduction
	F	0.278	-0.004	0.555	linear reduction
TAA	A	5.010	-0.162	0.837	linear reduction
	B	5.283	-0.142	0.747	linear reduction
	C	4.839	-0.099	0.587	linear reduction
	D	4.920	-0.029	0.082	no change
	E	5.251	-0.061	0.443	linear reduction
	F	5.058	0.045	0.431	linear increase
FRAP	A	6.840	-0.078	0.636	linear reduction
	B	6.824	-0.003	0.002	no change
	C	6.788	-0.114	0.788	linear reduction
	D	6.802	-0.059	0.573	linear reduction
	E	7.717	-0.069	0.569	linear reduction
	F	6.982	0.020	0.126	linear increase

Total phenolic content [TPC]; total flavonoid content [TFC]; total antioxidant activity [TAA]; ferric reducing antioxidant power [FRAP]; oven at 35ºC [A]; ambient temperature and light [B]; ambient temperature and intense ligh [C]; ambient temperature and in the dark [D]; fridge [E] and freezer [F]

When it comes to the TAA assay, oven storage (A) caused once again a quicker degradation, showing a statistical difference from original results after only 1 day. In this assay, light exposure was also a significant factor, with no significant difference between first and last day of storage for sample D (in the dark) while those at ambient (B) and high intensity light (C) showed signs of degradation after 6 days. Regarding FRAP, the decrease and linear fit of the degradation can be seen bellow. Results were statistically different after only one day for all samples except those at lower temperatures, with sample E showing statistical differences only after 7 days of storage.

Some storage conditions also promoted an alteration of sample colour. While the initial colour was yellow, exposure to non-refrigerated temperatures turned the colour into brown orange and brown. This change in colour happened quicker and more intensely on the samples on the oven and exposed to intense light (Figure VI–1).

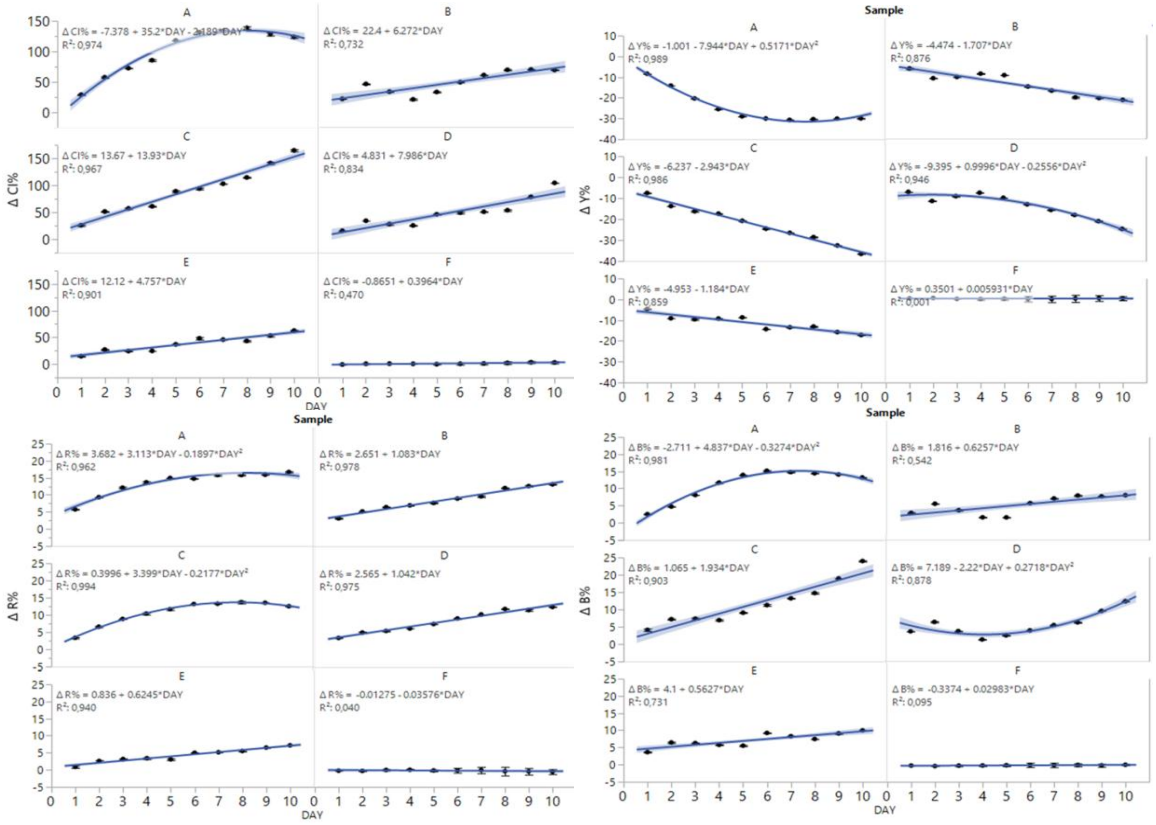

Figure VI–1. Changes in colour parameters during storage. Oven at 35ºC [A]; ambient temperature and light [B]; ambient temperature and intense light [C]; ambient temperature and in the dark [D]; fridge [E] and freezer [F]

Colour change was analytically measured in a spectrophotometer, which allowed the determination of the colour intensity, yellow, red and blue percentages. The ΔCI% behaviour is different from sample to sample. For sample A, the changes follow a quadratic curve, while for other samples a linear fit is adequate (Table VI–3). Sample A was also the only sample to reach a peak in ΔCI%, which happened on day 6. Between samples exposed to light, sample C (intense light) had a much quicker initial variation than any other sample. While sample A stopped increasing its colour intensity, the same did not succeed with sample C, which kept increasing the intensity and darkening. Sample E showed minimal variation while sample F showed no variation between the original CI and the final CI. Regarding Δyellow %, sample A variation is once again best explained by a quadratic curve, together with sample D, while

for other samples a linear fit can accurately describe the variations occurring during storage. Sample A was also the only sample to once again reach a peak of variation.

Table VI–3. Equations for the change in colour parameters during storage.

Parameter	Treatment	m^2	m	b	R^2	type
Δ CI%	A	2.189	35.200	-7.378	0.974	quadratic
	B	-	6.272	22.400	0.732	linear
	C	-	13.930	13.670	0.967	linear
	D	-	7.986	4.831	0.834	linear
	E	-	4.757	12.120	0.901	linear
	F	-	0.396	-0.865	0.470	linear
Δ Y%	A	0.517	-7.944	-1.001	0.989	quadratic
	B	-	-1.707	-4.474	0.876	linear
	C	-	-2.943	-6.237	0.986	linear
	D	0.256	1.000	-0.940	0.946	quadratic
	E	-	-1.184	-4.953	0.859	linear
	F	-	0.006	0.350	0.001	linear
Δ R%	A	-0.190	3.113	0.368	0.962	quadratic
	B	-	1.083	0.265	0.978	linear
	C	0.218	3.399	0.400	0.994	quadratic
	D	-	1.042	2.565	0.975	linear
	E	-	0.625	0.836	0.940	linear
	F	-	-0.036	-0.013	0.040	linear
Δ B%	A	-0.327	4.837	-2.711	0.981	quadratic
	B	-	0.626	1.816	0.542	linear
	C	-	1.934	1.065	0.903	linear
	D	0.272	-2.220	7.189	0.878	quadratic
	E	-	0.563	4.100	0.731	linear
	F	-	0.030	-0.337	0.095	linear

Variation in colour intensity [Δ CI]; variation in yellow [Δ Y]; variation in red [Δ R]; variation in blue [Δ B] Oven at 35ºC [A]; ambient temperature and light [B]; ambient temperature and intense ligh [C]; ambient temperature and in the dark [D]; fridge [E] and freezer [F]

For samples at ambient temperature, the variation was much quicker on sample C (intense light) than on sample B (ambient light), with sample D (dark) showing the slowest rate of variation. In fact sample D only started showing variation after 5 days in storage, about the same time sample A took to reach the maximum variation. Sample E showed once again minimal variation while sample D showed no significant variation during the storage period tested. The variation of red colour % with time is mostly similar to the inverse of what happened with Δyellow %. Samples A and C were once again the samples where the highest degree of variation was found, while sample E showed the least amount of variation and sample F showed no significant variation.

The kinetics of Δred % are best described with a quadratic curve for samples A and C, while the remaining samples can be well described with a linear fit. Samples A and C reached a maximum of variation after 6 days in storage. The results measured in the spectrophotometer are according to what was observed visually, with samples A and C changing colour to orange-brown much more quickly than the other samples. For Δblue % the kinetics of the variation were similar to those of yellow but inverted, with samples A and D being best described with a quadratic curve and sample C variation occurring quicker than either B or D.

By the end of the storage period, sample A had a colour very similar to samples B and D. On the other hand sample C, which had been under intense light, showed a darker brown colour. Sample E and F, which had been refrigerated showed no visual difference in colour from the original, however sample F increased its turbidity (Figure VI–2). This change in turbidity caused by prolonged storage in a freezer can be used as a quality control parameter to detect if the beverage has been previously frozen.

Figure VI–2. Colour change after 10 days of storage. From left to right, A, B, C, D, E and F.

It is known that phytochemical compounds can react differently to temperature and light according to their structure, with some compounds being more sensitive than others. From those present in the extract, compounds classified as phenolics showed the fastest degradation, although not very dissimilar from flavonoid degradation.

Flavonoids and anthocyanins are compounds with high antioxidant activity known to be both light and thermosensitive (Alighourchi & Barzegar, 2009; Mrmosanin et al., 2015). Other compounds known to be both light and thermosensitive and which degradation can be contributing for lower antioxidant activity are carotenoids and vitamins (Mgaya-Kilima, Remberg, Chove, & Wicklund, 2014; Wibowo et al., 2015). Mrmosanin et al. (2015) studied the storage temperature effect on the stability of catechins, procyanidins and flavonoid contents. They found all compounds were degraded quicker at the temperatures of 35°C than at 4 or 22°C, which is in accordance with results from the present work. When Alighourchi

and Barzegar (2009) studied the kinetics of anthocyanin degradation, they found that both light and temperature had negative effect, with the degradation being less noticeable at lower temperatures. They also noticed that with the degradation of anthocyanins the samples colour turned closer to brown. Similar results were verified in the present work, with those samples at ambient temperature being darker than refrigerated samples, and those exposed to intense light being the darkest of all. Other reasons for the browning of the beverages could include the degradation of ascorbic acid, caramelization of sugars and the Maillard reaction, which is a non-enzymatic reaction between reducing sugars and amino acids (Bharate & Bharate, 2014). The degradation of ascorbic acid is one of main reasons for orange juice browning, and its loss could also account for the decrease in antioxidant activity detected in the present work (Bharate & Bharate, 2014). It is a process involving the transformation from ascorbic acid into xylosone or other compounds which can then react with amino acids to form brown pigments (Bharate & Bharate, 2014). Mgaya-Kilima et al. (2014) tested a roselle-fruit blend, and noticed a decrease in anthocyanins and in vitamin C which was attributed to both enzymatic and non-enzymatic reactions in the presence of oxygen. They also noticed that TPC degradation was affected by storage temperature (higher degradation at higher temperatures), which is in agreement with the present work. On the other hand in their work FRAP assay results did not decrease substantially while in the present wok FRAP was the antioxidant assay which showed the most difference between storage periods. This degradation in the present work is likely due to the TPC degradation, since from a previous work it was concluded that TPC and FRAP were highly correlated. Considering results obtained, the changes in colour and turbidity can be used to detect abuses during storage of the obtained beverage, while FRAP is the best assay to detect changes in the antioxidant activity, since it was the most sensitive.

4.2.2 During in-vitro simulated digestion

Bioactive compounds can also lose their properties during digestion. In the digestive system they can be degraded due to the action of pH variation or enzymes, and it is therefore important to study the stability of extracts to the digestive process. For this reason a simulation of the digestive process was conducted with fresh extract, using enzymes and pH levels similar to those found on the digestive system. TPC content was reduced by approximately 20% due to the simulated digestive process. Similarly, FRAP was reduced by approximately 19% ($p<0.05$). On the other hand TAA showed a non-significant ($p>0.05$) increase of 1.3 %. The similar decrease in TPC and FRAP was expected, since these two

assays have been very well correlated. It is possible that the phenolic degradation can be reduced, if protective agents are introduced into the obtained drink. Similar results in degradation due to digestion have been seen by other researches in similar drinks and are mostly attributed to the action of acidic pH. The fact that TAA was not reduced after the simulated digestive process indicates that the compounds responsible for this activity are resistant to both pH variations that exist during digestion and to some of the stomach and pancreatic enzymes. When Mosele et al. (2016) studied the stability of different phenolic compounds present on *A. unedo* fruits, they found that at the end of the digestive process gallic, galloyl shikini and gallic acid hexonoside, catechin, epicatechin, dephinidin arabinoside and lutein were the only compounds that increased during digestion. On the other hand other gallic acid and gallotanins derivates decreased to almost zero and some cyanindins suffered an 80% reduction. Similar results were found by Murugan et al. (2016), which found a decrease in the antioxidant activities of *Phoenix loureirii* methanolic extract after a simulated *in-vitro* digestion. This decrease happened in all their tested methods, but in different proportions for peduncle and fruit extracts. It should be noted that when they tested the extracts with the FRAP assay, they found that while the final activity was reduced, there was an intermediate increase in activity after the gastric digestion. In their work they also studied the polyphenolic content decrease and found this decrease happened during both steps of the simulated digestive process. They also found not all compounds reacted in the same way to the simulated digestive process, and this reaction was also dependent on their original matrix. While gallic acid showed a decrease of almost 40% on the peduncle extracts, it only showed a decrease of 1.25% on the fruit extracts. On the other hand chlorogenic acid showed a decrease of 15% for the peduncle, but a decrease of almost 60% for fruits. Both p-coumaric and ferulic acids showed a 100% lost if the matrix was the fruit, but only 40% loss on the peduncle matrix. A decrease in antioxidant activity during the digestive process is expected and in accordance with other works, however even with the reduction experienced on the phenolic levels and FRAP assay, the beverage prepared in the current work still has a high amount of antioxidants present, which could translate into beneficial health effects to the consumers.

