INSTITUTO POLITÉCNICO DE BRAGANÇA
Escola Superior Agrária

Caracterização fitoquímica de folhas e frutos de *Arbutus unedo* L.

Ivo Vaz de Oliveira

Dissertação apresentada à Escola Superior Agrária de Bragança
para obtenção do Grau de Mestre em Qualidade e Segurança Alimentar

Orientado por

Prof. Doutora Paula Cristina dos Santos Baptista

Prof. Doutor José Alberto Cardoso Pereira

Bragança
2010

AGRADECIMENTOS

Deixo aqui os meus agradecimentos a todos que me ajudaram, de alguma maneira, a chegar ao fim desta etapa.

Na Escola Superior Agrária, gostaria de agradecer à Prof. Doutora Paula Cristina dos Santos Baptista e ao Prof. Doutor José Alberto Pereira pela orientação deste trabalho, pelo apoio, incentivo e acompanhamento, disponibilidade e ajuda ao longo do trabalho; Ao Prof. Doutor Albino Bento, pela simpatia e esforço para garantir as condições materiais e financeiras para o bom desenvolvimento deste trabalho.

À Doutora Paula Guedes, do Serviço de Toxicologia da Faculdade de Farmácia da Universidade do Porto pelas facilidades concedidas para a determinação dos compostos voláteis.

À Prof. Doutora Susana Casal, do Serviço de Bromatologia da Faculdade de Farmácia da Universidade do Porto, pelas facilidades concedidas para a determinação dos ácidos gordos e Vitamina E.

A todos os colegas do Laboratório de Agrobiotecnologia, Susana Pereira, Anabela Sousa, Ricardo Malheiro e Eric Pereira pela boa disposição, pelo bom ambiente de trabalho proporcionado e ajuda constante na realização da parte experimental deste trabalho.

À Ana Paula, Sofia e Soraia, pelo seu apoio e amizade demonstrada nestes anos todos.

À Sílvia, não só pela ajuda que me deu na parte experimental do trabalho, mas principalmente pelo apoio e incentivo constante.

Finalmente, à minha família, aos que estão perto, e aos que estão um bocadinho mais longe. Obrigado.

Trabalho financiado em parte pelo projecto ”**Prospecção e caracterização do medronheiro, *Arbutus unedo* L. em Trás-os-Montes com vista à sua valorização**” financiado pela Caixa de Crédito Agrícola Mútuo da Região de Trás-os-Montes e Alto Douro.

ÍNDICE

RESUMO

O medronheiro (*Arbutus unedo* L.) é um arbusto nativo da região mediterrânica, estando distribuído por todo o território nacional. Na medicina tradicional, diversas partes da planta têm sido usadas, nomeadamente as folhas, frutos, casca e raízes, no tratamento de uma grande diversidade de doenças. Acresce ainda o interesse social, ecológico e económico desta planta, sendo os seus frutos usados para a confecção de compotas e geleias, bem como no fabrico de aguardente de medronho.

Apesar de todas estas características, o medronheiro tem vindo lentamente a desaparecer, sendo de importância procurar meios para a sua valorização. Assim, este trabalho pretende contribuir para essa valorização, caracterizando diversas propriedades de diferentes partes deste arbusto.

Nas folhas, procedeu-se à avaliação da actividade antioxidante, usando três métodos distintos (Efeito bloqueador de radicais de 2,2-defenil-1-picril-hidrazilo (DPPH), poder redutor sobre o complexo Fe (III)/ferricianeto e efeito sequestrante de radicais superóxido) e quantificação dos fenóis totais, usando quatro tipos diferentes de extracção. Os extractos etanólicos apresentaram os melhores resultados nos métodos do DPPH e poder redutor (valores de EC_{50} de 232,7 µg/mL e 63,2 µg/mL, respectivamente), sendo também nestes extractos onde se encontrou a maior quantidade de fenóis totais (192,66±1.66 mg GAE/g extracto). No método do radical superóxido, os melhores resultados foram obtidos com a extracção metanólica (valor de EC_{50} de 6,9µg/mL).

Relativamente aos frutos, a quantificação dos fenóis totais mostrou que a presença destes compostos é mais elevada nos frutos com um estado de maturação intermédio (111mg GAES/g peso seco). Na avaliação da actividade antioxidante, os frutos maduros e intermédios apresentaram os melhores resultados no DPPH e poder redutor, respectivamente (EC_{50} de 0,25±0,02 mg/mL e 1,09±0,05 mg/mL). Os resultados obtidos no ensaio do DPPH estão fortemente correlacionados com o estado de maturação dos frutos, diminuindo os valores de EC_{50} conforme a sua maturação avança. Por outro lado, os valores de EC_{50} obtidos no ensaio do poder redutor estão correlacionados com a quantidade de fenóis totais encontrada nos diferentes estados de maturação. O perfil em ácidos gordos é semelhante entre os três estados de maturação, sendo os ácidos gordos mais importantes o alfa-linolenico, linoleico e oleico, representando os ácidos gordos polinsaturados a maior fracção (60%). Nos ácidos

gordos maioritários, foi possível obter uma forte correlação entre o seu teor e o estado de maturação dos frutos. Da análise da vitamina E, verificou-se a importância do isómero gama-tocotrienol na quantidade total desta vitamina, sendo que a sua quantidade decresce com o aumento da maturação do fruto. Para três dos isómeros, e para o total de vitamina E presente nos frutos foi possível observar uma forte correlação entre o estado de maturação das amostras e a quantidade detectada nesses mesmos frutos.

Na avaliação dos compostos voláteis do medronho, foi visível o predomínio de três classes químicas: álcoois, aldeídos e esteres. Estes três grupos representam a maioria dos compostos identificados nos três estados de maturação considerados neste trabalho. A quantidade total de compostos voláteis diminui à medida que a maturação dos frutos aumenta, sendo este comportamento visível em todos os grupos químicos presentes, com a excepção dos sesquiterpenos e monoterpenos. Os compostos maioritários identificados foram (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, (*E*)-2-hexenal e acido acético 4-hexen-1-yl ester. Outras classes químicas foram também identificadas, em quantidades reduzidas, nomeadamente derivados de norisoprenóides, sesquiterpenos e monoterpenos, alguns presentes apenas quando o fruto se encontrava no estado de máxima maturação. Os compostos responsáveis pelo cheiro verde característicos diminuem com o avanço da maturação, ao mesmo tempo que surgem compostos com aromas doces e frutados, responsáveis pelo cheiro que os frutos, quando maduros, apresentam.

ABSTRACT

The strawberry-tree (*A. unedo* L.) is a Mediterranean native shrub, being distributed throughout the Portuguese territory. Several parts of the plant are used in the folk medicine, namely the leaves, fruits, bark and roots, in the treatment of a large array of diseases. In addition, this plant as an social, ecological and economical interest, being its fruits used in the confection of jams and marmalades, as well as used for the production of alcoholic distillates.

Although presenting all those characteristics of interest, the strawberry-tree population are in decline, urging to find new ways for its valorisation. The goals of this work in to contribute to that valorisation, by characterizing several features of different parts of this shrub.

In the leaves, we proceeded to the evaluation of the antioxidant activity, using three different methods (reducing power of iron (III)/ ferricyanide complex assay, scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and scavenging effect on superoxide radicals) and the quantification of the total phenolics, using four different extraction methodologies. The ethanolic extracts achieved the best results in the DPPH and reducing power assays (EC_{50} values of 232.7 µg/mL and 63.2 µg/mL, respectively), being also in these extracts were the higher amount of total phenolics was found (192.66±1.66 mg GAES/g extract). In the superoxide radical assay, the best results were accomplished by the methanolic extracts (EC50 value of 6.9µg/mL).

Regarding the fruits, the total phenolic quantification showed that the amount of these compounds is higher when the fruits present an intermediate ripening stage (111 mg GAES/g of dry weight). When evaluating the antioxidant activity, ripe and intermediate fruits presented the best results in the DPPH and reducing power assays, respectively (EC_{50} of 0.25±0.02 mg/mL and 1.09±0.05 mg/mL). The results achieved in the DPPH assay are strongly related to maturation stage of the fruits, with the EC_{50} values decreasing as the maturation advances. By other hand, the values of EC_{50} of the reducing power assay were found to be correlated to the total phenolics amounts present in the fruits, the different ripening stages.

The fatty acid profile is very similar between the three ripening stages, being alfa-lilonenic, linoleic and oleic acids the major ones, representing polyunsaturated fatty acids the major fraction (60%) of all fatty acids. For the major fatty acids, a strong statistic correlation was found between their amounts and the maturation stage presented by the fruits. The vitamin E analysis showed that the gama-tocotrienol

vitamer is the most important, and that the vitamin E content decreases as the maturation of the fruit advances. For three of the isomers, and for the total amount of vitamin E present in the fruits, a strong correlation between the ripening stage of fruits and the amounts present in those fruits.

In the evaluation of the volatile compounds of the fruits, it was visible that three chemical classes are predominant: alcohols, aldehydes and esters. These three groups represent the majority of the identified compounds in the three maturation stages of the fruits, considered for this work. The total amount of volatiles decreases as the maturation of the fruits advances, being this pattern visible for all chemical groups present, with the exception of sesquiterpenes and monoterpenes. The most abundant identified volatiles were (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, (*E*)-2-hexenal e acetic acid 4-hexen-1-yl ester. Other chemical classes were also identified, in reduced amounts, as norisoprenóides derivatives, sesquiterpenes and monoterpenes, some of them only present when the fruit was fully ripe. The compounds that transmitr the characteristic green scent decrease with the advancing of the maturation, appearing, at the same time, compounds with sweet and fruity aroma, responsible for the odor presented by fruits when ripe.

Capítulo 1
Introdução Geral

1.1. INTRODUÇÃO GERAL

O medronheiro, *Arbutus unedo* L. é um arbusto distribuído por toda a bacia mediterrânica. Em Portugal, esta espécie encontra-se disperso por todo o País, sendo mais abundante no Sul. Apresenta elevado interesse na preservação da diversidade da flora e da paisagem, evita a erosão dos solos, uma vez que tem uma regeneração rápida após incêndios e é capaz de crescer em solos pobres, podendo ser usada na fitorremediação de solos contaminados com arsénico.

São várias as partes desta planta com aplicações quer na produção de geleias, compotas, aplicação em pastelaria e produção de aguardente para o caso dos frutos (medronho) e aplicação em fitoterapia de diferentes partes das plantas, nomeadamente folhas, frutos, cascas e raízes.

A nível nacional existe muito pouca informação acerca desta espécie. Os trabalhos desenvolvidos dizem respeito sobretudo à produção e caracterização de aguardentes de medronho e da composição dos frutos, com o objectivo de melhorar a produção desta bebida. Outros trabalhos abordam actividade biológica da planta por inteiro, ou apenas os frutos.

Neste sentido, com este trabalho pretendeu-se assim, contribuir para aumentar o conhecimento sobre esta planta, nomeadamente ao nível da composição química dos frutos e avaliação da actividade antioxidante de folhas e frutos. No caso dos frutos estudou-se também a influência do estado de maturação na composição química e actividade antioxidante, com especial referência para a composição em compostos voláteis. Assim, os objectivos específicos deste trabalho foram:

- Proceder a uma breve revisão bibliográfica da informação disponível sobre as diferentes partes desta espécie ao nível da composição química e actividade biológica, apresentada no Capítulo 2;
- Avaliar o potencial bloqueador de radicais livres das folhas de medronheiro, usando três métodos de avaliação de actividade antioxidante (Efeito bloqueador de radicais de DPPH, poder redutor sobre o complexo Fe (III)/ferricianeto e efeito sequestrante de radicais superóxido), e quatro solventes de extracção (água, etanol, metanol e éter dietilico), apresentado e discutido no Capitulo 3;
- Determinar a actividade antioxidante de frutos (medronhos), através dos métodos do efeito bloqueador de radicais de DPPH e poder redutor sobre o

complexo Fe (III)/ferricianeto, bem como a sua composição em ácidos gordos, através de GC/FID, e vitamina E, por HPLC/FD, em diferentes etapas de maturação dos frutos, discutida no Capítulo 4;

- Detecção e quantificação de compostos voláteis emitidos por medronhos com diferentes estados de maturação, usando a metodologia HS-SPME e cromatografia gasosa acoplada a espectroscopia de massa de armadilha de iões (GC/IT-MS), informação apresentada no Capítulo 5.

Capítulo 2
Arbutus unedo L. and its benefits on human health

Arbutus unedo L. and its benefits on human health

Journal of Food and Nutrition Research, **50** (2011), 73–85.

ABSTRACT

Arbutus unedo has been long used in folk medicine, throughout Mediterranean countries, with the employment of infusions and decoctions of almost all parts of this plant: leaves, fruits, barks and roots. The application of these traditional remedies arises from several health-promoting characteristics, the treatment of gastrointestinal and urological problems, hypertension and cardiac diseases, diabetes and as anti-inflammatory, among other interesting properties. Antioxidant ability of A. unedo shrub is also known, and antimicrobial activity has also been reported. Several compounds, present in the different parts of the plant, may be linked to these properties. Included in those are carotenoids, flavonoids, phenolics acids and vitamins (C and E). Other bioactive compounds may be found in the different parts of A. unedo, like terpenoids and organic acids. This review will focus on the known composition of the several parts of A. unedo, their antioxidant ability and traditional use, and the available data sustaining the rationality of the use of as part of folk medicine.

KEY-WORDS: *Arbutus unedo*; chemical composition, biological properties, folk medicine.

2.1. INTRODUCTION

In the recent years there has been an increased interest on natural products, leading to an extensive search for bioactive compounds, namely plant antioxidants, and their significance, in medicine, food industry and human nutrition (Liu and Ng, 2000).

Arbutus unedo L., the strawberry-tree, belongs to the family of Ericaceae and is an ever-green shrub (Figure 1A), native of the Mediterranean region.

Figure 1. *Arbutus unedo* tree (A), unripe (B) and ripe fruits (C)

In Europe, grows mostly in the Mediterranean basin (Portugal, Spain, France, Italy, Albania, Greece, Bosnia and Herzegovina, Croatia, Macedonia, Montenegro, Serbia and Slovenia) including the some of the Mediterranean islands (Balearic, Corsica, Sardinia, Sicily and Crete), mainly in coastal and inland areas were climate is adequate to its development. It has also been able to adapt to conditions of the south-western coast of Ireland (Torres *et al*., 2002) (Figure 2). Although occasionally reaching a height of 12 metres, it is normally between 1.5 to 3 metres tall (Celikel *et al*., 2008). The fruits are conspicuous, globular, orange-red when ripe, growing up to 2 cm across (Figure 1B and C), and the flower is a clump of little cream-colored lanterns. The

maturation phase of the fruits usually comprise two periods. The first starts in the middle of October and ends in the beginning of December, while the second one happens around New Year's Eve (Soufleros *et al*., 2005). The leaves are alternate, simple, with oblanceolate form and a dark green colour, leathery, short-stalked and toothed (Males *et al*., 2006).

Figure 2. Approximate distribution of *Arbutus unedo* L.

There are several qualities associated to this plant, such as ornamental, ecological and economical value, as well as therapeutic and medicinal properties. It's an important ornamental tree, producing red fruits and pinkish-white flowers, which appear during the winter months, simultaneously, which increases its value for planting and ornamental purposes (Males *et al.*, 2006). This plant also has an important role, from an ecological point of view. It helps maintain the diversity of the fauna, avoids erosion of the soils, it regenerates rapidly after fires, grows in poor soils (Gomes and Canhoto, 2009), and it may be used for phytoremediation, namely against arsenic contamination (Moreno-Jiménez *et al*., 2008).

The fruits, even though edible, are usually consumed as jams, marmalades or distilled into liquors (Soufleros *et al*., 2005). Strawberry-tree honey is popular for its strong and distinctly bitter taste and has also been subject to some studies. The production of jams and liquors, as well has the commerce of *A. unedo* based honey are a source of complementary resource of income, especially in rural areas (Alarcão-e-Silva

et al., 2001) attesting the economic implication of this shrub. There are several reports of the use of different parts of this plant in traditional medicine (Table 1).

Table 1. Traditional medicinal uses of different parts of *A. unedo* plant.

Part used	Medicinal use	References
Leaves	- Gastrointestinal disorders; - Urological problems; - Dermatologic problems; - Cardio-vascular application; - Kidney diseases; - Hypertension; - Cardiac diseases; - Diabetes; - Antihaemorrhoidal; - Diuretic; - Anti-inflammatory; - Anti-diarrheal.	Ziyyat *et al*., 1997 El-Hilaly *et al*., 2003 Leonti *et al*., 2009 Jouad *et al*., 2009 Cornara *et al*., 2009
Fruits	- Gastrointestinal disorders; - Urological problems; - Dermatologic problems; - Kidney diseases; - Cardio-vascular application.	El-Hilaly *et al*., 2003 Leonti *et al*., 2009 Cornara *et al*., 2009
Bark	- Gastrointestinal disorders, - Urological problems, - Dermatologic problems, - Cardio-vascular application.	Leonti *et al*., 2009
Roots	- Gastrointestinal disorders; - Urological problems; - Dermatologic problems; - Cardio-vascular application; - Hypertension; - Cardiac diseases; - Diabetes; - Diuretic; - Anti-inflammatory; - Anti-diarrheal.	Ziyyat *et al*., 1997 Novais *et al*., 2004 Leonti *et al*., 2009 Jouad *et al*., 2009

Fruits are used in folk medicine, due to several characteristics, such as antiseptic, diuretic and laxative (Ziyyat and Boussairi, 1998; El-Hilaly *et al*., 2003; Pallauf *et al*., 2008; Cornara *et al*., 2009; Leonti *et al*., 2009). The leaves of this shrub are used as an infusion, for their astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and more recently, in the therapy of some diseases, such as hypertension, diabetes, and in

the treatment of inflammatory diseases (Ziyyat *et al*., 1997; Ziyyat and Boussairi, 1998; Mariotto *et al*., 2008; Afkir *et al*., 2008).

The roots and bark of this plant are also used in traditional medicine, on gastrointestinal disorders, as well as for urological and dermatologic problems (Leonti *et al*., 2009; Novais *et al*., 2004), using decoction of the roots as method to prepare the "drug". Besides all this facts, this tree still keeps on being underexploited, mainly due to the high heterogeneity of the plants. All these described medicinal and therapeutical characteristics are linked to the composition of the different parts of the *A. unedo* tree, which include several biologically active compounds. This review will focus the chemical composition of different parts of *A. unedo*, with especial reference to leaf and fruit composition, and on the biological activity and health-promoting effects of extracts of this plant.

2.2. CHEMICAL AND ANTIOXIDANT CARACTERISTICS OF *ARBUTUS UNEDO*

Several chemical classes of antioxidants are identified in different parts of *A. unedo*. In Figure 3 are shown the principal antioxidant metabolites identified. The chemicals of the different vegetative parts are presented in the following points.

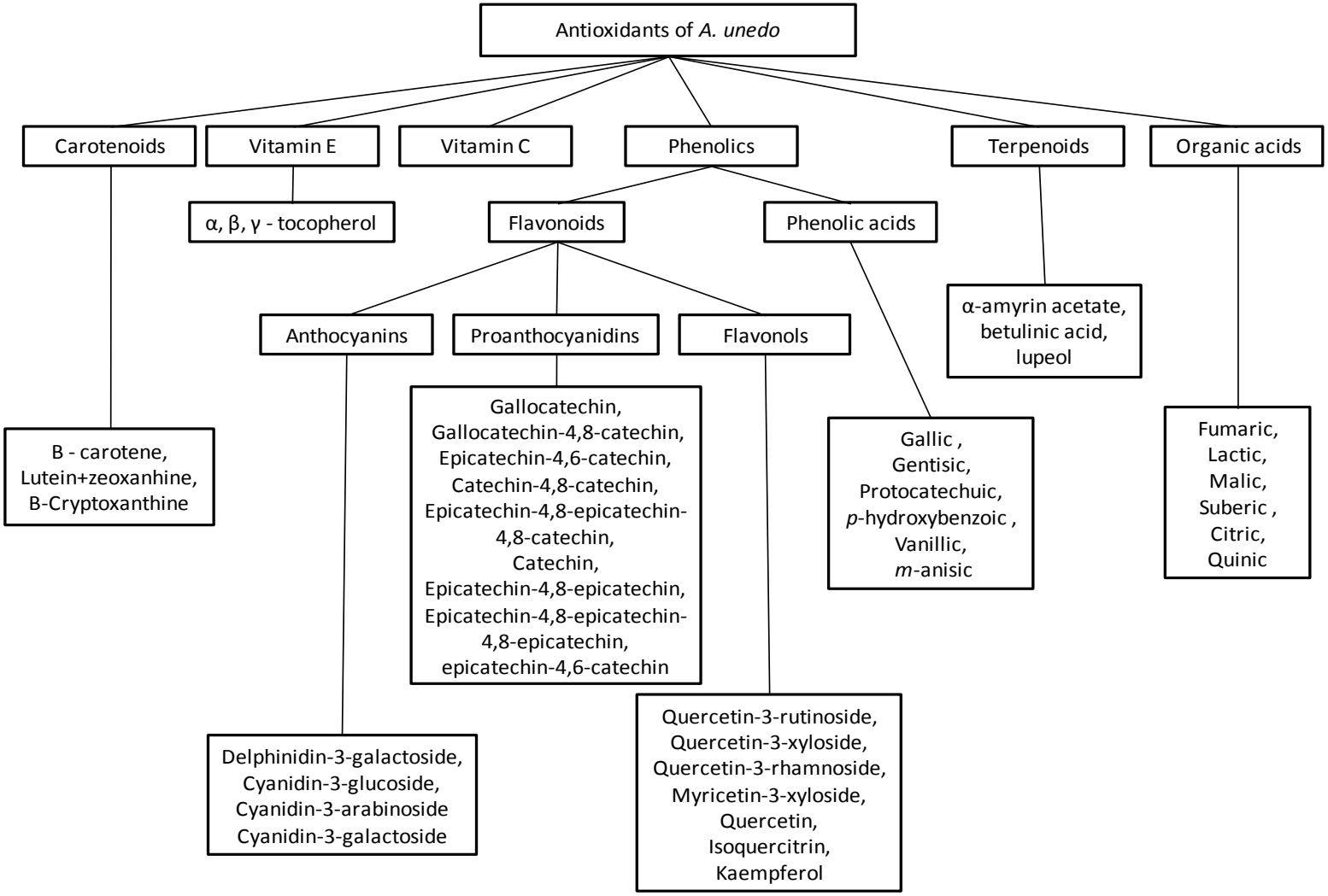


Figure 3. Antioxidant compounds present in the *Arbutus unedo* tree

2.2.1. Leaves

2.2.1.1. Leaves' chemical composition

Leaves of *A. unedo* contain several classes of phytochemical compounds, such as terpenoids, α-tocopherol, essential oils and phenolic compounds. The known terpenoids present in the leaves are α-amyrin acetate, betulinic acid and lupeol (Gaspar *et al*., 1997). The α-tocopherol amount present in the leaves of *A. unedo* varies, depending on the time of collection of the samples. The highest amount is found when the leaves are collected in March, reaching an amount of 0.01328%. Although this appears to be a very low amount, is very similar to the one present in the major industrial source of α-tocopherol, the soya bean (Kivçak and Mert, 2001). The composition of the essential oil of *A. unedo* leaves has already been disclosed by Kivcak *et al*., 2001a. The major compounds present in this essential oil are (E)-2-decenal, α- terpineol, hexadecanoic acid, and (E)-2-undecenal. The phenolic fraction of the leaves of the strawberry-tree includes a large variety of compounds: tannins, flavonoids (catechin gallate, myricetin, rutin, afzelin, juglanin, avicularin), phenolic glycosides (quercitrin, isoquercitrin, hyperoside) and iridoid glucosides (Sanjust *et al*., 2008; Legssyer *et al*., 2004; Males *et al*., 2006; Carcache-Blanco *et al*., 2006). Polyphenols have been identified and quantified by Fiorentino *et al*. (2007). This work allowed the identification of twelve compounds (arbutin, ethyl gallate, *p*-hydroxybenzoyl arbutin, galloyl arbutin, gallocatechin, catechin, kaempferol 3-*O*-α-L-ramnopyranoside, quercetin 3-*O*-α-L - ramno pyranoside, myricetin 3-*O*-α-L ramnopyranoside, kaempferol 3-*O*-β-D-arabinofuranoside, quercetin 3-*O*-β-D -arabinofuranoside and myricetin 3-*O*-β-D-arabinofuranoside). The major polyphenolic compound was found to be arbutin (62.7 mg in 100g of fresh leaves), followed by catechin (54.6 mg) and ethyl gallate (44.0 mg) The most important phenolics are represented in Figure 4. The amount of phenolic compounds present in the leaves, quantified using the Folin-Ciocalteau's phenol reagent, and performed in extracts obtained using different solvents, achieved as much as 192.66 mg of gallic acid equivalents (GAE´s) per gram of extract (Oliveira *et al*., 2009), or about 16 mg GAE's per gram of dry fruit (Tavares *et al*., 2010).

