UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

Valorization of wild olives (*Olea europaea* var. *sylvestris*) as potential source of functional ingredients

Janina Soraia Góis Diogo

DISSERTAÇÃO

Mestrado em Biologia Humana e Ambiente

2013

UNIVERSIDADE DE LISBOA

FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA ANIMAL

Valorization of wild olives (*Olea europaea* var. *sylvestris*) as potential source of functional ingredients

Janina Soraia Góis Diogo

Mestrado em Biologia Humana e Ambiente

Dissertation supervised by Dr Catarina Duarte, PhD and Dr Ana Viegas-Crespo, PhD

2013

Prof. Dr Maria Rosário Bronze, PhD, Associate Teacher, Head of Department of Toxicological Sciences and Bromatology, Pharmacy Faculty, Lisbon University, co-advised all the analytical component of this work.

Acknowledgments

Eu gostaria de agradecer a todas as pessoas que, directa ou indirectamente, me apoiaram e contribuiram para a realização deste trabalho, e sem as quais o mesmo não teria sido possível.

À minha orientadora, Dr Catarina Duarte, por me ter “acolhido” no laboratório, por ter confiado em mim e nas minhas capacidades, e por me ter ensinado imenso ao longo destes meses, um muito obrigado.

À Dr Rosário Bronze, por me ter guiado no desenvolvimento do meu trabalho e por estar sempre disponivel para responder às minhas dúvidas. E tal como a Dr Catarina, obrigado por terem partilhado toda a experiência a nível da investigação.

À professora Dr Ana Maria Viegas-Crespo, não só por ter aceito ser a minha orientadora interna, mas também por ter partilhado a toda a sua experiência durante as aulas. Obrigado.

À Sandra Silva, por ter sido uma referência a nível de conhecimento da matriz, e por me ter auxiliado em todo o procedimento de preparação da mesma.

Ao professor Dr Luis Vilas-Boas, por partilhar toda a sua sabedoria e experiência na investigação.

Ao Dr João Ferreira, agradeço a incansável explicação e apoio que me deu nas análises por HPLC-DAD/MSMS.

À Andreia, sem dúvida o meu braço direito, e o esquero também, em cada dia de trabalho. Pelo companheirismo, amizade, e por acreditar em mim enquanto investigadora, um GRANDE obrigado.

Ao Mário, obrigado por toda a paciência disponibilizada nos ensaios de actividade antioxidante, assim como, ao longo de cada dia de trabalho.

Às meninas da Química Analitíca, Joana, Maria e Elsa, agradeço o apoio em toda de análise química. E, pelo excelente ambiente de trabalho que me proporcionaram.

A todo o grupo do laboratório Nutraceuticals and Controlled Delivery, Joana, Inês, Vanessa, Cátia, Daniel, Agostinho, Susana, Bárbara, Sara e Liliana, obrigado pelo companheirismo, amizade e pelo excelente convivio de cada dia. Um especial obrigado à

Ana Nunes Nunes, pelas horas dedicadas, pela paciência, e pelo grande apoio que foi durante a realização dos ensaios de extracção supercritica.

À Dr Teresa Serra, por ser sempre receptivel, pelas boleias e partilha de conhecimento, obrigado. Agradeço igualmente à Dr Ana Nunes, pelo interesse demonstrado e apoio no meu trabalho.

À professora Dr Deodália Dias e à professora Dr Teresa Rebelo, pelo grande apoio nas minhas decisões e por acreditarem em mim como cientista.

Aos meus amigos desde sempre, João V. Pais, Patricia, Vanessa e Nicola pelos momentos de descontracção que me porpocionaram, e por estarem sempre disponiveis, sendo sempre vocês.

Ao Jorge, por ao longo deste tempo todo estares sempre ao meu lado, sendo paciente, amigo e o meu grande apoio nesta “viagem”.

Aos meus pais, por me permitirem viver aquilo em que acredito, apoiando-me sempre.

À minha avó, por todos os valores que me passa a cada dia, e pelo colo que me dá.

Aos meus tios e primos, pelos bons momentos em familia.

Abstract

The olives and olive oil are known to have many health-promoting effects, having been associated to a decrease in the risk of some diseases, due the presence of polyphenols, which have several bioactivities, mainly antioxidant capacity.

The wild olives tree, zambujeiro, which belongs to the Mediterranean forest, survives harsh environmental changes. It is expected that, similarly to the common olive tree, its fruits will have a composition that reflects that adaptation capacity.

In this work, extracts of wild and cultivar olive seeds and stones were characterized, regarding their phenolic content. Seed extracts were prepared using solvent mixtures and analyzed for the total phenols concentration (TPC) and by HPLC-DAD/MSMS. Methanol proved to be the best solvent for the extraction of these compounds. The phytochemical composition of the seed extracts yielded high TPC, mainly seicoiridoids.

Extracts of zambujeiro olive stones were prepared using three different methodologies (MeOH at reflux, refined olive oil, and conventional solvents). Extraction by methanol at reflux yielded a richer composition, when compared to the other two procedures, and was the only method that showed lignan compounds, such as Ac-Pr and OH-Pr. All the extracts showed a high TPC.

Antioxidant activity assays using the seed extracts showed a good scavenging activity against the peroxyl radical, whereas the stone extracts displayed a higher scavenging activity against hydroxyl radicals, while also preventing their formation.

The extracts obtained from the seeds revealed an antiproliferative effect in colon cancer cell line HT29, unlike to the extracts from cultivars' varieties. This assay was made in parallel by a co-laboratory.

In case this method is integrated as a compound recovery system from wild olives, it would require an initial 2-3 hour extraction with sc-CO_2,. The delipidated matix must then be extracted with PLE+25:65:10 (CO_2: EtOH: H_2O), during 15 min (incubation) plus 30 minutes.

Keywords: wild olives, phenols, lignans, antioxidant activity, high pressure technologies.

Resumo

As azeitonas e o azeite são conhecidos pelos seus efeitos benéficos na saúde. Como alimentos integrantes da dieta Mediterrânica, o seu consumo tem sido associado à baixa incidência de doenças cardiovasculares na região da bacia Mediterrânica. Esses efeitos benéficos têm sido associados não só ao conteúdo em gorduras monoinsaturadas, mas também à presença de uma fracção minoritária da qual constam os polifenóis. Os polifenóis são metabolitos secundários das plantas e a sua bioactividade tem sido referida através do seu efeito antimicrobiano, antiproliferativo, anti-envelhecimento, hipocolesterolémico, e o mais estudado, o seu efeito antioxidante.

Existe uma variedade de oliveira selvagem pertencente à flora Mediterrânica, comumente designada por zambujeiro (*Olea europaea*, subsp. *oleuropaea* var. *sylvestris*), e que também vive durante séculos. Tal como a oliveira comum (*Olea europaea* L.), também produz azeitonas. Sendo uma árvore que se adapta a grandes oscilações de temperatura e diferentes composições de solos, será de esperar que a sua azeitona reflicta essas adaptações através da sua composição fitoquímica.

No presente trabalho, as sementes e os caroços das azeitonas provenientes do zambujeiro, foram caracterizados pela primeira vez. Para a caracterização das sementes foram preparados extractos convencionais com diferentes misturas de de modo a investigar qual o melhor para a extração de compostos a partir dessa matriz, e a fim de comparar esses extractos com extractos de sementes de azeitonas provenientes de cultivares, nas mesmas condições de extracção. Depois, foi determinado o conteúdo total de fenóis e realizada a análise por HPLC-DAD/MSMS. Os extractos com metanol foram os melhores para a extracção dos compostos presentes nas sementes. Os valores de fenóis totais obtidos nos extractos das sementes do zambujeiro, foram elevados e similares aos obtidos pelos extractos das cultivares. A composição fitoquímica dos extractos das sementes é maioritariamente constituida por compostos seicoiridoids, tendo como composto maioritario o nuzenido.

Prepararam-se extractos de caroços do zambujeiro com três diferentes metodologias de forma a testar qual a melhor para extrair os compostos a aprtir dos caroços, que são um material maioritariamente hemicelulósico e linhoso. De entre as três metodologias aplicadas, a extracção com metanol a refluxo acima dos 65°C, sendo o processo mais agressivo, permitiu obter extractos com maior abundância de compostos. Os extractos obtidos a partir desta metodologia, foram os únicos nos quais foi possivel extrair compostos linhanos, como o Ac-Pr e o OH-Pr. Os extractos revelaram elevado teor de fenóis.

A bioactividade determinada por ensaios de actividade antioxidante (ORAC, HORAC E HOSC) a partir dos extractos de sementes revelou uma boa capacidade de resgate do radical peroxil. Por outro lado, os extractos de caroços tiveram melhor actividade contra o radical hidroxil (OH), tanto no seu resgate como na inibição da sua formação. Estes resultados poderão ser indicadores de uma diferente composição química entre a semente e o caroço.

O possível efeito antiproliferativo dos extractos de sementes das diversas cultivares, e do zambujeiro, foi testado numa linha celular de cancro do cólon colon cancer cell line HT29) através de um ensaio realizado em paralelo por uma colega de laboratório. Apenas o extracto de sementes de zambujeiro testado revelou uma capacidade antiproliferativa contra as células HT29, contrariamente aos extractos das variedades de cultivares.

Palavras-Chave: zambujeiro, fenóis, linhanos, actividade antioxidante, tecnologias de extracção a alta pressão.

Index

List of figures

List of tables

List of abbreviations, acronyms and symbols

Abbreviation	Full form
AAPH	2”, 2” –Azobis (2- amidinopropane) dihydrochloride
Ac-Pr	1 - acetoxypinoresinol
AFE	Accelerated fluid extraction
Ar	Argon
BHA	Butylated hydroxyl anisol
BHT	Butylated hydroxyl toluene
$C_6H_5NO_2$	Picolinic acid
$C_9H_8O_4$	Caffeic acid
CH_3CN	Acetonitrile
CH_2Cl	Dichloromethane
CoF_2	Cobalt floride tetrahydrate
CO_2	Carbon dioxide
DAD	Diode array detection
DNA	Deoxyribonucleic acid
ESE	Enhanced solvent extraction
Et2O	Diethyl ether
EtOH	Ethanol
$FeCl_3$	Iron (III) chloride
FL	Disodium fluorescein
GAE	Gallic acid equivalents
GRAS	Generally Recognized As Safe
HO	Hydroxyl radical
HO_2	Hydroxyperoxyl radical
H_2O_2	Hydrogen peroxide
H_2O	Water
HOCl	Hypochlorous
HORAC	hydroxyl Radical Scavenging Capacity
HOSC	Hydroxyl Radical Adverting Capacity
HPLC	High performance liquid chromatography
HPSE	High pressure solvent extraction
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
L	Liter

MeOH	Methanol
MS	Mass spectrometry
Na_2CO_3	sodium carbonate solution
NaCl	Sodium chloride
$NaH_2PO_4.H_2O$	Sodium dihydrogenphosphate
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic dehydrates
O_2^-	Superoxide radical
OH-Pr	Hydroxypinoresinol
O_2	Single oxygen
ORAC	Oxygen Radical Absorbance Capacity
PBS	Phosphate buffer solution preparation
PFE	Pressurized fluid extraction
PLE	Pressurized liquid extraction
Pr	Pinoresinol
PSE	Pressurized solvent extraction
ROS	Reactive oxygen species
RT	Retention time
sc-CO_2	Supercritical carbon dioxide
SCE	Supercritical extraction
SCF	Supercritical fluid
SPB	Sodium phosphate buffer
SFE	Supercritical Fluid Extraction
SFT	Supercritical Fluid Technology
TCA	Total chromatographic areas
TEAC	Total equivalents acid caffeic
TPC	Total phenols content
UV	Ultraviolet light

1 State of the Art

1.1 Nutraceuticals and Functional Ingredients

In the past few years, with the developments in biological and chemical sciences, and due to the cost for the treatment of chronic diseases, the main focus of medicine has switched from treatment to prevention (Kwak & Jukes, 2001).