4.3 Changes in acetylcholinesterase and butyrilcholinesterase inhibition

4.3.1 During storage

As seen before, leaves possess a high inhibitory activity towards ACHE and BCHE, both important in the Alzheimer's disease symptoms control. These activities can be related to

several compounds, two of which are phenolics and flavonoids. In a previous work, we tested several extracts from *A. unedo* tree parts, and found flowers and leaves to be the most powerful. The beverage obtained in this work, showed similar results regarding the IC_{50} of ACHE and BCHE to what was previously observed for pure leaf extract obtained at high temperature. This was despite a slight variation on the extraction conditions (higher solid/solvent ratio) which gives the obtained beverage the advantage of being at a much higher concentration, meaning even after some degradation of the bioactive compounds its action may still be strong enough to be of biological relevance. Since it was seen that the phenolic and flavonoid contents of the prepared beverage suffer a decrease during storage at inadequate storage conditions, it was deemed important to test if the same decrease is verified on the potential anti-Alzheimer's activity of the beverage. To this end the inhibitory activity towards the enzymes after 10 days in storage was tested and compared to the inhibitory activity of the original extract. Results show some storage conditions led to a decrease in inhibitory activity (Table VI–4).

Table VI–4. ACHE and BCHE inhibition after 10 days in storage under different conditions

	Inhibition			
Sample	ACHE %	Loss to original %	BCHE %	Loss to original %
Original	69.78 ± 0.40[a]	-	65.61 ± 0.41[a]	-
A	53.88 ± 0.40[b]	-22.79 ± 0.88	53.02 ± 0.29[b]	-19.19 ± 0.54
B	56.66 ± 0.80[b]	-18.80 ± 1.43	58.68 ± 0.44[c]	-10.56 ± 0.70
C	47.45 ± 0.80[c]	-32.00 ± 1.34	50.04 ± 0.59[b]	-23.18 ± 0.70
D	60.64 ± 0.80[d]	-13.11 ± 0.99	57.96 ± 0.51[c]	-11.65 ± 1.33
E	67.59 ± 0.34[a]	-3.13 ± 1.01	65.78 ± 0.22[a]	0.26 ± 0.87
F	68.85 ± 0.50[a]	-1.33 ± 0.71	65.23 ± 0.51[a]	-0.58 ± 0.84

Standard	Inhibition	
	ACHE %	BCHE %
Gallic acid	2.27 ± 0.49	11.53 ± 2.63
Chlorogenic acid	3.70 ± 0.56	12.72 ± 2.71
Caffeic acid	4.49 ± 0.56	10.77 ± 1.77
Quercetin	7.36 ± 1.81	16.86 ± 1.92
Citric acid	2.64 ± 0.00	9.00 ± 0.46
Ascorbic acid	2.55 ± 0.49	8.79 ± 0.90
Arbutin	4.21 ± 0.35	8.07 ± 0.48

Results are presented as mean ± standard deviation of three replicates. Loss to original calculated as % variation of the initial inhibition. Different letters in the same column mean statistically different results. Oven at 35ºC [A]; ambient temperature and light [B]; ambient temperature and intense ligh [C]; ambient temperature and in the dark [D]; fridge [E] and freezer [F].

Similarly to what succeeded with the antioxidant activity, samples stored in the freezer (F) showed no decrease in inhibitory activity. However, contrary to what succeeded for the

antioxidant activity, samples stored in the fridge (E) also did not show a significant decrease. On the other hand, samples stored in the oven (A) and under direct light (C) suffered significant changes, showing a decrease in their inhibitory activity towards the ACHE and BCHE enzymes. While there were significant differences between the samples subjected to ambient light (B) and those subjected to intense light (C), there was no difference between ambient light (B) and protected from light (D) storage conditions. Additionally, no difference regarding the degradation of inhibitory activities towards ACHE and BCHE for extracts stored in the dark or in the oven was detected but ACHE inhibitory activity degradation was quicker than that of BCHE for extracts stored under direct light. It is likely that the compounds degraded under direct light and in the oven had both antioxidant and anti-enzymatic activity, which would explain why the worse storage conditions for maintaining the antioxidant activity were also the worse conditions for maintaining the ACHE and BCHE inhibitory activities.

Several pure compounds, used as standards in antioxidant activity assays, were tested for their inhibition towards ACHE and BCHE. None of the tested compounds showed significant inhibitory activities. Most of the standard results obtained were in accordance with other authors. Quercetin however, does inhibit ACHE and BCHE according to Orhan et al. (2007), but at a much higher concentration than tested on this work. While the pure compounds at a concentration of 100 μg/mL showed nearly no inhibition on both enzymatic assays (Table VI–4), it is likely the inhibitory activity detected on the extract is due to a mix of compounds instead of a single compound.

4.3.2 During in vitro digestion

Not many studies exist regarding the effect a simulated *in-vitro* digestion on the enzymatic inhibitory activities of extracts towards ACHE and BCHE. Many focus only on the activities prior to digestion when comparing the potential of the plants in the treatment of the Alzheimer's disease. In this work, we tested the ACHE and BCHE inhibitory activity of the obtained extract and the impact the simulated *in-vitro* digestion process had on them. Results after the *in-vitro* digestion reveal that there is a decrease in the inhibitory activity. The inhibition decreased from 68% at 300 μg/mL to 46% for acetylcholinesterase and from 53 to 46% for butyrilcholinesterase, at the same concentration. This decrease is most likely linked to the decrease in phenolic content during the simulated digestion process, which was seen earlier. While the digestate shows a lower inhibitory activity than the crude extract, it should be noted that at a concentration of 300 μg/mL for approximately 50%, the digested drink still

shows promising results. For example the inhibition of five drinks prepared using medicinal plants and grapes was in the order of the 20%, despite having a much higher phenolic and flavonoid contents than the beverage of this work. The inhibition of 8 alcoholic beverages prepared in the same work was also in the order of 20% and much higher than the 3% inhibition of red wine, a beverage widely consumed in the world (Nanasombat, Thonglong, & Jitlakha, 2015). Also similar was the inhibition of 4 terebinth coffee brands (25 to 34%) at a concentration of 200 μg/mL (I. E. Orhan et al., 2012). The seeds used to obtain 4 coffees common in Turkey showed inhibitory activities between 10 and 50% at a concentration of 300 μg/mL, while their coffees inhibition ranged between 5 and 30% at the same concentration (Sekeroglu et al., 2012). On the other hand, when testing beverages composed by lemon juice enriched with other fruits, Girones-Vilaplana et al. (2012) found their IC_{50} to range from 8 to 20 mg/mL for both ACHE and BCHE, much higher than what was required in the current work. Also using fruits the same author found the inhibition of 5% juice from papaya, lemon, noni and their combinations to range from 7 to 20%. The inhibition decrease after digestion detected in the present work is contrary to what Murugan et al. (2016) found when testing the effect of simulated *in-vitro* digestion on the extracts of *P. loureirii*. They found the final inhibitory activity actually increased due to digestion, although its result after gastric digestion was higher than after pancreatic digestion. This increase was unexpected since it happened despite a decrease in the antioxidant and polyphenolic contents, which included the complete loss of p-Coumaric and ferulic acids present in fruit extracts, during the digestive process. It is possible that the solvent used in their work can account for the unexpected results, since methanol is capable of extracting other compounds which were not tested. On the other hand when Khadri et al. (2016) tested the effect of i*n-vitro* digestion simulated process on the inhibitory activity towards ACHE displayed by *Ruta chalepensis* and *Ruta montana* they found a reduction in the activities, with post digested activity ranging between 82 and 100% for *R. chalepensis* and between 86 and 100% for *R. montana* of the original activity, which they consider could be due to interference of the test reagents. In their work, and contrary to Murugan et al. (2016), they found pancreatic digestion had no effect in the reduction of the enzymatic inhibitory activity. They also found no degradation of bioactive compounds, which could explain the lack of severe reduction in antiacetylcholinesterase activity. Similar results were observed by Dias et al. (2015) which tested the effect of *in-vitro* digestion on the antioxidant and acetylcholinesterase inhibitory activities of *Erica australis* extracts, a plant from the same family as *A. unedo*. Their extracts showed an IC_{50} between 257 and 296 μg/mL for decoction and infusion respectively, which

was not statistically different after the simulated digestive process. These values are higher than those displayed in this work for *A. unedo* prior to the digestive process and similar after the digestive process. However, it should be noted that both authors used a much lower amount of ACHE and comparatively higher extract amount than the one used in the present work, meaning some degradation could have occurred but due to a higher extract/enzyme proportion it was not detected. Finally, when testing *Lavandula viridis* methanolic extracts, Costa et al. (2011) found its IC_{50} to range between 245 and 285 μg/mL. These values, while higher than those of the prepared beverage before digestion, are similar to those obtained after digestion. They also tested the *in-vivo* effect of the extract administration on the enzymatic activity in the mouse brain, and found that a peritoneal injection of extract at 50 mg/kg led to an inhibition of 87 and 68% for ACHE and BCHE respectively. Considering the obtained beverage showed a similar enzymatic inhibition after digestion, it is likely the beverage obtained in this work would show similar inhibitions when tested *in-vivo*. This would mean the obtained beverage has an effect that is powerful enough to be of biological significance.

5 Conclusion

When testing a mixture of fruits and leaves from *Arbutus unedo*, no synergism was detected. It was therefore decided to pick the best overall extract, the pure leaf, to continue with the work. Both storage conditions and *in-vitro* digestion can affect negatively the antioxidant activity of the extract. The worse storage conditions were oven at 35°C and intense light, which caused a severe loss in antioxidant activity and colour change. Storage in the fridge was able to maintain for the most part the antioxidant and colour of the original extract, while storage in freezer while maintaining the antioxidant activity, caused an undesired increase in turbidity. The simulated digestive process caused a decrease of approximately 20% in TPC and FRAP, but no appreciable difference in TAA. Similar results were found for inhibitory activities towards acetylcholinesterase and butyrilcholinesterase for the *in-vitro* digestion, which reduced the inhibition from 70 to 50% at the same concentration. Samples stored in the oven and under direct intense light also showed the most degradation in the inhibitory activity, however neither fridge nor freezer storage showed any degradation.

Overall the beverage obtained shows a high antioxidant and enzymatic inhibitory activity, even after the *in-vitro* digestion, which could reveal advantageous for the treatment of Alzheimer's disease symptoms.

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Production, optimization and stability of *Arbutus unedo* L. microcapsules rich in antioxidants

Ricardo Nunes[1] and Isabel S. Carvalho[2,3]

[1]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

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1 Abstract

This works aim was to encapsulate *Arbutus unedo* leaf and fruit extracts to obtain edible macrocapsules as novel products. Shape, integrity and stability were studied. Best encapsulation was obtained with a 2% alginate solution mixed in a 2:1 proportion with extract, 8% $CaCl_2$ solution and a permanence time of 10 minutes. From the three formulations studied, one with just fruit extract (A), one with fruit on the inside and leaf on the $CaCl_2$ solution (C) and one with leaf on the inside and fruit on the $CaCl_2$ solution, the best results regarding the antioxidant activity were obtained with formulation B (leaf as core and fruit as enrichment on the $CaCl_2$ solution). This solution capsules had a phenolic content of 2 mg GAE/mL and antioxidant activities of 2.4 and 2.6 mg AAE (TAA) and mg TE per mL (FRAP). On the other hand formulations A (fruit as core) and C (fruit as core and leaf as enrichment on the $CaCl_2$ solution) were more stable to degradation. Results show that both extracts can be successfully encapsulated, yielding a product rich in antioxidant activity, which can be added to other food products or consumed fresh.

KEYWORDS

Arbutus unedo, encapsulation efficiency, experimental design, response surface and neural network modelling, storage stability, antioxidant activity.

2 Introduction

The encapsulation method consists in enveloping one or more compounds in one or more materials. This envelopment has the goal of protecting the core (material to be encapsulated) from degradation caused by external factors, such as oxygen, heat and light. It also can protect from evaporation of volatile compounds and mask undesired tastes or odours from the consumers' palate (Bustamante et al., 2017, Comunian & Favaro-Trindade, 2016). Depending

on the material of the wall, it can also be used to improve bioavailability by preventing the degradation caused by the stomach pH. This technique is also used in the food-industry to develop novel products with added value. Compounds of interest to be encapsulated include fatty acids, phenolics and other antioxidants, vitamins, and bacteria that act as probiotics. Several techniques can be used to achieve the encapsulation (Comunian & Favaro-Trindade, 2016). These techniques can be split and classified according to the methodology used. Physical techniques include spray-drying, spray chilling, spray coating and the use of microfluidic devices. Physico-chemical techniques include coacervation, emulsions, solvent evaporation and molecular inclusion, and are mostly used to encapsulate lipophilic compounds (Comunian & Favaro-Trindade, 2016). Finally the encapsulation can also be achieved using the chemical method of polymerization, for which gelatin, pea protein, pectin, chitosan, gum Arabic or alginate can be used (Bustamante et al., 2017, Comunian & Favaro-Trindade, 2016, Santhalakshmy et al., 2015). In the polymerization method, two liquids which are free-flowing when separated create a polymer when joined together, becoming solid and inseparable. The technique used will influenced the final particle. If complex coacervation, spray coating, polymerization or molecular inclusion are used, then the obtained particle will be called a microcapsule, which can contain one or several nuclei. By using spray drying or spray chilling, the obtained particles will be of the microsphere type, a matrix mix where the desired compound is spread throughout the whole volume of the capsule, sometimes even on the surface. The release of the substances contained within the capsules can occur by diffusion or by digestion, depending on the physical properties of the encapsulating material (Comunian & Favaro-Trindade, 2016).