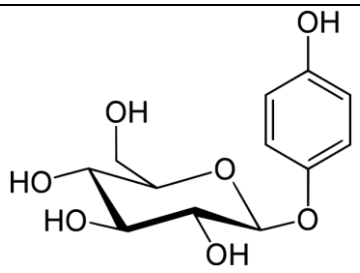
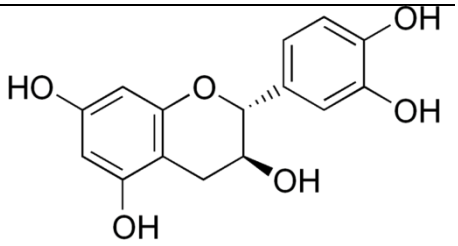
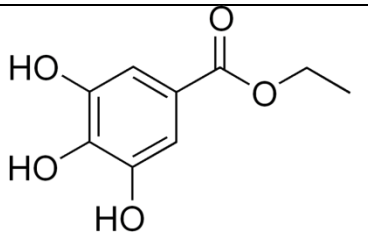
Phenolic compound	Chemical structure
Arbutin	
Catechin	
Ethyl gallate	

Figure 4. Chemical structures of the most abundant phenolic compounds in *Arbutus unedo* L.leaves

2.2.1.2. Leaves' antioxidant ability

Although presenting in their composition a wide variety of antioxidant compounds, as described before, the information available about the antioxidant ability of the leaves is scarce. An initial study was performed by Pabuçcuoğlu *et al* (2003), and a more comprehensive study was performed by Oliveira *et al*. (2009), existing a third study referring the antioxidant ability of leaves. The first one tested the antioxidant activity of ethanolic and methanolic extracts of the leaves, using the 2.2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) and related the activity to the flavonol glycosides and tannins present in the leaves. The second study, besides testing the antioxidant activity, using three different methods (Reducing power assay, scavenging effect on 2.2-diphenyl-1-picrylhydrazyl (DPPH) radicals and scavenging effect on superoxide radicals), also quantified the total phenolics of the leaves (Oliveira *et al*. 2009). Extracts achieved using ethanol presented the highest reducing power (EC_{50} of 232.7 $\mu g \cdot mL^{-1}$) and DPPH scavenging effect (EC_{50} of 63.2 $\mu g \cdot mL^{-1}$). In the scavenging

assay on superoxide radical, methanolic extracts obtained the best results (EC_{50} 6.9 $\mu g \cdot mL^{-1}$). The third work tested extracts of *A. unedo* leaves, using the Oxygen Radical Absorbance Capacity (ORAC) method. The antioxidant ability, using this method, was higher than the ones achieved with green tea extracts (Tavares *et al*., 2010). The results demonstrated that the leaves of *A. unedo* posses high scavenging effect against DPPH radical and high reducing power, as well as a potent effect in scavenging superoxide radical, one of the most important free-radical, precursor of several molecules associated to tissue damage through oxidation (Aruoma, 1996), and higher activity, when compared to samples considered to be highly effective, like green tea leaves.

2.2.2. Fruits

2.2.2.1. Fruits' chemical composition

By other hand, fruits of *A. unedo* have also been subject of some studies, regarding their chemical composition and antioxidant ability. Özcan and Hacıseferoğulları (2007) evaluated the chemical composition of the strawberry-tree fruits (Table 2). Besides moisture, the most important component of the fruits are sugars, representing from 42% to 52% of the total weight of dry fruits (Alarcão-e-Silva *et al*., 2001; Ayaz *et al*., 2000).]. From the total carbohydrates, when the fruit is unripe, saccharose is the major carbohydrate (87.7 ± 0.6 g per Kg of dry fruit), but as the fruits turn ripe, fructose becomes the most important carbohydrate present in these fruits (208 ± 2 g per Kg of dry fruit) (Alarcão-e-Silva *et al*., 2001). Protein is also present in considerable amount, 33.6 ± 0.12 g per kilogram, followed by ashes (28.2 ± 1.24) and fat (21 ± 1). The energetic value presented by these fruits is of 13682 ± 544 kj (Özcan and Hacıseferoğulları, 2007).

Table 2- Chemical composition of *Arbutus unedo* fruits (g/Kg).

	Özcan and Hacıseferoğulları [30]	Barros *et al*.* [31]
Moisture	537.2 ± 21.0	597.0 ± 26.7
Carbohydrates	-	938.3 ± 4.1
Proteins	33.6 ± 1.2	30.9 ± 0.8
Fat	21 ± 1.0	13.7 ± 4.0
Ash	28.2 ± 1.24	17.1 ± 0.9
Energy	13682 ± 544 kj·	16735.6 ± 48.9 kj·

"-" not reported
* – expressed per Kilogram of dry weight.

The fatty acid composition of *A. unedo* fruits was evaluated by Barros *et al.* (2010). Twenty-one fatty acids were identified and quantified, being α-linolenic acid (C18:3n3) is the one present in higher amounts (36.51 ± 0.64, relative percentage), and representing polyunsaturated fatty acids the most part of all the fatty acids (58.28 ± 0.54). Sugars, minerals, phenolic compounds, organic and phenolic acids, vitamins and carotenoids are also present in the *A. unedo* berries. The amount of sugar present in these fruits is variable between ripeness stages, being, when unripe (Alarcão-e-Silva *et al*., 2001), 14% of dry weight, and when ripe varying between 40.55% to 52.21 % of dry weight (Alarcão-e-Silva *et al*., 2001; Ayaz *et al*., 2000). From the total sugars, when the fruit is unripe, sucrose is the major sugar, but as the fruits turn ripe, fructose becomes the most important sugar present in these fruits (Alarcão-e-Silva *et al*., 2001).

The mineral content of these fruits was disclosed by Özcan and Hacıseferoğulları (2007). This study showed that these fruits are a very good source of some minerals, especially potassium (K), calcium (Ca), and phosphorus (P). The first one is present in the berry of *A. unedo* in very high amounts, 14909.08 ± 1687 mg/kg of dried weight, while calcium and phosphorus quantities are 4959.02 ± 15 and 3668.56 ± 339.69 mg/kg of dried weight, respectively.

There are several organic and phenolic acids present in the *A. unedo* fruits. The organic acids identified are fumaric (1.94 mg/g dry weight), lactic (0.84 mg/g), malic (0.84 mg/g), suberic (0.23 mg/g) and citric acids (0.01 mg/g), while gallic (10.7 mg/g dry weight), gentisic (1.9 mg/g), protocatechuic (0.6 mg/g), *p*-hydroxybenzoic (0.3 mg/g), vanillic (0.12 mg/g) and m-anisic (0.05 mg/g) are the phenolic acids identified in these fruits (Ayaz *et al*., 2000). Quinic acid is referred by Alarcão-e-Silva *et al*. (2001) as the most important one, both in unripe (7.35±0.03 g/100g dry matter) and ripe fruits (5.99±0.05 g/100g dry matter). By other hand, Ayaz *et al*. (2000) presents fumaric acid as the major organic acid (1.94±0.07 mg/g dry matter) present in the ripe *A. unedo* fruits.

The amount of vitamins and carotenoids in the fruits, although not being very high, should not be ignored. The total vitamin E content was determined by Barros *et al*. (2010), showing that the fruits present in their composition 23.46 ± 0.26 mg/100 g dry weight of total tocopherols, being α-tocopherol the most important form of vitamin E present, with 21.98 ± 0.18 mg/100 g dry weight. The isomer α-tocopherol has also been quantified in another work (Pallauf *et al*., 2008), where the amount of this form of

vitamin E was of 0.0237 ± 0.001 mg per 100 g edible portion. The amounts reported by those two authors are quite dissimilar, the different methodology used on those two works must account for such differences. Another vitamin has already been found in the berries of the strawberry tree, vitamin C. There are three known works that provide us with the quantification of this vitamin. The highest amount was found by Alarcão-e-Silva *et al*. (2001), whose samples contained 542 ± 11 mg of ascorbic acid per 100 g of dry matter of the fruit, while Barros *et al*. (2010) reach a lower value, of only 15.07 ± 0.77 mg of ascorbic acid per 100 grams of dry weight. The work of Pallauf *et al*. (2008) provides us with the amount of vitamin C present in 100 grams of edible portion of the strawberry-tree fruits. The amount found by those authors, of 5.50 ± 0.147 mg per 100 g edible portion, although considered low, in comparable to the quantity present in other fruits, like peaches, apples or plums.

Carotenoids are also present in the fruits, especially β – carotene. Total carotenoids were quantified by Pallauf *et al*. (2008), that reported an amount of these compound of 0.0647±0.014 mg per 100 g edible portion, being the most part quantified as lutein + zeaxanthine (0.0427±0.009) and the remaining as β – carotene (0.025±0.007). The content of this carotenoid in this fruits is also presented by Barros *et al*. (2010) and Alarcão-e-Silva *et al*. (2001). The first authors refer 1.07 ± 0.09 mg/100 g dry weight as the amount of β-carotene that is present in the strawberry-tree fruits, while Alarcão-e-Silva *et al*. (2001) refer the changes in the amount of β-carotene with fruit ripening, beginning with 38.1±3.7 mg/100 g of dry matter, when the fruit is unripe, to 70.9±5.2 mg/100 g of dry matter when the fruit is red mature.

The phenolic fraction present in the fruits of this shrub includes several chemical classes, such as tannins, flavonoids, ellagic acid derivatives and gallic acid derivatives. The total phenolics present was quantified in different studies: Barros *et al*. (2010) indicate an amount of 126.83 ± 6.66 mg GAE/g of extract while Heinrich (2005) report a value of 37.6 mg/g extract. By other hand, Fortalezas *et al*. (2010) indicate 16.46 ± 3.66 mg GAE per g of dry weight, Tavares *et al*. (2010) found about 18 mg GAE per g of dry fruit, while Alarcão-e-Silva *et al*. (2001) refer 15.5±0.6 mg catechin equivalents per g of dry weight as the amount of total phenolics present in the fruits. In the flavonoid class of compounds present in the berries, four different anthocyanins can be found: delphinidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside (Fortalezas *et al*., 2010; Pawloska *et al*., 2006; Pallauf *et al*., 2008) and cyanidin-3-galactoside (Figure 5). This last compound was found only by Pallauf *et al*. (2008), who

were able to achieve the separation of two anthocyanin isomers, which only differ on the sugar present in the anthocyanidin, in this case the glucoside and galactoside of cyanidin, that may co-elute, interfering both in the identification and quantification of these kind of compounds (Pallauf *et al*., 2008). Therefore, the statement of Pawloska *et al*., (2006), who declare cyanidin 3-glucoside has the major anthocyanin present in these fruits, with 0.39 mg/100 g of fresh fruits may not be entirely correct. Pallauf *et al*. (2008) claim that cyanidin-3-galactoside, the other isomer this anthocyanin is the major one, presenting the berries 2.84±0.935 mg per 100 g edible portion, while the glucoside isomer is present in a very much lower amount (0.12±0.025 mg per 100 g edible portion). The amount of these compounds was accessed by Alarcão-e-Silva *et al*. (2001), who quantified the total anthocyanin content of *A. unedo* fruits presenting two different stages of maturation. The quantity of anthocyanins increases as the maturation advances, passing from 0.25±0.02 mg/g dry matter when the fruit is unripe to 1.01±0.01 mg/g dry matter as the fruit becomes red mature. On another study, Fortalezas *et al*., 2010, quantified the total amount of anthocyanin, reaching a value of 76.26 ± 9.85 as mg of cyanidin 3-glucoside equivalents per 100 g of dry weight.

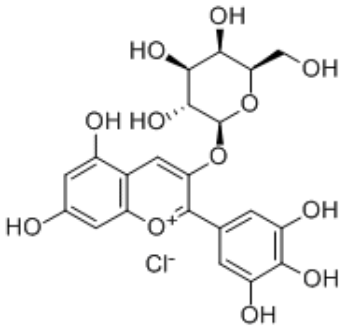
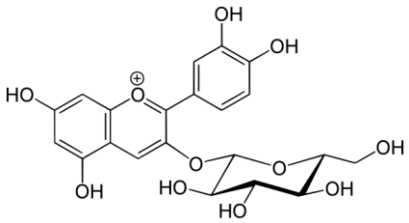
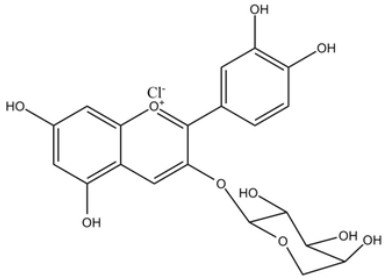
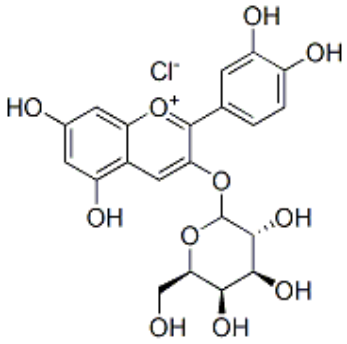
Anthocyanins	Chemical structure
Delphinidin-3-galactoside	
Cyanidin-3-glucoside	
Cyanidin-3-arabinoside	
Cyanidin-3-galactoside	

Figure 5. Chemical structures of the anthocyanins present in *Arbutus unedo* L. fruits

Alarcão-e-Silva *et al*. (2001) quantified the total amount of tannins in fruits with different maturation stages. According to those results, the amount of tannins decreases has the fruit become ripe, from 3.13±0.06 mg/g dry matter when the fruit is unripe to 1.75±0.02 mg/g dry matter when the fruit is red mature. As the taste of these berries is considered to be too astringent when the fruits are green, becoming edible when they reach the ripening stage, this bitter taste can be attributed to the high amount of tannins present, which are known to be astringent. Another chemical group within flavonoids,

the proanthocyanidins, are present in the fruits. Ten compounds were identified and quantified by Pallauf *et al*., (2008), including gallocatechin, gallocatechin-4,8-catechin, epicatechin-4,6-catechin, catechin-4,8-catechin, epicatechin-4,8-epicatechin-4,8-catechin, catechin, epicatechin-4,8-epicatechin, epicatechin-4,8-epicatechin-4,8-epicatechin, epicatechin-4,6-catechin. The total quantity of proanthocyanidins is 27.46 ± 0.989 mg per 100 g edible portion, being epicatechin-4,8-epicatechin-4,8-catechin (4.52±0.874 mg per 100 g edible portion) the individual compound that most contributes to the large presence of proanthocyanidins in these fruits, followed by catechin and gallocatechin (Figure 6). Furthermore, among fruits, the strawberry-tree berries are ones that present higher amount of these compounds (Pascual-Teresa *et al*., 2000). Belonging also to the flavonoids, flavonols are as well present. The most abundant are quercetin derivatives (quercetin-3-rutinoside, quercetin-3-xyloside and quercetin-3-rhamnoside), and myricetin-3-xyloside is the fourth flavonol identified and quantified by Pallauf *et al*., (2008). Other reports refer the presence of quercitrin, isoquercitrin and kaempferol (Males *et al*., 2006; Mazza and Miniati, 1993). The total amount of flavonols is 1.14±0.346 mg per 100 g edible portion, almost half of that quantity represented by quercetin-3-xyloside (0.52±0.031 mg per 100 g edible portion) (Pallauf *et al*., 2008).

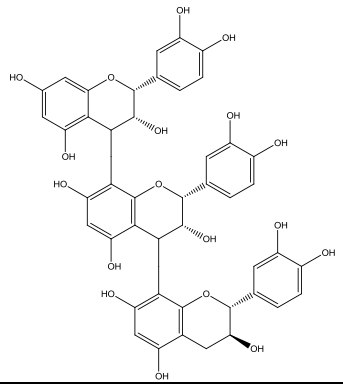
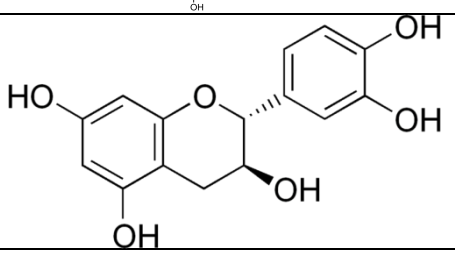
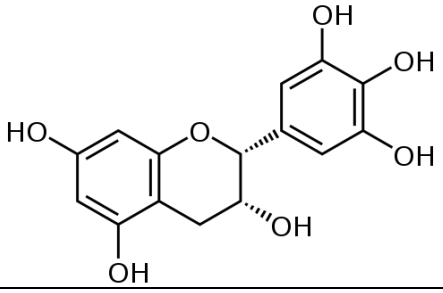
Proanthocyanidins	Chemical structure
Epicatechin-4.8-epicatechin-4.8-catechin	
Catechin	
Gallocatechin	

Figure 6. Chemical structures of the most abundant proanthocyanidins present in *Arbutus unedo* L. fruits

Other antioxidant compounds present in *A. unedo* are ellagic acid and gallic acid derivatives. The first ones are present in amounts similar to flavanols, (1.54±0.162 mg per 100 g edible portion), and five derivatives were found by Pallauf *et al*. (2008), namely ellagic acid glucoside, ellagic acid diglucoside, methylellagic acid rhamnoside + ellagic acid arabinoside and ellagic acid xyloside, being the first one present in higher amount (0.46±0.019 mg per 100 g edible portion). There are six derivatives of gallic acid found in the strawberry-tree fruits (*β*-D-glucogalline, 3-*O*-galloylquinic acid, gallic acid 4-*O*- *β*-D glucopyranoside, 5-*O*-galloylquinic acid, 5-*O*-galloylshikimic acid and 3-*O*-galloylshikimic acid).

Volatile composition of *A. unedo* berries as also been investigated. Besides studies on traditional distillate produced by the fermentation of strawberry tree fruits (Soufleros et al., 2005) strawberry-tree honey (Bianchi et al., 2005; de la Fuente et al., 2007) essential oils (Kahriman et al., 2010) and the emission of volatile organic compounds by the entire shrub (Owen et al., 1997; LLusià and Peñuelas, 2000;

Peñuelas and Llusià, 2001) one report is available on the volatiles emitted by the berries themselves (Oliveira et al., in press). Six chemical classes were found (alcohols, aldehydes, esters, norisoprenoids, sesquiterpenes and monoterpenes), comprising 41 volatile compounds. Alcohols are the main component of the volatile fraction of A. unedo fruits, followed by aldehydes and esters. The variation of volatiles was studied in three maturation stages of the fruits, and results show that all the chemical classes decreased their content from unripe to ripe stages, with the exception of sesquiterpenes and monoterpenes. These appear in higher amount in ripe fruits than in unripe fruits. The major compounds were (Z)-3-hexen-1-ol, 1-hexanol, hexanal, (E)-2-hexenal and acetic acid hexyl ester. Decrease of the compounds responsible by the characteristic green odors (alcohols, aldehydes and esters) are reported to be replaced by others, associated to flower and sweet scents (mainly monoterpenes and norisoprenoid compounds), present in ripe fruits of *A. unedo*.

2.2.2.2. Fruits' antioxidant ability

There are only four known studies regarding the antioxidant activity of the fruits of *A. unedo*. The most comprehensive one was performed by Barros *et al*. (2010). Four different methods to evaluate such capacity were tested by those authors: reducing power, scavenging activity on DPPH radicals, inhibition of ß-carotene bleaching and inhibition of lipid peroxidation in brain tissue (TBARS). Low EC_{50} values (extract concentration providing 0.5 of absorbance, for the reducing power assay and extract concentration providing 50% antioxidant activity, in the remaining three methods) where achieved four all the tested methods, (447.92±0.81, 410.80±0.93, 774.99±0.86 and 94.27±1.21 μg/mL, respectively), especially for the TBARS inhibition method.

Heinrich (2005) tested several antioxidant and enzyme inhibition properties when studying 127 consumed wild or semi-wild plants that includes *A. unedo* fruits. Fruits presented high antioxidant activity on the DPPH assay (over 50% of the control) and medium protective ability against hydrogen peroxide-induced DNA damage (between 25% to 50% of the control). On the enzyme inhibition tests, high activity (> 75% of control) was achieved on G-OH assay (protective ability against hydrogen peroxide-induced DNA damage) and medium activity on the test of the xanthine oxidase. The third known report about the antioxidant ability of strawberry-tree fruits was performe by Fortalezas *et al*. (2010), who accessed the antioxidant activity using the oxygen radical absorbance capacity (ORAC) method. Their result showed a slightly lower activity (11.66 ± 2.01 mmol Trolox Equivalents by 100 g of dry weight) than raspberry

extracts (15.37 ± 2.73 mmol Trolox Equivalents by 100 g of dry weight), used in that study as a control sample.

The work of Tavares *et al*. (2010) tested extracts of fruits, using only the ORAC method. The results obtained showed a more effective ability to counteract oxidative processes than extracts of blackberry, which is considered to be highly effective as an antioxidant source.

The entire shrub as also been subject of a study regarding its antioxidant ability. In that work, Andrade *et al*. (2009) quantified the total phenolic content (mg Gallic Acid Equivalents/g plant extract) and flavonoid content (mg Quercetin Equivalents/g plant extract), as well as the ability to scavenge DPPH radical, testing two different extraction methods (ethanol and acetone). The results showed a high amount of both phenolics (255.19±7.12, in the ethanolic extract and 334.46±31.83 for the acetone extract) and flavonoids (20.50±0.77, in the ethanolic extract and 23.37±0.67 for the acetone extract). The EC_{50} value of those extracts considerably low, especially the one achieved with the ethanolic extraction (7.85 μg/mL), proving the high antioxidant ability of this shrub.

2.3. *ARBUTUS UNEDO* L. EFFECTS ON HUMAN HEALTH

2.3.1. Leaves

Associated to leaves are some traditional uses, against some diseases, as mentioned before. There are several potential benefits to human health of *A. unedo* leaves, such as antioxidant activity, as described before, vasorelaxant effect, improvement of cardiovascular health, treatment or prevention of inflammatory diseases, among others. Some reports are available about specific effects of extracts of these leaves, which prove the rationality of the folk medicine. These works studied especially the vasorelaxant and anti-inflammatory properties of leaves' extracts. One example is the work of Legssyer *et al*. (2004), who tested extracts of leaves of *A. unedo*, using different solvents, to evaluate the vasorelaxant effect of those extracts on Male Wistar rats' aortic ring. The results of this work, proved that the leaves possessed a strong vasorelaxant activity, and that this activity is mainly due to the presence of oligomeric condensed tannins and catechin gallate.

Other works are available on the effects of the leaves, specially related to cardiovascular and inflammatory diseases. The findings of those works indicate that the

extracts of leaves are able to decrease the platelet hyperaggregability, which is an important factor in the pathogenesis of inflammatory diseases. Mekhfi et al. (2006) showed that extracts of *A. unedo* leaves were able to inhibit *in-vitro* thrombin-induced platelet aggregation, being this activity probably related with the presence of tannins in the leaves. This was further confirmed, and the action of the tested extracts was mainly due to the attenuation of the mobilization of $Ca2^{+}$ ions, decrease on the reactive oxygen species production and protein tyrosine phosphorylation, proving that extracts of this leaves may be used to treat or prevent inflammatory and cardiovascular diseases (El Haouari *et al*., 2007). By other hand, Mariotto *et al*. (2008) also proved the anti-inflammatory effects of leaves from strawberry-tree. Their results showed that the leaves are able to down-regulate one of the initial factors of the inflammatory process on inflamated lungs of mice, member of transcription factors, signal transducers and activators of transcription family (STAT's), and leading to an decrease of all the parameters associated to inflammation, therefore proving to be one promising source of compounds able to reduce lung inflammation. Furthermore, there is one know report about the antimicrobial activity of extracts of the leaves of *A. unedo*. Kivcak *et al*. (2001b) tested ethanolic extracts against Gram positive, Gram negative (*Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis*, *Salmonella thyphimurium*, *Enterobacter cloacae* and *Enterococcus faecalis*), as well as against yeasts (*Candida albicans*). The tested extracts proved to possess ability to inhibit the growth of all the tested bacteria.

By other hand, the activity of the leaves in the inhibition of enzymes linked to the appearing of several diseases, like rheumatoid arthritis, tumour cell invasion and metastasis (Tavares *et al*., 2010), and linked this activity to the presence of polyphenolic compounds in the tested extracts, namely gallic acid and its derivatives.

2.3.2. Fruits

As referred before, several health-promoting attributes are attributed to the fruits of *A. unedo*, and exploited in folk medicine. However, as far as we know, only one report studied such attributes. Heinrich (2005) studied extracts of these fruits on different parameters, such as enzyme inhibition tests (inhibition of xanthine oxidase, inhibition of myeloperoxidase-catalysed guaiacol oxidation, inhibition of acetylcholine esterase), inhibition of cytokine-induced cell activation (including the extracts' potential cytotoxicity), assays measuring the anti-proliferation and anti-diabetic activity and one assay investigating the extracts' effect on mood disorder-related biochemical

parameters. From all the assays carried out, the fruits present low activity in the anti-diabetic, mood disorder-related and anti-inflammatory assays. However, in the test of the anti-proliferative effect of the ethanolic extracts of the fruits showed a medium activity, when compared to the control, proving to posses some ability to inhibit DNA synthesis and cellular proliferation. Furthermore, some reports are available about the traditional uses of those berries, for different diseases. Those reports concern traditional uses given to *A. unedo* fruits, in different regions, and include a number of diverse health-promoting features to fruits. Furthermore, some reports are available about the traditional uses of those berries, for different diseases in several Mediterranean regions like Italy, Morocco and Turkey. That includes the use of fruits in the treatment of gastrointestinal disorders, dermatologic and urological problems and for cardio-vascular application (Leonti *et al*., 2009), the ability of raw fruits to reduce kidney diseases (El-Hilaly *et al*., 2003) and against gastritis (Cornara *et al*., 2009).