Plant-derived foods such as fruits, vegetables, legumes, whole grains, species, etc., appear to exert some beneficial effects in preventing the onset of some diseases. The direct impact of food on health has been observed in epidemiological studies, by relating the dietary habits and the risk of disease (Espín et al., 2007). Because of this, two new concepts appeared in the market: nutraceuticals and functional foods (Poejo, 2011). The term "nutraceuticals" was first used in 1989 by Stephen DeFelice, MD, as a hybrid term combining the words "nutrients" and "pharmaceutics" (Brower, 1998). Nutraceuticals are defined as diet supplements that deliver a concentrated form of a presumed bioactive agent (Espín et al., 2007). They are currently marketed in a range scale (Mahabir, 2013), in designs similar to those of drugs (pills, tablets, extracts, etc.) (Poejo, 2011). Another concept - "functional food" - was born in Japan in the 80's (Plaza et al., 2009). It is applied when it is satisfactory demonstrated that a food beneficially affects one or more functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or a reduction of the risk of disease. (Plaza et al., 2009; Espín et al., 2007).

The beneficial effects that some plant-derived foods have in reducing the risk of chronic diseases have been associated with the presence of secondary-metabolites - phytochemicals. Among the most common phytochemicals, polyphenols (anthocyanins, proanthocyanidins, flavonols, hydroxycinnamates, coumarins, ellagic acid and ellagitannins, isoflavones, lignans, etc.) are currently used as bioactive compounds, found in the nutraceuticals' and functional foods' markets (Espín et al., 2007).

Polyphenols have been studied in different food matrices, such as fruits, vegetables, nuts, seeds and beverages. Apples, berries, tea, cocoa, coffee, wine, chocolate, olive oil, olives and onions are common known sources of polyphenols in the human diet (Motilva & Macià, 2013) with bioactivities ranging through anticarcinogenicity, antimutagenicity, antiallergenicity, antiaging and antioxidant activity (Moure et al., 2001).

1.2 Antioxidant Activity

The interest in substances that exhibit antioxidant proprieties has been increasing (Brahmi et al., 2012). Polyphenol-rich foods, which act as dietary sources of antioxidants have received much attention due to their physiological effects on human health (Hayes et al., 2011).

Antioxidant activity is one of the most studied properties of polyphenols, which exhibit strong antioxidant activity by scavenging different families of reactive oxygen species (ROS). The ROS comprehends free radicals such as superoxide radical ($O_2^{-\bullet}$), hydroxyl radical ($HO^{\bullet}$), hydroperoxyl radical ($HO_2^{\bullet}$), peroxyl radical ($ROO^{\bullet}$) and alkoxyl radical ($RO^{\bullet}$), but also non-radical compounds, namely hydrogen peroxide (H_2O_2), single oxygen ($^1O_2$), and hypochlorous acid (HOCl) (Fink, 2002).

Oxidative stress results from the oxygen consumption during cell growth and leads to the generation of oxygen free radicals – oxygen-containing atoms or molecules capable of independent existence while possessing one or more unpaired electrons. Free radicals are involved in different biological functions, namely signal transduction, neurotransmission, smooth muscle relaxation, peristalsis, platelet aggregation, blood pressure modulation, immune system control, fagocytosis, learning and memory, production of energy, the regulation of cellular growth and signaling, the synthesis of important biological compounds and the metabolism of xenobiotics (Gomes et al., 2005). In case of overproduction or exhaustion of antioxidants, they can become deleterious. The reactive species can cause oxidative stress, which leads to cellular damage through the oxidation of bio-molecules, such as the lipids of cellular membranes, tissue proteins or enzymes, carbon hydrates and DNA. This damage may become irreversible, after a certain point (Gomes et al., 2005). Accumulated scientific evidence indicates that oxidative stress, namely the one resulting from reactive oxygen species $ROO^{\bullet}$, $HO^{\bullet}$, $O_2^{-\bullet}$ and $^1O_2$, is implicated in the pathophysiology or contributes to numerous degenerative conditions, including carcinogenesis, cardiovascular disease, inflammation, Alzheimer's disease, Parkinson's disease, diabetes, and aging (Davis et al., 2010).

Mechanisms involved in the association of ROS and oxidative stress with the development of diseases may include an alteration of important bio-molecules, such as oxidative modification of proteins, oxidation of lipids, strand breaks and modification of nucleic acids, modulation of gene expression through activation of redox-sensitive transcription factors, and modulation of inflammatory responses through signal transduction (Davis et al., 2010).

Antioxidants are responsible for neutralizing the ROS or for preventing their production in the first place (Gomes et al., 2005). Hence, recent research has been aiming at the identification of novel antioxidants from natural sources, such as fruits and vegetables, wine, tea, cocoa, olives and extra-virgin olive oil (Robards, 2003).

1.3 Procedures for the extraction of phytochemicals

Functional ingredients are commonly extracted from natural sources, such as plants, food industry by-products or marine microorganisms (Herrero et al., 2006). In order to obtain the new functional ingredients to be used by the food industry, selective and efficient extraction techniques are required (Plaza et al., 2009).

Various conventional extraction processes have been applied for the extraction of bioactive compounds from complex matrices (Shilpi et al., 2013). These conventional separation techniques, such as solvent and distillation extractions, have several drawbacks (Shilpi et al., 2013; Herrero et al., 2006) - they are known to leave trace amounts of organic solvents, and/or to cause thermal degradation of the interest compounds (Shilpi et al., 2013) and to have low selectivity and/or low extraction yields. Furthermore, they are time consuming, laborious, and need large amounts of solvents (Herrero et al., 2006).

A good alternative to the conventional extraction processes should be sustainable, with reduced energy requirements and low environmental impact (Ciftci, 2012). Two alternatives in the food industry have been the high pressure techniques, supercritical fluid technology (SFT) and pressurized solvent extraction (PSE). Both have been used to prepare samples and extract a wide variety of bioactive compounds from natural products.

SFT is mainly used to isolate bioactive non-polar compounds (e.g. from oils and dairy products, and algae and microalgae). However, not only non-polar bioactive compounds are extracted using supercritical fluids. The addition of small amounts of modifiers allows to obtain fractions enriched in polar compounds (e.g. phenolic compounds). The majority of the pressurized liquid solvent (PLE) developed applications are aimed at the extraction of contaminants from natural, food and environmental samples, but have also been proved useful in the extraction of bioactive compounds from natural matrices, such as anthocyanins, phenolic acids, flavonoids, or alkaloids. PLE has been widely compared to other extraction techniques for extraction of bioactive compounds and, generally, provides better results than these more conventional techniques (Herrero et al., 2013).

1.3.1 Principles of Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) consists of using solvents at temperatures above their boiling point, under high pressure in order to increase the extraction efficiency, with respect to extraction time, solvent consumption and extraction yields (Serra et al., 2010).

A pure component is considered to be in a supercritical state if its pressure and temperature are higher than the critical values (Pc and Tc respectively) **(Figure 1)** (Serra et al., 2010; Herrero et al., 2006).

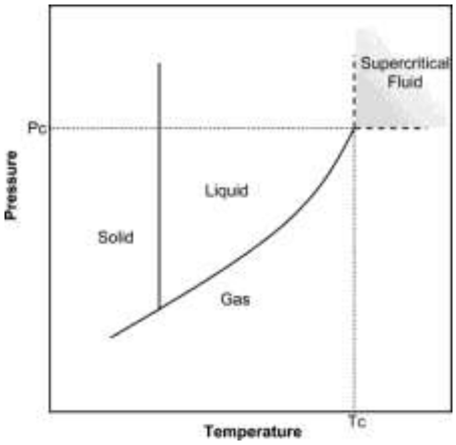

Figure 1 - Typical phase diagram for a pure compound (Herrero et al., 2006).

Under these conditions, many proprieties of the fluid stand between those of a gas and those of a liquid: while the density of a supercritical fluid is similar to that of a liquid, its viscosity is similar to that of a gas and its diffusivity is intermediate between both states (Herrero et al., 2006). The main feature of a supercritical fluid is that in the supercritical region liquid and gas are indistinguishable from each other, or as a state in which the fluid is compressible (i.e. similar behavior to a gas) even though possessing a density similar of a liquid and, therefore, similar solvating power (Azmir et al., 2013; Herrero et al., 2006). Supercritical fluids have low viscosity and relatively high diffusivity, which allows an easier transport than liquids, and can diffuse easily through solid material, therefore, favoring mass transfer. SFE is operated at room temperature (or close), so and it’s an ideal method for the extraction of thermolabile compounds (Azmir et al., 2013).

Another favorable property of supercritical fluids is that their selectivity is higher than liquid solvents, as their solvation power can be tuned by changing either the temperature and/or pressure, which allows to obtain determined target compounds in the extract by modulating these proprieties (Ciftci, 2012).

1.3.1.1 CO_2 as a supercritical fluid (sc-CO_2)

CO_2 is the standard solvent of choice for use in SFE with a critical point at 31.2ºC and 7.38 MPa (Shilpi et al., 2013). This is due to its characteristics: it's non-toxic, non-inflammable and inexpensive (Shilpi et al., 2013; Herrero et al., 2006), and it's a gas at room temperature, which allows supercritical operations at relatively low pressure and temperature, therefore being suitable for extracting thermolabile natural compounds. The extraction system provides a light- and oxygen-free environment, therefore minimizing the degradation and preserving the bioactive and antioxidant proprieties of extracts (Serra, 2009; Herrero et al., 2006).

Extracts from supercritical treatments with CO_2 are recognized as entirely natural, and all the products that are allowed for food applications have the denomination "Generally Recognized as Safe" (GRAS) (Shilpi et al., 2013).

Despite its advantages, CO_2 is less effective in extracting more polar compounds from natural matrices, due to its nonpolar and lipophilic nature and its inability to extract compounds of higher molecular weight (Shilpi et al., 2013; Herrero et al., 2006). In order to increase the solubility of such compounds in supercritical carbon dioxide and also to enhance the selectivity of the extraction, modifiers (also called co-solvents) may be added (Ciftci, 2012). Co-solvents such as ethanol, methanol, hexane, acetone, chloroform and water, are polar compounds that, when added in small amounts, can change the solvent proprieties of neat supercritical CO_2 (Shilpi et al., 2013; Herrero et al., 2006).

1.3.2 Pressurized Liquid Solvent (PLE)

Pressurized liquid solvent (PLE) may be known by several different names: pressurized fluid extraction (PFE), accelerated fluid extraction (AFE), enhanced solvent extraction (ESE) and high pressure solvent extraction (HPSE) (Serra et al., 2010).