Alginate is a polysaccharide of natural origin, obtained from marine plants, which can be used in the food field due to being edible and generally recognised as safe (Sudareva et al., 2016). It is used to protect compounds during stomachic digestion, due to its different behaviours depending on the pH it is subjected to, and since it is hydrophilic, it is ideal for encapsulating the aqueous extracts used in this work. It can also be used to encapsulate unstable materials by ionic gelation, due to its ability to form a gel at ambient temperature, when it comes into contact with Ca^{2+} or Mg^{2+} cations which will substitute Na^{+} in sodium alginate (Pasukamonset et al., 2016, Sudareva et al., 2016). In this work, the gelification that occurs between sodium alginate and $CaCl_2$ was used to form a protective wall surrounding the core where the *Arbutus unedo* L. extract to be protected was situated. This fruit bearing tree is a source of income for many small villages of the Portuguese interior, mostly used in the production of alcoholic beverages. Its leaves are known to possess antioxidant activity and are

used in traditional medicine to treat diabetes while its fruits are rich in vitamins and antioxidants (Oliveira et al., 2011, Barros et al., 2010, Delgado-Pelayo et al., 2016, Orak et al., 2011). In a previous work it was revealed that leaf extracts, and fruits to a lesser extent, also possess anti-Alzheimer potential, via the inhibition of acetylcholinesterase and butyrilcholinesterase. In this work we first study the parameters for obtaining an extract rich in antioxidants and the physical parameters for the encapsulation process to provide spherical particles, capable of preventing liquid leakage after being stored in an oven for 1 week are also studied. The encapsulation was then modelled using response surface methodology and neural networks analysis. Finally, we studied three different compositions (A- fruit extract as core, B- leaf extract as core and fruit as enrichment for the $CaCl_2$ solution, C- fruit extract as core and leaf as enrichment for the $CaCl_2$ solution) regarding their initial antioxidant activity and its degradation during a 6 week storage period. The goal was to obtain particles which can be consumed individually or as a novel food ingredient.

3 Material and methods

3.1 Reagents

All reagents used in this work were of analytical grade. Ammonium molybdate, Folin-Ciocalteu reagent, gallic acid, monosodium phosphate, quercetin, sodium phosphate dibasic and Trolox were all purchased from Sigma-Aldrich Co. Ltd (Poole, UK). Ascorbic, glacial acetic and hydrochloric acids, iron (III) chloride, methanol, sodium acetate, sodium carbonate, sulfuric acid, trichloroacetic acid, 2,4,6-tripyridyl-2-triazine (TPTZ), were purchased from VWR (Pennsylvania, USA). Absolute ethanol and aluminium chloride were purchased from Merck (Nottingham, UK).

3.2 Equipment

The spectrophotometer used was a UV/Vis spectrometer (T70+ UV/Vis Spectrometer) acquired from PG Instruments Ltd, England.

3.3 Plant material

The fruit and leaf samples were collected from the "Herdade da Corte Velada" in Portugal, GPS: N 37 11.536; W 8 40.820, during the fruiting period of November 2012. A total of 25 samples for leaves and 18 for fruits were collected from trees 3 to 4 years old spread in an area of 180 hectare. After collection samples were put in tagged plastic bags inside an isothermal container until arrival on the FSLAB. Leaves were dried in an incubator at 40ºC for 72 hours (Binder BD 53 incubator, Binder, Tuttilgen, Germany) while fruits were directly

put in the freezer at -20°C. When dried leaves were ground in a mortar and pestle followed by a kitchen grinder. A composite sample, made with 2 grams of each individual sample, was stored in a freezer until use.

3.4 Screening design

Similarly to a previous work, the effect of temperature, time, ratio and stir was studied using a screening design, with 4 factors on two levels and a slightly of centre point made on JMP 10© (Table VI–5). Temperature ranged between 20 and 60ºC; time between 30 and 180 minutes; solid: solvent ratio between 0.01 and 0.035 g/mL and stirring between 60 and 200 rpm. All extractions were performed using water. The results of the antioxidant activity for each extract were evaluated based on the amount of antioxidants per mL of solution.

Table VI–5. Screening design for determination of best extraction conditions

Set	Temperature (°C)	Time (min)	Ratio (g/mL)	Stirr
1	20	180	0.030	200
2	40	105	0.115	130
3	20	30	0.030	60
4	20	180	0.200	60
5	60	30	0.030	200
6	60	180	0.200	200
7	20	30	0.200	200
8	60	30	0.200	60
9	40	105	0.115	130
10	60	180	0.030	60

3.5 Total Phenolic Content

Total Phenolic Content (TPC) in samples was determined using a spectrophotometric procedure originally described by Singleton and Rossi (1965) using Folin-Ciocalteau's reagent, and a saturated sodium carbonate solution. Phenolic content was calculated using a gallic acid calibration curve and the results were expressed as mg GAE/mL (gallic acid equivalents per milliliter of solution).

3.6 Total Flavonoid Content

Total Flavonoid Content (TFC) was determined according to the method of Lamaison and Carnat (1990) using a 2% methanolic $AlCl_3.6H_2O$ solution. Flavonoid content was calculated using a quercetin calibration curve, and the results were expressed as mg QE (quercetin equivalents)/mL.

3.7 Total Antioxidant activity

Total Antioxidant Activity (TAA) of extracts was determined follwing the method proposed by Prieto (1998) using sulphuric acid, sodium phosphate and ammonium molybdate. Results were calculated from an ascorbic acid calibration curve, and expressed as mg AAE (ascorbic acid equivalents)/mL.

3.8 Reducing Power

Reducing Power (RP) was determined following the method previously described by Oyaizu (1986), using sodium phosphate buffer, potassium ferricyanide, trichloroacetic acid and ferric chloride. Results were calculated from a Trolox calibration curve, and expressed as mg TE (Trolox equivalents)/mL.

3.9 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined using the method previously described by Benzie and Strain (1996), using acetate buffer, TPTZ, HCl and $FeCl_3.6H_2O$. Results were calculated from a Trolox calibration curve, and expressed as mg TE (Trolox equivalents)/mL.

3.10 Encapsulation conditions

3.10.1 Capsule evaluation

Initially the minimum height and adequate angle that would yield round capsules were studied. This was necessary to prevent malformation of capsules due to hitting the bottom of the container containing the encapsulating agent. The stirring speed to prevent damaging the obtained capsules was also studied, together with the angle of the tip. After the adequate method for the formation of the capsules was established, a new experimental design was performed, to determine the best encapsulation conditions, using 2% of alginate, which was chosen based on preliminary tests. A higher concentration of alginate leads to a thick solution which is not easy to prepare and properly mix with the extract, while a lower % leads to a lower encapsulation efficiency (data not shown). In the design the % of the coencapsulation solution ($CaCl_2$) was set to range from 2 to 8%, the extract: alginate proportion was set to range from 1:1 to 1:2 and the time during which the capsules remain in the $CaCl_2$ solution was set to range from 10 to 30 minutes (Table VI–6). In addition, some capsules were also prepared with the addition of gelatine and compared with the original capsules. The evaluation of the capsules was based on the visual aspect and liquid leakage after 1 week.

Table VI–6. Encapsulation conditions studied

Code	Alginate (%)	$CaCl_2$ (%)	Extract: Alginate	Time (min)
1	2	8	1:2	10
2	2	8	1:2	20
3	2	8	1:2	30
4	2	5	1:2	10
5	2	5	1:2	20
6	2	5	1:2	30
7	2	2	1:2	10
8	2	2	1:2	20
9	2	2	1:2	30
10	2	8	1:1	10
11	2	8	1:1	20
12	2	8	1:1	30
13	2	5	1:1	10
14	2	5	1:1	20
15	2	5	1:1	30
16	2	2	1:1	10
17	2	2	1:1	20
18	2	2	1:1	30

3.10.2 Encapsulation efficiency

Encapsulation efficiency was determined based on the retention % of the total phenolic content after encapsulation with the following formula:

$$Efficiency\ (\%) = \frac{TPC\ of\ the\ encapsulate}{Theoretical\ TPC\ of\ the\ encapsulate}$$

3.10.3 Modelling

A model was constructed using the data of the encapsulation efficiency. To obtain this model JMP Pro 12 was used. Both response surface (RSM) and neural network (NNw) models were used. For both methods validation was performed using the holdback method, which kept 33% of the data for validation. The data for validation was chosen randomly by the software used.

3.11 Enriched encapsulation

Upon determining the best encapsulation condition from Table VI–6, new capsules were prepared. Three different compositions were studied, composition A was *A. unedo* fruits, composition B was based on the fruits but the $CaCl_2$ solution was enriched with leaf extract and composition C was based on the leaves but the $CaCl_2$ solution was enriched with fruit extract. The phenolic content and antioxidant activity were measured after macerating 18 capsules, the number 1 mL of solution yielded, and reported as mg of standard equivalent per

mL. The stability of these capsules phenolic content and antioxidant activities was then studied over a period of 6 weeks.

3.12 Scanning electron microscopy (SEM)

Capsule analysis was performed using a scanning electron microscope (SEM) from JEOL (JCM-6000Plus). Capsules were attached to a stub using a two-sided adhesive carbon tape and examined using an acceleration voltage between 5 and 15 kV at high vacuum.

3.13 Statistical analysis

For determination of statistical differences, ANOVA was performed on SPSS 22 at an α of 0.05, with the post-hoc Games-Howell or LSD after checking the variance homogeneity with Levene's test.

4 Results

From the results obtained from the screening design, the best extraction condition was by far condition 6 (
Table VI–7). For the encapsulation, several tip angles (A), distance between the tip and the $CaCl_2$ solution (B), distance from the surface of the solution to the bottom of the container (C), size and speed of the agitation bar (D) and diameter of the container (D) were studied. These all affected the obtained capsules and are important to determine before proceeding with the encapsulation process. The values for these parameters which were deemed adequate for the pilot assay were then used throughout the work (Figure VI–3).

Table VI–7. Results obtained from the screening design for best extraction conditions prior to encapsulation

Set	TPC (mg GAE/mL)	TAA (mg AAE/mL)	RP (mg TE/mL)	FRAP (mg TE/mL)
1	0.20 ± 0.01	1.11 ± 0.01	0.12 ± 0.01	0.17 ± 0.01
2	0.69 ± 0.03	2.95 ± 0.09	0.76 ± 0.03	0.77 ± 0.00
3	0.23 ± 0.03	0.79 ± 0.01	0.27 ± 0.03	0.32 ± 0.01
4	0.85 ± 0.01	3.17 ± 0.10	1.00 ± 0.07	0.81 ± 0.03
5	0.44 ± 0.01	0.79 ± 0.01	0.30 ± 0.02	0.33 ± 0.00
6	1.69 ± 0.06	4.46 ± 0.14	1.53 ± 0.14	1.35 ± 0.05
7	0.84 ± 0.02	2.30 ± 0.04	0.83 ± 0.08	0.59 ± 0.02
8	0.67 ± 0.03	2.63 ± 0.09	0.74 ± 0.05	0.57 ± 0.04
9	0.45 ± 0.02	1.65 ± 0.03	0.51 ± 0.01	0.45 ± 0.02
10	0.27 ± 0.03	0.59 ± 0.02	0.21 ± 0.02	0.24 ± 0.01

Results are presented as mean ± standard deviation of three replicates.

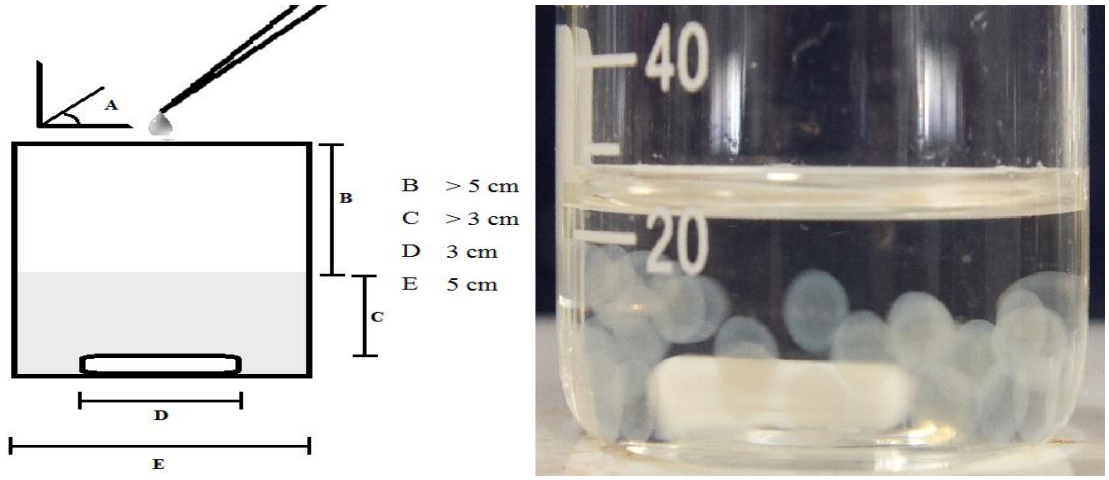

Figure VI–3. Physical parameters for the encapsulation process and photo of the capsules in suspension.