Other parts of this plant are also used in folk medicine. The roots are used for the treatement of several illnesses, which include abdominal pain, renal antispasmodic, bladder ailments, as an antihypercholesterolaemic or as a blood depurative (Novais *et al*., 2004), and for the treatment of diabetes, hypertension and cardiac disease (Jouad *et al*., 2001), as diuretic, astringent, anti-inflammatory and antidiarrheal against blennorrhagia (Ziyyat *et al*., 1997). Furthermore, Leonti *et al*. (2009) also found that *A. unedo* bark is used for the treatment of gastrointestinal disorders, dermatologic and urological problems and for cardio-vascular application.

Different studies sustain the traditional use of the roots in the treatment of hypertension. Ziyyat and Boussairi (1998), Ziyyat *et al*. (2002) and Afkir *et al*. (2008) studies all presented results proving the vasodilator effect of the extract of roots, on rats, regressing the development of hypertension. Furthermore, Mekhfi *et al*. (2004) proved the anti-aggregant properties of decocted roots, support the preventive and/or therapeutic properties linked to cardiovascular diseases and decreasing the risk of thrombosis.

In another study, Carcache-Blanco *et al*. (2006) proved that a compound present and isolated from the entire plant cause inhibition of carcinogenesis, using to different methods, (JB6 murine epidermal cell line assay and inhibition of the cyclooxygenase-2 (COX-2) enzyme), assays that represent major mechanisms of protection against tumour promotion.

As in the leaves, the ability of the fruits to inhibit enzymes associated to rheumatoid arthritis, tumour cell invasion and metastasis was also tested. In the same work, Tavares *et al*. (2010) also showed the capability that fruits present to deter such enzymes.

2.3.3. Antioxidants mechanisms of action

There are three major mechanism believed to be the way through which the antioxidants play protective role against oxidation agents: H-atom transfer, single electron transfer and metal chelation. The first mechanism involves the transfer of a hydrogen atom to the free radical (FR) (Equation 1). Therefore, this free-radical becomes harmless, while the antioxidant (ANTIOX), although becoming itself oxidized, it is also undamaging.

The second mechanism is called single electron transfer (Equation 2). It implies the donation of an electron from the antioxidant agent to the free radical. The free-radical anion is energetically stable with an even number of electrons, while the cation radical Antioxidant^{+} is also a less reactive radical species.

$$ANTIOX - OH + FR \cdot \rightarrow ANTIOX - O \cdot + FR - H \qquad \textit{Equation 1}$$

$$ANTIOX - OH + FR \cdot \rightarrow ANTIOX - OH^{-} + FR^{+} \qquad \textit{Equation 2}$$

The third mechanism, known as Transition Metals Chelation, is explained by the possibility that antioxidant may chelate transition metal (M^{n+}), leading to the formation of stable complexed compounds (Equation 3). This chelation prevents the metals from taking part in the reactions that may lead to the formation of free-radical, many of them considered the most dangerous ones.

$$ANTIOX + M^{n+} \rightarrow ANTIOX - M^{n+} \qquad \textit{Equation 3}$$

The antioxidant activity of flavonoids, and therefore, of a large part of the antioxidant present in *A. unedo*, have been related to both their reducing ability, as well as to their metal chelating properties (Gonçalves *et al*., 2005). By other hand, carotenoids are able to scavenge free-radicals by two different mechanisms: the above-mentioned electron transfer, and by another mechanism, known as the radical addition process, where the carotenoid molecule incorporates in its structure the free-radical

(Mortensen *et al*., 1997). Phenolics acids are able to act as antioxidant by two of the three described mechanism: H-atom transfer and single electron transfer. However, it appears that these compounds are more active as antioxidants when using the first mechanism (Leopoldini *et al*., 2004). The vitamins present in *A. unedo* are reducing agents that counteract free radicals using the three mechanisms described above (Pinchuk and Lichtenberg, 2002). Together they form one of the most efficient synergistic interactions among antioxidants. This interaction is especially important especially against lipid peroxidation of low density lipoproteins (LDL), plasma, and whole blood in vitro. As vitamin E is oxidized by a free-radical, the subsequent vitamin-E radical formed may be implicated in the initiation of another chain oxidation process by attacking polyunsaturated lipids. If vitamin C is present, it reduces vitamin E radical to vitamin E, prior to lipid oxidation caused by the vitamin E radical. Moreover, it has been proved that the simultaneous presence of those two vitamins can completely inhibit the oxidation of LDL and plasma lipids (Niki, 2010). The organic acids mechanism of action against antioxidant is believed to be inhibition of the radical reaction in the oxidation processes (Kikuzaki *et al*., 2002). As *A. unedo* presents in its composition a large number of different antioxidants, it could be and indicative that extracts of this plant may exert their antioxidant activity through one or more of the referred mechanisms.

2.4. CONCLUSION

Almost all parts of the *A. unedo* plant are used in the traditional medicine, in a different variety of forms and in several countries. Results achieved with extracts of several parts of the plant, both in animal and human cells support this traditional use, namely in the treatment of hypertension, cardiovascular diseases and diabetes. Those results open new possibilities for the use of this shrub, especially for the development of new drug to treat those illnesses, as well as in the food industry, due to their antioxidant ability. The beneficial effects associated to this tree are linked to polyphenolic compounds present in its composition. Further studies are needed to evaluate possible interactions between the intake of *A. unedo* and of other dietary constituents, the necessary dose of *A. unedo* ingestion that proves to be beneficial to humans, as well as to assess the bioavailability of those health-promoting compounds in humans. The *in vivo* antioxidant capacity, as well the safety of such compounds should also be examined.

2.5. REFERENCES

Afkir, S. - Nguelefack, T. B. - Aziz, M. - Zoheir, J. - Cuisinaud, G. - Bnouham, M. - Mekhfi, H. - Legssyer, A. - Lahlou, S. – Ziyyat, A.: Arbutus unedo prevents cardiovascular and morphological alterations in L-NAME-induced hypertensive rats. Part I:Cardiovascular and renal hemodynamic effects of Arbutus unedo in L-NAME induced hypertensive rats. Journal of Ethnopharmacology, 116, 2008, pp. 288–295.

Alarcão-E-Silva M. - Leitão A.E.B. - Azinheira H.G. - Leitão M.C.A.: The Arbutus Berry: Studies on its Color and Chemical Characteristics at Two Mature Stages. Journal of Food Composition and Analysis, 14, 2001, pp. 27-35.

Andrade, D. - Gil, C. - Breitenfeld, L. - Domingues, F. - Duarte, A.: Bioactive extracts from Cistus ladanifer and Arbutus unedo L. Industrial Crops and Products, 30, 2009, pp. 165-167.

Aruoma, O.: Assessment of potential prooxidant and antioxidant actions. Journal of the American Oil Chemists' Society, 73, 1996, pp. 1617-1625.

Ayaz, F. – Kucukislamoglu, M. - Reunanen., M.: Sugar, Non-volatile and phenolic acids composition of strawberry tree (Arbutus unedo L. var. ellipsoidea) fruits. Journal of Agricultural and Food Chemistry, 13, 2000, pp. 171-177.

Barros, L. - Carvalho, A. - Morais, J. - Ferreira, I.: Strawberry-tree, blackthorn and rose fruits: Detailed characterization in nutrients and phytochemicals with antioxidant properties. Food Chemistry, 120, 2010, pp.247-254.

Bianchi, F. - Careri, M. - Musci, M.: Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (Arbutus unedo L.) honey: Characterization of aroma compounds by dynamic headspace extraction and gas chromatography–mass spectrometry. Food Chemistry, 89, 2005, pp. 527-532.

Carcache-Blanco, E. - Cuendet, M. - Park, E.J. - Su, B.N. - Rivero-Cruz, J.F. - Farnsworth, N.R. - Pezzuto, J.M. - Douglas Kinghorn, A.: Potential cancer chemopreventive agents from Arbutus unedo. Natural Product Research, 20, 2006, pp. 327–334.

Celikel, G. - Demirsoy, L. - Demirsoy, H.: The strawberry tree (Arbutus unedo L.) selection in Turkey. Scientia Horticulturae, 118, 2008, pp. 115–119.

Cornara, L. - La Rocca, A. - Marsili, S. - Mariotti, M.: Traditional uses of plants in the Eastern Riviera (Liguria, Italy). Journal of Ethnopharmacology, 125, 2009, pp. 16-30.

de la Fuente, E. - Sanz, M. - Martínez-Castro, I. - Sanz, J. - Ruiz-Matute, A.: Volatile and carbohydrate composition of rare unifloral honeys from Spain. Food Chemistry, 105, 2007, pp. 84-93.

El Haouari, M. - López, J. - Mekhfi, H. - Rosado, J. - Salido, G.: Antiaggregant effects of Arbutus unedo extracts in human platelets. Journal of Ethnopharmacology, 113, 2007, pp. 325-331

El-Hilaly, J. - Hmammouchi, M. - Lyoussi, B.: Ethnobotanical studies and economic evaluation of medicinal plants in Taounate province (Northern Morocco). Journal of Ethnopharmacology, 86, 2003, pp. 149-158.

Fiorentino, A. - Castaldi, S. - D'Abrosca, B. - Natale, A. - Carfora, A. - Messere, A. - P., Monaco.: Polyphenols from the hydroalcoholic extract of Arbutus unedo living in a monospecific Mediterranean woodland. Biochemical Systematics and Ecology, 35, 2007, pp. 809-811.

Fortalezas, S. - Tavares, L. - Pimpão, R. - Tyagi, M. - Pontes, V. - Alves, P. - McDougall, G. - Stewart, D. - Ferreira, R. - Santos, C.: Antioxidant properties and neuroprotective capacity of strawberry tree fruit (Arbutus unedo). Nutrients, 2, 2010, pp.14-229.

Gaspar, E. - Neves, H. - Noronha, J.: Application of HPLC-PBMS to the identification of unknown components in a triterpenoid fraction of Arbutus unedo fruits. Journal of High Resolution Chromatography, 20, 1997, pp. 417-420.

Gomes, F. - Canhoto, J.: Micropropagation of strawberry tree (Arbutus unedo L.) from adult plants. In Vitro Cellular & Developmental Biology - Plant, 45, 2009, pp. 72-82.

Gonçalves, C. - Dinis, T. - Batista, M.: Antioxidant properties of proanthocyanidins of Uncaria tomentosa bark decoction: a mechanism for anti-inflammatory activity. Phytochemistry, 66, 2005, pp. 89-98.

Heinrich, M.: Understanding local Mediterranean diets: A multidisciplinary pharmacological and ethnobotanical approach. Pharmacological Research, 52, 2005, pp.353-366.

Jouad, H. - Haloui, M. - Rhiouani, H. - El Hilaly, J. - Eddouks, M.: Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the North centre region of Morocco (Fez–Boulemane). Journal of Ethnopharmacology, 77, 2001, pp. 175-182.

Kahriman, N. - Albay, C. - Dogan, N. - Usta, A. - Karaoglu, S. - Yayli, N.: Volatile constituents and antimicrobial activities from flower and fruit of Arbutus unedo L. Asian Journal of Chemistry, 22, 2010, pp. 6437-6442.

Kikuzaki, H. - Hisamoto, M. - Hirose, K. - Akiama, K. - Taniguchi, H.: Antioxidant properties of ferulic acid and its related compounds. Journal of Agricultural and Food Chemistry, 50, 2002, 2161-2168.

Kivcak, B. - Mert, T. - Denizci, A.: Antimicrobial activity of Arbutus unedo L. FABAD Journal of Pharmaceutical Sciences, 26, 2001, pp. 125-128.

Kivçak, B. - Mert, T.: Quantitative determination of α-tocopherol in Arbutus unedo by TLC-densitometry and colorimetry. Fitoterapia. 72, 2001, pp. 656-661.

Kivcak, B. - Mert,T. - Demirci, B. - Baser, K.: Composition of the essential oil of Arbutus unedo. Chemistry of Natural Compounds, 37, 2001, pp. 445-446.

Legssyer, A. - Ziyyat, A. - Mekhfi, H. - Bnouham, M. - Herrenknecht, C. - Roumy, V. - Fourneau, C. - Laurens, A. - Hoerter, J. - Fischmeister, R.: Tannins and catechin gallate mediate the vasorelaxant effect of Arbutus unedo on the rat isolated aorta. Phytotherapy Research, 18, 2004, pp. 889-894.

Leonti, M. - Casu, L. - Sanna, F. - Bonsignore, L.: A comparison of medicinal plant use in Sardinia and Sicily—De Materia Medica revisited? Journal of Ethnopharmacology, 121, 2009, pp. 255-267.

Leopoldini, M. - Marino, T. - Russo, N. - Toscano, M.: Antioxidant properties of phenolic compounds: H-atom versus electron transfer mechanism. The Journal of Physical Chemistry A, 108, 2010, pp. 4916-4922.

Liu, F. - Ng, T. B.: Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sciences, 66, 2000, pp. 725-735.

LLusià, J. - Peñuelas, J.: Seasonal Patterns of terpene content and emission from seven Mediterranean woody species in field conditions. American Journal of Botany, 87, 2000, pp. 133-140.

Males, Z. - Plazibat, M. - Vundac, V.B. - Zunta, I.: Qualitative and quantitative analysis of flavonoids of the strawberry tree – Arbutus unedo L. (Ericaceae). Acta Pharmaceutica, 56, 2006, pp. 245-250.

Mariotto, S. - Esposito, E. - Di Paola, R. - Ciampa, A. - Mazzon, E. - Carcereri de Prati, A. - Darra, E. - Vincenzo, S. - Cucinotta, G. - Caminiti, R. - Suzuki, H. - Cuzzocrea, S.: Protective effect of Arbutus unedo aqueous extract in carrageenan-induced lung inflammation in mice. Pharmacological Research, 57, 2008, pp. 110–124.

Mazza, G. - Miniati, E.: Anthocyanins in fruits, vegetables and grains. Boca Raton : CRC Press, 1993. 362 pp. ISBN 0849301726.

Mekhfi, H. - El Haouari, M. - Bnouham, M. - Aziz, M. - Ziyyat, A. - Legssyer, A.: Effects of extracts and tannins from Arbutus unedo Leaves on rat platelet aggregation. Phytotherapy Research, 20, 2006, pp.135-139.

Mekhfi, H. - El Haouari, M. - Legssyer, A. - Bnouham, M. - Aziz, M. - Atmani, F. - Remmal, A. - Ziyyat, A.: Platelet anti-aggregant property of some Moroccan medicinal plants. Journal of Ethnopharmacology, 94, 2004, pp. 317–322

Moreno-Jiménez, E. - Peñalosa, J. - Carpena-Ruiz, R. - Esteban, E.: Comparison of arsenic resistance in Mediterranean woody shrubs used in restoration activities. Chemosphere, 71, 2008, pp. 466-473.

Mortensen, A. - Skibsted, L. - Sampson, J. - Rice-Evans, C. - Everett, S.: Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. FEBS Letters, 418, 1997, pp. 91-97.

Niki, E.: Assessment of antioxidant capacity in vitro and in vivo. Free Radical Biology & Medicine, 49, 2010, pp. 503-515.

Novais, M. - Santos, I. - Mendesa, S. - Pinto-Gomes, C.: Studies on pharmaceutical ethnobotany in Arrabida Natural Park (Portugal). Journal of Ethnopharmacology, 93, 2004, pp. 183-195.

Oliveira, I. – Baptista, P. – Malheiro, R. – Casal, S. – Bento, A. – Pereira, J.: Influence of strawberry tree (Arbutus unedo L.) fruit ripening stage on chemical composition and antioxidant activity. Food Research International, in press, DOI: 10.1016/j.foodres.2011.02.009.

Oliveira, I. - Coelho, V. - Baltasar, R. - Pereira, J. - Baptista, P.: Scavenging capacity of strawberry tree (Arbutus unedo L.) leaves on free radicals. Food and Chemical Toxicology, 47, 2009, pp. 1507-1511.

Oliveira, I. – Pinho, P.- Malheiro, R. –Baptista, P. – Pereira, J.: Volatile profile of Arbutus unedo L. fruits trough ripening stage. Food Chemistry, in press.

Owen, S. - Boissard, C. - Street, R. - Duckham, S. - Csiky, O. - Hewitt, C.: Screening of 18 Mediterranean plant species for volatile organic compound emissions. Atmospheric Environment, 31, 1997, pp. 101-117.

Özcan, M. - Hacıseferoğulları, H.: The Strawberry (Arbutus unedo L.) fruits: Chemical composition, physical properties and mineral contents. Journal of Food Engineering, 78, 2007, pp. 1022-1028.

Pabuçcuoğlu A. - Kıvçak, B. - Baş, M. - Mert, T.: Antioxidant activity of Arbutus unedo leaves. Fitoterapia, 74, 2003, pp. 597-599.

Pallauf, K. - Rivas-Gonzalo, J.C. - Castillo, M.D. - Cano, M.P. - Pascual-Teresa, S.: Characterization of the antioxidant composition of strawberry tree (Arbutus unedo L.) fruits. Journal of Food Composition and Analysis, 21, 2008, pp. 273-281.

Pascual-Teresa, S. - Santos-Buelga, C. - Rivas-Gonzalo, J.: Quantitative analysis of flavan-3-ols in Spanish foodstuffs and beverages. Journal of Agricultural and Food Chemistry, 48, 2000, pp.5331-5337.

Pawlowska, A. - Leo, M. - Braca, A.: Phenolics of Arbutus unedo L. (Ericaceae) fruits: Identification of anthocyanins and gallic acid derivatives. Journal of Agricultural and Food Chemistry, 54, 2006, pp.10234-10238.

Peñuelas, J. - Llusià, J.: Seasonal patterns of non-terpenoid C6-C10 VOC emission from seven Mediterranean woody species. Chemosphere, 45, 2001, pp. 237-244.

Pinchuk, I. - Lichtenberg, D.: The mechanism of action of antioxidants against lipoprotein peroxidation, evaluation based on kinetic experiments. Progress in Lipid Research, 41, 2002, pp. 279-314.

Sanjust, E. - Mocci, G. - Zucca, P. - Rescigno, A.: Mediterranean shrubs as potential antioxidant sources. Natural Product Research, 22, 2008, pp. 689-708.

Soufleros, E. - Mygdalia, S. - Natskoulis, P.: Production process and characterization of the traditional Greek fruit distillate ‘‘Koumaro’’ by aromatic and mineral composition. Journal of Food Composition and Analysis, 18, 2005, pp. 699-716.

Spano N. – Piras I. – Ciulu M.: Reversed-phase liquid chromatographic profile of free amino acids in strawberry-tree (Arbutus unedo L.) honey. Food Composition and Additives, 92, 2009, pp. S73-S83.

Spano, N. - Piras, I. - Ciulu, M. - Floris, I. - Panzanelli, A. - Pilo, M. - Piu, P. - Sanna, G.: Reversed-phase liquid chromatographic profile of free amino acids in strawberry-tree (Arbutus unedo L.) honey. Journal of AOAC International, 92, 2009, pp. 1145-1152.

Torres, J. - Valle, F. - Pinto, C. - García-Fuentes, A. - Salazar, C. - Cano, E.: Arbutus unedo L. communities in southern Iberian Peninsula mountains. Plant Ecology, 160, 2002, pp. 207-223.

Ziyyat, A. - Boussairi, E.: Cardiovascular effects of Arbutus unedo L. in spontaneously hypertensive rats. Phytotherapy Research, 12, 1998, pp. 110–113.

Ziyyat, A. - Legssyer, A. - Mekhfi, A. - Dassouli, A. - Serhrouchni, M. - Benjelloun, W.: Phytotherapy of hypertension and diabetes in oriental Morocco. Journal of Ethnopharmacology, 58, 1997, pp. 45–54.

Capítulo 3
Scavenging Capacity of Strawberry Tree (*Arbutus unedo* L.) Leaves on Free Radicals

Scavenging Capacity of Strawberry Tree (*Arbutus unedo* L.) Leaves on Free Radicals

Food and Chemical Toxicology, **47** (2009), 1507-1511

ABSTRACT

Despite strawberry tree (*Arbutus unedo* L.) leaves had a long use in traditional medicine due to its antiseptic, diuretic, astringent and depurative properties, the potential of their antioxidant activity are still lacking. Our study goals to assess the antioxidant and free radical scavenging potential of water, ethanol, methanol and diethyl ether extracts of *A. unedo* leaves. Total phenols content was achieved spectrophotometrically using Folin-Ciocalteu reagent with gallic acid as standard. Antioxidant activity was evaluated using three different methods: reducing power of iron (III)/ ferricyanide complex assay, scavenging effect on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals and scavenging effect on superoxide radicals by using the PMS-NADH-nitroblue tetrazolium system. Ethanol extracts of *A. unedo* leaves were the highest in reducing power (IC_{50} 232.7 µg/mL) and DPPH scavenging effect (IC_{50} 63.2 µg/mL) followed by water extracts (with IC_{50} of 287.7µg/mL and 73.7µg/mL, respectively); whereas diethyl ether extracts were the lowest. In the scavenging on superoxide radical assay, methanol extracts obtained the best results (IC_{50} 6.9µg/mL). For all the methods tested the antioxidant activity was concentration dependent. In accordance with antioxidant activity, highest total phenols content were found in ethanol, followed by water, methanol and diethyl ether extract. The results indicated that *A. unedo* leaves are a potential source of natural antioxidants.

KEY-WORDS: *Arbutus unedo* L. leaves; extraction method; total phenols; antioxidant activity; superoxide scavenging.

3.1. INTRODUCTION

The importance of oxygen-derived free radicals, commonly named reactive oxygen species (ROS), in health and disease is now recognized by every branch of medicine and biological science. ROS are chemically reactive molecules that are derived from the successive reduction of molecular oxygen to H_2O. They included free radicals, such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($HO^{\bullet}$), and non-free-radical species, such as H_2O_2. Singlet oxygen ($^{-}O_2$) species are also forms of activated oxygen, among others (Aruoma, 1996; Gülcin, 2007). The excessively produced ROS can injure cellular biomolecules such as proteins, carbohydrates, nucleic acids and lipids causing cellular and tissue damage (Aruoma, 1996; Pulido *et al*., 2000). Overwhelming evidence indicates that ROS play a role in most major health problems of the industrialized world, including cardiovascular diseases, cancer, diabetes, neurological diseases, and atherosclerosis and are believed to be a major factor in aging (Finkel and Holbrook, 2000).

In recent years, the increasing interest around 'natural' products has encouraged the scientific community to obtain information about natural plant antioxidants and its importance in medicine, human nutrition and food industry (Liu and Ng, 2000; Wang, 2006). It has been demonstrated that plants contain many natural antioxidants compounds such as carotenoids, vitamins, phenols, flavonoids, dietary glutathionine, and endogenous metabolites (Larson, 1988); which have been identified as a free radical or active oxygen scavengers (Zheng and Wang, 2001). Therefore, an appropriate dose of antioxidants derived from plants in the human diet can help to avoid the risk of contracting diseases where ROS are involved in its pathogenesis. Taking the traditional application form of *Arbutus unedo* L. into consideration, phytochemicals contents and in particular their antioxidant activity, are of considerable interested to investigated from the point of view of its use as a potential therapeutic agent against a wide range of human disease. Also, provide new scientific information for the further development of modern herbal medicines.

A. unedo, the strawberry tree, belongs to the Ericaceae family, and it's native of the Mediterranean climate (Celikel *et al*., 2008). In Portugal, the strawberry tree is mainly implanted in the south, being however present throughout all of the country in a dispersed way (Pedro, 1994). This species have been traditionally used as food, by using the arbutus berries in the production of alcoholic beverages, jams, jellies and

marmalades (Alarcão-e-Silva *et al*., 2001; Pallauf *et al*., 2008); and as phytopharmaceuticals. For example, the fruits are well known in folk medicine as antiseptic, diuretic, and laxative, while the leaves are used as astringent, diuretic, urinary anti-septic, antidiarrheal, depurative and more recently in the therapy of hypertension, diabetes and in the treatment of inflammatory diseases (Ziyyat *et al*., 1997; Ziyyat and Boussairi, 1998; Mariotto *et al*., 2008; Afkir *et al*., 2008). Indeed, phytochemical studies showed that leaf extracts contains several phenolic compounds, like tannins, flavonoids, phenolic glycosides, among others (Males *et al*., 2006; Fiorentino *et al*., 2007), as well as α-tocopherol (Kivçak and Mert, 2001). The composition of the berries is relatively well-known when compared to leaves. They contain several antioxidant molecules namely phenolic compounds (e.g. anthocyanins, gallic acid derivatives, tannins and flavonoids), vitamin C, vitamin E and carotenoids (Ayaz *et al*., 2000; Alarcão-e-Silva *et al*., 2001; Pawlowska *et al*., 2006; Males *et al*., 2006; Pallauf *et al*., 2008).

Based on their antioxidant composition, and while a detailed study is still lacking, we could reasonably anticipate a high-antioxidant activity of both berries and leaves of *A. unedo* (Wang and Lin, 2000). In a preliminary study, Pabuçcuoğlu *et al*. (2003) verified the antioxidant activity of *A. unedo* leaves ethanol and methanol extracts by $ABTS^{\bullet+}$ [2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] method.