The concept of PLE refers to the application of high pressure to maintain a solvent in a liquid phase, beyond their normal boiling point. High pressure and temperatures provide a faster extraction and consequently, decrease the solvent requirement (Azmir et al., 2013). The extraction high temperature can promote higher analyte solubility, by increasing both the solubility and the mass transfer rate. Furthermore, it can also decrease the viscosity and the surface tension of solvents, thus improving the extraction rate.

PLE involves the use of H_2O and/or organic solvents at considerable temperatures (40-200°C) and pressures (3.3-20.3 MPa) for extraction of compounds (Seabra et al., 2012). PLE can represent an attractive option to extract polar compounds from organic pollutants, bioactive compounds from marine sponges, and from different food matrices (Seabra et al., 2012).

Mass transfer and solvating proprieties can be improved by using gas-expanded liquids (usually obtained upon the dissolution of CO_2 in water or in an organic solvent). Furthermore, the utilization of a mixture of two or three miscible solvents, with different polarities and with different abilities to establish specific interactions with the target compounds, is a procedure that can enhance the selectivity for target compounds from plant matrices (Seabra et al., 2012).

Solvents with different polarities such as CO_2, ethanol (EtOH) and H_2O can be mixed in different proportions, which allows to obtain homogeneous solvent mixtures with polarities that go from the one of the less polar CO_2 to the one of the most polar H_2O (Seabra et al., 2012).

1.4 Phytochemical analysis methodologies

The preparation of natural extracts is commonly used to screen natural matrices for bioactive compounds. General screening of compounds is made, in order to determine total phenol content. The standard methodology for this is the colorimetric Folin-Ciocalteau assay. The Folin-Ciocalteau reagent is not specific and detects all phenolic groups found in the extracts. This method is based on the reducing power of phenolic hydroxyl groups, which, upon reaction with the Folin-Ciocalteau reagent under basic conditions, leads to the formation of phenolate anion, through the dissociation of a proton. The phenolate ion reduces the reagent to form a blue complex that has an absorption maxim at 765 nm. Simple phenols have absorption maximum between 220 and 280 nm wavelengths.

In order to analyze the phenolic content in natural extracts, high performance liquid chromatography (HPLC) technique is widely applied, for both the separation and quantification of these compounds (Naczk & Shahidi, 2004). The separation of different classes of phenolic compounds is achieved through the introduction of a reverse phase column, which enhances the process. Usually, diode array detector (DAD) is used for food phenols detection. HPLC coupled with mass spectrometry (MS) has commonly been used for structural characterization of phenols. Electrospray ionization mass spectrometry (ESI/MS) has been employed for the structural confirmation of phenols in peaches, nectarines, olives, grape seeds, cocoa, olive oil, etc (Naczk & Shahidi, 2004). The extracts are screened and some retrieved parameters, such as retention time (RT), UV spectra, MS data are analyzed and compared against those of known compounds, using commercial and in-house databases (Butler, 2004).

The antioxidant activity from natural extracts can and must be evaluated with different tests. Most of the chemical methods are based on the ability to scavenge different free radicals. Tests measuring the scavenging activity with different challengers, such as superoxide radical ($O_2^{\cdot}$) and hydroxyl radical ($OH^{\cdot}$) have been developed (Moure et al., 2001). The Oxygen Radical Absorbent Capacity (ORAC) assay measures the ability of the antioxidant species present in the samples to inhibit the oxidation of disodium fluorescein (FL), catalyzed by peroxyl radicals. Many reports have investigated the implementation of ORAC to natural extracts from food matrices, such as cherries, olives and oil seeds. In HORAC assay, a sample's capacity to prevent hydroxyl radical production is evaluated using FL as the probe. This assay also measures the scavenging capacity of antioxidants present in the samples, against hydroxyl radicals. There are few studies that apply the HORAC and HOSC assays to evaluate the antioxidant activity from natural matrices (Price et al., 2006).

2 Olives as a source of bioactive compounds

2.1 Olive tree (*Olea europaea* L.)

Olive trees are the most extensively cultivated fruit crop in the world, particularly widespread throughout the Mediterranean Basin region (Matos *et al.*, 2010; Loumou & Giourga, 2003). The olive tree that is present along the Mediterranean is designated *Olea europaea*, subspecies *europaea*, which includes the wild variety (*Olea europaea*, subsp. europaea, var. *sylvestris*) and cultivars variety (*Olea europaea*, subsp. europaea, var. europaea). It belongs to the botanic family Oleacea, subfamily *Oleideae*, which comprises other species such as the jasmine (*Jasminum fruticans* L.), the ash (*Fraxinus excelsior* L. and *F. augustifolia* L.), and the mastic (*Phillyrea angustifolia* L.) (Esteve et al., 2012).

The olive tree (*Olea europaea* L.) is the main species of the botanic family *Oleacea* to be cultivated, in order to obtain the pulp and oil of its fruit, the olive (Gulfraz, 2006), while it is the only one in this family known to produce edible fruits (Esteve et al., 2012). The *Oleacea* family includes approximately 30 genera and 600 species (Silva et al., 2010), which spread throughout Europe, Asia, Oceania and Africa (Bracci *et al.,* 2011).

The olive tree (*Olea europaea* L.) is one of the most economically relevant species, from those cultivated in the Mediterranean basin. In 2005, 96% of the world production of olives occurred in this region (MADRP, 2007). Currently, there are over 2000 cultivars of a huge diversity along the Mediterranean (Bartolini et al. 2002).

Olea europaea subsp. *europaea* var. *sylvestris* is commonly designated as zambujeiro. Although, zambujeiro contributes to the Mediterranean forest, it seems well adapted to hard environments such as cold, salt, drought, poor soils, and high temperatures (Chiappetta & Muzzalupo, 2012; Loureiro et al., 2007). As far as we know, there are no published reports focusing on the wild olive fruit characterization. Since the common olives from cultivated olive trees are mainly used in the production of olive oil and table olives, it might be relevant to study the wild variety in terms of its phytochemical composition and compare it to the common cultivar olives. We were unable to find any records regarding the distribution of zambujeiro trees in Portugal, or in other Mediterranean countries.

Phenols content and antioxidant activity of solvents and SFE extracts from zambujeiro olive leaves were investigated in Greece, by Lafka et al. (2013). Different solvents were tested and ethanol was found to be the most effective solvent, since its extract exhibited the highest antiradical activity, among other solvents and SFE extracts. In addition, the ethanol extract also showed a higher antioxidant capacity, when compared to that of synthetic and natural food antioxidants, such as butylated hydroxytoluen (BHT) and vitamin E (Lafka et al., 2013).

2.1.1 Bioactive compounds present in olives

The phenolic compounds are one of the most widely occurring groups of phytochemicals (Prosek & Golc, 2005). They are secondary plant metabolites, and they have an important role in plant growth and reproduction, providing an efficient protection against pathogens and predators, while also contributing to the color and sensory characteristics of fruits and vegetables (Ignat et al., 2011).

The phenolic compounds are one of the most widely occurring groups of phytochemicals (Prosek & Golc, 2005). They are secondary plant metabolites, and they have an important role in plant growth and reproduction, providing an efficient protection against pathogens and predators, while also contributing to the color and sensory characteristics of fruits and vegetables (Ignat et al., 2011).

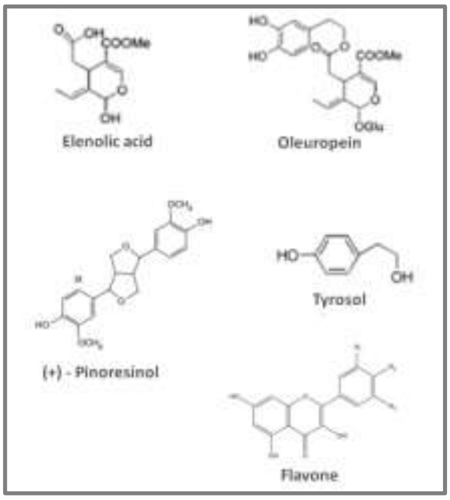

Figure 2 - Phenol compounds structures. Some of the compounds presents in a phenolic fraction of olive oils and fruits. Adapted from Ryan et al. (2002) and Robards et al., (1999).

There are many reports that refer an important role of natural phenols in the protection against some diseases, such as atherosclerosis, brain dysfunction and cancer (Ignat et al., 2011), and in the modulation of pathways associated with inflammation, enzyme induction and their ability to modulate oxidative stress (Andjelkovic et al., 2006; Martinez-Dominguez et al., 2001). Furthermore, polyphenols have many industrial applications: they have been reported as having excellent proprieties as food preservatives, and may be used as natural colorants in foods, or in the production of paints, paper or cosmetics (Ignat et al., 2011).

Olive tree cultivars provide two byproducts – olive oil and table olives – which are an important part of the Mediterranean diet. They have a high content of phytochemicals (Tofalo et al., 2012). Along the centuries, the potential beneficial effects of olive oil for health have been recognized, especially in Mediterranean region. Therefore, the low incidence of cardiovascular diseases in this region has been attributed to the ingestion of olive oil, which is rich in monounsaturated fatty acids, mainly oleic acid, and to the presence of some minor components such as tocopherols, carotenoids and phenolic compounds (Esteve et al., 2012; Fabiani et al., 2006; Soler-Rivas et al., 2000). Table olives result from a natural fermentation process that confers them a more enjoyable taste. The worldwide demand for this product has been increasing, due to its sensorial, chemical and nutritional characteristics, as well as to its known high content in monounsaturated fatty acids and, although in smaller concentrations, tocopherols and phenolic compounds (Bautista-Gallego *et al.*, 2011).

The high concentration of phenolic compounds present in olives, particularly *ɵ*-diphenol oleuropein, is responsible for their unique flavor (Charoenprasert & Mitchell, 2012; Soler-Relvas et al., 2000). Olive fruits contain several groups of polyphenols:

flavonoids, phenolic acids, phenolic alcohols, seicoiridoids and lignans (Alagna et al., 2012; Ignat et al., 2011; Vinha et al., 2005). The phenol composition is different in each part of the fresh olive fruit (peel, pulp, stone or seed) (Silva et al., 2006), although, the overall content may be affected by several factors, such as the maturation period, cultivar and season (Charoenprasert and Mitchell, 2012). The phenolic acids are the less complex group found in olives (Ignat et al., 2011). The predominant phenolic acids found in olive leaves and seeds include caffeic, chlorogenic, ρ-coumaric, homovanillic, and vanillic acids (Ryan et al., 2002).

The olive pulp has been more extensively studied than the ones of the other olive tissues. In the pulp, the main phenolic compounds of its composition are oleuropein and hydroxytyrosol. hydroxytyrosol, tyrosol and their glycosidic forms (Silva et al., 2006). These compounds can also be found in other olive tissues, such as the seeds, and also in olive tree leaves (Charoenprasert & Mitchell, 2012). Hydroxytyrol has a cathecol moiety and is generated from the hydrolysis of oleuropein. Several studies reported it as being a potential antioxidant agent, with beneficial health effects, such as immunostimulation, antimicrobial activity, and inhibition of atherosclerotic plaques. Tyrosol, which is a metabolite of ligstroside (Charoenprasert & Mitchell, 2012), and hydroxytyrosol have been suggested to play a role in the prevention of oxidative damage, in cells (Brenes et al., 2000).