From the 18 different compositions studied, only four showed adequate results both in regards to the visual appearance and the integrity of the capsule. These were all obtained when using a 1:2 extract: alginate proportion (A, B, H and I) (Table VI–8).

Table VI–8. Evaluation of the obtained capsules

Code	Capsules/ mL	10 capsules (mg)	Average weight (mg)	Aspect	Integrity	Encapsulation efficiency (%)
1	20	55.0	5.50	+++	+++	98
2	16	63.1	6.31	+++	++	82
3	16	66.7	6.67	++	++	84
4	19	29.5	2.95	++	++	75
5	20	59.0	5.90	++	++	76
6	20	32.5	3.25	++	++	81
7	19	17.9	1.79	++	++	61
8	18	23.3	2.33	++	+++	64
9	20	21.0	2.10	++	+++	66
10	20	49.0	4.90	++	+	36
11	20	41.5	4.15	+	+	41
12	20	41.5	4.15	++	+	46
13	20	34.0	3.40	+	+	38
14	20	37.0	3.70	++	+	41
15	19	32.6	3.26	++	+	44
16	20	19.0	1.90	++	++	68
17	19	22.1	2.21	++	++	74
18	19	17.4	1.74	+	+++	81

Aspect: + deformed capsule, ++ slightly deformed capsule, +++ spherical capsule; Integrity: + major liquid leakage, ++ slight liquid leakage, +++ no liquid leakage. Integrity was evaluated after 14 days in an oven at 60°C.

The amount of capsules obtained by millilitre of solution ranged from 16 to 20. On the other hand the weight of 10 capsules ranged from 17.4 to 66.7 mg, equalling to 1.74 to 6.67 mg per capsule, while the efficiency ranged between 36 and 98%.

Out of both models, NNw was better for the data obtained on the encapsulation conditions and encapsulation efficiency. It identified time was a significant factor and had a higher R^2 on the training and validation data than RSM.

Following the results obtained, three formulations using *Arbutus unedo* plant parts were prepared and tested using the conditions of encapsulation 1. Formulation A consisted only in fruit extract mixed with alginate and dropped into a $CaCl_2$ solution. For solutions B and C, $CaCl_2$ was enriched with extract. For solution B, the core was leaf extract and enrichment was performed using fruit extract, while for solution C the core was fruit extract and enrichment was performed with leaf extract. The obtained capsules were then evaluated for their phenolic content and antioxidant activity over a period of 6 weeks. Overall solution B always had the highest initial and final results, but also presented the highest degradation rate.

5 Discussion

In this work, only water was used as a solvent. This choice was made based on the fact that the obtained capsules are to be edible so they can be mixed with other food products or consumed as is. While it would be possible to extract with one solvent and later replace it with water, this process would add a step which was deemed to be unnecessary. The screening design results were evaluated based on the phenolic content and antioxidant activity per mL instead of per gram of dry weight because for the encapsulation process, it is preferable to have a more concentrated extract, so that even after some losses the obtained capsules are still rich in antioxidant activity.

The fact that extraction condition 6 provided the best results was expected, since a higher ratio and time should yield a higher extract concentration. This evaluation also served to prove that there is no degradation regarding the phenolic content and antioxidant activities when applying a higher temperature and a longer extraction time. This degradation could happen due to the light, temperature and oxygen sensitivity of the phytochemical compounds and vitamins. In fact, such degradation was seen previously when testing for the anti-Alzheimer activity of the fruit extracts, in which case the extraction obtained at 60°C couldn’t even inhibit 50% of the enzymatic activity of either acetylcholinesterase or butyrilcholinesterase, in clear contrast with the extractions obtained at lower temperatures.

Following the results obtained for the best extraction conditions, the next step was the determination of the physical parameters of the material for proper encapsulation of the obtained extract. The encapsulation material chosen was alginate which crosslinks with $CaCl_2$. This combination was chosen due to its stability at low pH, which enables it to pass through the stomachic digestion mostly unchanged, and high swelling observed at higher pH, ideal for the intestinal release (Pillay & Fassihi, 1999).

When evaluating the physical parameters of the equipment used in the encapsulation, it was possible to conclude that all parameters had influence. By increasing the angle of the tip, it was possible to obtain smaller sized capsules due to the reduced surface tension that can cause the droplet to adhere to the dispenser tip when the angle is lower. This in turn means that a higher amount of liquid is necessary for the gravity to be strong enough to overcome the surface tension and release the droplet from the tip. The solution flow rate was also important, since if it was too fast the individual droplets would not form. Parameter B was important since it influences the form of the obtained capsule. If the distance was too small, then a "tailing" would occur, due to the surface tension not having enough time to recover from being connected with the tip to form a rounder capsule. On the other hand if the distance was too big, the droplet might gain too much speed which again prevents a spherical form, giving instead a flatter part on the bottom. In addition the extra speed means the impact with the surface of the $CaCl_2$ solution will increase, causing the formation of sprinkles and deforming the droplet upon impact. Similarly, the distance C also required a minimum, but unlike B it had no maximum. The minimum distance required is due to the speed of the capsule formation when alginate contacts $CaCl_2$. If the distance was too small, then the unfinished capsule will reach the bottom and impact with enough force to cause a deformation, which would then remain unchanged while the encapsulation process continues, and appear in the final capsules. Parameter D was important regarding once again the form of the obtained capsules. Both the size and the speed needed to be adequate. If the bar was too small or moving too slow, then the capsules won't be flowing around the container and will instead be quiet on the bottom, which prevents a uniform encapsulation process and yields non spherical capsules. On the other hand, if the bar is moving too quickly, then the kinetic energy would be enough to cause a deformation on the capsules upon impact. Finally, parameter E is related to parameter D and to the maximum number of capsules that can be on the container at the same time at any point of the encapsulation process. Gelatine was also used on the initial capsule formulations as a filler agent. This addition had an effect on the lower concentration of alginate which could be used. Without gelatine the concentration

needed to be 2% minimum, while with gelatine it could be 1.5%, however the $CaCl_2$ solution needed to be at least 5%, to prevent malformation and extensive liquid leakage (data not shown). This is in agreement with Xie et al. (2016) observation when optimising oyster hydrolysate encapsulation.

Additionally, when using a 2% $CaCl_2$ solution, it is imperative that the alginate is at 2%, otherwise even after 30 minutes in solution the capsules will not finish the wall formation process (Figure VI–4).

Figure VI–4. Photograph of a spherical (left) and deformed capsule (right).

When Xie et al. (2016) prepared and optimised liposome beads in alginate containing oyster hydrolysate, they concluded that the optimum conditions were 0.5 M $CaCl_2$ and 3% alginate. The differences from our work are likely due to the matrix difference and the use of ethanol instead of water. This dependency of alginate % and encapsulation is in agreement with what Nagpal et al. (2012) observed when encapsulating ibuprofen. Additionally, Pasukamonset et al. (2016) studied the encapsulation of *Clitoria ternatea* and found that bellow 1.5% $CaCl_2$ and 1% alginate there was no capsule formation, likely due to low concentration of carboxyl groups to form the gel. The $CaCl_2$ % also affects the release and swelling of the capsules when resuspended in simulated gastric fluid, with higher concentrations leading to a lower release (Sudareva et al., 2016). Visually, the increase in contact time from 10 to 20 minutes caused in some cases a darkening in the final capsules, likely due to a larger part of the extract being retained. Between 20 and 30 minutes no colour change was detected.

The amount of capsules obtained by millilitre of solution ranged from 16 to 20. On the other hand the weight of 10 capsules ranged from 17.4 to 66.7 mg, equalling to 1.74 to 6.67 mg per capsule, which is in agreement with results obtained by Pillay & Fassihi (1999) at similar conditions. This difference in weight is related both with the amount of extract encapsulated and the proportions of alginate and $CaCl_2$, which interfere in the formation of the capsule wall, causing it to be thinner or thicker. On the other hand the efficiency ranged between 36 and 98%. These values are much broader than those obtained by Pasukamonset et al. (2016) and Xie et al. (2016), which obtained efficiencies in the order of 80 to 85%, likely

due to the matrix difference and encapsulation processes used. On the other hand when Lotfipour et al. (2012) encapsulated bacteria, their efficiency ranged between 98 and 100%, likely due to the pores on the surface of the capsules being too small for the bacteria to diffuse through them like smaller molecules can. It has been reported by Gombotz & Wee (1998) that the pores on the surface of alginate calcium beads which allow for the diffusion of molecules range from 5 to 200 nm, depending on the technique and conditions used, indicating that the lower efficiency of some formulations was either due to capsule malformation or larger pores being present. In fact when looking at one of the formulations with an electron microscope, it was possible to see large crevasses on its surface, from which the extract could have leaked (Figure VI–5).

Figure VI–5. Photographs obtained with electron microscope. A- capsule with visible crevasses, B- Detail of the largest crevass.

The largest of these had approximately 350 µm in length and 10 µm in width, although most of the large crevasses were smaller than 150 µm. These crevasses can also be formed during aggressive drying, which is the reason why we didn't dry the capsules using high temperature.

RSM indicated extract: alginate alone and its interaction with $CaCl_2$ as significant factors. With this methodology time was not a significant factor. While the RSM R^2 was 0.92, its AdjR^2 was only 0.85, and the validation R^2 was only 0.37, indicating that while the model is fit for the training data it is not fit for the validation data. Similar R^2 was obtained by Xie et al. (2016) when they used RSM for the optimization of their encapsulation process, but no validation data is available on that work. On the other hand the model obtained with NNw had an R^2 of 0.94 for the training data and 0.99 for the validation data, indicating a much better fit for all the data, including that which was not available for the model construction. This fit was maintained even after changing the validation data and was the average of 1000 modelling runs. The NNw model was accurate in predicting almost all of the encapsulation efficiency

data. The exception was point 15, which was an outlier on the predicted plot, and the reason R^2 is not higher on the training data (Figure VI–6). Using NNw, time was also identified as an important factor, similarly to what Pillay et al. (1998) had found when encapsulating indomethacin in alginate capsules, when they used times of contact with $CaCl_2$ solution between 30 minutes and 72 hours. However when Blandino et al. (1999) studied the evolution of wall thickness with time they found that at 30 min the thickness is already very close to the maximum, which indicated such a broad variation of time was not required. Additionally, when Lotfipour et al. (2012) tested different contact times when preparing capsules to protect bacteria from stomach pH, they also found both alginate and $CaCl_2$ % have a significant influence, together with the hardening time, which is in agreement with the model obtained.

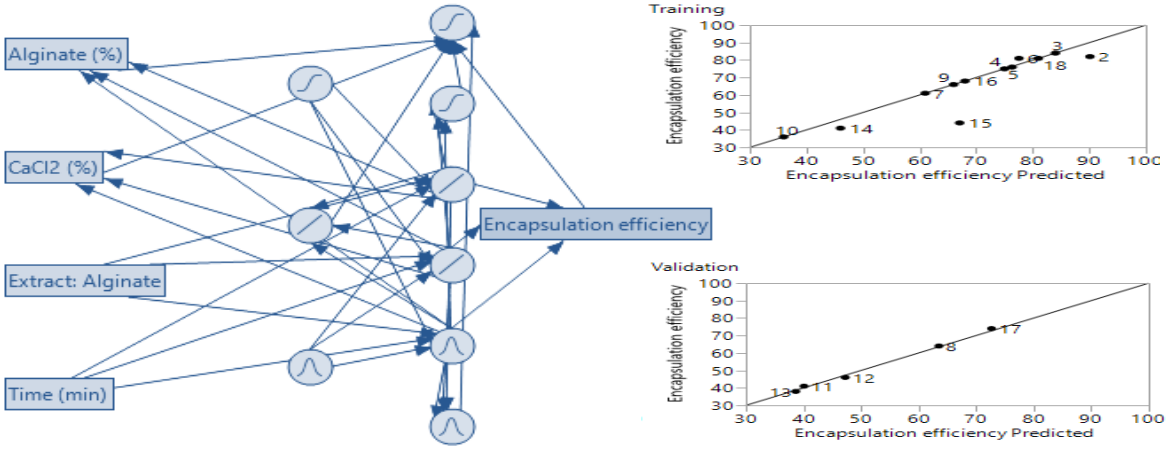

Figure VI–6. Neural network modelling.

Given the increased method robustness, which is evident in the R^2 for both training and validation data and $adjR^2$, the response surface curves for encapsulation efficiency presented were obtained using NNw instead of RSM (Figure VI–7).