Therefore, the aim of this work is to allow a better knowledge of the antioxidant capacity of leaves from *A. unedo*. For this purpose, total phenols content, reducing power and radical scavenging activity [DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and $O_2^{\bullet-}$ scavenging] of leaves extracts were evaluated. The effect of the solvent polarity, used in the extracting procedure, on the yields and antioxidant activity has also discussed. The solvent systems tested included water, absolute methanol, ethanol and diethyl ether. It is expected from this study to assess the potentially of *A. unedo* leaves as a source of natural antioxidant for pharmaceutical and food application.

3.2. MATERIALS AND METHODS

3.2.1. Samples

The leaves of *Arbutus unedo* L. were collected in January of 2009, in the Natural Park of Montesinho (Bragança, northeastern region of Portugal). The samples were immediately frozen and freezed dried (Ly-8-FM-ULE, Snijders) prior to extraction.

3.2.2 Extract preparation

Three powdered sub samples (~ 5 g; 20 mesh) were extracted with 250 mL of boiling water for 45 min and filtered through Whatman nº 4 paper. The extracts (water extracts - WE) were then evaporated under vacuum (rotary evaporator Büchi R-210), and dissolved in phosphate buffer (KH_2PO_4, pH 7.4) to a final concentration of 50mg/mL.

For ethanolic (EE), methanolic (ME) and diethyl ether extraction (DEE), 1.5g of lyophilized leaves was extracted three times with 25mL of the tested solvents and filtered through Whatman nº 4 paper. The extracts were then evaporated under vacuum (rotary evaporator Büchi R-210), dissolved in phosphate buffer (KH_2PO_4, pH 7.4) to a final concentration of 50mg/mL and stored in the dark at 4ºC for further use.

3.2.3. Antioxidant Activity

3.2.3.1. Reagents.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was obtained from Fluka. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Methanol and n-Hexane were obtained from Panreac (Spain). Diethyl ether and 96 % ethanol were purchased from Riedel-de Haën. Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

3.2.3.2. Total phenols.

Total phenols quantifications were achieved according to Singleton and Rossi (1965), with minor modification. Thus, 1mL of the extract solution was mixed with 1mL of Folin-Ciocalteu's phenol reagent. The mixture was shaken vigorously and left to stand for 3 min. After that, 1mL of a saturated solution of sodium carbonate was added and the total volume was adjusted to 10 mL with distilled water. The reaction

was kept in the dark for 90 minutes, after what the absorbance was read at 725 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. Galic acid was used as standard, being the results expressed in mg of gallic acid equivalents (GAE)/g of extract.

3.2.3.3. Reducing power assay.

The reducing power was determined according to a described procedure (Berker *et al*., 2007). Various concentrations of sample extracts (1 mL) were mixed with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min. After incubation 2.5 mL of 10% tricloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge (Centorion K24OR- 2003), for 8 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against the correspondent extract concentration.

3.2.3.4. Scavenging effect on DPPH radicals.

The capacity to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a method reported before (Oyaizu, 1986). Various concentrations of sample extracts (0.3 mL) were mixed with 2.7 mL of methanolic solution containing DPPH radicals ($6x10^{-5}$ mol/L). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH-AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract has been added at a particular level and ADPPH is the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against extract concentration.

3.2.3.5. Scavenging effect on superoxide radicals.

The superoxide radical was determined by the PMS-NADH generating system, as described by Fernandes *et al*., (1999) with minor modifications. Briefly, 150µL of the extract solution were mixed with 150µL of NADH (166µM), 450µL of NBT (86µM) and 150µL of PMS (16.2µM) (final concentrations in 900µL; all the components of the mixture were dissolved in phosphate buffer, KH_2PO_4, 19 mM, pH 7.4). The changes of absorbance at 560 nm were recorded during 3 min at 560 nm in a PG Instruments Ltd. T70 UV/VIS spectrometer, and the data acquisition was achieved in UV-WIN5 software V 5.0.5. Data was expressed as ΔA_{560} nm/min. The scavenging activity on superoxide radicals was calculated as follows: ((ΔA_{560} nm/minblank - ΔA_{560} nm/minsample) / ΔA_{560} nm/minblank) x 100%. EC_{50} stands for the concentration of half-inhibition.

3.2.4. Statistical analysis

For each extraction solvent, six independent extractions were performed and in each extraction all assays were carried out in duplicate. Results are shown as solvent mean values and standard deviation. The differences between solvents in each parameter were analysed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SAS v. 9.1.3 program. The regression analysis between total phenols contents and IC_{50} values for reducing power, scavenging activity on DPPH assay and superoxide radicals was conducted using the same statistical package.

3.3. RESULTS AND DISCUSSION

Antioxidant capacities of plant extracts are largely dependent of the extract composition and conditions of the test system. The measure of antioxidant capacities are influenced by many factors, which cannot accurately and quantitatively described with one single method. To avoid this in the present work the antioxidant properties of *A. unedo* leaves were measured using three different assays: the reducing power assay, the scavenging effect on DPPH radicals and the scavenging effect on superoxide radicals, and four different extraction solvents with different polarities (water, methanol, ethanol and diethyl ether). The extraction yield and total phenols content were also evaluated in order to correlate it with the antioxidant potential.

The obtained yields, as well as the extraction yield for the different tested solvents, are showed in Table 1. The solvent used for *A. unedo* leaves extract preparation showed significant different ($p<0.05$) capacities to extract leaf compounds and probably different composition of the extracts. Extraction yield varied between 2.86% in the diethyl ether extracts (DEE), the lowest, to 32.14 % in the aqueous extracts (WE), the highest.

Table 1. Extraction yield (Mean ± SD) of *Arbutus unedo* leafs extracts. In each column different letters mean significant differences ($p<0.05$).

Extraction method	Yield (g)	Extraction (%; w/w)
WE	1.62 ± 0.13 a	32.14 ± 2.64 a
ME	0.34 ± 0.02 b	22.85 ± 1.01 b
EE	0.23 ± 0.02 c	15.03 ± 0.86 c
DEE	0.04 ± 0.00 d	2.86 ± 0.13 d

It is well-known that plant phenolics, in general, are the highly effective free radical scavengers and antioxidants. The content of total phenols in *A. unedo* leaf extracts was determined spectrometrically using the Folin-Ciocalteau reagent and calculated as Gallic Acid Equivalents (GAE). The highest quantity of total phenols was achieved in ethanolic extracts (192.66±1.66 mg GAE/g extract) and the lowest in diethyl ether extracts (14.93±0.54 mg GAE/g extract) (Table 2).

Table 2. Contents of total phenols (Mean ± SD) in *Arbutus unedo* leaf extracts. In the column different letters mean significant differences ($p<0.05$).

Extraction method	Total phenols (mg GAE/g of extract)
WE	172.21 ± 6.29 a
ME	149.28 ± 5.33 b
EE	192.66 ± 1.66 c
DEE	14.93 ± 0.54 d

In our study we observe that the solvent used in the extraction of leaf samples had a significant effect ($p<0.05$) on the total phenols content of the extracts. The Folin-Ciocalteau reagent was been extensively used to determine the total phenols content of a varied number of matrixes, however some criticisms are appoint to these methodology. The Folin-Ciocalteau also reacts with other non-phenolic reducing compounds such sugars, amino acids and ascorbic acids that are quantified as phenols. To avoid this Costa *et al*. (2009) use the same methodology to determine the total reducing capacity of the extracts. In our work we observed that EE showed the higher total phenols or higher total reducing capacity that are in total accordance with the highest antioxidant activity obtained by the reducing power and DPPH assays. A few works were developed concerning the phenolic characterization of *A. unedo* leaves. Males *et al*. (2006) study the flavonoidic composition of samples collected in Croatia and identify quercitrin, isoquercitrin, hyperoside and rutin. And Craccache-Blanco *et al*. (2006) reports the identification of (-)-catechin in samples collected in Turkey. More recently Fiorentino *et al*. (2007) refers the identification of twelve phenolic compounds in leaves collected in Central Italy, namely arbutin, ethyl gallate, p-hydroxybenzoyl arbutin, galloylarbutin, (+)-gallocatechin, catechin, kaempferol 3- *O*-α-L–ramnopyranoside, quercetin 3-*O*-α-L-ramnopyranoside, myricetin 3- *O*-α-L–ramnopyranoside, kaempferol 3-*O*-β-D–arabinofuranoside, quercetin 3-*O*-β-D–arabinofuranoside, and myricetin 3-*O*-β-D-arabinofuranoside.

In the reducing power assay, the extracts prepared by *A. unedo* leaves displayed a concentration-dependent antioxidant potential, with the exception of DEE extracts (Figure 1). In this assay, the presence of reducers (antioxidants) in the extracts causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form, leading to a color change of the test solution changes from yellow to various shades of green and blue,

depending to the reducing power capacity of each tested extracts. Therefore, Fe^{2+} concentration can be monitorized by measuring the formation of Perl's Prussian blue at 700 nm. Increased absorbance at 700 nm indicates an increase in reducing power. The reducing capacity of a prepared extract, in this case prepared with *A. unedo* leaves, may serve as a significant indicator of its potential antioxidant activity. Accordingly, *A. unedo* leaves contain high antioxidant properties which could react with free radicals to stabilize and terminate radical chain reactions. High values of reducing power at low concentrations, at μg/mL level, were obtained with ethanolic (EE), aqueous (WE) and methanolic (ME) extracts, while the diethyl ether extracts (DEE) presented almost null reactions. Significantly differences (p<0.05) were observed in the IC_{50} (the concentration required to provide 0.5 of absorbance) values calculated for the different extracts (Table 3). EE exhibited the strongest capacity (IC_{50} at 232.7 ± 12.7 μg/mL), while ME was the less active (IC_{50} at 496.8 ± 25.4 μg/mL) (Figure 1 and Table 3). These results reveal that *A. unedo* leaf extracts are an electron donor and could react with free radicals, convert them to more stable products, and determinate radical chain reaction.

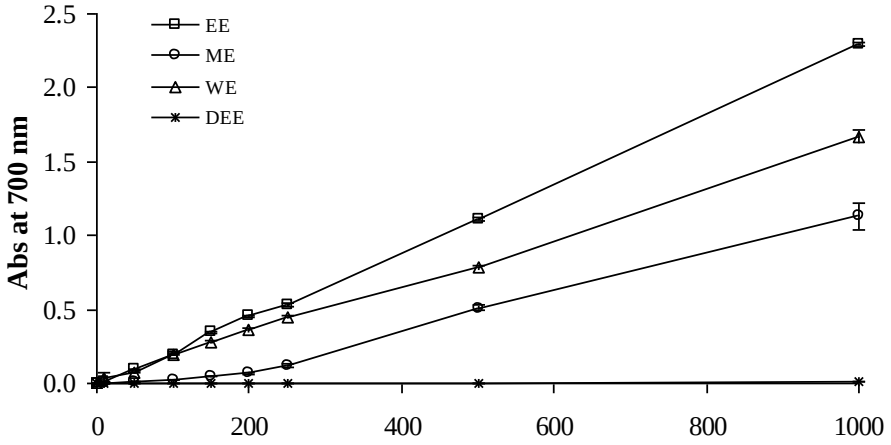


Figure 1. Reducing power values for the extracts obtained with different extraction methods. Each value is expressed as mean ± standard error. (EE – ethanol extraction; ME – methanol extraction; WE – water extraction; DEE - diethyl ether extracts).

The scavenging activity on DPPH radicals assay is extensively used as a basic screening method for testing the antiradical activity of different plant materials such as leaf shots, *calli*, leaves, inflorescences and fruits, among others. DPPH is a stable free radical that possesses a characteristic absorption maximum between 515 and 517 nm, which is diminished in the presence of a compound (i.e. antioxidants) capable of reducing it to its hydrazine form by hydrogen/electron donation. Free radical scavenging

is one of the known mechanisms by which antioxidants inhibit lipid oxidation (Ferreres *et al*., 2007). In this assay, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wavelength. *A. unedo* leaves exhibited strong free radical scavenging activity on DPPH assay at very low concentrations of the aqueous (WE), methanolic (ME) and ethanolic (EE) extracts (Figure 2). For example, at 150 µg/mL of tested extract the scavenging effects was 88.84%, 78.59% and 87.78% for WE, ME and EE respectively. In opposite diethyl ether extracts presented very low activity (Figure 2). As in the reducing power assay, significantly differences ($p<0.05$) were observed in the IC_{50} values calculated for the different extracts on DPPH assay and follows similar behavior, with the lowest value obtained by ethanol extracts (63.2±6.6 µg/mL), followed by water extracts (73.7±6.3 µg/mL) and methanol extracts (99.9±5.9 µg/mL) (Table 3).

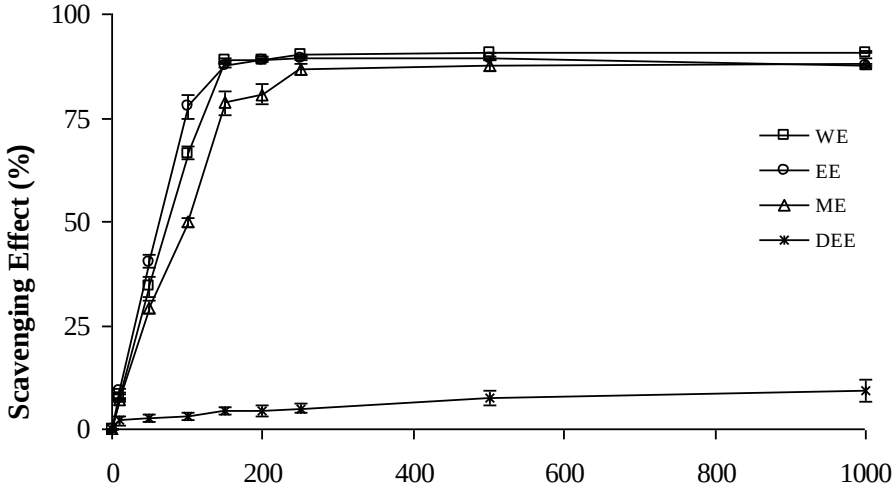


Figure 2. Scavenging activity on DPPH radicals (%) of the extracts obtained with different extraction methods. Each value is expressed as mean ± standard error. (EE – ethanol extraction; ME – methanol extraction; WE – water extraction; DEE - diethyl ether extracts).

Superoxide anion ($O_2^{\bullet-}$), the one-electron reduced form of molecular oxygen, is one of the most representative free radicals. It is a precursor to active free radicals that have the potential of reacting with biological macromolecules and thereby inducing tissue damage (Aruoma, 1996; Pulido *et al*., 2000). In cellular oxidation reactions, superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of cell-damaging free radicals and oxidizing agents (Aruoma, 1996). In the present work, the scavenging effects of *A. unedo* leaves extracts

on superoxide radical was assessed by using the PMS-NADH-nitroblue tetrazolium system (Fernandes *et al*., 1999). In this method, superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitroblue tetrazolium (NBT). The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. In the present work, marked inhibitory effect of *A. unedo* leaves WE, ME and EE on superoxide radicals were in a dose-dependent manner (Figure 3). High inhibitions were observed at very low extract concentrations, as example at 100 µg/mL of tested extract the scavenging effects on superoxide radical were found to be 48.36%, 63.72% and 35.82% for WE, ME and EE respectively (Figure 3).

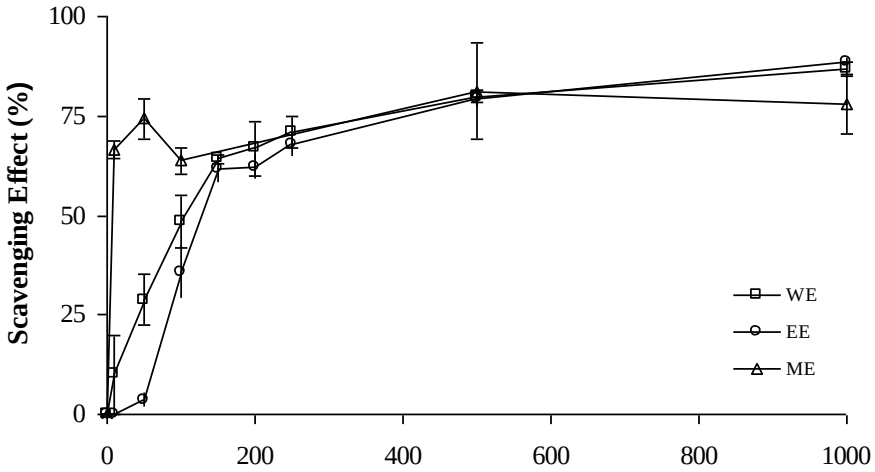


Figure 3. Superoxide anion inhibition (%) of the extracts obtained with different extraction methods. Each value is expressed as mean ± standard error. (EE – ethanol extraction; ME – methanol extraction; WE – water extraction; DEE - diethyl ether extracts)

At very low extract concentrations methanolic extracts posses the strongest scavenging effect, however beyond concentrations of 200 µg/mL similar results was obtained for all extracts. Results for diethyl ether extracts are not present, due to the lack of activity of radical scavenging. Significantly differences ($p<0.05$) were observed for IC_{50} values (Table 3). The lowest IC_{50} value, that corresponds to the highest scavenging activity of superoxide radicals, was observed for methanolic extracts (6.9 ± 0.8 µg/mL) followed by aqueous extracts (100.7 ± 23.8 µg/mL) and the highest values was obtained for ethanolic extracts (125.1 ± 11.1 µg/mL) (Table 3).

Table 3. IC_{50} values (Mean ± SD) obtained in the antioxidant activity assays of *Arbutus unedo* leaf extracts. In each column different letters mean significant differences ($p<0.05$).

	IC_{50} values (µg/mL)		
Extraction method	**Reducing Power**	**DPPH scavenging activity**	**Superoxide anion scavenging activity**
WE	287.7 ± 15.6 a	73.7 ± 6.3 a	100.7 ± 23.8 a
ME	496.8 ± 25.4 b	99.9 ± 5.9 b	6.9 ± 0.8 b
EE	232.7 ± 12.7 c	63.2 ± 6.6 c	125.1 ± 11.1 c
DEE	ND	ND	ND

ND - not done

Different works have reported good linear correlations between antioxidant/antiradical activity tests and Folin–Ciocalteau assay (Kim *et al*., 2008; Oliveira *et al*., 2008; Oliveira *et al*., 2009). In our work if we combine IC_{50} values and total phenols of each sample and each extraction solvent, with the exception of DEE, a significantly negative linear correlation was obtained for reducing power assay (Equation: IC_{50} µg/mL = -5.825*Total Phenols mg GAE/g extract + 1337; determination coefficients 0.853; $p<0.001$). In the same way, for DPPH assay, and independently of the extraction solvent samples with higher total phenols showed the strongest free radical-scavenging effect (lower IC_{50} values), being established a significantly negative linear correlation between the total phenols content and IC_{50} values on DPPH assay (Equation: IC_{50} µg/mL = -0.832*Total Phenols mg EGA /g extract + 221.577; determination coefficients 0.855; $p<0.001$). Contrary for the superoxide assay a significantly positive linear correlation was obtained for total phenols and IC_{50} values (Equation: IC_{50} µg/mL = 2.676*Total Phenols mg GAE/g extract – 381.07; determination coefficients 0.867; $p<0.001$). The apparent contradiction in the results concerning to superoxide assay is not surprising; in our work we use different solvents with different polarities that probably extract different classes of compounds. Various polyphenolic phytochemicals may react with free superoxide radicals in different ways, depending of its chemical structure, and thus lead to different scavenging activities (Chung *et al*., 2003). Those authors have verified that aglycones are more effective on superoxide radical scavenging activity than their glycosides. On the other hand, different plant extracts could present different behaviour according the antioxidant evaluation methodologies. And also in the extracts a synergies (or antagonisms) among the antioxidants in the mixture could occur.

In conclusion, it is well-known that free radicals are one of the causes of several diseases, such as Parkinson's disease, coronary heart disease, and cancer. Our results demonstrated for the first time that *A. unedo* leaves extracts not only exhibited excellent free radical scavenging activities but were also potent in scavenging superoxide radical. The obtained results could be beneficial for the development of herbal extracts from *A. unedo* leaves for pharmaceutical application or food supplements in order to prevent and treat illnesses and improve patients' overall health.

3.4. REFERENCES

Afkir, S., Nguelefack, T. B., Aziz, M., Zoheir, J., Cuisinaud, G., Bnouham, M., Mekhfi, H., Legssyer, A., Lahlou, S., Ziyyat, A., 2008. *Arbutus unedo* prevents cardiovascular and morphological alterations in L-NAME-induced hypertensive rats. Part I: Cardiovascular and renal hemodynamic effects of *Arbutus unedo* in L-NAME-induced hypertensive rats. J. Ethnopharmacol. 116, 288-295.

Alarcão-e-Silva, M., Leitao, A.E.B., Azinheira, H.G., Leitão, M.C.A., 2001. The Arbutus Berry: Studies on its Color and Chemical Characteristics at Two Mature Stages. J. Food Compos. Anal. 14, 27-35.

Aruoma, O., 1996. Assessment of Potential Prooxidant and Antioxidant Actions. J. Am. Oil Chem. Soc. 73, 1617-1625.

Ayaz, F. A., Kucukislamoglu, M., Reunanen, M., 2000. Sugar, Non-volatile and Phenolic Acids Composition of Strawberry Tree (*Arbutus unedo* L. var.ellipsoidea) Fruits. J. Food Compos. Anal. 13, 171-177.

Berker, K., Güçlü, K., Tor, I., Apak, R., 2007. Comparative evaluation of Fe (III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents. Talanta 72, 1157-1165.

Carcache-Blanco, E.J., Cuendet, M., Park, E.J., Su, B.N., Rivero-Cruz, J.F., Farnsworth, N.R., Pezzuto, J.M., Douglas Kinghorn, A., 2006. Potential cancer chemopreventive agents from *Arbutus unedo*. Nat. Prod. Res. 20, 327 -334.

Cavaco, T., Longuinho, C., Quintas, C., Carvalho, I., 2007. Chemical and microbial changes during the natural fermentation of strawberry tree (A*rbutus unedo* L.) fruits. J. Food Biochem. 31, 715-725.

Celikel, G., Demirsoy, L., Demirsoy, H., 2008. The strawberry tree (*Arbutus unedo* L.) selection in Turkey. Scientia horticulturae, 118, 115-119.

Chun, O.K., Kim, D.-O., Lee, C.Y, 2003. Superoxide radical scavenging activity of the major polyphenols in fresh plums. J. Agric. Food Chem. 51, 8067-8072

Costa, R.M., Magalhães, A.S., Pereira, J.A., Andrade, P.B., Valentão, P., Carvalho, M., Silva, B.M., 2009. Evaluation of free radical-scavenging and antihemolytic activities of quince (*Cydonia oblonga*) leaf: A comparative study with green tea (*Camellia sinensis*). Food Chem. Tox. 47, 860-865.

Fernandes, E., Borges, F., Milhazes, N., Carvalho, F. D., Bastos, M. L., 1999. Evaluation of superoxide radical scavenging activity of gallic acid and its alkyl esters using an enzymatic and a non-enzymatic system. Toxicol. Lett. 109, 42.

Ferreres, F., Sousa, C., Valentão, P., Seabra, R.M., Pereira, J.A., Andrade, P.B., 2007. Tronchuda cabbage (*Brassica oleracea* L. var. *costata* DC) seeds: phytochemical characterization and antioxidant potential. Food Chem. 101, 549–558.

Finkel T., Holbrook N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 39-247

Fiorentino, A., Castaldi, S., D`Abrosca, B., Natale, A., Carfora, A., Messere, A., Monaco, P., 2007. Polyphenols from the hydroalcoholic extract of *Arbutus unedo* living in a monospecific Mediterranean woodland. Biochem. Syst. Ecol. 35, 809-811.

Fridovich, I., 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? Ann.NY Acad.Sci. 893, 13-18.

Gülcin, I., 2007. Comparison of in vitro antioxidant and antiradical activities of L-tyrosine and L-Dopa. Amino Acids 32, 431-438.

Kim, M.Y., Seguin, P., Ahn, J.K., Kim, J.J., Chun, S.C., Kim, E.H., Seo, S.H., Kang, E.Y., Kim, S.L., Park, Y.J., Ro, H.M., Chung, I.M., 2008. Phenolic compound concentration and antioxidant activities of edible and medicinal mushrooms from Korea. J. Agric. Food Chem. 56, 7265–7270.

Kivçak, B., Mert, T., 2001. Quantitative determination of α-tocopherol in *Arbutus unedo* by TLC-densitometry and colorimetry. Fitoterapia 72, 656-661.

Larson, R.A., 1988. The antioxidants of higher plants. Phytochemistry, 27, 969-978.

Liu, F., Ng, TB., 2000. Antioxidative and free radical scavenging activities of selected medicinal herbs. Life Sc. 66, 725.735.

Liu, Q., Yao, H., 2007. Antioxidant activities of barley seeds extracts. Food Chem. 102, 732-737.

Males, Z., Plazibat, M., Vundac, V.B., Zunta, I., 2006. Qualitative and quantitative analysis of flavonoids of the strawberry tree – *Arbutus unedo* L. (Ericaceae). Acta Pharm. 56, 245-250.

Mariotto, S., Esposito, E., Di Paola, R., Ciampa, A., Mazzon, E., Carcereri de Prati, A., Darra, E., Vincenzo, S., Cucinotta, G., Caminiti, R., Suzuki, H., Cuzzocrea, S., 2008. Protective effect of *Arbutus unedo* aqueous extract in carrageenan-induced lung inflammation in mice. Pharmacol. Res. 57, 110-124.