Oleuropein and ligstroside belong to a very specific group of coumaric-like compounds denominated seicoiridoids, which characterize the *Oleaceae* family (Silva et al., 2010; Soler-Rivas et al., 2000). These compounds are produced from the secondary metabolism of terpenes, as precursors of various alkaloids, and they are characterized by having a combination of elenolic acid or its derivatives, such as oleosides, in their structures (Silva et al., 2010; Silva et al., 2006).

The most common seicoiridoids in olives are oleuropein, ligstroside, demethyloleuropein and nüzhenide (Silva et al., 2010). Oleuropein is an ester consisting of hydroxytyrosol and elenolic acid, and is reported as the major phenolic compound in olive pulp and leaves, although it has been found throughout the fruit (peel, pulp and seed) (Soler-Rivas et al., 2000). Oleuropein is reported to have hypocholesterolemic and hypoglycaemic activities and to be a potent antioxidant with anti-inflammatory proprieties (Cardoso et al., 2005). In olive seeds, however, the main phenolic compound is nuzhenide (Silva *et al.*, 2010), which is rarely found in other tissues of olive. Olive seeds also contain other phenolic compounds, such as phenolic alcohols (hydroxytyrosol, tyrosol and their glucosides), phenolic acids (caffeic, chlorogenic, homovanillic, and vanillic acids) (Charoenprasert & Mitchell, 2012). In general, seicoiridoids play an important role in the prevention of atherosclerosis through the inhibition of low-density lipoprotein peroxidation.

There are reports that refer the anticancer activity by seicoiridoids and their contribution to the nutritional prevention of osteoporose (Alagna et al., 2012).

Lignans are another phenolic group that was found in olive oil and olives (Alagna et al., 2012). These are one of the major classes of phytosterols, which have a similar activity to estrogen, as well as antioxidant activity (Lowcock et al., 2013).

The demand for the identification of lignans has increased, due to recent studies which showed that lignans from several vegetal matrices, such as sesame and olive oil, are in vitro antioxidants and display high antioxidant capacities, similar to those of hydroxytyrosol and oleuropein derivatives (López et al., 2008). Many other biological activities of lignans were referred, for example, their chelating activity and protection against LDL oxidation. Furthermore, studies carried out in flaxseed, another relevant source of lignans, showed that these compounds have the capacity to inhibit cancerous cell growth in skin, breast, prostate, colon, and lung tissue. Due to their chemical similarity with estrogen, lignans are possibly able to bind to estrogen-receptors, which could eventually negatively affect the promotion of breast cancer (Lowcock et al., 2013).

The most common lignan compounds found among the phenolic compounds, in olive oil, are pinoresinol (Pr) and acetoxypinoresinol (Ac-Pr) (López et al., 2008). However these are rarely found in olive fruits being that only two research groups have cited the presence of lignans in the endocarp of the fruit of some Italian varieties (López et al., 2008; Bonoli et al., 2004).

2.1.2 Antioxidants from olives

Various studies reported the presence of phenols in olives and their beneficial effects in human health. These include antimicrobial (Obied et al., 2007), anti-viral, anti-ischemic, anti-inflammatory, hypolipidemic (Petti & Scully, 2009), cardioprotective (Covas, 2007), and antioxidant capacity (Cioffi et al., 2012). The higher antioxidant capacity of phenols, such as hydroxytyrosol and other seicoiridoids derivatives is apparently due to their o-dihydroxy structure (catechol) charactheristic (de Pinedo et al., 2007). Antioxidant proprieties of olive phenols, including oleuropein, hydroxytyrosol, tyrosol, apigenin-7-glucoside and luteolin-7-glucoside, are higher than typical antioxidants, such as vitamin C, E or BHT (Peralbo-Molina & de Castro, 2013).

Although the antioxidant activity of polyphenols from olive oil and leafs are backed by several studies, few refer the antioxidant activity or other biological effects of olive stone and seeds. It is possible that the aforementioned olive phenols' effects could also be observed in zambujeiro olive fruit.

2.1.3 High pressure processes to recover compounds from olives

As previously stated, the main phenolic compounds found in olive fruits are seicoiridoids, phenolic acids and alcohols, and lignans. As far as we know, the few studies on the extraction of seicoiridoids and lignans were mainly in olive fruits and using high pressure technologies. There is no published data, that we are aware of, describing the phytochemical content of zambujeiro olive fruits.

2.1.3.1 Extraction of seicoiridoids

Şahin and Bilgin (2012) studied the extraction of oleuropein from olive tree leaves, by means of SFE. Their aim was to evaluate the extraction yield and the extract oleuropein content, using SFE with pure CO_2, SFE with CO_2 modified by water and SFE modified by ethanol. The experimental results obtained by using sc-CO_2 alone were not satisfactory and showed no significant differences. Generally, the addition of a small amount of water enhanced the extraction efficiency significantly. By increasing the molar ratio of water, the amount of extract rises, especially at high temperatures. It was found that sc-CO_2 modified by water, at a flow rate of 1 ml/min and at dense conditions (300 bar, 100°C), sharply increased the solubility, when compared to the one obtained when using sc-CO_2 alone. However, during the experiments with high levels of water, the authors observed that, due to the high moisture content in plant material, the pores of the matrix became clogged. On the other hand, the experiments with low levels of water required too much time to soak the solid sample. Therefore, the overall extraction time rose significantly (Şahin & Bilgin, 2012).

No statistically significant differences were observed among the extract and oleuropein quantities at low temperatures (50°C), or between the values of the solvent system with 1 ml/min water flow rate, at the pressure points of 100 and 200 bar. At 100°C, the solvating power of the fluid system significantly increases, as previously observed by Floch et al., 1998, who studied the effect of the extraction temperature on the amount of phenols extracted from olive leaves at constant pressure (334 bar). Le Floch et al. (1998) observed that the extraction yield increase at 100°C.

At 300 bar and 100°C, CO_2 modified by water with a flow rate of 1 ml/min was found to give the best results with an extract yield of 233.68 mg/g dried leaf, and an oleuropein yield of 10.91 mg/g dried leaf (1.09%). Practically no oleuropein was obtained with pure CO_2. At the optimum conditions (300 bar and 100°C), the CO_2 modified by water yielded an extract nearly 11 times higher than that of the pure CO_2.

CO_2 modified by ethanol at 1ml/min flow rate and 300 bar was found to give the best result with the oleuropein yield of 2.90 mg/g dried leaf at 50°C, and an extract yield of

220.24 mg/g dried leaf at 100°C, nearly the same amount as the system including water as co-solvent. At the optimum extract obtaining conditions (100°C and 300 bar), the CO_2 modified by ethanol yielded an extract nearly 10 times higher than that of the pure CO_2. In case of the optimum oleuropein obtaining conditions (50°C and 300 bar), the CO_2 modified by ethanol yielded an oleuropein nearly 26 times higher than that of the pure CO_2. Water makes the matrix swell, which opens the pores, distorts the matrix-solute diffusion process (Le Floch, 1998).

2.1.3.2 Extraction of lignan compounds

The other interesting compounds present in olive oil are the lignans, which have a dibenzocyclooctadiene skeleton. The hydroxyl groups in lignans confer lower solubility than those compounds without such a group.

Choi and coworkers (1998) observed that optimum conditions for lignans extractions in *S. chinensis* (a Chinese plant with berries fruit) were determined at 60ºC and 340 bar. However, the lignans extraction yields showed no clear dependence on temperature or pressure: under 40ºC and 136bar the lignans could be sufficiently recovered (yield was 85% of that obtained under optimum conditions). These results indicate that the lignans can be extracted with sufficient efficiency under mild conditions which avoids possible degradation of components and decreases the process cost. At 80ºC and 130bar, the yields were much lower than under any other conditions. Possibly because the density of carbon dioxide under these conditions was distinctly lower than when under the other tested conditions. Regarding the extraction time, the authors observed that when the pressure increased, the extraction time was reduced.

Ethanol-water mixtures and carbon dioxide at high pressures have been used in the recovery of lignan compounds. Pasquini and coworkers (2005) described the extraction of lignans from *Pinus taeda* wood chips, by combining the use of ethanol-water mixtures and CO_2 at high pressures. Ethanol-water mixtures varied from 50 to 100% ethanol and the reaction times varied from 30 to 150 min. Most of the lignan removal occurred during the first 30 min of reaction. The effect of pressure (147 – 232 bar) and temperature (142 – 198°C) on the yield and recover of lignans was studied. The best results were obtained at 160 bar and 190°C, at which the yield and the lignan content retrieved from wood chips were 43.7 and 4.9%, respectively. Then, yields and lignan content showed, a much greater influence of temperature than pressure. The last set of experiments was the determination of the best ethanol-water ratio and its effect on extraction yield and lignan content. Ethanol content, ranged from 30 to 100%. The utilization of 100% of ethanol did not lead to extensive delignification. The addition of water promotes the delignification reactions but reduces the ability of the solvent to dissolve lignans. The results from the study of co-solvent composition demonstrated that the best compromise between the two requisites for a good delignification (nucleophilicity and solubility) was obtained with a 1:1 ethanol-water mixture.

3 Aims

The *Olea europaea* var. *sylvestris*, commonly known as zambujeiro tree, is widely spread throughout the Portuguese forest. However, the fruits from this tree are not used in the production of olive oil or table olives. It is a tree that adapts to high temperatures and different soils, so, it is possible that it has a rich phytochemical composition that allows such capacity. As far as we know, there are not studies focusing on that.

In this work we aimed at **1)** investigating the chemical composition of zambujeiro olive seeds, using different extractant solutions and comparing it with the ones from cultivar olive seeds, through adequate analysis and bioactivity determination methodologies; **2)** investigating the composition and presence of lignans in zambujeiro olive stones, using different methodologies and studying their antioxidant activity; **3)** developing a GRAS process to recover phytochemical compounds from zambujeiro olive fruits and investigating their antioxidant activity.

4 Materials and Methods

4.1 Chemicals

EtOH 96% (AGA, Lisbon, Portugal), water purified by means Milli-Q from Millipore (Molsheim, France) unit was used, distillation water (ITQB, Lisbon, Portugal),

For phytochemical characterization: Acetonitrile, methanol, acetone and EtOH were from Riedel-de-Häen (Seelze, Germany); phosphoric acid and Folin Ciocalteau reagent were acquired from Panreac (Barcelona, Spain). Water purified by means of Milli-Q from Millipore (Molsheim, France) unit. Sodium carbonate (Na_2CO_3) was purchased from Sigma-Aldrich (St Quentin Fallavier, France), gallic acid was purchased from Fluka (Germany); diethyl eter (Merck, Darmstadt, Germany), dichloromethane (Lab-Scan, Dublin, Ireland), oleuropein, tyrosol and luteolin-7-glucoside were purchased from Extrasynthese (Genay, France), hydroxytyrosol from Cayman (Michigan, USA), and caffeic acid was from Aldrich (Steinheim, Germany) Folin-Ciocalteau phenol reagent was purchased from Pronalab (Portugal) for total phenols quantification.