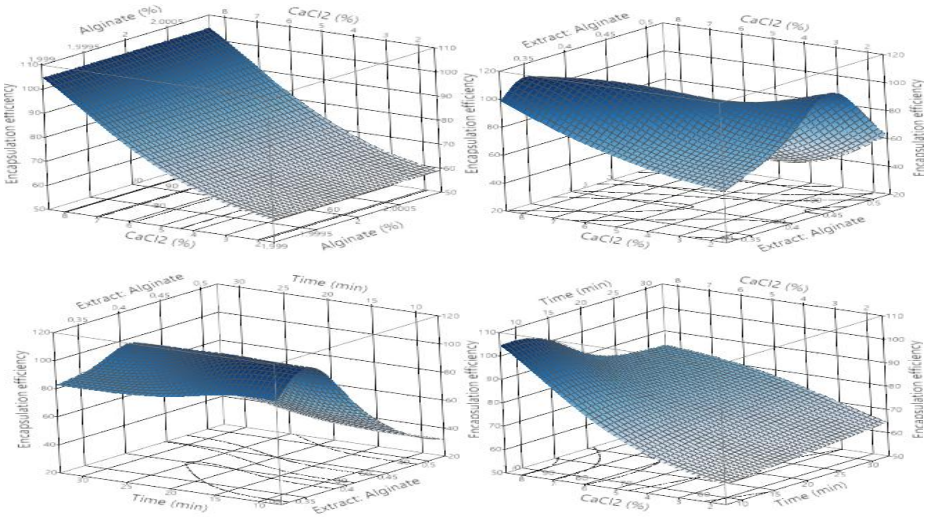

Figure VI–7. Response surfaces for the best predicted encapsulation efficiency.

Following the results obtained, three formulations using *Arbutus unedo* plant parts were prepared and tested using the conditions of encapsulation 1. Regarding phenolic content, formulation B showed the highest content, followed by formulation C and A (Figure VI–8).

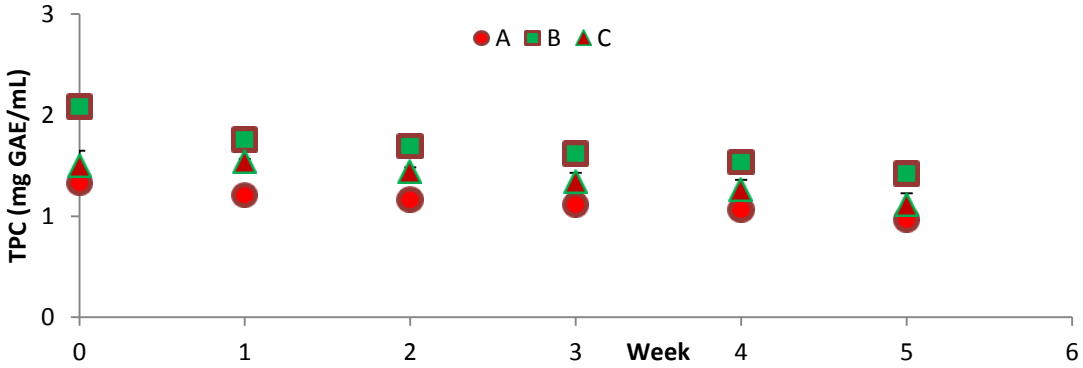

Figure VI–8. Evolution of TPC on the three capsule formulations during storage.

However, while initially formulation C presented a higher phenolic content than A, by the end of the storage period, due to the higher stability displayed by formulation A, the phenolic content between formulations A and C was no longer statistically different ($p<0.05$). While formulation B showed the highest degradation rate, especially from week 0 to week 1, its phenolic content was still significantly higher than the other formulations. The higher stability of formulation A is likely due to the extract being entrapped inside the capsule, which forms a protective barrier against degradation by oxygen. On the other hand, formulations B and C both have extract on the outer part of the wall, since it was in the $NaCl_2$ solution, which is mostly unprotected from degradation. The difference between formulations B and C indicates that leaf extract is much more stable to the storage conditions used. This is likely due to the different compounds present in fruit and leaf extracts, namely vitamins which are easily degraded by oxygen (Zerdin et al., 2003). It should also be noticed that while TPC measures the phenolic content, there are other compounds which can react with it, including ascorbic acid which is present in the fruits of *A. unedo* tree (Everette et al., 2010). The ascorbic acid present in the capsule outer wall can then be oxidized into dehydroascorbic acid, which can itself react with amino acids to form Strecker aldehydes. Some of these aldehydes do not react with the Folin reagent explaining the decrease observed (Bharate & Bharate, 2014). The total antioxidant activity (TAA) of the tested formulations showed similar results to those of TPC (Figure VI–9). Formulation B was always the best formulation, followed by formulations C and A. Similarly to TPC, by the end of the storage period, TAA of formulation C was statistically similar to that of formulation A. However, the degradation of the capsules total antioxidant activity happened at a much lower rate, and only during the first week in storage.

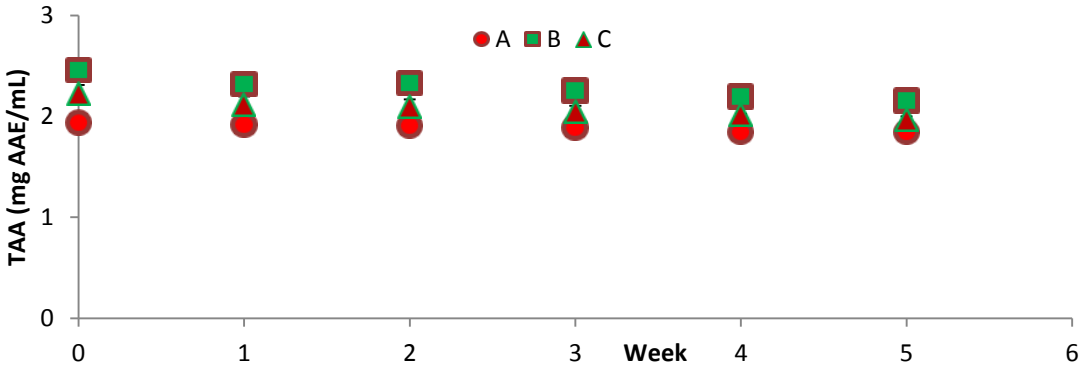

Figure VI–9. Evolution of TAA on the three capsule formulations during storage.

From the first week onwards there was no appreciable degradation of the TAA, with the results being statistically similar for weeks 1, 2, 3, 4 and 5 for each individual capsule formulation. The difference in degradation behaviour indicates that phenolics are not the only compounds responsible for the TAA of the capsules, which is in agreement with what was seen in previous works. It also indicates that the compounds responsible for this activity are much more stable to the storage conditions, namely the presence of oxygen, or they are degraded into compounds which still display antioxidant activity, such as the by-products of ascorbic acid degradation, which only loses vitamin activity after lactone hydrolysis, forming 2,3-diketogulonic acid (Bharate & Bharate, 2014). The FRAP degradation of the three tested formulations was similar to both TPC and TAA (Figure VI–10).

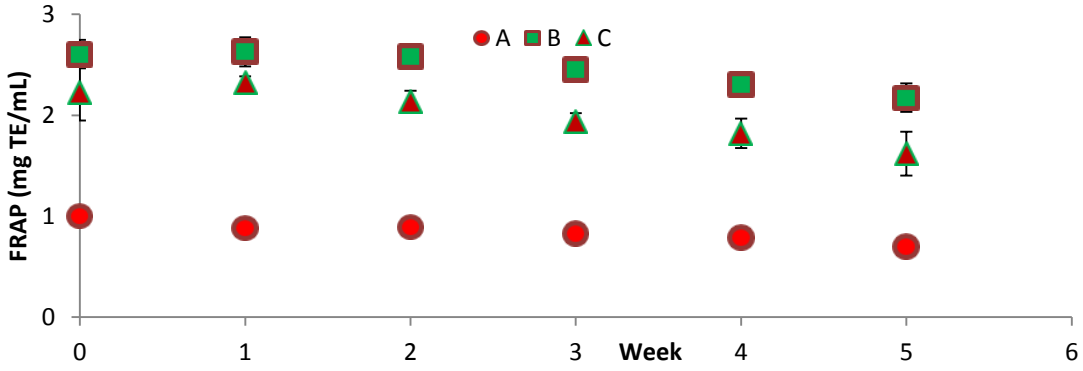

Figure VI–10. Evolution of FRAP on the three capsule formulations during storage.

There was a much steeper decrease from week 0 to week 1 for formulation B, similarly to what happened in TPC, and once again the order was B, C, A. It should however be noted that the difference between formulation A and the remaining formulations was much higher on FRAP than on the previous assays. Moreover, formulations A and C showed the best stability, with no statistical difference being detected between FRAP of week 0 and FRAP of week 5, in clear contrast with formulation B, which after only one week in storage already showed statistical differences comparatively to the initial activity. This relation between TPC and

FRAP was already explored previously (data not shown), and was detected in several works involving these two assays, therefore was expected to happen in the present work as well.

Overall the obtained capsules displayed a good antioxidant activity which remained stable for samples A and C. The choice of formulation should take into consideration the desired storage period and antioxidant activity. If a short storage period is acceptable, then formulation B would be the most suitable, since even after some degradation it was still the formulation that presented the best antioxidant activity. On the other hand, for a longer storage period formulations A or C should be chosen, since these formulations, especially formulation A, showed a much higher resistance to degradation when stored under aerobic conditions.

6 Conclusion

This work studied the extraction and encapsulation of bioactive compounds with antioxidant activity. The best extraction was obtained when using solvent at 60°C, for 180 minutes under an agitation speed of 200 rpm with a 0.2 solid/solvent ratio. After determining the encapsulation equipment parameters which yielded spherical capsules under normal conditions, encapsulation conditions were studied further. The best capsules were obtained when using 2% alginate solution mixed in a 2:1 proportion with extract, and dropped and maintained in 8% $CaCl_2$ solution for 10 minutes. From the three formulations studied, one with just fruit extract (A), one with leaf on the inside and fruit on the $CaCl_2$ solution (B) and one with fruit on the inside and leaf on the $CaCl_2$ solution (C), the best results regarding the antioxidant activity were obtained with formulation B. On the other hand formulations A and C were more stable to degradation. Overall the results show that both extracts can be successfully encapsulated, yielding a product rich in antioxidant activity, which can be added to other food products or consumed fresh.

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Physical characterization, antioxidant and anti-Alzheimer activity of spray-dried microparticles obtained from fruit and leaf extracts of strawberry tree (Arbutus unedo L.)

Ricardo Nunes[1], Filipa Guerreiro[2,3], Ana Grenha[2,3] and Isabel S. Carvalho[1]

[1]MeditBio, Faculty of Sciences and Technology - University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[2]Center for Biomedical Research (CBMR), Faculty of Sciences and Technology, University of Algarve, 8005-139 Faro, Portugal

[3] Centre for Marine Sciences (CCMar), University of Algarve, 8005-139 Faro, Portugal

1 ABSTRACT

Arbutus unedo fruits are known for their richness in antioxidant compounds and vitamins and leaves have anti-Alzheimer potential. In this work we study the effect spray-drying has on the antioxidant activity of a fruit extract, the properties of the obtained leaf and fruit powders at different maltodextrin concentrations, process temperatures and with or without gelatine addition. We also study the reconstituted extract anti-Alzheimer potential and stability to *in vitro* digestion. The spray-drying process had an efficiency between 46 and 80%, and yielded particles smaller than 20 μm with equivalent or higher antioxidant activity than the original extract. Inlet temperature significantly affected the yield of the powder. Fruit and leaf powders had similar physical characteristics, although leaf powder was slightly yellowish while fruit powder was white. Both powders had an extremely high solubility but a low flowability due to the clumping and their high hygroscopicity. The anti-Alzheimer potential was maintained during the process (10% maltodextrin leaf extract inhibition of 62 and 68% for acetylcholinesterase and butyrilcholinesterase at 1.16 mg/mL) but suffered a decrease during *in vitro* digestion (stomachic step for acetylcholinesterase and both steps for butyrilcholinesterase). The obtained powder could be used to increase antioxidant activity and confer anti-Alzheimer potential to other beverages or food products due to its solubility and physical properties.

KEYWORDS

Arbutus unedo, encapsulation, spray-drying, *in-vitro* digestion, Anti-Alzheimer.

2 Introduction

The strawberry tree (*Arbutus unedo* L.) is an evergreen fruit bearing fruit belonging to the Ericaceae family, present in the Mediterranean area. Its fruits are rich in phenolics with antioxidant activity, vitamin E and minerals beneficial for health (Oliveira et al. 2011) while leaves have anti-aggregant and anti-inflammatory properties (Mekhfi et al. 2006, Mariotto et

al. 2008). The fruits are available in great number but only during autumn in the period from middle of October to end of November. Not only is the fruiting period short, many trees bear fruit only every other year. In addition to this, because the fruits take a whole year to develop and mature, any climatic conditions that are improper can have repercussions in the yield of the fruits. For these reasons it is important to study how to properly preserve them after collection and to study ways to use them after collection, other than the preparation of fermented beverages. The effect of drying on the antioxidant activity of fruits, using both lyophilization (freeze drying) and an oven (heat drying) was previously studied by our group (unpublished data). This time another kind of drying is being tested, spray-drying. This is a technique commonly used to transform liquids into solids, usually called dry powders or microparticles (Comunian and Favaro-Trindade 2016). The approach is usually intended to improve the shelf-life and stability of a product. It can also be used to achieve a higher concentration of phytochemicals after resuspension of the particles and to facilitate storage. In addition, sensitive phytochemicals can be protected from degradation by light, heat and oxidation (Pereira et al. 2013, Ravichandran et al. 2014). The addition of matrix materials that serve as carrier agents has the advantage of increasing the soluble solids in solution, necessary for a higher efficiency of the process, increasing glass transition temperature, which is important in sugar rich solutions to prevent caramelization, and increasing heat protection of thermosensitive compounds (Ravichandran et al. 2014).