Mohsen, S., Ammar, S., 2009. Total phenolic contents and antioxidant activity of corn tassel extracts. Food Chem. 112, 595-598.

Naczk, M., Shahidi, F., 2006. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. J. Pharm. Biomed. Anal. 41, 1523-1542.

Oliveira, I., Sousa, A., S Ferreira, I.C.F.R., Bento, A., Estevinho, L., Pereira, J. A., 2008. Total phenols, antioxidant potential and antimicrobial activity of walnut (*Juglans regia* L.) green husks. Food Chem. Tox. 46, 2326-2331.

Oliveira, I., Valentão, P., Lopes, R., Andrade, P.B., Bento, A., Seabra, R., Pereira, J.A., 2009. Phytochemical characterization and radical scavenging activity of *Portulaca oleracea* L. leaves and stems. Microchem. J. doi:10.1016/j.microc.2009.02.006.

Oyaizu, M., 1986. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn J. Nutr. 44, 307-315.

Pabuçcuoğlu A., Kıvçak, B., Baş, M., Mert, T., 2003. Antioxidant activity of *Arbutus unedo* leaves. Fitoterapia 74, 597-599.

Pallauf, K., Rivas-Gonzalo, J.C., Castillo, M.D., Cano, M.P., Pascual-Teresa, S., 2008. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. J. Food Compos. Anal. 21, 273-281.

Pawlowska, A. M., De Leo, M., Braca, A., 2006. Phenolics of *Arbutus unedo* L. (Ericaceae) Fruits: Identification of Anthocyanins and Gallic Acid Derivatives. J. Agric. Food Chem. 54, 10234-10238.

Pedro, J., 1994. Carta da distribuição de figueira e medronheiro - Notícia Explicativa. Ministério do Ambiente e Recursos Naturais, Direcção Geral do Ambiente. Lisboa.

Pulido, R., Bravo, L., Saura-Calixto, F., 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agric. Food Chem. 48, 3396-3402.

Rice-Evans, C., Miller, N., Bolwell, P., Bramley, P., Pridham, J., 1995. The Relative Antioxidant Activities of Plant-Derived Polyphenolic Flavonoids. Free Radical Res. 22, 375-383.

Sanjust, E., Mocci, G., Zucca, P., Rescigno, A., 2008. Mediterranean shrubs as potential antioxidant sources. Nat. Prod. Res. 22, 689-708.

Sassi, A.B., Harzallah-Skhiri, F., Aouni, M., 2007. Investigation of Some Medicinal Plants from Tunisia for Antimicrobial Activities. Pharm. Biol. 45, 421-428.

Shi, J.; Gong, J., Liu, J., Wu, X., Zhang, Y., 2009. Antioxidant capacity of extract from edible flowers of Prunus mume in China and its active components. LWT-Food Sci. Technol. 42, 477-482.

Singleton, V. L., Rossi, J. A. Jr., 1965. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144-158.

Wang, S.Y., 2006. Effect of Pre-harvest Conditions on Antioxidant Capacity in Fruits. Acta Hortic. 712, 299-305.

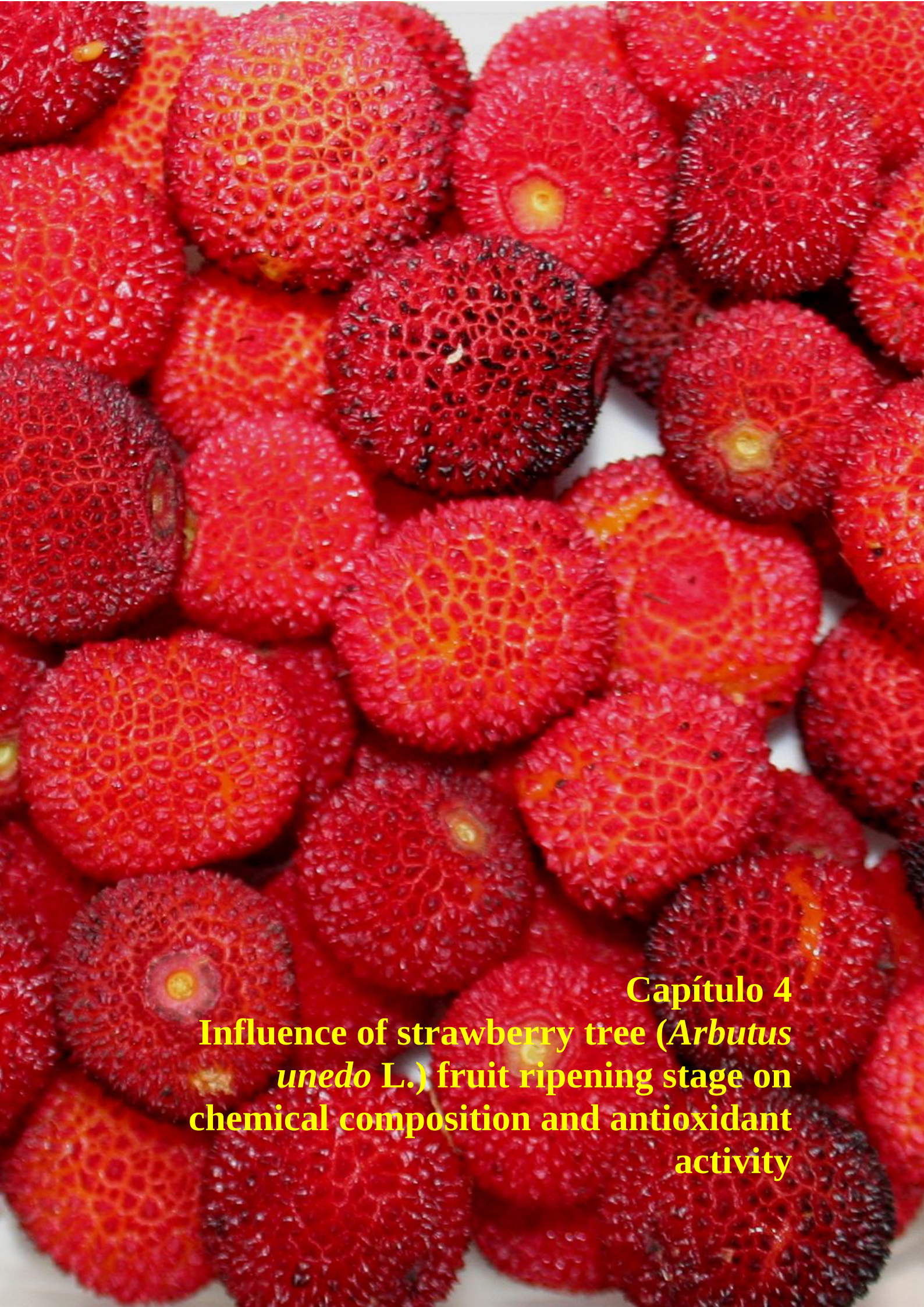
Wang, S., Lin, H., 2000. Antioxidant Activity in Fruits and Leaves of Blackberry, Raspberry, and Strawberry Varies with Cultivar and Developmental Stage. J. Agric. Food Chem. 48, 140-146.

Zhao, B., Hall III, C., 2008. Composition and antioxidant activity of raisin extracts obtained from various solvents. Food Chem. 108, 511-518.

Zheng, W., Wang, S. Y., 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49, 5165-5170.

Ziyyat, A., Boussairi, E., 1998. Cardiovascular effects of *Arbutus unedo* L. in spontaneously hypertensive rats. Phytother. Res. 12, 110-113.

Ziyyat, A., Legssyer, A., Mekhfilt, A., Dassouli, A., .Serhrouchni, M., Benjelloun, W., 1997. Phytotherapy of hypertension and diabetes in oriental Morocco. J. Ethnopharmacol. 58, 45-54.



Capítulo 4
Influence of strawberry tree (*Arbutus unedo* L.) fruit ripening stage on chemical composition and antioxidant activity

Influence of strawberry tree (*Arbutus unedo* L.) fruit ripening stage on chemical composition and antioxidant activity

Food Research International (2011), doi:10.1016/j.foodres.2011.02.009

ABSTRACT

Arbutus unedo is a widespread shrub with economic importance, derived from the use of its berries in the production of alcoholic beverages and in folk medicine. This work intends to evaluate for the first time the effect of fruit ripening stage on antioxidant activity, total phenolic content, fatty acid profile and tocopherol composition. Ripe fruits shown higher yield (45.04±2.23 %) when compared to other fruit ripening stages. By contrast, total phenols contents was higher in the unripe and intermediate stage of ripeness (108 and 111mg GAES/g dry fruit, respectively, against 60mg/g when ripe). Ripe and intermediate fruits shown the lower EC_{50} values on the DPPH radicals (0.25±0.02 mg/mL) and reducing power assay (1.09±0.05 mg/mL), respectively. A significant correlation was established between antioxidant activity and fruits ripening stage. Fatty acid profiles were very similar between the ripening stages, being alfa-linolenic, linoleic and oleic, the three major ones. Polyunsaturated fatty acids (PUFA) represent as much as 60% of the total fatty acids, with a highly favourable omega 3/omega 6 ratio. From the analysis of the vitamin E vitamers, the most important was γ-tocotrienol, with a clear reduction in the total free vitamin E content with ripening.

These results highlight that the fruits of intermediate ripeness can be regarded important sources of biologically active compounds with a fatty acid profile rich in omega-3 PUFA, properly supplemented with high vitamin E amounts.

KEY-WORDS: *Arbutus unedo* L. fruits; ripening stage; fatty acids; tocopherols; total phenols; antioxidant activity.

4.1. INTRODUCTION

Strawberry tree, *Arbutus unedo* L., is a small shrub, found all over Southern Europe, appearing in Portugal mainly in the south, although it can be found in sparse distribution throughout all of the country (Pedro, 1994). *A. unedo* produces red spherical fruits, of bitter taste until they reach a ripe state, in autumn. The consumption of fresh fruits is rare, being commonly used in the production of alcoholic beverages, or consumed as jellies, jam or marmalade (Pawlowska *et al*., 2006; Simonetti *et al*., 2008).

Several reports highlight the beneficial health effects of these fruits, such as antiseptic, diuretic, and laxative effects (Pallauf *et al*., 2008; Ziyyat and Boussairi, 1998). *A. unedo* mature fruits present in their composition high amounts of sugars, ranging from 42% to 52% of dry weight (Alarcão-E-Silva *et al*., 2001; Ayaz *et al*., 2000), as well as minerals, being especially rich in potassium and calcium (Özcan and Haciseferoğullari, 2007). Strawberry tree fruits presents also several important antioxidant compounds, including vitamins (tocopherols, ascorbic acid and carotenoids) (Alarcão-E-Silva *et al*., 2001; Ayaz *et al*., 2000; Pawlowska *et al*., 2006; Pallauf *et al*., 2008), as well as different phenolic compounds, namely gallic acid (Ayaz *et al*., 2000; Pawlowska *et al*., 2006) protocatechuic acid, gentisic acid, *p*-hydroxybenzoic acid, vanillic acid, *m*-anisic acid (Ayaz *et al*., 2000), arbutin, ß-D-glucogalline, gallic acid 4-*O*-ß-D-glucopyranoside, 3-*O*-galloylquinic acid, 5-*O*-galloylquinic acid, 3-*O*-galloylshikimic acid, and 5-*O*-galloylshikimic acid (Pawlowska *et al*., 2006).

Many of the biological functions attributed to the consumption of strawberry tree fruits could be due to their rich composition of those antioxidant compounds. It is well known the arbutus berries employed, for a long time, in traditional and popular medicine (Mariotto *et al*., 2008). In fact, the use of antioxidant in human nutrition and food industry has gain importance over the last years. Human cells hold mechanisms that maintain a low level of free radicals and reactive oxygen species (ROS) (Valko *et al*., 2007), such as enzymes (superoxide dismutase, glutathione peroxidase or catalase), as well as non-enzimatic molecules (Vitamin C, Vitamin E, glutathione, carotenoids and flavonoids) (Abdel-Hameed, 2009). Nevertheless, some external factors can increase the production of free radicals, like exposure to environmental pollutants (Prasad *et al*., 2009) leading to oxidative stress. Therefore, when the cell's defenses aren't enough, the intake of antioxidant compounds can provide additional protection, counteracting the

damaging effects of free radicals and reactive oxygen species (ROS) by breaking radical chain reactions, limiting or preventing cellular damage. Artificial antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ), due to their suspected toxic and carcinogenic effects, have been gradually restricted (Prasad *et al*., 2000; Sasaki *et al*., 2002). This fact has lead to an increased demand for natural antioxidants, especially phenolic compounds, found in vegetables, fruits and medicinal plants (Abdel-Hameed, 2009), due to their wide range of biological effects, including antioxidant properties by free radical or active oxygen scavenger's activities (Zheng and Wang, 2001), but also anti-inflammatory, anti-allergic, and antibacterial properties (Pawlowska *et al*., 2006). Thus, an appropriate dose of antioxidants derived from plants in the human diet could help reducing the risk of several diseases.

Although the chemical composition of strawberry tree fruits is relatively known, as far as we know no works were developed on how ripening stages influences fruit composition and their antioxidant activity. These fruits do not attain maturity simultaneously, coexisting in the same tree from yellow/green to red mature fruits. The most frequent situation during picking is to collect all fruits together when the majority is fully ripe. Hence, one objective of this work is to access the antioxidant ability of *A. unedo* fruits along ripening, by evaluation of total phenols content, reducing power and radical scavenging activity on DPPH radicals. Also, aware of the inexistence of composition data on the seeds, namely regarding their lipid composition, a detailed study on the fatty acid composition and vitamin E was performed.

4.2. MATERIALS AND METHODS

4.2.1. Samples

Samples were collected in the Natural Park of Montesinho, in the region of Trás-os-Montes, North-eastern Portugal. The berries were collected in November of 2009, separated by ripening stage (Unripe, Intermediate and Ripe, Figure 1 a, b, and c, respectively). Samples were frozen and freeze dried (Ly-8-FM-ULE, Snijders) prior analysis.

a) Unripe b) Intermediate c) Ripe

Figure 1. *Arbutus unedo* ripening stages considered in this work

4.2.2. Antioxidant Activity

4.2.2.1. Reagents.

2.2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Sigma (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was obtained from Fluka. Ethanol (96 %) was purchased from Riedel-de Haen. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). Water was treated in a Mili-Q water purification system (TGI Pure Water Systems, USA).

4.2.2.2. Extraction conditions.

A fine dried powder (20 mesh) of fruit sample (1.5 g) was extracted using 25mL of 96% ethanol for 30 minutes at room temperature (EE30m). These conditions presented the most promising results, after a previously performed assay, testing several different solvents and extraction conditions (data not shown). The extractions were performed in triplicate, filtered through Whatman no. 4 paper, and evaporated under vacuum, at 40ºC (rotary evaporator Büchi R-210). All the extracts were redissolved in water to a final concentration of 50 mg/mL, and analyzed for total phenols, scavenging effect on DPPH radicals, and reducing power assay.

4.2.2.3. Total phenolics.

Total phenols quantification was achieved according to Singleton and Rossi (Singleton and Rossi, 1965), with minor modification. Thus, 1mL of sample extract was mixed with 1mL of Folin-Ciocalteu's phenol reagent. The mixture was shaken vigorously and left to stand for 3 minutes. Then, 1mL of a saturated solution of sodium carbonate was added and the total volume was adjusted to 10mL with distilled water. The mixture was kept in the dark for 90 minutes, followed by absorbance reading at 725 nm. Gallic acid was used as standard, being the results expressed in mg of gallic acid equivalents (GAE)/g of extract.

4.2.2.4. Scavenging effect on DPPH radicals.

The ability to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was monitored according to a method reported before (Oyaizu, 1986). Various concentrations of sample extracts (0.3mL) were mixed with 2.7mL of methanolic solution containing DPPH radicals ($6x10^{-5}$mol/L). The mixture was shaken vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH-AS)/ADPPH] × 100, where AS is the absorbance of the solution when the sample extract was added and ADPPH is the absorbance of the DPPH solution. The extract concentration providing

50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against the extract concentration.

4.2.2.5. Reducing power assay.

The reducing power was determined according to a previously described procedure (Berker *et al*., 2007). Various concentrations of sample extracts (1 mL) were diluted with 2.5 mL of 200 mmol/L sodium phosphate buffer (pH 6.6) and mixed with 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid (w/v) were added and then the mixture was centrifuged at 1000 rpm in a refrigerated centrifuge (Centorion K24OR-2003, 4ºC), for 8 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionised water and 0.5 mL of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance registered at 700 nm against the correspondent extract concentration.

4.2.3. Chemical composition

For fatty acids and vitamin E analysis, crude oil was obtained from the seeds of *A. unedo* fruits, extracted with light petroleum ether (b.p. 40–60 ºC) in a Soxhlet apparatus, and the remaining solvent was removed by vacuum distillation, always protected from light and at room temperature.

4.2.3.1. Fatty acid composition

Fatty acids were evaluated as their methyl esters, after alkaline transesterification with methanolic potassium hydroxide solution (ISO 5509: 2000) and extraction with n-heptane. The fatty acid profile was analyzed with a Chrompack CP 9001 Chromatograph equipped with a split-splitless injector, a FID, an autosampler Chrompack CP-9050 and a 50m x 0.25 mm i.d. fused silica capillary column coated with a 0.19µ film of CP-Sil 88 (Chrompack). Helium was used as carrier gas at an internal pressure of 120kPa. The temperatures of the detector and injector were 270ºC and 250ºC, respectively. The split ratio was 1:50 and the injected volume 1µL. The

results are expressed in relative percentage of each fatty acid, calculated by internal normalization of the chromatographic peak area (ISO 5508: 1990). The fatty acid methyl esters standard mixture (Supelco 37 FAME Mix) was used for identification (Sigma, Spain).

4.2.3.2. Tocopherol composition

Tocopherols were evaluated following an international standard (ISO 9936: 2006) with some modifications (Amaral *et al*., 2005). Tocopherols and tocotrienols standards (α, β, γ and δ) were purchase from Calbiochem (La Jolla, San Diego, CA) and 2-Methyl-2-(4,8,12-trimethyltridecyl)chroman-6-ol (tocol) was from Matreya Inc. (Pleasant Gap, PA). A 20 mg amount of extracted fat was blended with an appropriate amount of internal standard (tocol) in a 1.5 mL volume of n-hexane and homogenized by stirring. Sample preparation was conducted in dark and tubes containing the samples were always wrapped in aluminum foil. The mixture was centrifuged for 5 minutes at 13000g and the supernatant analyzed by HPLC. The liquid chromatograph consisted of a Jasco integrated system (Japan) equipped with an AS-950 automated injector, a PU-980 pump, an MD-910 multiwavelength diode array detector and an FP-920 fluorescence detector (λ exc= 290 nm and λ em= 330 nm), connected in series. The chromatographic separation was achieved on a Supelcosil TM LC-SI (3 μm) 75 x 3.0 mm (Supelco, Bellefonte, PA), operating at constant room temperature (21ºC). A mixture of n-hexane and 1,4-dioxane (98:2) was used as eluent, at 0.7 mL/min. Data were analyzed in the Borwin PDA Controller Software (JMBS, France). Tocopherols (α, β, γ, and δ) were identified by chromatographic comparisons with authentic standards, by co-elution and by their UV spectra. Quantification was based on the internal standard method, using the fluorescence signal response.

4.2.4. Statistical Analysis

For each extraction methodology, three independent extractions were performed and for each one all the assays were carried out in duplicate. Results are shown as solvent mean values and standard deviation. The same procedure was performed when using the fruits presenting different maturation stages. For fatty acid and vitamin E analyses, four determinations were executed in each maturation stage.

A regression analysis, using Excel from Microsoft Corporation, was established between the different maturation stage and the fatty acids data and tocopherols and tocotrienols compositions. Principal components analysis (PCA) and one-way ANOVA were performed using SPSS software, version 17.0 (SPSS, Inc.). Principal components analysis (PCA) was applied for reducing the number of variables (22 variables corresponding to the fatty acids profile; 7 variables corresponding to the vitamin E determination; and 32 variables corresponding to fatty acids profile, vitamin E determination, antioxidant potential and phenolic content all together) to a smaller number of new derived variables (principal component or factors) that adequately summarize the original information, i.e., the three different maturation stages, unripe, intermediate and ripe. Moreover, it allowed recognizing patterns in the data by plotting them in a multidimensional space, using the new derived variables as dimensions (factor scores). An analysis of variance (ANOVA) with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 17.0 (SPSS, Inc.). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov-Smirnov with Lilliefors correction (if $n>50$) or the Shapiro-Wilk`s test (if $n<50$), and the Levene´s tests, respectively. All dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending if the requirement of the homogeneity of variances was fulfilled or not. The main factor studied was the influence of maturation stage on fatty acids profile, tocopherols and tocotrienols compositions, antioxidant activity, total phenolic content and extraction yield from *A. unedo* fruits. If a statistical significant effect was found, means were compared using Tukey´s honestly significant difference multiple comparison test or Dunnett T3 test also depending if equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

4.3. RESULTS AND DISCUSSION

4.3.1 Antioxidant ability of A. unedo fruits with different ripening stages

The extraction method (EE30m) was applied to the samples with different ripening stages (Figure 1a, b and c). Table 1 show an overall view of the achieved EC_{50} values for the reducing power and DPPH radical assay, as well as the extraction yield and total phenols content.

Table 1. Reducing power and scavenging effect EC_{50} values (mg/mL), and total phenols content (mg/g) of *Arbutus unedo* fruits presenting different ripening stages (mean ± SD).

	Unripe	Intermediate	Ripe	*P* - value
Extraction yield (%)	24.77±3.84 a	43.53±5.52 b	45.04±2.23 b	0.002
Total phenolics (mg GAE/g of extract)	25.35±2.51 a	48.26±4.49 b	26.81±2.44 a	< 0.001
Reducing Power (EC_{50}) mg/mL	2.00±0.05 b	1.09±0.05 a	1.50±0.10 c	< 0.001
DPPH (EC_{50}) mg/mL	0.58±0.03 c	0.37±0.02 b	0.25±0.02 a	< 0.001

Extraction yield of the unripe fruits was 24.8±3.8 %, that significantly increasing for the intermediate fruit (43.5±5.5 %), this last similar to the ripe fruits (45.0±2.2). The total phenols content presented a different evolution pattern: while both unripe and ripe fruits presented similar values (25.4±2.5 and 26.8±2.4 mg GAE/g of extract, respectively), the intermediate stage of ripening presented significantly higher amounts of total phenols, almost doubling the formers compounds in their composition (48.3±4.5 mg GAE/g of extract). When reported for the fruit, the differences are clearer, with 6.3 mg in the unripe stage, 21.0 in the intermediate and 12.1 in the ripe fruits, all expressed as GAE/g dry fruit. These values are in accordance with those observed by Alarcão-e-Silva *et al*. (2001) who reported 15.5±0.6 mg catechin/g of dry matter in unripe fruits, and 14.6±0.9 on ripe fruits, taking into account the different extraction conditions and standard for quantification. The individual phenols present in the *A. unedo* berries have already been identified and quantified. Ayaz *et al*. (2000) identified six phenolics acids, namely gallic, protocatechuic, gentisic, p-hydroxybenzoic, vanillic and m-anisic acid, being the most important one gallic acid (10.7±0.04 mg/g dry matter), followed by gentisic (1.9±0.11) and protocatechuic acids (0.6±0.03). Pawlowska *et al*. (2006) also found seven gallic acid derivatives that were identified and characterized as arbutin, ß-

D-glucogalline, gallic acid 4-*O*-ß-D-glucopyranoside, 3-*O*-galloylquinic acid, 5-*O*-galloylquinic acid, 3-*O*-galloylshikimic acid, and 5-*O*-galloylshikimic acid. The DPPH assay results present a concentration-dependent activity (Figure 2). The tested extracts, obtained for the three ripening stages, showed similar behaviours, with the extracts reaching the higher activity at a concentration of 2mg/mL, from which point forward it remained constant.

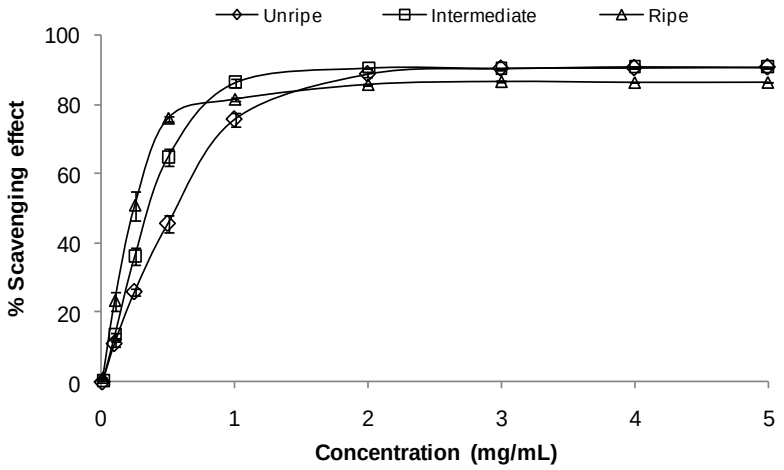


Figure 2. Scavenging activity on DPPH radicals (%) of the extracts obtained for fruits presenting different ripening stages. Each value is expressed as mean ± standard error.