Chemicals used for antioxidant activity assays were: 2”, 2” - Azobis (2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox), caffeic acid ($C_9H_8O_4$), cobalt floride tetrahydrate (CoF_2), hydrogen peroxide (H_2O_2) and picolinic acid ($C_6H_5NO_2$) were purchased from Sigma-Aldrich (St Quentin Fallavier, France), Iron (III) chloride ($FeCl_3$) was acquired from Sigma-Aldrich (St Quentin Fallavier, France). Disodium fluorescein (FL) was from TCI Europe (Antwerp, Belgium). Sodium chloride (NaCl), potassium chloride (KCl) and monopotassium phosphate (KH_2PO_4) were from Sigma-Aldrich (St Quentin Fallavier, France) and sodium phosphate dibasic dehydrate ($Na_2HPO_4.2H_2O$) from Riedel-de-Häen (Seelze, Germany) were used for phosphate buffer solution preparation (PBS). Sodium phosphate dibasic dehydrates ($Na_2HPO_4.2H_2O$) and sodium dihydrogenphosphate ($NaH_2PO_4.H_2O$) from Riedel-de-Häen (Seelze, Germany) were used for sodium phosphate buffer (SPB).

Solvents used for high pressure extractions were: carbon dioxide (99,5%) from Praxair (Spain), ethanol (99,5%) from Panreac Quimica S.A. (Spain) and distilled water.

4.2 Preparation of extracts for phenolic compounds analyze

Olive seeds of six olive trees cultivars (*Olea europaea* L.) were provided by Sovena and studied: “Picual”, “Arbosana”, “Arbequina”, “Cobrançosa”, “Koroneiki” and from *Olea europaea* var. *sylvestris* commonly know by zambujeiro. The olives from zambujeiro olive tree were collected at the National Agronomic Station in Oeiras, Portugal.

The olive pulps were removed and the stones were broken, in order to remove the intact seed. The extraction was achieved as previously described (Silva *et al.*, 2010; Vinha *et al.*, 2005) with some modifications. Briefly, each sample of seeds (2 g) was milled and extracted by solid-liquid extraction with a mixture of a solvent: MeOH:water or EtOH:water or acetone:water (80:20,v/v) or water 100%. Samples were then thoroughly mixed by vortexer during 5 min and, then, centrifuged for 15 min at 5000 rpm (Eppendorf Centrifuge 5804R). After two successive extractions (2 x 10 ml), the supernatant was filtered and concentrated using a rotary evaporator and re-dissolved in the same solvent mixture, respective of each extraction. All samples were filtered through a 0.45 µm filter Acrodisc® (Pall, USA) and stored at -20ºC until analysis.

In order to characterize zambujeiro olives stone (*Olea europaea* var. *sylvestris*), the pulp was removed and the intact stone was broken and milled. Samples (20 g) were thoroughly mixed with one of the four aforementioned solvents (i.e. MeOH:water, EtOH:water, acetone:water and water 100%) on vortexer, during 5 min and then centrifuged for 15 min at 7000 rpm (Eppendorf Centrifuge 5804R). After two successive extractions (2 x 40 ml), samples results of the supernatant was filtered and concentrated by rotary evaporator and re-dissolved in one of the respective solvents.

4.3 Preparation of extracts for the analysis of lignan compounds

In order to determine the presence of lignan compounds in olive stones, we carried out two methods of conventional extraction (in duplicate): extraction with refined olive oil overnight and extraction with MeOH at reflux. For the first one, we left 5 g samples of milled olive stones overnight, in refined olive oil (10 g), with stirring in a 30ºC bath. In the next day, we extracted 2g aliquots by liquid-liquid extraction, with MeOH 100% (3 ml). Then, the samples were homogenized with a vortexer, for 15 min and centrifuged at 7000 rpm during 30 min. After two extractions (2 x 3 ml) the liquid obtained was dried under reduced pressure (30ºC), re-dissolved in MeOH 80% (2 ml) and filtered through a 0.45 µm filter Acrodisc® (Pall, USA), for chromatographic analysis.

The extraction with MeOH at reflux was carried out as described previously by (López et al., 2008) with some modifications. The extraction was carried out as summarized in Figure 3, at about 65ºC. Three aliquots (3 x 25 ml), each in duplicated, of the obtained methanol solution (final volume of 200 ml) were concentrated and re-dissolved in 5 ml of $MeOH/CH_3CN$/acidic H_2O (pH 2.5 by HCOOH) 6:2:2 (v/v/v) for the HPLC/DAD/MS analysis. The extraction procedure was also carried out with sesame seeds, to use their extract as a standard for the identification of lignans.

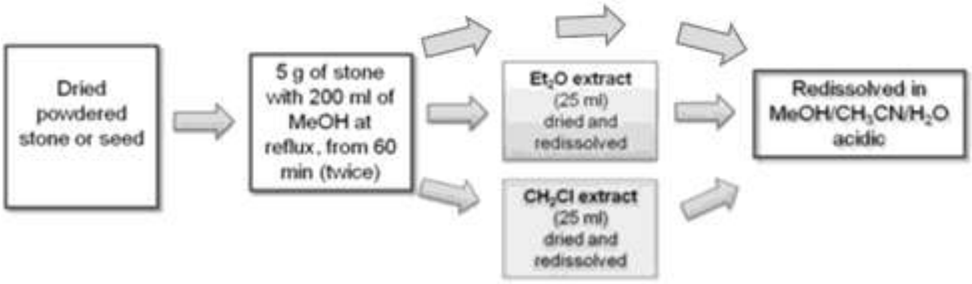

Figure 3 - Extraction with MeOH reflux at 65ºC adapted from López et al. (2008).

4.4 Phytochemical characterization

4.4.1 Phenols content

Folin-Ciocalteau

The total concentrations of phenolic compounds present in the extracts were determined using the Folin-Ciocalteau colorimetric method (Singleton & Rossi, 1965). Briefly, 3500 µl samples appropriately diluted in distilled water were oxidized with 100 µl of Folin-Ciocalteau reagent. The reaction was neutralized with 400 µl of sodium carbonate solution (Na_2CO_3 35%) and the samples were incubated at room temperature for 60 min. After that, the absorbance was measured at 765 nm wavelength, using a spectrophotometer (Beckman DU® - 70 spectrophotometer, USA). Gallic acid was used as standard (1 – 6 ppm) for the calibration curve, $y = 0.0842x + 0.0241$, $r^2 = 0.9997$. The results were means of triplicates (mg of gallic acid equivalents per litre of extract – mg GAE/L). The calibration curve obtained for TPC determination in stone extracts and in high pressure extracts was $y = 0.0011x + 0.0117$, $r^2 = 0.9997$ and $y = 0.0012x + 0.0042$, $r^2 = 0.9994$, respectively.

4.4.2 HPLC-DAD analysis

HPLC analysis of phenolic compounds was carried out using a surveyor apparatus from Thermo Finnigan, with a diode array detector (DAD) (Thermo Finnigan-Surveyor, San Jose, CA, USA). Separations were performed at 35ºC in a LiChrospher C18 (5 µm, 250 nm x 4 mm i.d.) column from Merck, with a guard column of the same type. The samples were injected using a 20 µl loop. Separations were carried out with a flow rate of 700 µl/min and the mobile phase consisted of a gradient mixture of eluent A (phosphoric acid (0.1%)) and eluent B (phosphoric acid (0.1%) – acetonitrile – water 5:400:595, v/v/v). The following eluents gradient was used: 0 – 15 min from 0 to 20% of eluent B; 25 – 70 min from 20 to 70% eluent B; 70 – 75 min with 70% eluent B; 75 – 85 min from 70 to 100% of eluent B; 85 – 90 min, with 100% eluent B. Diode array detection was performed between 200 and 800 nm wavelength. The data acquisition system used was the Chromequest version 4.0 (Thermo Finnigan – Surveyor, San Jose, CA, USA). Chromatograms recorded at 240 nm, 280 nm and 360 nm were used to analyze seicoiridoids, total phenols and flavonoids. Identification of compounds was done by comparing retention time (RT) and spectra.

In high pressure extracts, the analysis by HPLC-DAD was modified due to the fact that the compounds were only present in the end of the current method (see annex 1) previously applied for olive seeds and stones extracts. In order to get the compounds of interest to appear earlier and reduce the duration of the process, the eluents gradient was changed to: 0 min from 20 only eluent A; 20 – 40 min 44.4% eluent B; 40 – 56 min with 100% eluent B; 56 – 70 min with 100% of eluent A.

4.4.3 HPLC-DAD/MS/MS analysis

Some of the compounds present in the samples, mainly seicoiridoids, were identified by HPLC MS/MS as previously described (Silva et al., 2010). Briefly, the analysis were performed on a Waters® Alliance 2695 (Waters), equipped with a quaternary pump, solvent degasser, auto sampler and column oven, coupled to a Photodiode Array Detector Waters 996 PDA (Waters), with a detection range from 210 to 600 nm wavelengths. A pre – column (RP – 18, 5 µm) and reversed phase column (RP – 18 Synergy, 2.5 µm, 100 x 2.00 mm, Max – RP from Phenomenex, USA) operating at 35ºC were used for the separation. The mobile phase consisted of water/formic acid (A) (99:0.5, v/v) and acetonitrile (B) at a 0.30 ml/min flow rate and 5 µl injection volume. The eluting conditions applied were 10 minutes of a linear gradient from 99 to 98% of eluent A; 5 minutes of isocratic elution with 98% of eluent A; a linear gradient during 72 minutes from 98 to 70% of eluent A. The column was re-equilibrated with 99% of eluent A during 16 minutes. A triple quadrupole mass spectrometer MicroMass® Quattro micro (Micromass, Waters) outfitted with electrospray source ionization (ESI) was used in tandem. The source temperature was 120ºC, capillary voltage was 3.0 kV and cone voltage was 30 V. The compounds were ionized in negative mode and spectra of column eluate were recorded in the range of 100 – 1000 m/z. High purity nitrogen (N2) was used both as drying and as a nebulizing gas. Ultrahigh – purity Argon (Ar) was used as collision gas. For the MS/MS experiments, different collision energies (eV) were applied: 10 eV, 20 eV and 30 eV. MassLynx software was used for data acquisition and processing.

4.5 Antioxidant activity evaluation

In order to evaluate the antioxidant activity of the olive extracts three methodologies were used: 1) Oxygen Radical Absorbance Capacity (ORAC), to evaluate the antioxidant capacity of the samples towards peroxyl radicals; 2) Hydroxyl Radical Adverting Capacity (HORAC) to evaluate the hydroxyl radical prevention capacity of the sample and 3) hydroxyl radical (HO) scavenging capacity (HOSC).

4.5.1 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay measures the ability of the antioxidant species, present in the samples, to inhibit the oxidation of disodium fluorescein (FL) catalyzed by peroxyl radicals. This assay was carried out as previously described by Huang et al. (2002), modified for the FL800 microplate fluorescent reader (Bio – Tek Instruments, Winooski, VT, USA), as described by Feliciano et al. (2009).

Briefly, in a black 96-well microplate, 150 µl of disodium fluorescein (4 x 10^{-3} mM) and 25 µl of the appropriate sample dilution were added. Phosphate buffer (75 mM, pH 7.4) was used to prepare AAPH and FL solutions and was used as blank. Trolox solutions of 5, 10, 20, 30 and 40 µmol/L were used as control standards. The microplate was put in a fluorescent reader and allowed to incubate at 37ºC, for 10 minutes. The reaction was started with 25µl of AAPH, (153 mM) added through the injector. Fluorescence emitted by the reduced form of FL was measured using an FL800 microplate fluorescent reader (Bio-Tek Instruments, Winooski, VT, USA) and recorded every 1 minute, at 485 ± 20 nm excitation and 530 ± 25 nm emission wavelengths, for a period of 30 minutes. All samples, including the blank and the controls, were analyzed in triplicates. Final ORAC values were calculated by a regression equation between the trolox concentration and the net area under the FL decay curve and were expressed as trolox equivalents' antioxidant capacity per L of extract.