While different materials can be used to achieve encapsulation, lipids, proteins (gelatine, soy), Arabic gum and polysaccharides derived from starch are among the most common (Comunian and Favaro-Trindade 2016). Maltodextrin is a starch hydrolysate which has matrix forming properties, making it suitable as wall material. In addition to caramelization protection, maltodextrin has the advantage of being a slow release starch, which is suitable for diabetics. In this work, we studied the effect of maltodextrin addition and subsequent spray-drying of a strawberry tree fruit extract, evaluated the physical properties of dry powders obtained from leaf and fruit and the antioxidant activity before and after spray-drying. Finally, we investigate the acetylcholinesterase and butyrilcholinesterase inhibitory activity before and after the process and after *in vitro* digestion, to determine the potential and benefits that would come from adding these powders to beverages or food products.

3 Methodology

3.1 Material collection and treatment

Fruits and leaves of *Arbutus unedo* were collected from 18 different trees in *Herdade da Corte Velada*, Algarve, Portugal, in a clear and dry day during November 2014 and stored in a thermal container at 4°C. After arriving at the lab the fruits were dried in an oven (Binder, Germany) at 45°C for 120 h while leaves were dried at 45°C for 48 h. After drying, samples were ground to a powder in a kitchen mill and 2 g from each fruit or leaf sample powder belonging to each tree were pooled together. The pooled samples (one for fruits and one for leaves) was transferred into a falcon and stored at -20°C until extraction.

3.2 Extraction

The extraction was performed on a hot plate with stirring, using the conditions detailed in Table VI–9. The previously prepared samples were added to distilled water at the indicated temperature and that temperature was maintained during the extraction process. When finished the extract was filtered and centrifuged. 50 mL of each supernatant were collected and the maltodextrin and/or gelatine powder were added and mixed (Table VI–10). After dissolution the extracts were centrifuged once again and the supernatant was collected for injection in the spray-dryer.

Table VI–9. Extraction conditions for the spray-dry process.

	Temperature (°C)	Time (min)	Ratio (g/mL)	Stir (rpm)
Fruit	45	30	0.05	200
Leaf	60	30	0.05	200

Table VI–10. Composition and spray-dry temperature of the atomized samples.

	Maltodextrin (%)	Gelatin (%)	Spray drying T (°C)	Discharge (µL/s)
Fruit 1_10 (1)	10	0	140	237
Fruit 2_10 (2)	10	0	160	185
Fruit 2_15 (3)	15	0	160	164
Leaf_10 (4)	10	0	160	206
Leaf_9+1 (5)	9	1	160	182

3.3 Spray drying process

The spray-drying process was carried out on a Buchi mini spray-dryer B-290 (Buchi, Switzerland) operating in open cycle mode. As indicated in Table 2, the operating parameters

were: inlet temperature of 140 ºC or 160°C, aspirator set to 80%, feed rate between 9.6 and 14.2 mL/min and spray flow rate of 473 L/h.

The yield of the spray-drying process was calculated based on the ratio between the amount of dry powder obtained from each sample and the initial solids in solution, according to the formula below:

$$Process\ yield\ (\%) = \frac{\text{Obtained powder (g)}}{\text{Initial solids in solution (g)}} \times 100$$

3.4 Physical properties

Physical properties were determined according to Santhalakshmy et al. (2015). These properties were determined on the day of the spray-dry process and after a storage of 5 days in a freezer to evaluate changes due to storage.

3.4.1 Moisture

Moisture content was determined by difference after drying each sample in an oven at 100 ºC. Process was considered finished when there was no more change in weight.

$$Moisture\ content\ (\%) = \frac{\text{Initial weight (g)} - \text{Final weight (g)}}{\text{inital weight (g)}} \times 100$$

3.4.2 Turbidity

The powder was resuspended in distilled water with the help of a vortex (5 min. 5000 rpm) and its turbidity was calculated against a blank, with the same method used for the determination of water quality regarding solids in suspension, using a UV-Vis spectrophotometer set at 900 nm.

3.4.3 Bulk and tapped density

A known quantity of spray-dried *Arbutus unedo* fruit spray-dryed extract was loaded into a 5 mL graduated cylinder. The volume occupied by the powder was used to calculate bulk density (ρB). The graduated cylinder was then tapped for 1 minute, on the side (ρTs), or on the bottom (ρTb), and the new density called tapped density (ρT).

3.4.4 Particle density (ρP)

A known weight of spray-dryed extract was transferred into a 5 mL graduated cylinder and 3 mL of n-hexane was added. After suspending all the particles using agitation, 1 mL of n-hexane was used to wash the cylinder walls. The total volume occupied by the particles and the n-hexane was measured. The density was calculated by the following formula:

$$\rho P = \frac{\text{Weight of sample (g)}}{\text{Total Volume (ml)} - 4}$$

3.4.5 Porosity and flowability

The porosity (ε) of the spray-dryed sample was calculated using the results obtained for ρP and ρT. The flowability of the pray-dryed sample was calculated using the results obtained for ρT and ρB, using the Carr index formula:

$$\varepsilon = \frac{\rho P - \rho T}{\rho P} \times 100 \qquad Carr\ Index = \frac{\rho T - \rho B}{\rho T} \times 100$$

3.4.6 Cohesiveness (Hausner ratio)

The cohesiveness parameter of the obtained spray-dried extract was determined using the Hausner ratio, which is calculated using the results from ρT and ρB using the formula bellow:

$$Hausner\ ratio = \frac{\rho T}{\rho B}$$

3.4.7 Wettability

The wettability was determined by counting the time a 0.1 g of powder takes to become fully submersed in 30 mL of distilled water at 25°C.

3.4.8 Solubility

Briefly, 0.5 g of powder were mixed into 30 mL distilled water and shaken in a vortex at 1500 rpm for 5 minutes. After homogenization the sample was transferred into Eppendorfs and centrifuged at 3000 rpm for 5 minutes. An aliquot totalling 25 mL of the supernatant was transferred into pre-weighted petri dishes and put in an oven at 105 °C to dry for 5 hours. The solubility was calculated as the weight difference.

3.4.9 Higroscopicity

A total of 0.5 g of sample was placed in a pre-weighted petri dish and put in a sealed contained container at 25 °C containing saturated NaCL solution. After sealing with a cover, the container was covered with parafilm. Samples were weighted after one week. The higroscopicity was calculated with the difference in weight and expressed as grams of adsorbed moisture per 100 grams of dry powder.

$$Higroscopicity = (\text{final weight} - \text{initial weight}) \times 200$$

3.4.10 Particle saturation in water

To determine the amount of powder needed to saturate a solution, a known weight was added into 1 mL of distilled water and shaken. When the powder was fully dissolved, more powder varying in weight between 2 and 4 mg was added. This procedure was repeated until the powder could no longer be completely solubilized. The results are expressed as mg powder/mL of distilled water.

3.5 Antioxidant activity

3.5.1 Total Phenolic Content

Total Phenolic Content (TPC) in samples was determined using a spectrophotometric procedure originally described by Singleton and Rossi (Singleton and Rossi 1965). Phenolic content was calculated using a gallic acid calibration curve and the results were expressed as mg GAE/g dw (gallic acid equivalents per gram of dry weight).

3.5.2 Total Flavonoid Content

Total Flavonoid Content (TFC) was determined according to the method of Lamaison and Carnat (1990). It was calculated using a quercetin calibration curve, and the results were expressed as mg QE (quercetin equivalents)/g dw.

3.5.3 Total Antioxidant Activity

Total Antioxidant Activity (TAA) of extracts was determined using a spectrophotometer and the method proposed by Prieto et al. (1998). Results were expressed as mg AAE (ascorbic acid equivalents)/g dw.

3.5.4 Ferric-Reducing Antioxidant Power

Ferric-Reducing Antioxidant Power (FRAP) was determined using the method previously described by Benzie and Strain (1996). Results were calculated from a Trolox calibration curve and expressed as mg Trolox/g dw.

3.6 Acetylcholinesterase and butyrilcholinesterase inhibition

Both enzymatic inhibitory activities promoted by the extracts were evaluated following the method reported by (Sancheti, Sancheti and Seo 2013), but with volumes adjusted for Eppendorf use. For both enzymes 100 µL of the prepared enzyme (ACHE solution 0.03 U/mL, BCHE 0.1 U/mL) in 7.2 mM with 0.1 mM EDTA 7.2 pH phosphate buffer were mixed with 50 µL of test sample and 900 µL of Tris-Hcl buffer (50 mM, pH 8). After incubation for 30 min at 4°C, 100 µL of a 0.3 mM 5,5-dithio-bis(2-nitrobenzoic acid) and 100 µL of a 1.8

mM for ACHE and 6.8 mM for BCHE acetyltiocholine iodide solution were added and incubated for 20 min at 37°C. The absorbance was then read at 412 nm in a T70 + UV-VIS spectrophotometer from PG instruments (UK). Inhibition was calculated using the following equation:

$$Inhibition\ (\%) = \frac{Acontrol - Asample}{Acontrol} \times 100$$

The concentration of extract correspondent to 50% inhibition (IC_{50}) was determined, when possible, from concentration versus inhibition curves.

3.7 In-vitro digestion

The in vitro digestion of the samples was performed following the method described by (Liu, Glahn and Liu 2004).The digested sample volume was adjusted to 4.5 mL and stored until analysis in the following day.

3.8 Statistical analysis

Mean values were analysed using ANOVA and the LSD or Games-Howell post-hoc tests with p value set to 0.05. The software used to conduct the statistical analysis was SPSS 22 (SPSS Inc, Chigaco).

4 Results and discussion

The effect of maltodextrin and gelatine addition, the drying temperature (inlet temperature) on the spray-dryer, the spray-drying process and *in vitro* digestion on the antioxidant and anti-Alzheimer activity of the extracts were studied. Due to the high sugar content of the fruit extract, the addition of dextrin or other agent was imperative in order to prevent the caramelization of the sugars. The encapsulation of a fruit solution without maltodextrin was attempted, but no powder was produced, caramelization being observed instead. Considering that spray-drying temperature is known to affect the characteristics of the final product (Mishra, Mishra and Mahanta 2014), fruit extract solutions were spray-dried at two different spray-dryinlet temperatures were tested, 140 ºC and 160°C, in presence of 10% maltodextrin. It was found that both drying temperatures allowed the encapsulation of the extract and maintained the antioxidant activity. This was in disagreement with a work conducted by (Kha, Nguyen and Roach 2010) who found that spray-drying temperatures between 120ºC and 170°C have a slight effect on the antioxidant activity of the powders measured with the TAA and DPPH assays. Nevertheless, in our study, processing the samples at 160°C led to a much higher yield, 72% instead of 48% (Table VI–11). This is likely due to improved drying of the droplets formed in the spray-drier and a reduced powder deposition on the walls, one of the

major sources of loss during spray-drying, as reported by Maury et al. (2005) and Aliakbarian et al. (2015). Following this observation, the temperature of 160 ºC was selected to continue the studies. When maltodextrin amount was increased to 15%, the fruit powder characteristics were equivalent to those obtained with 10% maltodextrin (Table VI–11 and Table VI–12). Although the recovered mass was higher, due to more solids in suspension, the yield did not increase. It was also decided to test the addition of gelatine. Gelatine has the advantage of being protein based instead of starch based, which could improve the stability during the digestive process and decrease the possibility of an increased glycaemia due to the resuspended powder ingestion. When testing the addition of gelatine to the solution, leaf extract had to be used instead of fruit extract. This was necessary due to fruit extracts losing some antioxidant activity when subjected to higher temperatures, a step that would need to be performed for the dissolution of gelatine. In fact, even on the leaf extract the addition of gelatine revealed itself to cause problems, although there were advantages as well.

Table VI–11. Yield, moisture, saturation, solubility and turbidity of the powder.

Sample	Day	Yield (%)	Moisture (%)	Saturation (mg/mL)		Solubility (%)	Turbidity (Abs)
				Standing	Agitation		
1	1	48.18	3.3 ± 0.6	24.0 ± 1.0	198.7 ± 1.5	99.87 ± 0.15	0.053 ± 0.006
	5		6.0 ± 0.1	24.3 ± 0.6	203.7 ± 1.5	99.87 ± 0.06	0.058 ± 0.004
2	1	72.52	3.0 ± 0.1	22.3 ± 0.6	200.0 ± 3.6	99.90 ± 0.10	0.051 ± 0.004
	5		6.0 ± 0.1	24.7 ± 1.5	203.7 ± 5.7	99.93 ± 0.06	0.049 ± 0.005
3	1	68.99	2.7 ± 0.2	24.0 ± 1.0	199.3 ± 5.1	96.60 ± 5.89	0.050 ± 0.006
	5		4.7 ± 0.2	24.0 ± 1.0	200.0 ± 5.3	99.92 ± 0.00	0.054 ± 0.004
4	1	70.53	3.0 ± 0.2	22.7 ± 0.6	199.7 ± 6.4	99.90 ± 0.10	0.049 ± 0.004
	5		4.9 ± 0.2	22.0 ± 0.0	195.3 ± 0.6	99.87 ± 0.06	0.053 ± 0.006
5	1	80.07	3.4 ± 0.0	23.7 ± 1.5	209.0 ± 1.7	98.87 ± 1.27	0.049 ± 0.003
	5		4.2 ± 0.1	23.0 ± 1.7	201.0 ± 8.9	99.00 ± 0.98	0.053 ± 0.007

Sample: 1-Fruit 1_10; 2-Fruit2_10; 3-Fruit 2_15; 4-Leaf_10; 5-Leaf 9+1Results are presented as mean ± SD of 6 repetitions with the exception of yield (two repetitions).