Although presenting similar behaviour, the EC_{50} values, accomplished by the extracts are significantly different (Table 2), and a decrease is visible as ripeness increases. A strong negative linear correlation was established between those two factors (R^2= 0.788, P< 0.001). This correlation proves that as the ripening stages increase (from unripe to ripe), the amount of fruits extract necessary to achieve 50% of inhibition decreases. Furthermore, the values of EC_{50} for the DPPH assay are significantly different (Table 1). These results may be linked to the increase of anthocyanins content, responsible for the colour of the fruits, as they become ripe. These compounds are known as potent antioxidants (D'Archivio *et al*., 2007.) and their presence increases substantially, from 0.25±0.02 mg/g, when the fruits are unripe, to 1.01±0.01 mg/g when the berries are red mature (Alarcão-E-Silva *et al*., 2001). Furthermore, the increase on the content of sugars, which occur during fruit ripening, might be also a factor contributing for these results, as some sugars can act as reducing agents, namely fructose. The amount of sugar present in these fruits is variable between ripeness stages. When unripe they represent 14% of the dry weight of the fruits (Alarcão-E-Silva *et al*., 2001) and when ripe correspond to as much as 43% (Alarcão-E-Silva *et al*., 2001) or 52% of dry weight (Ayaz *et al*., 2000). From the total sugars, when

the fruit is unripe, sucrose is the major sugar, but as the fruits turn ripe, fructose becomes the most important sugar present in these fruits (Alarcão-E-Silva *et al*., 2001).

Our results are in agreement with the ones achieved by Heinrich (2005) where it was showed that extracts at a concentration of 2 mg/mL presented antioxidant activity on the DPPH assay, reaching over 50% of the control.

When analysing the results for the reducing power assay, a concentration-dependent activity was noticeable (Figure 3).

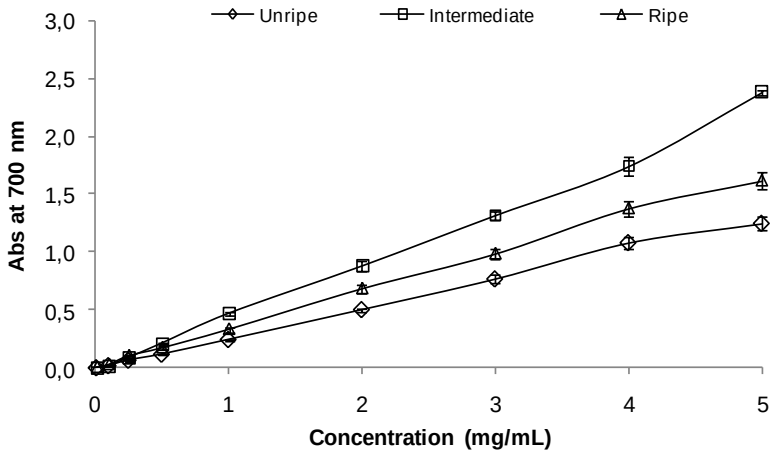


Figure 3. Reducing power values of the extracts obtained for fruits presenting different ripening stages. Each value is expressed as mean ± standard error.

Furthermore, a significantly negative linear correlation was established between EC_{50} reducing power values and ripening stage (R^2 = 0.256; *P* = 0.032). The fruits presenting higher amounts of total phenols (intermediate stage of maturation) achieved a lower EC_{50} value for this method (1.09±0.05 mg/mL). This relationship is valid for the other two ripe stages. The unripe fruits, presenting the lowest content of total phenols, present the higher value of EC_{50} (2.00±0.05 mg/mL) in the reducing power assay, and the ripe fruits, with an in-between phenolic content, also presented an intermediate EC_{50} values (1.50±0.10 mg/mL). When comparing the results achieved with the strawberry-tree fruits to other berries, known for their antioxidant activity, promising uses come about to *A. unedo* fruits. Comparing our results with previous published data (Pantelidis *et al*., 2007) who quantified the total phenols content of several berries, it is visible that the amount of these compounds is very similar to the one of other berries, as gooseberries, raspberries and blackberries cultivars. For instance, the highest content uncover on the referred work was of 2611±69 mg GAE/100g dry weight, on a *Rubus ideaus* x *Rubus fructicosus* hybrid fruits.

4.3.2. Lipid composition

In table 2 results for the quantification of the 15 main fatty acids are shown. The major fatty acids, present in the three maturation stages, are $C_{18:3n3}$, that significantly increase with the fruit maturation (α-linolenic acid, ranging from 36.9% in the unripe stage, to 41.6% in the intermediate stage and 43.1% when the fruits are ripe), followed by $C_{18:2n6}$ that presented a contrary behaviour and decrease significantly (linoleic acid, ranging from 20.14% in the unripe stage, to 18.8% when the fruits are ripe) and $C_{18:1n9}$ (oleic acid, ranging from 29.4% the unripe stage, to 26.8% in the ripe fruits seeds). With the exception of the above-mentioned fatty acids and $C_{16:0}$ and $C_{18:0}$, all other fatty acids appear in amounts lower than 1%. The most representative fatty acids reported for other berries (bilberry, cranberry, rose hip, strawberry, elder, and black currant) are also linoleic, linolenic and oleic acids (Helbig *et al*., 2008.) disclosed in this as the most representative fatty acids present in *A. unedo* fruits, although not in this order. Polyunsaturated fatty acids (PUFA) were the major fraction of the fatty acids, representing at least 52% of the total fatty acids, with a highly favourable ω3/ω6 ratio, due to the richness in α-linolenic acid. Monounsaturated fatty acids (MUFA) also represent a considerable part of the fatty acids, with percentages varying from 27.0% in the ripe stage to 33.5% in the unripe fruits. Saturated fatty acids (SFA) were present in low percentages, and kept almost constant along ripening. When comparing to other berries, *A. unedo* fruits present a similar profile, with the SFA present in low quantity, and PUFA representing the majority of the fatty acid present. Other berries, such as bilberry, cranberry, rose hip, strawberry, elder, and black currant, present, in their seed press residue, high amounts of MUFA and PUFA, mainly ω3 and ω6 (Helbig *et al*., 2008). The amounts of MUFA ranged from 12.46 mol% of fatty acid methyl esters (FAME), in *Ribes nigrum* L. to 23.55 mol% of FAME in *Vaccinium myrtillus* L.

Table 2. Fatty acid composition (percent) of oil extracted from analyzed stages of ripening of *Arbutus unedo* fruits (mean ± SD). SFA - saturated fatty acids, MUFA - Monounsaturated fatty acids, PUFA - polyunsaturated fatty acids.

	Unripe	**Intermediate**	**Ripe**	***P***	$\mathbf{R^2}$
$\mathbf{C_{14:0}}$	0.07±0.02 b	0.04±0.00 a	0.04±0.00 a	0.008	0.525
$\mathbf{C_{15:0}}$	0.04±0.00 b	0.03±0.00 a	0.03±0.00 a	< 0.001	0.814
$\mathbf{C_{16:0}}$	6.73±0.21 b	5.99±0.01 a	5.92±0.05 a	< 0.001	0.753
$\mathbf{C_{16:1n9}}$	0.09±0.01 b	0.06±0.00 a	0.05±0.00 a	< 0.001	0.782
$\mathbf{C_{16:1n7}}$	0.11±0.03 a	0.09±0.00 a	0.09±0.00 a	0.245	0.132
$\mathbf{C_{18:0}}$	4.67±0.23 b	3.72±0.03 a	3.78±0.01 a	0.002	0.653
$\mathbf{C_{18:1n9}}$	29.38±1.82 b	27.61±0.09 a,b	26.75±0.17 a	0.005	0.568
$\mathbf{C_{18:2n6}}$	20.14±0.64 b	19.22±0.03 a	18.84±0.11 a	< 0.001	0.696
$\mathbf{C_{20:0}}$	0.24±0.01 b	0.22±0.00 a	0.22±0.00 a	0.003	0.603
$\mathbf{C_{18:3n6}}$	0.08±0.01 a	0.09±0.00 a,b	0.10±0.00 b	0.009	0.513
$\mathbf{C_{18:3n3}}$	36.90±1.75 a	41.55±0.23 b	43.07±0.16 b	< 0.001	0.827
$\mathbf{C_{20:1n9}}$	0.02±0.02 a	0.03±0.01 a	0.03±0.00 a	0.189	0.166
$\mathbf{C_{20:2n6}}$	0.04±0.00 a	0.05±0.01 a	0.05±0.00 a	0.637	0.0231
$\mathbf{C_{22:1n9}}$	0.09±0.05 a	0.05±0.01 a	0.05±0.00 a	0.113	0.231
$\mathbf{C_{24:0}}$	0.08±0.04 a	0.06±0.05 a	0.04±0.02 a	0.083	0.271
SFA	12.64±0.86 b	10.09±0.02 a	10.04±0.07 a	0.001	0,676
MUFA	33.49±2.55 b	27.89±0.10 a	27.00±0.19 a	0.001	0.710
PUFA	52.47±4.26 a	60.86±0.26 b	62.01±0.26 b	0.001	0.670
ω3/ω6	1.59±0.13 a	2.15±0.01 b	2.27±0.01 b	< 0.001	0.840
trans	0.25±0.07 b	0.14±0.05 a	0.12±0.02 a	0.004	0.587

[a,b]Means within a line with different superscripts differ, $P < 0.05$.

PUFA fraction of the fatty acid content was lowest in *Vaccinium myrtillus* L. 64.16 mol% of FAME and highest in one sample of 79.18 mol% of FAME *Ribes nigrum* L. Saturated fatty acids were the minor fraction, with amount inferior, in all

berries, to 10 mol% of FAME (Helbig *et al*., 2008). Table 3 also shows the correlation between ripening stage and the individual fatty acids content. For some fatty acids, and giving special attention to the major ones, very significant correlations were established between ripeness and fatty acid content. For $C_{18:3n3}$, the major fatty acid, a *P* value of < 0.001 was achieved, and for $C_{18:1n9}$ and $C_{18:2n6}$, the *P* values are 0.005 and < 0.001, respectively, proving the extreme influence of the maturation on the fatty acid content of *A. unedo* fruits. For the first one, an increase of its content is noticeable, as the ripeness increases, also proved by the obtained linear correlation (R^2 = 0.827). For the other two major fatty acids, a negative linear correlation was achieved, proving that their amount decreases with the ripening of the fruit (R^2 = 0.568 for $C_{18:1n9}$ and, R^2 = 0.696 for $C_{18:2n6}$).

Regarding the vitamin E family of compounds quantification, results expressed in terms of mean values and standard deviations for each vitamer, with regard to every stage of maturation, are presented in table 3. Higher amount of vitamin E was found on the unripe fruits, and the content decreases as the maturation increases. The most important constituent is γ-tocotrienol. For all the vitamers, a strong significant relationship was observed with the ripening stages.

Table 3. Tocopherol and tocotrienol composition (mg/Kg) of oil extracted from analyzed stages of ripening of *Arbutus unedo* fruits (mean ± SD).

	Unripe	**Intermediate**	**Ripe**	***P***	**R^2**
α-tocopherol	271.47±45.99 b	278.83±5.80 b	32.16±1.55 a	< 0.001	0.698
α-tocotrienol	7.97±1.08 b	1.82±0.33 a	ND	< 0.001	0.887
ß-tocopherol	4.06±0.47 b	4.51±0.09 b	0.39±0.20 a	0.002	0.639
γ-tocopherol	68.89±8.82 b	31.85±0.48 a	25.09±0.51 a	< 0.001	0.819
γ-tocotrienol	1013.88±90.33 b	615.70±87.61 a	498.96±12.18 a	< 0.001	0.840
δ-tocopherol	2.31±1.14 a	1.71±0.73 a	ND	0.002	0.628
Total	1368.58±147.82 c	934.43±95.04 b	556.60±14.55 a	< 0.001	0.938

For α-tocotrienol, γ-tocopherol and γ-tocotrienol, a negative correlation was established (*P* <0.001, R^2 = 0.887, *P* < 0.001, R^2 = 0.819 and *P* < 0.001, R^2= 0.840,

respectively). α-Tocotrienol and δ-tocopherol content decreases at such extent, that the amount present in the ripe stage is not detectable. The total amount of vitamin E vitamers is significantly different between ripening stages ($P < 0.001$). In addition, a very strong negative linear correlation was obtained between the total amount of vitamin E and the ripeness presented by the fruits ($R^2 = 0.938$). Additionally, when performing a PCA using the content of the vitamers of vitamin E, a clear separation of the three stages of ripening was observed (Figure 4A) the same was observed using the fatty acids profile (Figure 4B), with the models explaining respectively 95.8% and 81.6% of all variance detected in the experimental data. In comparison with other berries, *A. unedo*, especially when unripe, presents higher vitamin E amounts. Recent works (Helbig *et al*., 2008) reported that the seeds of *Sambucus nigra* L. contain around 1224 mg of total vitamin E per kg of extracted oil (1153 mg of tocopherols and 71 of tocotrienols). The strawberry-tree fruits analyzed contain, when unripe, an amount of total vitamin E of 1369 mg/Kg of oil.

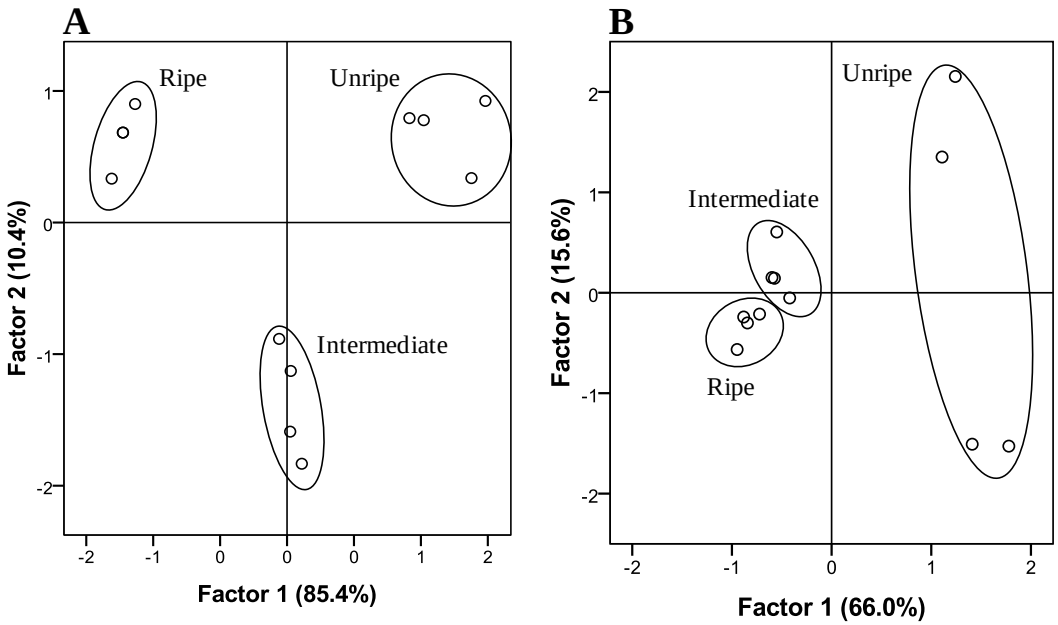


Figure 4. Principal component analysis using tocopherols and tocotrienols composition (**A**) and fatty acids (**B**) data of *Arbutus unedo* fruits, presenting different ripening stages. The PCA factors explain 95.8% (**A**) and 81.6% (**B**) of the total variance.

Finally, a PCA was performed using all the parameters studied (data of fatty acid, vitamin E vitamers, total phenolics and EC_{50} values of the tested antioxidant methods). Using all this values, an evident differentiation of the ripening stages of the fruits is

possible, explaining 87.7% of the variance of the experimental data using only three principal component factor scores (Figure 5).

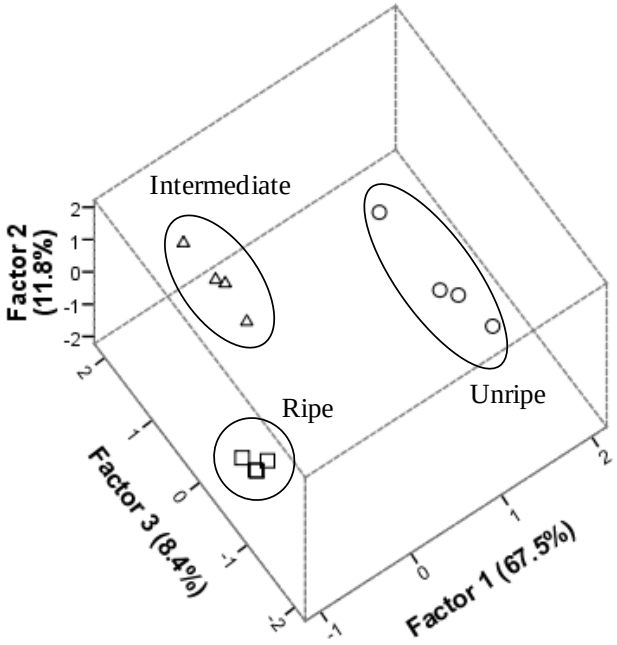


Figure 5. Principal component analysis using tocopherols and tocotrienols composition, fatty acid profile, total phenolics and EC_{50} values of the tested antioxidant methods of *Arbutus unedo* fruits, presenting different ripening stages The PCA factors explain 87.7% of the total variance.

The results achieved in this work may open the possibility of the use of *A. unedo* fruits as source of antioxidant compounds, particularly those of intermediate ripeness, and highlight their richness of their seeds in omega 3 fatty acids and vitamin E. They contain a considerable amount of phenolic compounds, as well as important antioxidant activity against free-radicals, which combined with the fact that these berries are a good source of vitamins, as niacin, vitamins C and A (Alarcão-E-Silva *et al*., 2005) and flavonoids, carotenoids and vitamin E (Pallauf *et al*., 2008), make them a promising sources of bioactive compounds.

4.4. REFERENCES

Abdel-Hameed, E. 2009. Total phenolic contents and free radical scavenging activity of certain Egyptian Ficus species leaf samples. Food Chem. 114, 1271-1277.

Alarcão-E-Silva, M., Leitão, A.E.B., Azinheira, H.G., Leitão, M.C.A. 2001. The Arbutus Berry: Studies on its Color and Chemical Characteristics at Two Mature Stages. J. Food Compos. Anal. 14, 27-35.

Amaral, J.S., Casal, S., Oliveira, M.B., Seabra, R.M. 2005. Development and evaluation of a normal phase liquid chromatographic method for the determination of tocopherols and tocotrienols in walnuts. J. Liq. Chromatogr. Relat. Technol. 28, 785–795.

Ayaz, F., Kucukislamoglu, M., Reunanen., M. 2000. Sugar, Non-volatile and Phenolic Acids Composition of Strawberry Tree (*Arbutus unedo* L. var. ellipsoidea) Fruits. J. Agric. Food Chem. 13, 171-177.

Berker, K., Güçlü, K., Tor, I., Apak, R. 2007. Comparative evaluation of Fe (III) reducing power-based antioxidant capacity assays in the presence of phenanthroline, batho-phenanthroline, tripyridyltriazine (FRAP), and ferricyanide reagents. Talanta. 72, 1157-1165.

D'Archivio, M., Filesi, C.; Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R. 2007. Polyphenols, dietary sources and bioavailability. Ann. Ist. Super. Sanità. 43, 348-361.

Heinrich (co-ordinater of the Local Food-Nutraceuticals Consortium), M., 2005. Understanding local Mediterranean diets: A multidisciplinary pharmacological and ethnobotanical approach. Pharmacol. Res. 52, 353-366.

Helbig, D., Böhm, V., Wagner, A., Schubert, R., Jahreis, G. 2008. Berry seed press residues and their valuable ingredients with special regard to black currant seed press residues. Food Chem. 111, 1043-1049.

ISO 5508: 1990. Animal and vegetable fats and oils. Analysis by gas chromatography of methyl esters of fatty acids.

ISO 5509: 2000. Animal and vegetable fats and oils – Preparation of methyl esters of fatty acids.

ISO 9936: 2006. Animal and vegetable fats and oils – Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography.

Mariotto, S, Esposito, E, Di Paola, R, Ciampa, A, Mazzone, E, Carcereri de Prati, A, Darra, E., Vincenzo, S., Cucinotta, G., Caminiti, R., Suzuki, H., Cuzzocrea, S.

2008. Protective effect of *Arbutus unedo* aqueous extract in carrageenan-induced long inflammation in mice. Pharmacol. Res. 57, 110-124.

Oyaizu, M. 1986. Studies on products of browning reactions: Antioxidative activities of products of browning reaction prepared from glucosamine. Jpn. J. Nutr. 44, 307-315.

Özcan, M., Haciseferoğullari, H. 2007. The strawberry (*Arbutus unedo* L.) fruits: Chemical composition, physical properties and mineral contents. J. Food Eng. 78, 1022-1028.

Pallauf, K., Rivas-Gonzalo, J.C., Castillo, M.D., Cano, M.P., Pascual-Teresa, S. 2008. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. J. Food Compos. Anal. 21, 273-281.

Pantelidis, G., Vasilakakis, M., Manganaris, G., Diamantidis, G. 2007. Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. Food Chem. 102, 777-783.

Pawlowska, A. M., De Leo, M., Braca, A. 2006. Phenolics of *Arbutus unedo* L. (Ericaceae) Fruits: Identification of Anthocyanins and Gallic Acid Derivatives. J. Agric. Food Chem. 54, 10234-10238.

Pedro, J. 1994. Carta da distribuição de figueira e medronheiro - Notícia Explicativa. Ministério do Ambiente e Recursos Naturais, Direcção Geral do Ambiente. Lisboa.

Prasad, K., Yang, B., Yang, S., Chen, Y., Zhao, M., Ashraf, M., Jiang, W. 2009. Identification of phenolic compounds and appraisal of antioxidant and antityrosinase activities from litchi (*Litchi sinensis* Sonn.) seeds. Food Chem. 116, 1-7.

Sasaki, Y., Kawaguchi, S., Kamaya, A., Ohshita, M., Kabasawa, K., Iwamaa, K., Taniguchi, K., Tsuda, S. 2002. The comet assay with 8 mouse organs: results with 39 currently used food additives. Mutat. Res. 519, 103-119.

Simonetti, M., Damiani, F., Gabrielli, L., Cossignani, L., Blasi, F., Marini, F., Montesano, D., Maurizi, A., Ventura, F., Bosi, A., Damiani, P. 2008. Characterization of triacylglycerols in *Arbutus unedo* L. seeds. Ital. J. Food Sci. 20, 49-56.

Singleton, V. Rossi, J. 1965. Colorimetric of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am. J. Enol. Vitic. 16, 144-158.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M., Mazur M., Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.

Zheng, W., Wang, S. 2001. Antioxidant activity and phenolic compounds in selected herbs. J. Agric. Food Chem. 49, 5165-5170.

Ziyyat, A. Boussairi, E. 1998. Cardiovascular effects of *Arbutus unedo* L. in spontaneously hypertensive rats. Phytother. Res. 12, 110-113.

Capítulo 5
Volatile profile of *Arbutus unedo* L. fruits through ripening stage

Volatile profile of *Arbutus unedo* L. fruits through ripening stage

Food Chemistry. In press, DOI: 10.1016/j.foodchem.2011.03.084.

ABSTRACT

Arbutus unedo L. is a shrub largely distributed throughout the Mediterranean basin, and its fruits are used in the production jams and marmalades and beverages, as well as in the traditional medicine. In this work, for the first time, the volatile compounds of *A. unedo* were evaluated. The effect of ripening of the fruits was also evaluated, using headspace-solid phase microextraction (HS-SPME) combined with gas chromatography/ion trap-mass spectrometry (GC/IT-MS). Overall, 41 volatile compounds were identified, and grouped in 6 chemical classes (alcohols, aldehydes, ester compounds, norisoprenoids derivates, sesquiterpenes and monoterpenes. Alcohols are the main component of the volatile fraction of *A. unedo* fruits, followed by aldehydes and esters. All the chemical classes decreased their content from unripe to ripe stages, with the exception of sesquiterpenes and monoterpenes that appear in higher amount in ripe fruits than in unripe fruits. The main volatile compounds identified were (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, (*E*)-2-hexanal and acetic acid hexyl ester, all formed through the lipoxygenase pathway (LOX). The green odors are progressively replaced by flower and sweet sensations, due to the decreasing of the main chemical classes identified (alcohols, aldehydes and ester compounds) and to the more perceptible sensations associated with the minor compounds (mainly monoterpenes and norisprenoids derivates) that compose *A. unedo* fruits.

KEY-WORDS: *Arbutus unedo* L. fruits; volatile composition, HS-SPME GC/IT-MS: ripening stage.