4.5.2 Hydroxyl Radical Adverting Capacity (HORAC)

The HORAC assay was based on the method reported by Ou et al. (2002), modified for the FL800 microplate fluorescence reader (Bio-Tek Instruments, Winooski, VT, USA), as described by Serra *et al.* (2010). In this assay, the hydroxyl radical prevention capacity of a sample was evaluated, using FL as the probe. The hydroxyl radical was generated by a Co(II)-mediated Fenton like reaction and, similarly to the previous assay ORAC, the fluorescence decay curve of FL was used to quantify the HORAC values. Briefly, 30 µl of appropriated dilutions of samples were added to 170 µl of FL (9.28 x 10^{-8} M). The reaction was started by the addition of 40 µl of H_2O_2 (0.206 M) to the mixture, plus 60µl of CoF_2 (1.15 mM), in a 96-well microplate, at 37ºC. Fluorescence emitted by the reduced form of FL was measured and recorded every 1 minute, during 60 minutes. The FL800 microplate fluorescence reader was used with fluorescence filters for 485 ± 20 nm excitation and 530 ± 25 nm emission wavelengths, and controlled by software Gen5. Caffeic acid solutions of 50, 100, 150, 200 and 250 µM were used as standards. The data was expressed as µmol of total caffeic acid equivalents (TEAC) per L of extract and all samples and blank were analyzed as triplicates.

4.5.3 Hydroxyl Radical Scavenging Capacity (HOSC)

The HOSC assay measures the scavenging capacity of antioxidant samples against hydroxyl radicals. The hydroxyl radical was generated by combining $FeCl_3$ with H_2O_2, as described by Moore et al. (2006), and measured using the FL800 microplate fluorescence reader. Briefly, 170 µl of disodium fluorescein (9.28 x 10^{-8} M), 30 µl of the appropriate sample dilution, 40µl of H_2O_2 (0.1990 M) and 60 µl of $FeCl_3$ (3.43 mM) were added, in a black 96-well microplate. Sodium phosphate buffer (75 mM, pH 7.4) was used to prepare FL solution. 5, 10, 15, 20 and 30 µmol/L Trolox solutions were used as control standards and were prepared with an acetone:water (50:50 v/v) solution. Fluorescence emitted by the reduced form of FL was measured in an FL800 microplate fluorescent reader (Bio-Tek Instruments, Winooski, VT, USA) and recorded every 1 minute at 485 ± 20 nm excitation and 530 ± 25 nm emission wavelengths, for a period of 60 minutes, at 37ºC. All samples, including the blank (acetone:water 50:50 (v/v) solution and the controls, were analyzed in triplicates. Final HOSC values were calculated by a regression equation between the Trolox concentration and the net area under the FL decay curve and were expressed as Trolox equivalents' antioxidant capacity per L of extract.

4.6 High pressure extraction

The extractions obtained from high pressure technologies were carried out using the TharSFC500 (Pittsburgh, PA USA) apparatus, which is schematically represented in Figure 4, and the coupled software (Thar Instruments Process Suite).

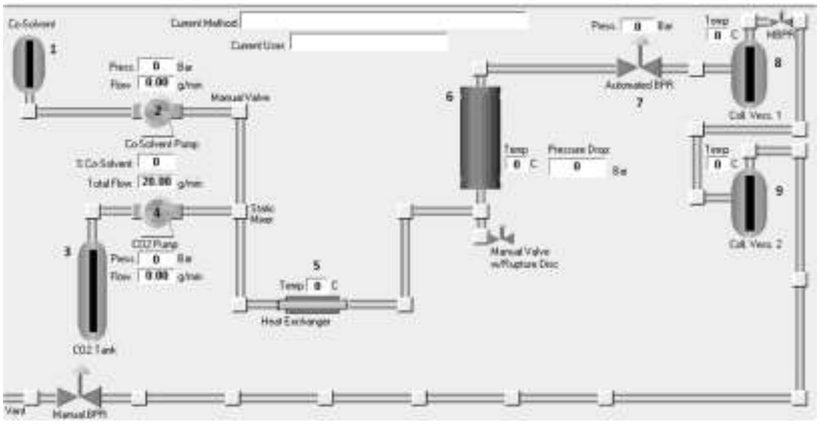

Figure 4 – Schematic diagram of the employed high pressure extraction apparatus. Legend: **1)** co-solvent reservoir; **2)** co-solvent pump; **3)** CO_2 cylinder; **4)** CO_2 pump; **5)** Heat exchanger; **6)** extraction cell; **7)** back-pressure pump; **8)** recovering vessel (1); **9)** recovering vessel (2).

4.6.1 SFE with (CO_2+EtOH)

Supercritical CO_2 (99.5 %) was delivered to the extraction cell using a high pressure liquid compressor (maximum pressure of 300 bar), and EtOH (99.5%) was delivered by a high pressure liquid pump. A stainless steel extraction cell was filled with milled olives and a filter was placed on each of both endings of the cell, in order to achieve a uniform distribution of the solvent flow, as well as to prevent obstructions inside the line. The pressure was maintained by a back-pressure regulator and measured by a pressure transducer. Extracts were recovered in a recovering flask, placed in an ice bath. The extraction methodology involved **1)** a first continuous batch of CO_2, in order to remove the lipophilic fraction from olive fruits (15 min static + 6h dynamic period, with collections every 30 minutes), and **2)** a second step with the fat-free matrix, for 3 hours, to extract polar compounds with CO_2 plus different percentages of EtOH (25, 50 and 75%). The exit tubing line was always cleaned with EtOH at the end of each extraction. The selection of the operating conditions, including the time of the process, pressure (250 bar), temperature (50ºC) and flow (20 g/ml), was based on previous studies. According to Serra et al. (2010), a pre-treatment of raw material with supercritical CO_2 is required to efficiently remove lipophilic and nonpolar substances, thus making phenols more available for a second extraction. EtOH-containing extracts were concentrated at 40ºC, under vacuum and in the dark and keep at -18ºC until further analysis.

4.6.2 PLE with (CO_2+EtOH+H_2O)

Supercritical CO_2 (99.5 %) was delivered to the extraction cell using a high pressure liquid compressor (maximum pressure of 300 bar), and different mixtures of EtOH (99.5%) plus water, were delivered by a high pressure liquid pump. A stainless steel extraction cell was filled with milled olives and a filter was placed on each of both endings of the cell, in order to achieve a uniform distribution of the solvent flow, as well as to prevent obstructions inside the line. Pressure was maintained by a back-pressure regulator and measured by a pressure transducer. Extracts were recovered in a recovering flask, placed in an ice bath. The extraction methodology involved **(1)** a first continuous batch of CO_2 in order to remove the lipophilic fraction from olive fruits (15 min static + 6h dynamic period with collections off every 30 minutes), and **(2)** a second step with the fat-free matrix, for 3 hours, to extract polar compounds with mixtures of CO_2:EtOH:H_2O (25:65:10 and 40:50:10). The exit tubing line was cleaned with EtOH after each extraction. The selection of the operating conditions, including the time of the process, pressure (250 bar), temperature (50ºC) and flow (20 g/ml), was based on previous studies. According to Serra et al. (2010), a pre-treatment of raw material with supercritical CO_2 is required to efficiently remove lipophilic and nonpolar substances, thus making phenols more available for a second extraction. EtOH-containing extracts were concentrated at 40ºC, under vacuum and in absence of light and keep at –18ºC until further analysis. Extracts obtained from a mixture with water were concentrated at 40ºC, under vacuum and, after, were freeze-dried. Assays were made in duplicate to determine experimental error of the yield values.

5. Results and discussion

The main aim of this work was to valorize wild olives (*Olea europaea* var. *sylvestris*) as a potential source of functional ingredients, through the analysis of their chemical composition and screening of their bioactivity potential. Since we weren't able to obtain the pulp from these olives, we focused our work on the stone and seed. First, we took the seeds, previously separated from the stones, from zambujeiro and five cultivar varieties of olives and prepared conventional extracts, by using different solvent combinations, in order to determine the best extractant composition **(section 5.1.1.)**.

Olive stones have been referred as a rich source of valuable compounds due to their chemical and physical properties. However, only a few studies have been dedicated to analyze them. Therefore, we attempted to characterize the zambujeiro olive stones, by using three different extraction procedures **(section 5.1.2.)**.

We then proposed to assess the bioactivity potential of the previously obtained extracts. Therefore, the extracts were analyzed for antioxidant activity by three different assays – ORAC, HORAC e HOSC **(section 5.2.1.)**. In order to further investigate the potential health effects of olive seeds extracts, a lab colleague incubated the colon cancer cell line HT29 with the obtained ethanolic seed extracts from the five cultivar and zambujeiro varieties and evaluated their anticancer potential **(Section 5.2.2.)**. As far as we know, this was the first time that extracts of zambujeiro olive seeds were tested for anticancer potential.

Finally, we obtained extracts from zambujeiro olives using clean technologies, including SFE with different CO_2 plus EtOH mixtures, and PLE, with the utilization of different CO_2/EtOH/H_2O mixtures. In a first approach, we characterized the obtained extracts, by determining the total phenol content and the chemical composition using HPLC-DAD. We also evaluated the antioxidant activity of these high-pressure extracts, through ORAC and HORAC assays **(section 5.3.)**.

5.1. Phytochemical characterization of olive seeds and stones

Olive oil and olives have previously been reported as sources of phenolic compounds. The olive fruit is a drupe, which can be structurally divided into three parts: (1) an outer skin layer, called epicarp; (2) the pulp or flesh, called mesocarp, and (3) the stone, called the woody endocarp. Several previous reports have studied the chemical composition of all the three parts of the common olives (*O. europaea* var. *europaea*). However, we were unable to find any reports on the chemical composition of the zambujeiro olives.

5.1.1. Olive seeds

In this work, extracts of the seeds from zambujeiro and from 5 cultivars (“Arbosana”, “Arbequina”, “Picual”, “Koroneiki” and “Cobrançosa”) (Figure 5) were prepared, using four different extracting solutions – water 100%, EtOH:water, acetone:water and MeOH:water, all in 80:20 (v/v) – in order to determine which one allowed a better extraction of phenolic compounds, from these samples. In order to compare the phytochemical composition of zambujeiro seeds with cultivars, the obtained extracts (n =24) were screened for their phenols content and analysed by HPLC-DAD.

Firstly, the olive seeds’ extracts were analyzed for their total phenolic content through the colorimetric Folin-Ciocalteau assay. Afterwards, the extracts were analyzed by HPLC-DAD to determine the main phenolic compounds, as well as to quantify the total content and compare it between the six studied olive varieties. A complementary HPLC-

DAD/MS/MS analysis was carried out to confirm the compounds present in the chromatographic profile obtained by HPLC-DAD analysis.