The addition of gelatine substantially increased the production yield, from 70 to 80% and decreased the final powder hygroscopicity. This is in agreement with other authors, who defend that a single encapsulation material may not be enough and a mixture should be used instead (Mahdavi et al. 2016). However, due to the high temperature required for the dissolution of the gelatine, care had to be taken to prevent burning and subsequent sticking of the solution to the glass flask. Additionally, only 1% of gelatine was used when initially the concentration was to be 6%, a proportion which according to other authors can be used in spray-drying (Maji et al. 2007).

Table VI–12.Physical properties of the powders obtained.

Sample	Day	Density (g/mL)			
		ρB	ρTb	ρTs	ρP
1	1	0.80 ± 0.01	1.00 ± 0.01	0.90 ± 0.02	1.02 ± 0.03
	5	0.33 ± 0.01	0.69 ± 0.03	0.50 ± 0.00	1.05 ± 0.05
2	1	0.80 ± 0.00	0.99 ± 0.01	0.91 ± 0.02	1.02 ± 0.02
	5	0.30 ± 0.00	0.50 ± 0.01	0.43 ± 0.00	1.04 ± 0.04
3	1	0.80 ± 0.00	1.00 ± 0.01	0.92 ± 0.05	1.03 ± 0.03
	5	0.25 ± 0.00	0.36 ± 0.00	0.35 ± 0.00	1.03 ± 0.02
4	1	0.80 ± 0.01	1.00 ± 0.01	0.89 ± 0.00	1.03 ± 0.03
	5	0.25 ± 0.00	0.36 ± 0.01	0.35 ± 0.01	1.05 ± 0.03
5	1	0.80 ± 0.00	1.00 ± 0.00	0.91 ± 0.01	1.03 ± 0.02
	5	0.24 ± 0.00	0.33 ± 0.00	0.32 ± 0.00	1.04 ± 0.02
		ε		Carr index	
		εTb	εTs	CIb	CIs
1	1	0.01 ± 0.02	0.12 ± 0.01	20.00 ± 0.00	10.67 ± 1.15
	5	0.34 ± 0.02	0.52 ± 0.02	52.06 ± 2.08	34.22 ± 1.54
2	1	0.02 ± 0.03	0.10 ± 0.03	19.33 ± 1.15	12.00 ± 2.00
	5	0.52 ± 0.02	0.59 ± 0.01	40.00 ± 0.00	30.00 ± 0.00
3	1	0.03 ± 0.03	0.11 ± 0.02	19.33 ± 1.15	12.00 ± 4.00
	5	0.65 ± 0.01	0.66 ± 0.01	31.25 ± 0.00	28.13 ± 0.00
4	1	0.03 ± 0.02	0.14 ± 0.02	20.00 ± 0.00	9.73 ± 0.46
	5	0.65 ± 0.02	0.67 ± 0.01	30.21 ± 1.80	27.08 ± 1.8
5	1	0.03 ± 0.02	0.12 ± 0.00	20.00 ± 0.00	11.33 ± 1.15
	5	0.68 ± 0.01	0.69 ± 0.01	29.41 ± 0.00	26.47 ± 0.00
		Hausner ratio		Wettability	
		HR Tb	HR Ts	90%	100%
1	1	1.25 ± 0.00	1.12 ± 0.01	129.3 ± 1.5	239.7 ± 2.5
	5	2.09 ± 0.09	1.52 ± 0.04	140.7 ± 3.1	237.0 ± 8.7
2	1	1.24 ± 0.02	1.14 ± 0.03	137.3 ± 5.7	733.0 ± 7.2
	5	1.67 ± 0.00	1.43 ± 0.00	147.0 ± 6.2	728.7 ± 12
3	1	1.24 ± 0.02	1.14 ± 0.05	139.0 ± 7.8	746.0 ± 5.6
	5	1.45 ± 0.00	1.39 ± 0.00	148.0 ± 7.0	735.3 ± 8.1
4	1	1.25 ± 0.00	1.11 ± 0.01	135.0 ± 4.0	751.7 ± 10
	5	1.43 ± 0.04	1.37 ± 0.03	139.0 ± 7.9	739.7 ± 2.5
5	1	1.25 ± 0.00	1.13 ± 0.01	132.3 ± 3.8	499.7 ± 16
	5	1.42 ± 0.00	1.36 ± 0.00	138.3 ± 7.5	496.3 ± 16.6

Results are presented as mean ± SD of 6 repetitions. Sample: 1-Fruit 1_10; 2-Fruit2_10; 3-Fruit 2_15; 4-Leaf_10; 5-Leaf 9+1. ρB-bulk density; ρTb- tapped density on the bottom; ρTs- tapped density on the side; ρP- particle density; εTb-porosity tapped on bottom; εTs- porosity tapped on side; CIb- Carr Index tapped on bottom; Cis- Carr Index tapped on side; HR Tb- Hausner ratio tapped on bottom; HR Ts- Hausner ratio tapped on side. Results are presented as mean ± SD of 6 measurements.

While it is possible to circumvent the heating of the obtained extracts by previously preparing a highly concentrated gelatine solution, this is not without disadvantages, namely the need to quickly use the solution, since a highly concentrated gelatine solution quickly solidifies at room temperature. In addition, it is possible that the intended proportion of gelatine may not be achieved due to the saturation of the water in which the gelatine solution would be prepared. Therefore, further works should be conducted with either low gelatine proportion or no gelatine at all.

Visually the powders were identical, although the fruit spray-dry powders were mostly white, while those of leaves had a slightly yellow colour (Figure VI–11). Colour did not change with the temperature used in the spray-drying process, but it did slightly change with the increase in maltodextrin %.

Figure VI–11. Powders obtained from spray drying. A-fruit 10% MD; B-fruit 15% MD; C-leaf 9+1%; D-leaf 10% MD.

The difference in colour between fruit and leaf powders was much more visible after the hygroscopy assay, with fruit powder presenting a light yellow colour while that from leaves presented a dark yellow-orange colour (Figure VI–12).

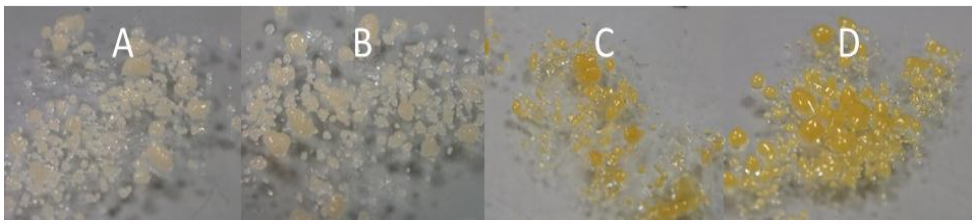

Figure VI–12. Powders after hygroscopy assay. A-fruit 10% MD; B-fruit 15% MD; C-leaf 9+1%; D-leaf 10% MD.

It is clear that some of the powder properties change during storage, namely the bulk (ρB) and tapped densities (ρT), porosity (ε), flowability (CI) and cohesiveness (HR). On the other

hand other properties like the turbidity of the resuspended powder, its solubility and the particle density (ρP) remain mostly constant. This change is likely related to moisture absorption or to the sugars present, which cause the powder to form clumps. While these clumps are easily desegregated their presence causes an increase in apparent density, porosity and cohesiveness as well as a decrease in the flowability of the powders. On the other hand the particle density which is measured with a polar solvent remains the same because the clumps will be destroyed during the process and therefore their presence doesn't affect the outcome of the determination. For the same reason the solubility and turbidity of the powder doesn't change, since the clumps will be dissolved in water, just like the unclumped powder is, although it may take some more time, as it can be seen by the wettability results.

While visually the powders appear to have large particles due to clumping, seen under an electron microscope it is clear that there are differentiated particles (Figure VI–13). For fruit capsules obtained with maltodextrin, the size of these particles was between 3 and 18 μm, with most particles having sizes between 6 and 10 μm. Leaf extracts also obtained with maltodextrin had a similar size range, between 4 and 16 μm with most particles having sizes between 8 and 5 μm. On the other hand, the use of gelatine allowed the formation of smaller particles, between 12 and 5 μm, with most particles measuring between 7 and 5. The appearance of the capsules was also slightly different, with fruit capsules surface being smoother with a slight depression.

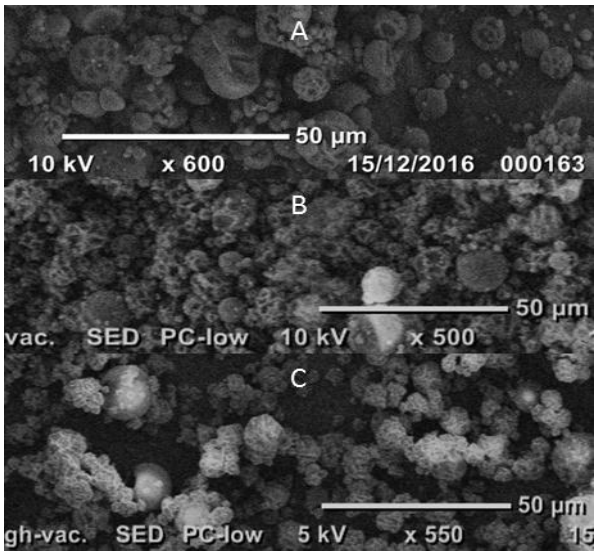

Figure VI–13. Size and aspect of the particles obtained by spray-drying. A- fruit 10%, B- leaf 10%, C- leaf 9+1%.

Compared to a jamum (*Syzygium cumini*) juice powder, the powder obtained from *A. unedo* samples presented a higher yield, although this was possibly to the absence of a matrix material in the processing of jamum juice. Similar bulk and tapped densities but lower

particle density, higher porosity and similar flowability after 5 days in storage were observed (Santhalakshmy et al. 2015). Compared to the gac fruit (*Momordica cochinchinensis*) aril powder obtained by (Kha et al. 2010) had similar bulk density, while compared to a amla (*Emblica officinalis*) powder the obtained powder has a much lower hygroscopicity and similar bulk density (Mishra et al. 2014).

Overall none of the carrier agents or the spray-drying process led to a decrease in antioxidant activity when compared to the original extracts. In addition, the spray-drying process did not lead to a decrease in either ACHE or BCHE inhibitory activity, proving the short period during which the extracts are subjected to high temperatures is not enough to degrade the compounds responsible for the enzymatic inhibition. When subjected to an *in vitro* digestion, the total phenolic content remained constant, for all extracts, while the TAA of the fruits increased for the spray-dried sample (data not shown). This increase was not verified on the extract with the addition of the maltodextrin but not spray-dried, therefore it is not the maltodextrin that is responsible for the increase in antioxidant activity. Similar tendency was verified on the FRAP of the spray-dried fruit extract, which significantly increased. The spray-drying process also prevented some of the FRAP loss occurring during *in vitro* digestion. Similarly to our work, (Hartiati and Mulyani 2015) also found no effect on phenolic content caused by maltodextrin addition to a sinom beverage (a mix of peeled fresh turmeric and tamarind leaves extracts).

Regarding ACHE and BCHE inhibitory activity, fruit extracts displayed some enzymatic inhibition while leaf extracts displayed strong inhibition. The spray-drying process maintained the BCHE inhibition for both leaf extracts and increased the ACHE inhibition of the leaf extract using maltodextrin and gelatine as carrier agents. On the other hand, the spray-drying process slightly diminished the ACHE inhibition of the 10 and 15% maltodextrin fruit extracts, likely due to the temperature used during the process, since the compounds that confer the ACHE inhibitory activity to the fruit extract are easily destroyed due to heating, as seen in an earlier work conducted where the fruits are extracted at different temperatures. The BCHE inhibitory activity remained unaltered, indicating the compounds responsible for both inhibitions are different, which is in agreement with previous observations.

After subjecting the resuspended powders and original extracts to an *in vitro* digestion, only leaf extracts spray-dried with 10% maltodextrin were powerful enough to still exhibit inhibitory activity. Extracts using 9% maltodextrin and 1% gelatine showed no inhibition after the digestive process, and much lower before, likely due to the high temperature used for the dissolution of gelatine. On the other hand, the lack of inhibition from the fruit extracts

may simply be due to the dilution performed during the *in vitro* digestion, since the final solution will have the extract diluted 8 and 10 times for stomach and pancreatic digestions respectively, at which point even the undigested extracts presented no inhibition. For the 10% maltodextrin leaf comparison before and after each digestion step, all digestate concentrations were adjusted to 1/10 the concentration of the original extract solution.

The addition of 10% maltodextrin did not protect the leaf extract from the digestive process regarding ACHE and BCHE inhibitory activities (Table VI–13). For ACHE the loss in inhibitory activity occurred during the stomach digestion step, while for BCHE both steps of the *in vitro* digestion led to a loss of inhibition. While the spray-drying process prevented some of the BCHE inhibitory activity loss occurring during the pancreatic step it had no effect during the stomachic step of the digestion.

Table VI–13. Enzymatic inhibition of the extracts and powders.