5.1. INTRODUCTION

Arbutus unedo is a small evergreen shrub, spread throughout the Mediterranean, found in Portugal mainly in the south, but distributed in all of the country (Pedro, 1994). Besides the ornamental importance, resulting from the largely appreciated pinkish-white flowers, it also produces red spherical fruits. These fruits, although not usually consumed directly, are used in the preparation of alcoholic beverages, as well as jam or marmalade (Pawlowska *et al*., 2006; Simonetti *et al*., 2008). They are used in those preparations, when ripe, presenting a strong red color and a tasteful flavor. Furthermore, there's a large use of these fruits in traditional medicine, in the treatment of several diseases, such as gastrointestinal disorders, dermatologic and urological problems and for cardio-vascular application (Leonti *et al*., 2009), kidney diseases (El-Hilaly *et al*., 2003) and gastritis (Cornara *et al*., 2009). Other reports also point additional beneficial effect, such as antiseptic, diuretic and laxative effects (Pallauf *et al*., 2008; Ziyyat and Boussairi, 1998). The chemical composition of the fruits of *A. unedo* has already been subject of some studies. Besides moisture, the major component, these fruits also present high amounts of sugars, ranging from 42% to 52% of dry weight (Alarcão-E-Silva *et al*., 2000) and minerals, particularly potassium and calcium (Özcan and Haciseferoğullari, 2007). Other constituents of strawberry tree fruits includes antioxidant compounds, such as vitamins (tocopherols, ascorbic acid and carotenoids) (Alarcão-E-Silva *et al*., 2001; Ayaz *et al*., 2000; Pawlowska *et al*., 2006; Pallauf *et al*., 2008) and several phenolic compounds, (Ayaz *et al*., 2000; Pawlowska *et al*., 2006). Volatile compounds are formed during the development of fruit ripening and they may be involved in ecological interactions, reducing herbivorous attack, or implicated in the attraction mechanism of insects, for pollination purposes (Kessler and Baldwin, 2002). Flavour is strongly affected by volatile compounds, which is very important to the final global quality attributed to a certain food product. Furthermore, a relationship between volatiles that contribute to the flavour and influence the organoleptic properties and the benefits to human health has already been established (Maarse, 1991). Despite all the studies conducted on the *A. unedo* fruit, there is no information available about the volatile compounds of this berry. The only published works about volatile compounds of *A. unedo* concerns its traditional distillate produced by the fermentation of strawberry tree fruits (Soufleros *et al*., 2005), strawberry-tree honey (Bianchi *et al*., 2005; de la Fuente *et al*., 2007), essential oils (Kahriman *et al*., 2010), and the emission of volatile

organic compounds by the entire shrub (Owen *et al*., 1997; Llusià and Peñuelas, 2000; Peñuelas and Llusià, 2001).

The main goal of this study is to evaluate the volatile profile during the maturation of the *A. unedo* berries, helping to understand the significance of those compounds in the ripening process of this fruit. For this purpose, three different maturation stages were considered (unripe, intermediate and ripe), and the volatile compounds were determined using headspace solid-phase microextraction (HS-SPME) combined with gas chromatography/ion trap-mass spectrometry (GC/IT-MS).

5.2. MATERIALS AND METHODS

5.2.1 Standards

All chemical used were of analytical grade and were obtained from several suppliers: 3-methyl-1-butanol, 2-methyl-1-butanol, (*Z*)-3hexen-1-ol, (*E*)-2-octen-1-ol, trans-2-decen-1-ol, hexanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-nonadienal, (*E*)-2-octenal, nonanal, (*E*)-2-nonenal, salicylic acid methylester, hexanoic ethyl ester, decanoic acid ethylester, caryophyllene, limonene, linalool and α-terpineol were obtained from Sigma-Aldrich (St. Louis, MO, USA); 1-hexanol was from Fluka (Buchs, Switzerland); benzaldehyde, β-ionone and (*E*)-2-decenal were from SAFC (Steinheim, Germany); and eucalyptol and acetic acid hexylester were from Extrasynthèse (Genay, France).

5.2.2. Samples

Samples were collected in the Natural Park of Montesinho, in the region of Trás-os-Montes (North-eastern Portugal). Berries in different ripening stages (unripe, intermediate and ripe, Figure 1 a, b, and c, respectively) were collected separately to sterile plastic bags. Samples were immediately taken to the laboratory, stored in refrigerator and processed within few hours.

a) Unripe b) Intermediate c) Ripe

Figure 1. *Arbutus unedo* ripening stages considered in this work

5.2.3. SPME Fibers

Several commercial fibers can be used to extract volatile compounds. According to bibliography, recommendations of supplier (Supelco, Bellefonte, PA) and using the experience of other works performed in the same laboratory (Guedes de Pinho *et al.*, 2009) the fiber used was coated with divinylbenzene/polydimethylsiloxane (DVB/PDMS), 65μm.

5.2.4. HS-SPME

For each ripening stage, approximately 1 g of *A. unedo* fruits, previously thawed, was putted into a 15 mL vial with the addition of 3 mL of water. The vial was then sealed with a polypropylene cap with PTFE/silicon septum (Supelco). This mixture was stirred (280 rpm) at 40 ºC for 5 minutes. Then, the DVB/PDMS fiber was exposed to the headspace and samples were stirred for 20 minutes (280 rpm at 40º C). Afterward, the fiber was pulled into the needle sheath, the SPME device was removed from the vial and inserted into the injection port of the GC system for thermal desorption. After 1 minute, the fiber was removed and conditioned in another GC injection port for 10 minutes, at 250 ºC. The HS-SPME analyses were performed in triplicate. The same procedure was performed with a control sample containing only water.

5.2.5. Gas Chromatography-Ion Trap-Mass Spectrometry Analysis

HS-SPME analyses were performed using a Varian CP-3800 gas chromatograph equipped with a Varian Saturn 4000 mass selective detector and Saturn GC-MS workstation software version 6.8. A VF-5 ms (30 m × 0.25 mm × 0.25μm) column from Varian was used. A Stabilwax-DA fused-silica (60 m × 0.25 mm × 0.25 μm) column (Restek, USA) was used to check the identity of some compounds found in the first column. The injector port was heated to 220 ºC. The injections were performed in splitless mode. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. The oven temperature was set at 40 ºC for 1 min, then increased at 2 ºC/min to 220 ºC, and held for 30 min. All mass spectra were acquired in electron impact (EI) mode. Ionization was maintained off during the first minute. The ion trap detector was set as follows: the transfer line, manifold, and trap temperatures were 280, 50 and 180 ºC, respectively. The mass ranged from m/z 40 to 350, with a scan rate of 6 scan/s. The emission current was 50 μA, and the electron multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25000 μs, with an ionization storage level of m/z 35. Analyses were performed in full-scan mode.

Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic standards analyzed under the same conditions and by comparison of the retention indices (as Kovats indices) with literature data. MS fragmentation patterns were compared with those of pure compounds, and mass spectrum database search was performed using the National Institute of Standards and Technology (NIST) MS 05 spectral database. Confirmation was also conducted

using a laboratory-built MS spectral database, collected from chromatographic runs of pure compounds performed with the same equipment and conditions. For quantification purposes, each sample was injected in triplicate, and the chromatographic peak areas (as kcounts amounts) were determined by a reconstructed full-scan chromatogram using for each compound some specific quantification ions: these corresponded to base ion (m/z 100% intensity), molecular ion (M+), and another characteristic ion for each molecule. Some peaks that are co-eluted in full-scan mode (resolution value < 1) can be integrated with a value of resolution > 1.

5.3. RESULTS AND DISCUSSION

The analysis of the volatile composition of *A. unedo* fruits presenting different ripening stages, performed using HS-SPME and GC/IT-MS, allowed the identification of a total of 41 compounds. The qualitative and quantitative (peaks areas/1000) data are shown in Table 1, and chromatographic profiles of each ripening stage are presented in Figure 2.

The identified volatile compounds were distributed by several chemical classes, including: 10 alcohols (1-10), 10 aldehydes (11-20), 10 esters (21-30), 2 norisoprenoid derivates (31-32), 4 sesquiterpenes (33-36) and 5 monoterpenes (37-41) (Table 1). The three ripening stages considered in this work showed a similar chromatographic profile, although differences can be pointed out, regarding the compounds present, as well as the amounts of each individual and chemical class of volatiles. The unripe and ripe fruits present similar number of volatiles (27 and 29 identified compounds, respectively), while fruits with intermediate maturation presented a lower number of volatiles (22).

The volatile compounds present in higher amounts (higher peak areas) were (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, (*E*)-2-hexenal, acetic acid 4-hexen-1-yl ester and acetic acid hexyl ester. The first four compounds are known as "green leaf volatiles" (GLV) (Matsui, 2006), and together with (*E*)-2-hexen-1-ol, are related to green notes and fresh green odours characteristically presented by fruits and vegetables (Poll and Lewis, 1986; Jensen *et al*., 2001). The GLV compounds are present in all three ripening stages (except (*E*)-2-hexenal in ripe fruits), reporting high amounts. Meanwhile, these volatile compounds decrease their content during fruit ripening, once that the fruits are passing from yellow-green to red (Figure 1), according to the maturation degree.

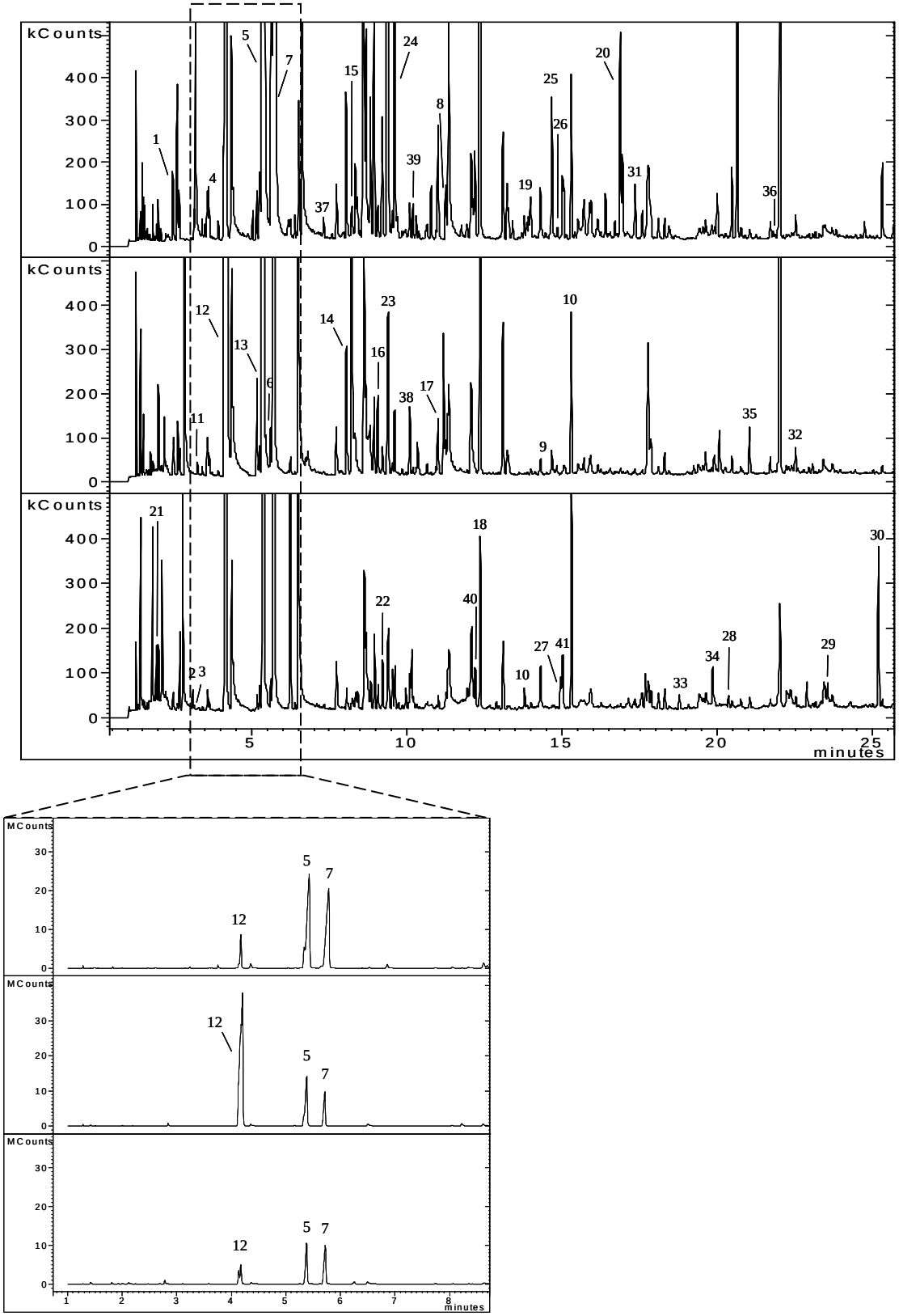


Figure 2. Chromatographic profile of *Arbutus unedo* fruits presenting different ripening stages, by HS-SPME using divinylbenzene/PDMS fiber. Identification numbers correspond to those in Table 1.

Alcohols are the major chemical class present in all three maturation stages, with their amount decreasing as the maturation of the fruit advance (Table 1). However, the relative abundance of these compounds does not follow the same trend (Figure 3A). When passing from the unripe to the intermediate maturation, the relative abundance decreases, from 82.9% to 61.7%. When reaching full ripeness, the abundance of this chemical group reaches its maximum, with an abundance of 87.6%. Belonging to this chemical class, 10 compounds were identified, including the major component of the volatiles of *A. unedo* fruits, (*Z*)-3-hexen-1-ol. The higher amount of this compound was found in the unripe fruits (32105±1747), decreasing in the two following maturation stages, to 13183±2054 and 8119±1052, in the fruits presenting intermediate and ripe maturation, respectively (Table 1). This volatile compound was also identified in the study of volatile profile of strawberries and their derivate products (Barron and Etiévant, 1990; Hakala *et al*., 2001; Hamilton-Kemp *et al*., 1996). 1-Hexanol is also present in high amounts (from 15425±148 to 5284±824), following the same tendency of (*Z*)-3-hexen-1-ol, with its quantity decreasing as the maturation of the fruit advances. Two other alcohols, 1-penten-3-ol and (*E*)-2-octen-1-ol present the same behaviour, with decreasing quantities along the maturation. Some compounds, like (*Z*)-2-penten-1-ol, are present in the unripe stage of maturation, after which are not detected, when the fruits are in the intermediate ripening stage, to reappear again, when the analysed samples were ripe. 1-Nonanol follows the inverse trend, only being detected in the intermediate stage of maturation. 3-Methyl-1-butanol and 2-methyl-1-butanol were only detected in ripe fruits, while the (*E*)-2-decen-1-ol amount is the only one that increases with the passing of the maturation stages.

Aldehydes represent the second major group of volatile compounds identified from *A. unedo*. Ten compounds belonging to this chemical class were identified, being hexanal the most abundant. Aldehydes reach the highest amount when the fruits were analysed with intermediate maturation, presenting, in this situation, two and twelve times the amount present in unripe and ripe fruits.

Table 1. Volatile profile of *Arbutus unedo* fruits under different ripening stages.

Chemical classes	Compound	RT (min)	QI (*m/z*)[a]	ID[b]	A/1000 ± SD[e]		
					Unripe	Intermediate	Ripe
Alcohols							
1	1-Penten-3-ol	2.473	57/67	MS[c] (86.4/92.0)	78.8 ± 44.8	51.3 ± 1.7	41.3 ± 9.3
2	3-Methyl-1-butanol	3.102	55/70	S[d]/MS (78.3/80.1)	n. d.	n. d.	27.7 ± 8.6
3	2-Methyl-1-butanol	3.158	56/70	S/MS (68.5/86.5)	n. d.	n. d.	1.6 ± 0.0
4	(*Z*)-2-Penten-1-ol	3.632	57/68	MS (85.4/87.5)	57.7 ± 9.8	n. d.	14.4 ± 1.8
5	(*Z*)-3-Hexen-1-ol	5.396	57/67/82	S/MS (85.8/87.6)	32105 ± 1747	13183.3 ± 2054.5	8119 ± 1052
6	(*E*)-2-Hexen-1-ol	5.622	41/57/67	MS (87.9/91.4)	345.6 ± 38.4	119.4 ± 69.4	45.8 ± 2.5
7	1-Hexanol	5.721	56/69	S/MS (82.5/82.9)	15425 ± 148	9997 ± 131	5284 ± 824
8	(*E*)-2-Octen-1-ol	11.253	57/67/81	S/MS (81.3/83.3)	100.1 ± 40.6	n. d.	n. d.
9	1-Nonanol	14.299	41/55/70	MS (81.7/86.5)	n. d.	24.6 ± 6.7	n. d.
10	*(E)*-2-Decen-1-ol	15.293	67/81/95/109	S/MS (86.7/87.4)	188.9 ± 34.1	193.6 ± 55.6	229.1 ± 4.4
			Σ of Alcohols		**48301.1**	**23569.2**	**13762.9**
Aldehydes							
11	(*E*)-2-Pentanal	3.400	55/83	MS (81.4/83.5)	27.8 ± 1.8	12.1 ± 0.6	n. d.
12	Hexanal	4.220	41/55/67/82	S/MS (80.1/81.5)	2720 ± 81	12708 ± 5066	887 ± 25
13	(*E*)-2-Hexenal	5.317	41/55/69/83	S/MS (82.4/89.8)	860.3 ± 16.2	287.9 ± 67.6	n. d.
14	(*Z*)-2-Heptenal	8.045	55/70/83	MS (87.5/91.4)	192.4 ± 13.3	219.5 ± 54.6	16.7 ± 1.9
15	Benzaldehyde	8.229	77/105	S/MS (83.0/87.9)	73.4 ± 6.6	n. d.	n. d.
16	(*E*,*E*)-2,4-Nonadienal	8.949	57/95	S/MS (81.5/85.5)	58.8 ± 17.4	25.0 ± 7.2	9.2 ± 1.0
17	(*E*)-2-Octenal	11.005	70/93	S/MS (86.3/87.1)	69.8 ± 11.5	29.8 ± 4.4	5.1 ± 0.3
18	Nonanal	12.359	57/81/95	S/MS (85.0/87.4)	1149 ± 582	127.4 ± 52.2	157.2 ± 25.6
19	(*E*)-2-Nonenal	13.986	43/55/70/83	S/MS (81.3/85.0)	70.7 ± 20.6	9.7 ± 1.3	n. d.
20	(*E*)-2-Decenal	16.873	70/83	S/MS (85.8/89.5)	198.0 ± 27.8	n. d.	n. d.
			Σ of Aldehydes		**5420.2**	**13419.4**	**1075.2**
Esters							
21	Acetic acid, ethyl ester	1.926	43	S/MS (76.9/83.8)	n. d.	n. d.	81.7 ± 15.3
22	Hexanoic acid, ethyl ester	9.211	43/73/88	S/MS (78.0/85.0)	n. d.	n. d.	53.8 ± 10.2
23	Acetic acid 4-hexen-1-yl ester	9.398	43/67/82	MS (87.3/87.7)	3172 ± 404	967.0 ± 40.3	224.3 ± 44.5
24	Acetic acid, hexyl ester	9.606	43/73/88	S/MS (86.8/87.5)	647.2 ± 79.7	318.1 ± 19.9	100.9 ± 2.4
25	Butanoic acid, 4-hexen-1-yl ester	14.670	43/67/82	MS (89.3/90.0)	447.2 ± 112.5	212.2 ± 46.4	n. d.

26	Butanoic acid, hexyl ester	14.857	43/71/89	MS (76.0/82.2)	27.3 ± 7.9	n. d.	n. d.
27	Salicylic acid, methyl ester	14.944	92/120/152	S/MS (88.5/90.6)	n. d.	n. d.	70.0 ± 27.6
28	Decanoic acid, ethyl ester	20.358	88/157	S/MS (73.4/80.1)	n. d.	n. d.	9.6 ± 1.3
29	Dodecanoic acid, methyl ester	23.542	74/87	MS (73.1/82.8)	n. d.	n. d.	27.7 ± 3.2
30	Dodecanoic acid, ethyl ester	25.184	73/88	MS (78.6/78.9)	n. d.	n. d.	137.3 ± 9.0
			Σ of Esters		**4293.7**	**1497.3**	**705.3**
Norisoprenoid derivates							
31	Ionone	17.348	93/121/177	MS (78.7/79.0)	67.8 ± 11.3	8.7 ± 0.2	9.0 ± 0.2
32	β-Ionone	22.515	177	S/MS (86.6/87)	51.4 ± 26.5	33.6 ± 2.3	5.4 ± 1.7
			Σ of Norisoprenoid derivates		**119.2**	**42.3**	**14.4**
Sesquiterpenes							
33	α-Gurjunene	18.767	161/189/204	MS (81.1/83.3)	n. d.	n. d.	12.7 ± 0.6
34	Sesquiterpene-like compound	19.848	189/204	MS (74.1/82.5)	n. d.	n. d.	13.1 ± 1.5
35	Caryophyllene	21.031	91/133	S/MS (85.3/86.6)	n. d.	52.6 ± 27.3	5.7 ± 0.8
36	(*Z*)-β-Farnesene	21.802	41/69/93	MS (80.3/84.9)	5.2 ± 0.8	n. d.	n. d.
			Σ of Sesquiterpenes		**5.2**	**52.6**	**31.5**
Monoterpenes							
37	1-R-α-Pinene	7.324	93	MS (73.5/77.4)	8.9 ± 8.6	n. d.	n. d.
38	Limonene	10.094	67/93	S/MS (86.2/82.1)	42.6 ± 2.2	36.7 ± 8.9	20.5 ± 1.2
39	Eucalyptol	10.199	81/93/139	S/MS (83.3/86.5)	27.5 ± 2.0	8.0 ± 1.2	n. d.
40	Linalool	12.195	71/93	S/MS (76.3/83.9)	n. d.	n. d.	47.4 ± 6.3
41	α-Terpineol	15.014	59/81/93/121	S/MS (83.4/87.6)	n. d.	n. d.	84.0 ± 9.3
			Σ of Monoterpenes		**79.0**	**44.7**	**151.9**

n. d. – not detected; RT – Retention Time (minutes)

[a]Quantification ions; [b]Identification method (fit/retrofit values, %); [c]Tentatively identified by NIST 05; [d]Identified by comparison with reference compound; [e]Area expressed as arbitrary units, S.D. = standard deviation of three assays.

The relative percentage (Figure 3A) of aldehydes presents the same behaviour as their total amount, increasing from unripe to intermediate maturation, and then showing a decrease when the ripe fruits are analysed. Hexanal and (*Z*)-2-heptenal also showed that their presence increases from unripe to intermediate fruits, followed by a decline in their quantities in ripe fruits. Five compounds ((*E*)-2-pentanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-nonadienal, (*E*)-2-octenal and (*E*)-2-nonenal) present the same trend, with their quantity decreasing with the maturation of the fruits, and, in two cases ((*E*)-2-pentanal and (*E*)-2-hexenal) completely disappear in the ripe fruits. Benzaldehyde, nonanal and (*E*)-2-decenal were only present in the unripe fruits. This trend in the amount of aldehydes has already been observed, by Robertson *et al*. (1995). When evaluating the changes of the volatile composition of the red raspberry (*Rubus idaeus*), it was noticeable that the relative abundance of these compounds increase when the fruit passes from green to pink, and them suffer a decrease in their amount when passing to red fruits.

Esters were the third chemical group present in high amounts. Ten compounds were identified belonging to this group, with acetic acid 4-hexen-1-yl ester demonstrating to be the major one. The total amount of these compounds decreases with the ripening of *A. unedo* fruits, going from 4293.7 in the unripe state, to 1497.3 and 705.3 in the intermediate and ripe stages of maturation, respectively. The same pattern can be described for the main ester found, acetic acid 4-hexen-1-yl ester, whose quantities go from the initial 647.2±79.7, when testing unripe fruits, to 100.9±2.4 when using ripe fruit. Regarding the relative abundance of these compounds, their highest value is reached in the unripe fruits, decreasing in the following stage, presenting a increase of their abundance in the ripe fruits (Figure 3A). Two other compounds are present in higher quantities in the unripe fruits, decreasing for the two other stages of maturation: acetic acid hexyl ester and butanoic acid 4-hexen-1-ylester. In the case of ethyl ester, salicylic acid methylester, decanoic acid ethyl ester, dodecanoic acid methyl ester and dodecanoic acid ethyl ester, all these esters are only present in the ripe fruit, becoming acetic acid 4-hexen-1-yl ester the major ester when the fruit present the last stage of maturation.