Figure 5 - Olive fruits from cultivars “Arbosana”, “Arbequina”, “Picual”, “Koroneiki” and “Cobrançosa” and wild olives, zambujeiro. The cultivars show up a similar form with a thin skin of mix of colors. The zambujeiro fruits are smaller than cultivar fruits, and they have a scratchy skin with much less pulp, almost nonexistent.

5.1.1.1. Total phenols content

Figure 6 represents the total phenolic content, as determined by colorimetric method. The values ranged from 249 to 906 mg GAE/L extract, varying in response to different solvents used (Table 1). In general, highest values were exhibited by methanol extracts (ranging of 622.4 ± 19.3 to 906.8 ± 9.4 mg GAE/L extract) followed by EtOH (ranging of 366.5 ± 4.9 to 879.8 ± 10.8 mg GAE/L extract) and acetone (ranging of 251.0 ± 18.7 to 627.6 ± 13.3 mg GAE/L extract) extracts. The water extracts obtained the lowest values, ranging between 250.0 ± 2.2 and 566.9 ± 5.5 mg GAE/L of extract.

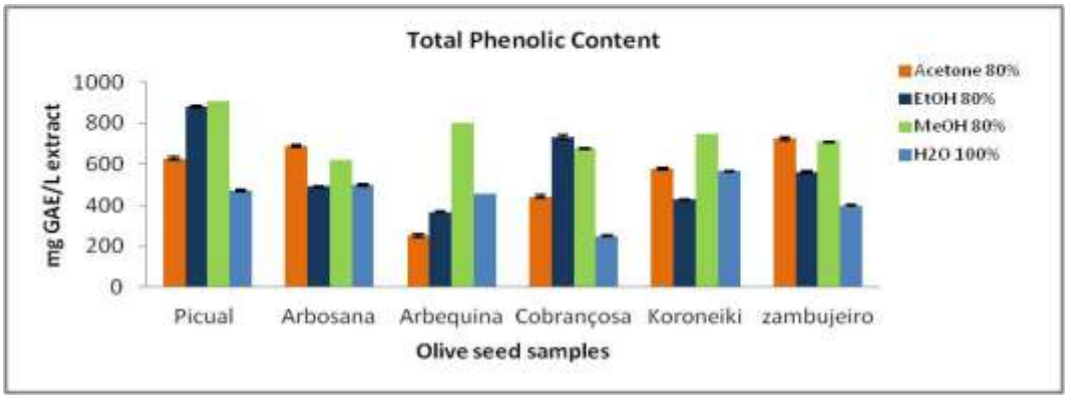

Figure 6 - Total Polyphenol content of extracts obtained from olive seeds, expressed in mg of gallic acid equivalent L of extract (mg GAE/L extract).

The values ranged from 249 to 906 mg GAE/L extract, varying in response to different solvents used (Table 1). In general, highest values were exhibited by methanol extracts (ranging of 622.4 ± 19.3 to 906.8 ± 9.4 mg GAE/L extract) followed by EtOH (ranging of 366.5 ± 4.9 to 879.8 ± 10.8 mg GAE/L extract) and acetone (ranging of 251.0 ± 18.7 to 627.6 ± 13.3 mg GAE/L extract) extracts. The water extracts obtained the lowest values, ranging between 250.0 ± 2.2 and 566.9 ± 5.5 mg GAE/L of extract.

Table 1

Phenols content obtained though the colorimetric method (TPC) and by total chromatographic areas, and nuzhenide content

Sample ID	TPC (mg/L)	Nüzenide content[1]		TCA	
		240nm	280nm	240nm	280nm
ZW	399,20	1019,17	446,50	4133,97	221,43
ZA	726,84	3054,14	1500,54	10797,39	288,53
ZE	560,96	1094,71	969,45	8625,52	199,21
ZM	708,86	2714,51	1244,95	9088,36	283,14
ABW	458,04	513,15	247,63	3572,35	242,66
ABA	251,02	548,09	277,13	3818,87	154,11
ABE	366,52	279,81	145,03	2077,28	118,83
ABM	800,73	823,90	406,75	4643,91	295,98
KW	566,85	843,43	413,60	3943,96	301,20
KA	576,17	878,06	584,97	3256,70	186,16
KE	427,19	514,51	220,34	1701,27	113,71
KM	749,63	862,34	473,48	3649,46	336,81
ASW	499,71	528,67	267,40	2724,69	267,91
ASA	691,30	1040,81	523,81	4780,60	238,88
ASE	491,08	419,77	310,70	3006,63	211,24
ASM	622,44	1031,81	473,02	3810,16	270,45
PW	471,93	674,93	597,77	2838,91	321,68
PA	627,63	1467,09	719,64	4046,19	256,87
PE	879,82	1569,47	758,26	4733,69	379,22
PM	906,80	1770,33	943,11	4691,35	433,03
CW	249,93	592,91	290,27	4288,55	229,95
CA	442,32	814,78	263,82	3204,11	136,52
CE	732,02	988,79	473,86	6645,88	384,92
CM	677,27	933,00	465,90	5294,57	274,85

[1] nuzhenide content was determined through a calibration curve with TCA values ($y = 20798x - 5828.1$, $r^2 = 0.9994$, at 280nm, and $y = 93246x – 28282$, $r^2 = 1$, at 240nm). **TCA** (Total chromatographic areas values), **TPC** (total phenolic content expressed as mg GAE/L extract).

These results are in agreement with the results obtained by Kostas et al. (2009) and by Brahimi et al. (2012), in which it was shown that the highest amount of total phenolic content in olive leaves was isolated when methanol was used, unlike with other solvents such as hexane, ethyl acetate, etc. These values are similar to the TPC values of other olive seeds as was show by Othman et al., (2008), but they were lower than those found by Silva et al. (2010). These differences maybe be due to variability in the stage of maturity of the fruits that contain the seeds, when they were harvested. Thus, we can conclude that the MeOH:H_2O (80:20 v/v) solution was the best of the tested solvents combinations, to extract phenolic compounds from olive seeds.

When compared to other food matrices, olive seeds have, as an example, approximately three times more phenols content than the pumpkin seeds (Zujko and Witkowska, 2013). The TPC values are also higher than the ones reported for olive oil (Bubonja – Sorjê, 2011, Sáncez et al., 2007), which average 196 ± 19 mg/Kg (Owen et al., 2000). According to Alu'datt et al. (2011), the higher TPC values in olive seeds, when compared to the phenols values in olive oil, is probably due to the process of olive oil extraction and the respective temperatures applied which may lead to the degradation of some compounds.

As far as we know, there are few studies characterizing olive seeds, regarding their total phenolic content, particularly from zambujeiro, on which we couldn't find any reports.

The total phenolic content of an extract is influenced by their solubility in the solvent or solvents used during the extraction process (Naczk and Shahidi, 2006). Solvent polarity may influence the solubility of various constituents present in olive seeds, but several other factors may also have an effect on the phenolic content, such as the maturation stage of the olives. The main phenolic compound, presents in olive seeds from immature olives is different from that in mature olives. According to Charoenprasert and Mitchell (2012), tyrosol glucosid is the major phenolic present in olive seeds from immature olives, and nuzhenide is the predominant phenolic compound in seeds from mature olives.

5.1.1.2. Identification of phenolic compounds in extracts

The extracts of the six varieties of olive seeds were analyzed by HPLC-DAD. All extracts exhibited similar chromatographic profiles when, compared between each other and between each extractant solution used, at 280nm **(Figure 7)**. However, the values of the total chromatographic areas (TCA) were slightly different **(table 1, section 5.1.1.1., page 25)**.

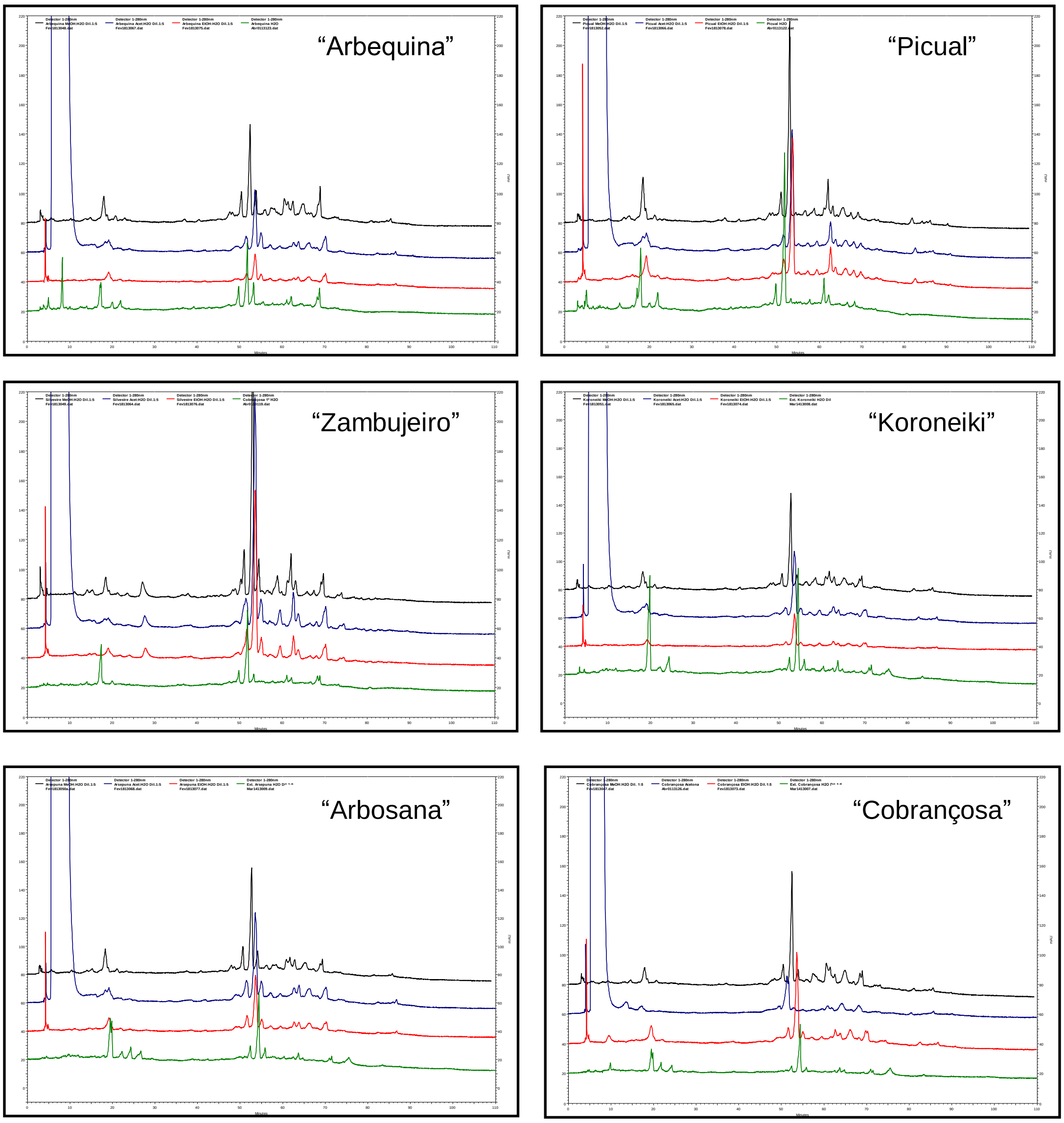

Figure 7 - Chromatografic profiles of the 4 different extractant solutions from 6 olive seeds varieties: **H_2O**, **EtOH**, **acetone** and **MeOH** at 280 nm.