			Leaf 10%	Leaf 9+1%	Fruit 10%	Fruit 15%
A	Activity of the enzymes (%)	ACHE	38.04 ± 1.32		82.70 ± 1.21	
		BCHE	32.72 ± 0.93		85.57 ± 1.64	
B		ACHE	38.65 ± 0.62	90.36 ± 0.48	83.81 ± 0.56	82.70 ± 0.48
		BCHE	32.19 ± 0.36	83.01 ± 0.50	85.63 ± 0.51	85.15 ± 0.00
C		ACHE	40.81 ± 0.40	78.57 ± 1.26	86.90 ± 0.49	86.03 ± 1.15
		BCHE	35.86 ± 0.36	81.80 ± 0.82	86.92 ± 0.44	87.15 ± 0.50
D		ACHE	59.80 ± 0.70	n.i.	n.i.	n.i.
		BCHE	60.81 ± 0.35	n.i.	n.i.	n.i.
E		ACHE	50.53 ± 0.33	n.i.	n.i.	n.i.
		BCHE	69.96 ± 1.09	n.i.	n.i.	n.i.

A- without carrier agent, B- with carrier agent, C- after spray dry, D- after digestion (stomach), E- after digestion (pancreas). Results are presented as mean ± SD of six repetitions.

This lack of protection can be explained by the high digestibility of the carrier agent used. While more resilient maltodextrin formulations have been produced (Brouns et al. 2007), the use of resilient materials may lead to the inability to make the extracted powder available for intestinal absorption. Despite the loss of activity occurring during the digestive process, the obtained 10% maltodextrin leaf powder still displayed great inhibitory activity. After the *in vitro* digestion of a 10 times dilution obtained from a 116 mg/mL concentration of powder (therefore at 1.16 mg/mL since the digestion assay equals a dilution of factor 10 of the original solution), the solution still achieved 40 and 30% inhibition of the ACHE and BCHE enzymes respectively. Therefore, the concentration required is low enough to be easily achievable and possibly of biological significance. While the spray-drying process did not confer substantial added stability during the *in vitro* digestion process, the high solubility and

saturation concentration make it possible to achieve concentrations, up to 200 μg/mL of powder, for even greater inhibitions ACHE and BCHE, and easier addition to other products. Additionally, the transformation into powder also allows for better stability against environmental factors, easier transportation and commercialization.

5 Conclusion

The characteristics of *A. unedo* powders obtained using spray-drying technique show that while the increase of maltodextrin from 10 to 15% does not have a significant effect, the addition of maltodextrin is required due to the high sugar content of the extracts. The addition of gelatine revealed to be detrimental to the inhibitory activities of the solution, due to the heating process required for the gelatine powder dissolution. After the spray-drying process, the 10% leaf maltodextrin powder exhibited potent ACHE and BCHE inhibitory activities, which were comparable to those of the original solution. This activity was still strong enough to inhibit 40% of the ACHE and 30% of the BCHE activities, even after an *in-vitro* digestion process with the solution diluted 10 times. The loss during the *in-vitro* digestion occurred during the stomachic step for ACHE and during both the stomachic and pancreatic steps for BCHE. Overall the obtained powder is highly soluble, has good anti-Alzheimer potential and has the advantages of easily allowing an increase of concentration up to 200 μg/mL for even higher inhibition. It also facilitates storage and transportation when compared to a liquid product.

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Discussion of Chapter VI

Using all the knowledge acquired from the previous chapters, in this final chapter the goal was to capitalize on the beneficial properties displayed by *A. unedo* plant parts. To that end, three different products were developed, using *A. unedo* as a basis. The first product was a beverage. Initially a mix of leaf and fruit was studied, since fruits sweet taste could counter the slight bitterness of leaves. However upon trying different mixtures it was found that the mixtures obtained from the different extracts had lower antioxidant activity than expected, indicating an antagonistic effect. This was true for the aqueous extracts, however when using ethanol on the extraction, the obtained mixtures actually displayed higher antioxidant activity than expected. This was particularly noticeable when the solvent used was 50% ethanol: water (v:v) (data not shown). This solvent had also previously provided extracts with higher antioxidant activity and better tyrosinase inhibition. However, since the objective was the direct consumption of the beverage, and using 50% ethanol would make it inappropriate for direct consumption, a decision was made to use only water extracts. Therefore the second part of the beverage study was conducted using pure leaf extract, which had previously shown the best properties. After obtaining the beverage, its stability under adverse conditions was studied. The conditions that caused the greatest loss of quality were direct intense light and storage in the oven at 35°C. These losses in quality were accompanied by the browning of the beverage, a factor which could be used for quality control. On the other hand storage in the fridge maintained most of the properties, including the colour, and storage in the freezer showed no difference from the original extract, other than an increase in turbidity which could be used as quality control. The obtained drink had high antioxidant activity and when properly stored maintained the ACHE and BCHE inhibitions. These inhibitions were similar to those obtained in an earlier work. It did however loose some of this activity when subjected to an *in-vitro* digestion. However, even after the loss, the drink diluted 50 times was still able to inhibit approximately 50% of both enzymes activity. This drink's performance was above or on par with other drinks in the literature. Therefore, the obtained drink has high potential to be used as a functional beverage to ameliorate the symptoms of Alzheimer's disease, however further work, namely *in-vivo* assays should be conducted to assess its actual benefits.

The second product studied was a macrocapsule with the extracts obtained. Both its formulation and stability were investigated. This macrocapsule would use the leaf or fruit extract, a % of alginate as the core and a % of $CaCl_2$ solution to react with alginate and create the outer wall. The physical parameters influencing the macrocapsule visual aspect were also studied. Out of the 18 iterations tested, only two were perfect from the visual aspect and 4 had

good integrity. It was concluded that the addition of gelatin would not bring many added benefits and that the alginate solution should be at 2%. From the three formulations studied, the encapsulation of a leaf extract core with outer fruit extract achieved the best results in antioxidant activity. However this was also the formulation which was least stable, while the simple fruit capsules were the most stable but with the least antioxidant activity. The degradation of TPC occurred mostly during the first week of storage, while FRAP values decreased mainly after 3 weeks in storage. Despite this, the capsules obtained still presented high antioxidant activity after 5 weeks in storage, a substantial increase in stability when compared to the 2 weeks of the leaf beverage studied before, therefore proving that macroencapsulation is a good technique to increase stability against degradation. The obtained macrocapsules can be consumed fresh on their own or after dried in conjugation with other food products, presenting either way a high antioxidant activity which could be beneficial for the consumers. Their size can be easily altered if so desired by changing the width of the dispensing needle and the angle of dispensation. It is also possible to make their colour more attractive by adding food grade colouring to the alginate.

The last product studied was a powder of microparticles obtained using a spray-drier. This technique is widely used to change liquids into powders, which have the advantage of being easier to store, transport, more stable to degradation that the beverages from which they are obtained and highly soluble. Initially a fruit solution without carrier agent was used for the powder creation, however this proved to be incompatible with the process. Due to the high sugar content and the submission to high temperatures, the sprayed extract would caramelize and stick to the walls of the drying chamber, therefore no powder could be retrieved. This problem was solved by the addition of maltodextrin. This carrier agent is easily soluble in water, is a slow release sugar, therefore being appropriate for diabetic people, increases solids in solution which in turn increases the process efficiency, and increases the vitrification temperature therefore preventing the caramelization of the sugars in solution. Afterwards an increased % of maltodextrin was used, but the results were not substantially better. For the leaf powder creation an extract obtained according to the best conditions seen before for anti-Alzheimer potential was mixed with 10% maltodextrin or 9% maltodextrin + 1% gelatin. The addition of gelatin required the heating of the extract for its dissolution, which was revealed to be undesirable. All obtained powders were easily resuspended in water, achieving concentrations of 200 mg/mL. The addition of maltodextrin or the spray-drying process didn’t cause a reduction in their antioxidant or anti-Alzheimer properties. The obtained powders were then subjected to an *in-vitro* digestion, an important step when testing extracts since the

stomach and intestinal pH or the enzymes present can degrade the compounds with beneficial activity. Out of the resuspended extracts only the 10% maltodextrin leaf was strong enough to still show anti-Alzheimer potential. This however may not have been simply to the simulated digestive process but also due to the concentration in which the extract will end after the digestion, since the total volume will be 10 times larger, thus being equivalent to a 10 times dilution, and at this dilution even the other undigested resuspended extracts showed no inhibitory activity. The 10% maltodextrin leaf resuspended extract however was capable of inhibiting 40% of the ACHE and 30% of the BCHE activities after the *in-vitro* digestion, compared to 60 and 70% before the digestive process. This loss occurred during the stomachic digestion for ACHE and during both steps for BCHE. Still, given the low concentration the extract was in (resuspended as 116 mg per mL) and the capability of increasing the concentration of the resuspended powder extract, the leaf powder obtained has remarkable potential to be used in the amelioration of Alzheimer disease symptoms.

Overall the new products studied, particularly those having leaf extract as basis showed good antioxidant activity and anti-Alzheimer potential. Additionally the beverage could also be used in the glycaemic control since as seen before leaf extracts have a high inhibitory activity towards α-glucosidase. The macrocapsules and spray-dried powder can be added to other foods or beverages to provide additional antioxidant activity and increase the value of the products regarding their anti-Alzheimer potential.

CHAPTER VII - FINAL REMARKS

In this chapter we summarize the results obtained and the main conclusions

Through the bibliographic review it was possible to understand that the interest in *Arbutus unedo* tree started to grow after 2008. Currently two main themes are being studied. The first theme is related to the plant physiology, where leaves and trees are the main focus of research. The second theme is related to medicinal properties, and here both leaves and fruits are studied. On the other hand very few works exist that study the flowers.

Analysing the characteristics of the trees, it was possible to see immediately that there were differences among them. Additionally, a preliminary extraction showed differences among the samples antioxidant activity, although leaves always had a higher antioxidant activity than fruits. It also showed that there was no significant advantage in using soxhlet extraction when compared to a simple hot plate extraction. Using a screening design it was possible to improve the antioxidant results obtained in the preliminary extraction and understand that some factors have a great effect on the extract antioxidant activity while others can be overlooked. Additionally it was also possible to understand that storing the leaves in a freezer for more than 6 months is not advised due to a loss in antioxidant activity, while storing the fruits under the same conditions led to an increase in this same antioxidant activity.

From all the plants studied, *Arbutus unedo* was the one with the highest antioxidant activity. It also showed good antibacterial activity and some selectivity towards cancer cell lines. This was likely due to the two main phenolic compounds detected using HPLC, gallic acid and quercetin. Given that these compounds are known to possess medicinal properties and this plant is sometimes used in traditional medicine for diabetes, a preliminary assay was conducted using α-glucosidase and α-amylase enzymes, which revealed a high inhibition of α-glucosidase. This work was then expanded, revealing that while extraction conditions have some influence on the results, plant part plays a much larger role, with leaves having overall the highest inhibitory activity, followed by flowers and finally fruits. Their mechanisms of inhibition are also different. The potential of *Arbutus unedo* extracts in preventing dementia related diseases (Alzheimer and Parkinson) was also investigated, due to the presence of arbutin, a compound known to exert inhibitory activity towards Parkinson related enzymes. This work showed that while the aqueous extracts inhibited the enzymes related with Alzheimer, with leaves showing the highest inhibitory activity, the inhibition of tyrosinase caused by the extracts was quite low, although it was possible to improve it after using ethanol in the extraction.

The proximal analysis of leaf and fruit samples revealed that leaves possess more protein, ashes and crude fat, while fruits possess more carbohydrates. The main fatty acids detected on the lipophilic fraction were α-linoleic and α-linolenic, although acid oleic was also highly present. Leaves and fruits lipophilic fraction was rich in vitamin E, mostly α-tocopherol which accounted for over 90% of the vitamers detected. Drying the fruit in the oven did not lead to a loss of fatty acid or vitamin E contents.

When developing a beverage, it was ultimately concluded that the inclusion of fruit extract to a leaf extract led to lower antioxidant activity than expected, which would likely translate into lower medicinal properties as well. The beverage was then submitted to a storage test, which analysed the change in colour and antioxidant activity and to an *in-vitro* digestion. It was concluded that exposure to light lead to high degradation, while fridge storage was adequate. Additionally it was found that while the *in-vitro* digestion lead to a decrease in antioxidant and inhibitory activities, the digested beverage still exhibited high antioxidant and inhibitory activities.

The best conditions for obtaining capsules made with fruit and leaf extracts were 2% alginate in a 2:1 proportion with extract in 8% $CaCl_2$ for 10 minutes. The obtained capsules would not leak, have adequate shape and were mostly stable if adequately stored. The highest antioxidant activity was obtained using leaf extract as core and fruit extract as enrichment, although using fruit as core and leaf extract as enrichment yielded even more stable capsules.

When using the spray-dryer, the addition of maltodextrin was extremely important. Without it the sugars present in the extracts would caramelize due to the high temperatures used, preventing the collection of any powder. While gelatin decreased the hygroscopy and size of the particles, its addition carries problems which need to be taken into consideration. The spray-dried particles obtained from leaf and fruit extracts were easily soluble in water and maintained their antioxidant activity. Leaf particles were better both in antioxidant activity and anti-Alzheimer potential, and were in fact the only particles that showed inhibitory activity after the *in-vitro* digestion.

Overall this work helps to close the gap on the knowledge from *Arbutus unedo* L. especially in regards to flowers and shows the potential use of its leaf and flower extracts in the control of chronic diseases. It also shows new products which could be an alternative to the current economic model which consists mainly in the production of alcoholic beverages or jams.