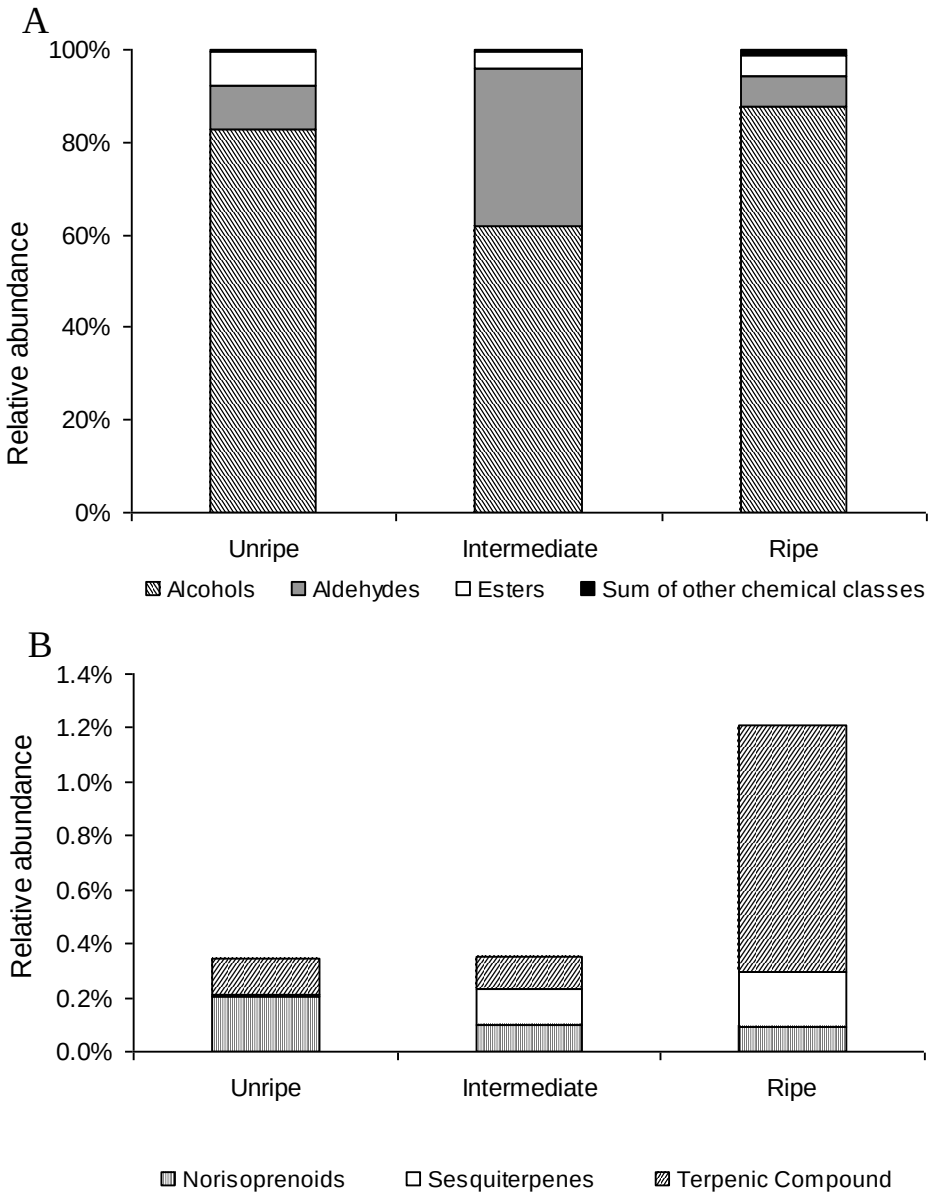


Figure 3. Changes in the relative abundance of the chemical classes identified with the increasing maturation of *Arbutus unedo* fruits (the sum of other chemical classes correspond to those represented in Figure 3B).

The most abundant volatile compounds identified are C6 compounds, considered GLV. Belonging to the three above mentioned chemical classes, those compounds are also known to be produced when leaves or fruits are crushed or otherwise injured (Matsui, 2006), and are synthesised via the lipoxygenase (LOX) pathway from C18 polyunsaturated fatty acids including linoleic acid and linolenic acids (Figure 4).

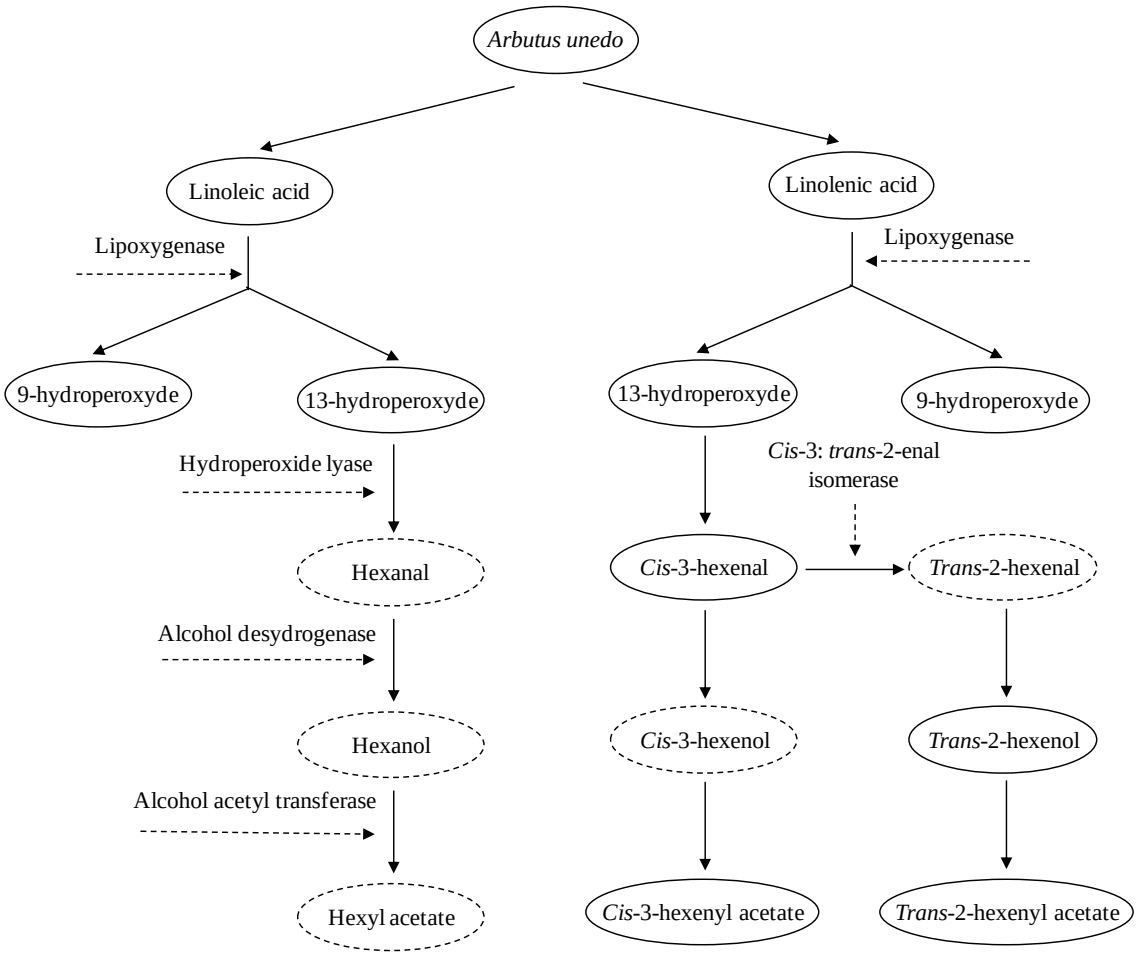


Figure 4. Volatile formation in *Arbutus unedo* by the lipoxygenase pathway. The most abundant volatiles in the fruits are marked as dotted circles and enzymes with dotted arrows.

With the rise of LOX activity, the hydroperoxidation of linoleic and linolenic acids occur leading to the formation of two groups of compounds: the 9-hydroperoxides and 13-hydroperoxides (Feussner and Wasternack, 2002). Meanwhile, LOX shows regiospecificity for the Δ-13 position for both linoleic and linolenic fatty acids, yielding 75-90% of Δ-13 fatty acid hydroperoxides. Even more, the LOX enzyme is more active in linolenic acid than linoleic acid (Salas *et al*., 1999) which can explain the biogenesis of six-carbon unsaturated volatile compounds, which are the main and most abundant volatile compounds in *A. unedo* fruits during ripening. It is known that LOX activity is lower in ripe strawberries, rather than in unripe or turning ones, although, in some conditions, in turning fruits presents is maximum of activity (Leone *et al*., 2006). This fact may account for the results obtained for alcohols, aldehydes and esters, whose formation is dependent of the LOX pathway. Furthermore, another study (Ménager *et al*., 2004) also conducted using strawberries, showed that relative proportions of C6

volatile compounds decreases as the maturation of the fruit reaches its full ripeness. These C6 volatile compounds are the major constituents of the most abundant chemical classes: (*Z*)-3-hexen-1-ol is the most important alcohol, while hexanal is the main aldehyde (Table 1). This work also refers the same behaviour of the esters, to the one observed in our work, appearing these compounds especially after the fruit had reached an advanced stage of maturation. Once formed, both hydroperoxide forms are cleaved by hydroperoxide lyase (HPL) forming volatile aldehydes and oxoacids. C6 aldehydes and C12 ω-oxoacids are formed from the 13-hydroperoxides of linoleic and linolenic acid. From 9-hydroperoxides are formed C9 aldehydes and C9 ω-oxoacids. Hexanal is the main C6 volatile compound formed from 13-hydroperoxides of linolenic acid, and (*Z*)-3-hexenal is formed from linolenic acid. These compounds are reduced to alcohols by the action of alcohol dehydrogenase, forming respectively hexanol and (*Z*)-3-hexenol (leaf alcohol), two of the most abundant alcohols identified. (*Z*)-3-Hexenal also suffers the action of cis-3 and trans-2-enal isomerase, forming one of the most abundant aldehydes in the unripe and intermediate stages of *A. unedo*, (*E*)-2-hexenal (leaf aldehyde). Once formed, alcohols are esterified by the action of alcohol acetyl transferase that catalyses the formation of acetate esters trhough acetyl-CoA derivates (Kalua *et al*., 2007). (*Z*)-3-Hexenol and hexanol form respectively (*Z*)-3-hexenyl acetate (leaf ester) and hexyl acetate, being this last one of the most abundant ester compounds in the early stages of maturation process.

All the other chemical classes are present in very low amounts, less than 0.91% (Figure 3B). It is noticeable that norisoprenoid derivates decrease their presence as the maturation increases. This pattern was also found in Monastrell grapes (Salinas *et al*., 2004), where the amount of β-ionone, one of the norisoprenoids found in *A. unedo*, decreases with the progress of the fruit ripening, and furthermore, the total amount of these compounds was found to decrease in white Portuguese grapes, with the ripening of the fruits (Coelho *et al*., 2007). This reduction in the amount is due to the possibility that these compounds may suffer glycosylation during ripening of the fruit (Nunes *et al*., 2008). Sesquiterpenes and monoterpenes show inverse behavior; while the first chemical class increases their amount from unripe to intermediate fruits, after which their presence is lower, monoterpenes quantities reduces from unripe to intermediate maturation, presenting in the ripe stage the highest quantity. Sesquiterpenes quantities are also known to show the same behavior in other fruits, like in Kensington Pride' mango fruit (Lalel *et al*., 2003), while Robertson *et al*. (1995) proved that monoterpenes

quantity decrease from green to pink fruits of Rubus idaeus, increasing when the fruits become red. In *Myrtus communis* var. *italica*, monotepenes also show variation with the increase of the maturation of the fruits, with a decrease when in intermediate stage of maturation, followed by a raise in the amount of these compounds when ripe (Aidi Wannes *et al*., 2009). Although the presence patterns of these two chemical classes can be found elsewhere, the large majority of fruits present in their volatile composition higher amount of mono- and sesquiterpenes when fully ripe. These differences between the amounts present of such compounds may be accounted by the specific composition and the expression of terpene synthases, the enzymes playing the key role in the biosynthesis of those compounds (Sharon-Asa *et al*., 2003). Terpenes and sesquiterpenes, if present in quantities that make them perceptible, are associated to sweet and flowery odors (Coelho *et al*., 2006), which are detectable when smelling the ripe berries of *A. unedo*. Although not present in high amounts, when comparing to the other chemical classes, such as alcohols, aldehydes and esters, the decrease of the amounts of the referred compounds, which were covering all the other odors, may allow the perception of those sweet and flowery scents.

The identified volatiles show a great influence in the aromatic perception of the fruits of *A. unedo*. The GLV's give the unripe berries the usual green scent, while terpenes and sesquiterpenes may largely contribute to the sweet and flowery aroma that the ripe fruits of this shrub produce.

5.4. CONCLUSIONS

Alcohols are the main volatile compounds found in the three ripening stages, followed by aldehydes and esters. During the ripening stages, the amounts of the mentioned chemical classes decreased. The majority of the most abundant volatile compounds identified, (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, (*E*)-2-hexenal and acetic acid 4-hexen-1-yl ester also decrease during ripening. These results may be related to the reduction of the activity of LOX enzymes during ripening, responsible for the formation of the main volatile compounds identified in the fruits of *A. unedo*. Volatile profile of *A. unedo* fruits is also composed by minor compounds belonging to chemical classes as norisoprenoids derivates, sesquiterpenes and monoterpenes. Some of these compounds are only present when the fruits are fully ripe. With the reduction of alcohols, aldehydes and ester compounds, the perceptible green sensations associated to this kind of volatile compounds are replaced by flower and sweet attributes, given by some of the minor compounds identified in the ripe fruits.

To the author's knowledge, this is the first known report of the volatile profile of the *A. unedo* fruits.

5.5. REFERENCES

Aidi Wannes, W., Mhamdi, B., Marzouk, B. 2009. Variations in essential oil and fatty acid composition during *Myrtus communis* var. *italica* fruit maturation. Food Chem. 112, 621-626.

Alarcão-E-Silva, M., Leitão, A.E.B., Azinheira, H.G., Leitão, M.C.A. 2001. The Arbutus Berry: Studies on its Color and Chemical Characteristics at Two Mature Stages. J. Food Compos. Anal. 14, 27-35.

Ayaz, F., Kucukislamoglu, M., Reunanen., M. (2000). Sugar, Non-volatile and Phenolic Acids Composition of Strawberry Tree (*Arbutus unedo* L. var. ellipsoidea) Fruits. J. Food Compos. Anal. 13, 171-177.

Barron, D., Etiévant, P.X. 1990. The volatile constituents of strawberry jam. Z Lebensm Unters Forsch. 191, 279-285.

Bianchi, F., Careri, M., Musci, M. 2005. Volatile norisoprenoids as markers of botanical origin of Sardinian strawberry-tree (*Arbutus unedo* L.) honey: Characterization of aroma compounds by dynamic headspace extraction and gas chromatography–mass spectrometry. Food Chem. 89, 527-532.

Coelho, E., Rocha, S., Delgadillo, I., Coimbra, M. 2006. Headspace-SPME applied to varietal volatile components evolution during *Vitis vinifera* L. cv. 'Baga' ripening. Anal. Chim. Acta. 563, 204-214.

Coelho, E., Rocha, S., Barros, A., Delgadillo, I., Coimbra, M. 2007. Screening of variety- and pre-fermentation-related volatile compounds during ripening of white grapes to define their evolution profile. Anal. Chim. Acta. 597, 257-264.

Cornara, L., La Rocca, A., Marsili, S., Mariotti, M. 2009. Traditional uses of plants in the Eastern Riviera (Liguria, Italy). J. Ethnopharmacol. 125, 16-30.

de la Fuente, E., Sanz, M., Martínez-Castro, I., Sanz, J., Ruiz-Matute, A. 2007. Volatile and carbohydrate composition of rare unifloral honeys from Spain. Food Chem. 105, 84-93.

El-Hilaly, J., Hmammouchi, M., Lyoussi, B. 2003. Ethnobotanical studies and economic evaluation of medicinal plants in Taounate province (Northern Morocco). J. Ethnopharmacol. 86, 149-158.

Feussner, I., Wasternak, C. 2002. The lipoxygenase pathway. Annu. Rev. Plant Biol. 53, 275-297.

Guedes de Pinho, P., Gonçalves, R. F., Valentão, P., Pereira, D. M., Seabra, R. M., Andrade, P. B., Sottomayor, M. 2009. J. Pharm. Biomed. Anal. 49, 674-685.

Hakala, M., Ahro, M., Kauppinen, J., Kallio, H. 2001. Determination of strawberry volatiles with low resolution gas phase FT-IR analyzer. Eur. Food Res. Technol. 212, 505-510.

Hamilton-Kemp, T.R., Archbold, D.D., Loughrin, J.H., Collins, R.W., Byers, M.E. 1996. J. Agric. Food Chem. 44, 2802-2805.

Jensen, K., Christensen, L.P., Hansen, M., Jørgensen, U., Kaack, K. 2001. Olfactory and quantitative analysis of volatiles in elderberry (*Sambucus nigra* L.) juice processed from seven cultivars. J. Sci. Food Agric. 81, 237-244.

Kahriman, N., Albay, C., Dogan, N., Usta, A., Karaoglu, S., Yayli, N. 2010. Volatile Constituents and Antimicrobial Activities from Flower and Fruit of *Arbutus unedo* L. Asian J. Chem. 22, 6437-6442.

Kalua, C.M., Allen, M.S., Bedgood, D.R., Bishop, A.G., Prenzler, P.D., Robards K. 2007. Olive oil volatile compounds, flavour development and quality: A critical review. Food Chem. 100, 273-286.

Kessler, A., Baldwin, I. 2002. Plant responses to insect herbivory: the emerging molecular analysis. Annu. Rev. Plant Biol. 53, 299-328.

Lalel, H., Singh, Z., Tan, S. 2003. Aroma volatiles production during fruit ripening of 'Kensington Pride' mango. Postharvest Biol. Technol. 27, 323-336.

Leone, A., Bleve-Zacheo, T., Gerardi, C., Melillo, M., Leo, L., Zacheo, G. 2006. Lipoxygenase Involvement in Ripening Strawberry. J. Agric. Food Chem. 54, 6835-6844.

Leonti, M., Casu, L., Sanna, F., Bonsignore, L. 2009. A comparison of medicinal plant use in Sardinia and Sicily—De Materia Medica revisited? J. Ethnopharmacol. 121, 255-267.

LLusià, J. Peñuelas, J. 2000. Seasonal Patterns of terpene content and emission from seven Mediterranean woody species in field conditions. Am. J. Bot. 87, 133-140.

Maarse, H. 1991. Volatile Compounds in Foods and Beverages, Illustrated Edition, CRC Press.

Matsui, K. 2006. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr. Opin. Plant Biol. 9, 274-280.

Ménager, I., Jost, M., Aubert, C. 2004. Changes in Physicochemical Characteristics and Volatile Constituents of Strawberry (Cv. Cigaline) during Maturation. J. Agric. Food Chem. 52, 1248-1254.

Nunes, C., Coimbra, M., Saraiva, J., Rocha, S. 2008. Study of the volatile components of a candied plum and estimation of their contribution to the aroma. Food Chem. 111, 897-905.

Owen, S., Boissard, C., Street, R., Duckham, S., Csiky, O., Hewitt, C. 1997. Screening of 18 Mediterranean plant species for volatile organic compound emissions. Atmos. Environ. 31, 101-117.

Özcan, M., Haciseferoğullari, H. 2007. The strawberry (*Arbutus unedo* L.) fruits: Chemical composition, physical properties and mineral contents. J. Food Eng. 78, 1022-1028.

Pallauf, K., Rivas-Gonzalo, J.C., Castillo, M.D., Cano, M.P., Pascual-Teresa, S. 2008. Characterization of the antioxidant composition of strawberry tree (*Arbutus unedo* L.) fruits. J. Food Compos. Anal. 21, 273-281.

Pawlowska, A., De Leo, M., Braca, A. 2006. Phenolics of *Arbutus unedo* L. (Ericaceae) Fruits: Identification of Anthocyanins and Gallic Acid Derivatives. J. Agric. Food Chem. 54, 10234-10238.

Pedro, J. (1994). Carta da distribuição de figueira e medronheiro - Notícia Explicativa. Ministério do Ambiente e Recursos Naturais, Direcção Geral do Ambiente. Lisboa.

Peñuelas, J. Llusià, J. 2001. Seasonal Patterns of non-terpenoid C6-C10 VOC emission from seven Mediterranean woody species. Chemosphere, 45, 237-244.

Poll, L. Lewis, M.J. 1986. Volatile components of elderberry juice. LWT. 19, 258-262.

Robertson, G., Griffiths, D., Woodford, J., Birch, A. 1995. Changes in the chemical composition of volatiles released by the flowers and fruits of red raspberry (*Rubus idaeus*) cultivar Glen Prosen. Phytochemistry. 38, 1175-1179.

Salas, J.J., Williams, M., Harwood, J.L., Sanchez, J. 1999. Lipoxygenase activity in olive (*Olea europaea*) fruit. J Am Oil Chem Soc. 76, 1163-1168.

Salinas, M., Zalacain, A., Pardo, F., Alonso, G. 2004. Stir Bar Sorptive Extraction Applied to Volatile Constituents Evolution during *Vitis vinifera* Ripening. . Agric. Food Chem. 52, 4821-4827.

Sharon-Asa, L., Shalit, M., Frydman, A., Holland, D., Or, E., Lavi, U., Lewinsohn, E., Eyal, Y. 2003. Citrus flavor and aroma biosynthesis: isolation, functional

characterization, and developmental regulation of Cstps1, a key gene in the production of the sesquiterpene aroma compound valencene. Plant J. 36, 664-674.

Simonetti, M., Damiani, F., Gabrielli, L., Cossignani, L., Blasi, F., Marini, F., Montesano, D., Maurizi, A., Ventura, F., Bosi, A., Damiani, P. 2008. Characterization of triacylglycerols in *Arbutus unedo* L. seeds. Ital. J. Food Sci. 20, 49-56.

Soufleros, E., Mygdalia, S., Natskoulis, P. 2005. Production process and characterization of the traditional Greek fruit distillate ‘‘Koumaro’’ by aromaticand mineral composition. J. Food Compos. Anal. 18, 699-716.

Ziyyat, A., Boussairi, E., (1998). Cardiovascular effects of *Arbutus unedo* L. in spontaneously hypertensive rats. Phytother. Res. 12, 110-113.

Capítulo 6
Discussão geral e conclusões

6.1. DISCUSSÃO GERAL E CONCLUSÕES

O medronheiro, especialmente as suas folhas e os seus frutos, são ricos em diversos compostos de interesse, quer medicinal, quer industrial. Apesar disso, esta planta não tem tido a atenção que lhe é devida. Quando comparando com outros produtos de origem vegetal, existem escassos estudos sobre as características e propriedades dos extractos de folhas e frutos de medronheiro. Assim, este trabalho permitiu aprofundar o conhecimento sobre estas matrizes, numa tentativa de valorização desta planta.

As folhas, para além de apresentarem na sua composição uma quantidade considerável de fenóis totais, possuem também uma notável capacidade para bloquear radicais livres, bem como para inibir a formação do radical superóxido, um dos mais importantes no processo oxidativo em células. Esta capacidade de reduzir a actividade dos radicais livres está associada à presença de compostos fenólicos, que possuem comprovada actividade antioxidante. Este facto reveste-se de elevada importância, devido à evidência de que estes radicais estão envolvidos na génese de várias doenças, tais como cancro, doenças coronárias e degenerativas. A extracção de compostos com capacidade antioxidante foi mais efectiva quando se usou etanol como solvente de extracção, relativamente a extractos aquosos, metanólicos ou usando éter dietílico.

Os medronhos apresentam também características de interesse, nomeadamente de capacidade bloqueadora de radicais livres, tendo na sua composição uma quantidade importante de compostos fenólicos. Aplicando uma metodologia de extracção usando etanol a 96% durante 30 minutos a frutos apresentando diferentes estados de maturação, foi visível, que quando os frutos se apresentam num estado intermédio de maturação, os valores de EC_{50} foram mais baixos, enquanto neste estado o teor em fenóis totais foi superior. Foi ainda possível obter uma correlação com significado estatístico entre o estado de maturação que os frutos apresentavam e a sua capacidade antioxidante. Estes frutos são também especialmente ricos em ácidos gordos ómega-3 e vitamina E, o que aumenta o seu valor biológico.

A avaliação dos compostos voláteis permitiu pela primeira vez identificar e quantificar estes compostos nos frutos de medronheiro. Foram identificados 41 diferentes compostos voláteis, pertencentes a 6 grupos químicos. Destes, os presentes em maior quantidade, nos três estados de maturação avaliados, foram os álcoois, seguidos pelos aldeídos e esteres. Os compostos maioritários foram o (*Z*)-3-hexen-1-ol,

1-hexanol, hexanal, (*E*)-2-hexanal e o ácido acético 4-hexen-1-yl ester. Os quatro primeiros compostos, conhecidos pelo seu cheiro característico a verde, juntamente com (*E*)-hexen-1-ol serão os responsáveis pelo cheiro a fresco e verde que os medronhos em estados iniciais de maturação apresentam. Durante a maturação do fruto foi visível um decréscimo da quantidade de voláteis emitidos pelos frutos, muito provavelmente devido à diminuição da actividade das enzimas envolvidas na síntese destes compostos. Para além dos compostos maioritários já referidos, os medronhos possuem também na sua composição outros grupos químicos de compostos voláteis, como norisoprenoides, sesquiterpenos e monoterpenos. Estes, apesar de presentes em baixas quantidades, quando comparados com os grupos maioritários, estão associados a aromas florais e doces. Estes odores tornam-se perceptíveis quando o fruto se encontra completamente maduro, devido a diminuição dos teores dos voláteis associados ao cheiro verde, bem como devido à presença de compostos minoritários apenas nos medronhos maduros.

Este trabalho permitiu uma caracterização de folhas e frutos do medronheiro, quer ao nível do potencial antioxidante, bem como da composição química. A avaliação da actividade antioxidante das folhas, a um nível detalhado, bem como da composição em voláteis dos frutos, são trabalhos inovadores, tendo sido efectuados pela primeira vez no decurso deste trabalho prático. Os resultados obtidos mostram que o *A. unedo* é uma árvore com potencial para ser uma fonte de compostos bioactivos contra os radicais livres, bem como de vitaminas, importantes componentes da alimentação humana. Existe ainda a possibilidade de aumentar o conhecimento sobre o valor desta árvore, efectuando estudos semelhantes ao apresentados neste trabalho, usando como matrizes as raízes e cascas do medronheiro, já descritas como possuindo propriedades medicinais, usadas na medicina popular, bem como possivelmente explorando a mais-valia que as flores poderão encerrar.