The highest TCA values were obtained in the methanol extracts, for all varieties except Cobrançosa, for which the highest TCA was obtained in the ethanolic extract. At 360 nm wavelength, profiles did not show any peaks.

Given that the obtained chromatographic profiles of the extract were similar we chose to show the methanolic extracts' profiles of all varieties, with the identified compounds (Figure 8). The identification of the peaks was made by HPLC-DAD/ESI/MSMS analysis, and the molecular ions are present in table 2 **(section 5.1.1.2., page 29)**.

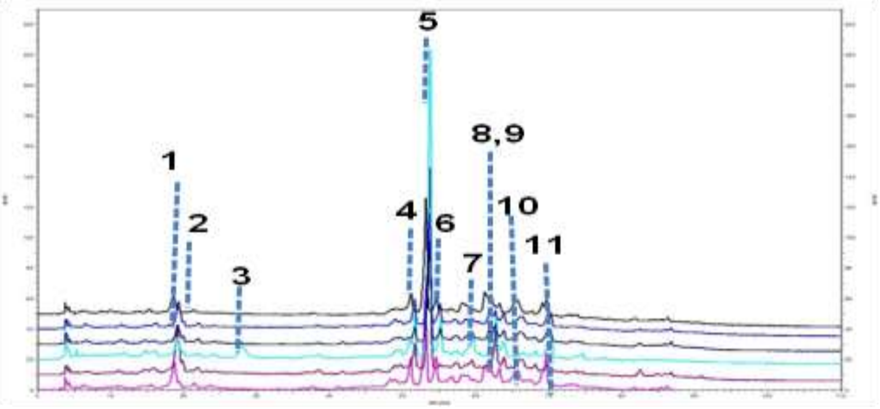

Figure 8 - Chromatographic profiles of methanolic extracts from olive seeds of 6 varieties, obtained at 280 nm: **Arbequina**, **Picual**, **zambujeiro**, **Koroneiki**, **Arbosana** and **Cobrançosa**. The identification of compounds is represented in table 2.

Picual and zambujeiro were the ones which had the highest and more salient peak (5), which was also confirmed by the total area of the peak in both varieties **(Figure 7 and table 1, section 5.1.1.1., page 25)**. This peak, as identified by HPLC-DAD/ESI/MS/MS, represents nuzhenide (m/z 685) (table 2), the main and characteristic compound of olive seeds, as previously described by other authors using the same matrix (Silva *et al.*, 2010), or others matrices belonging to the *Oleaceae* family. The zambujeiro variety was the only one that showed the peak number 3 at the retention time (RT) 27 min. The ion found in that peak was 403 m/z, which correspond to the characteristics of the molecular ion that has been identified in literature for elenolic acid glucoside, also known as 11-metil oleoside. This compound results from the degradation of oleuropein and is accumulated during ripening (Silva et al., 2010).

The ion observed in MS, which eluted at RT corresponding to the peak 2, is a hydroxytyrosol glycoside (315 m/z), as confirmed by the presence of the respective fragment at 153 m/z. The presence of hydroxytyrosol glycoside can be explained by the loss of 224 units (one-half of dried elenolic acid) from oleuropein (539 m/z). Peaks number 4 and 6 represent two isomers of one compound firstly described by Sandra et al. (2010) with the m/z ion at 715, but that has not been identified yet. The detection of the ion 715 m/z at negative mode yielded the fragments through MS^2: 385 and 553.

Table 2

Chromatographic peaks of zambujeiro olive fruit conventional and high pressure extracts and results by MS.

1 Compound A was designed by Sandra et al. (2010) who identified in olive seeds previously.

Peak number	RT (min)	Main negative ions (*m/z*)			Identification
		[M - H]$^-$	MS^2 Fragmentos	λ_{max}	
1	8.5	341	-	242	Ac caffeic glucoside
2	26	315	(135,179)	219,273	Hydroxytyrosol glucoside
3	36	403	(223,403)	278	11 – methyl oleoside
4	78	715	(385,451,553)		Compound A[1]
5	82	685	(223,299,523,685)		Nuzhenide
6	84	715	(329,385,451,553)		Isomer of compound AI
7	86	525	(317,447,525)	242	Demethyloleuropein
8	89	789			n.i.
9	91	1071		240,280	Nuzhenide (11-methyl oleoside)
10	100	1458			Di (methyl oleoside) of Nuzhenide
11	102	297			n.i.
12	81	299	227	276	Tyrosol glucoside
13	30	179	(135)	240,320	Caffeic acid
14	35	415	(123,153,415)	230,280	1 – Acetoxypinoresinol
15	44	373		225,280	Hydrpxypinoresinol
16	44.3	539	(223,275,307,377,539)	282	Oleuropein
17	69	295			n.i.
18	89	375	(100)		(+) – cycloolivil

The peak number 9 corresponds to nuzhenide 11-methyl oleoside with a molecular mass of 1072, the mass spectrum in the negative mode was observed at 1071 m/z. This compound is 386 mass units bigger more than nuzhenide, which result from the addition of a unit 11 to the last methyl-oleoside molecule. The occurrence of isomers of this compound in the sample is likely since the 1071 ion m/z is detected at different retention times. There were obtained from the respective ions of the ion fragmentation MS^2. The peak number 10 corresponds to a di-(methyl oleoside) of nuzhenide, which is characterized by the successive difference of 386 mass units with increasing retention time. 341 m/z ion was found in peak number 1, which is referred in literature as corresponding to caffeic acid glucoside. However, we cannot be sure about this, because the ions fragments were not obtained due to the low energy applied. We are unable to identify peaks number 8 and 11 and to find any references in the literature about them. In a future experiment various other energy collisions should be tested in order to obtain more fragments, and other techniques shoud be applied, in order to identify a possible molecular structure.

In conclusion, in order to extract phenolic compounds from olive seeds, a conventional extraction with MeOH:H2O (80:20 v/v) as extractant solution be applied. We found nuzhenide to be the most abundant compound in the olive seed extract, which may indicate that the olives used were overall in a advanced maturation stage. The fact that the nuzhenide concentrations differed among the 6 tested olive varieties may be due to different average maturation stages among the bacthes used from each variety. Therefore, it's not possible to conclude that one variety is richer in nuzhenide than another, unless the maturation stage of each olive variety batch is controlled.

5.1.2. Olive stones

The olive stone is a lignocellulosic material, with hemicelluloses, cellulose and lignan as its main compounds. The most common use of olive stones is as biofuel. Despite the environmental benefits of using this biomass as fuel, some problems remain such as air pollution (i.e. the release of carbon monoxide, nitrogen oxides, and particulates such as soot and ash produced by combustion process) (Esteve et al., 2012).

Regarding the recovery of lignan compounds, López el al. (2008) stated that not all the common extraction methodologies allow the isolation of the lignans from the olive stones. These authors observed that the extraction at reflux with methanol at 65°C was the only method, from those tested, that allowed to identify the presence of lignans in olive stones. Thus, in order to explore the potential of olive stone from zambujeiro olive fruits were prepared and analyzed extracts using three different experimental methodologies: 1) extraction at reflux with methanol, as described by López et al. (2008); 2) the same conventional procedure used on the seeds, with the four different extractant solutions; and 3) an overnight extraction with refined olive oil, at 30°C **(section 4.3.)**. The obtained extracts were then analyzed by HPLC-DAD and colorimetric Folin-Ciocalteau method, in order to compare each extracts' phytochemical composition

In this step, unlike what was performed with the seeds' extracts, we didn't compare the phytochemical composition of zambujeiro olive stones with the ones of the cultivar varieties, because we didn't have enough samples of cultivars to perform the analysis.

5.1.2.1. Total Phenol Content

The total phenolic content values obtained from stone extracts, ranged from 103.6 ± 9.3 to 599.7 ± 20.1 mg GAE/L extract **(Figure 9 and Table 3).**

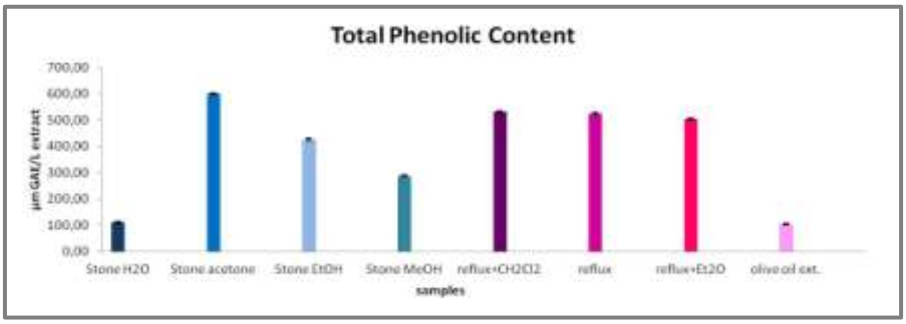

Figure 9 – Total phenolic content of olive stone extracts prepared using different procedures.

Table 3

General chemical composition of olive stones extracts prepared with different procedures.

Procedure extraction	TCA		TPC
	240nm	280nm	(mg GAE/L extract)
MeOH reflux	342396818	115704607	525.1
MeOH reflux + Et_2O	318150539	111661238	504.0
MeOH reflux + CH_2Cl	319699240	107911786	523.4
Extraction w/ refined olive oil	30856795	19226346	103.6
Conventional w/ water	4920664	5787252	112.4
Conventional w/ EtOH 80%	31314188	16313121	426.3
Conventional w/ MeOH 80%	32406086	16891888	287.2
Conventional w/ acetone 80%	39293163	20197838	599.7

The highest values of TPC were obtained in the conventional extract with acetone, followed by the three reflux extracts, with the mean values 599.7 ± 20.1, 532.4 ± 31.8, 525.1 ± 33.6, and 504 ± 5.9 GAE/L extract, respectively. The extractions with refined olive oil, and the conventional procedure with water, retrieved the lowest values, 103.6 ± 9.3 and 112.4 ± 13.8 mg GAE/L of extract. It is possible that, methanol at reflux with high temperatures (65°C) enhances the extraction of phenol compounds by being a more aggressive process, and therefore, promoting the cleavage of bonds between these compounds and the matrix.

5.1.2.2. Identification of lignans compounds

The lignans may be present in olive oils in higher (e.g., from cv. Arbequina) or lower concentrations (e.g., from cv. Picual) (López et al., 2008). There are not so many papers reporting the analysis of lignans from olive stone extracts. According to López et al. (2008), the determination of lignans in pulp and freeze-dried stone were low, probably due to the extraction procedure. After testing four extraction procedures, López and coworkers conclude that the extraction with MeOH at reflux allowed an easy detection of acetoxypinoresinol (Ac-Pr), pinoresinol (Pr) and hydroxypinoresinol (OH-Pr) by using MS technique.

Once again, as far as we know, there are no studies focusing on the detection of lignans in olive stone from zambujeiro by HPLC-DAD/MS. In order to characterize the zambujeiro olive stones, three different methodologies were applied and the obtained extracts were analyzed by HPLC-DAD/MS/MS at 280 nm and 240 nm wavelengths. All the obtained chromatographic profiles from each extract were, then, compared among each other, using a refined olive oil extract, as a blank solution **(Figure 10)**.

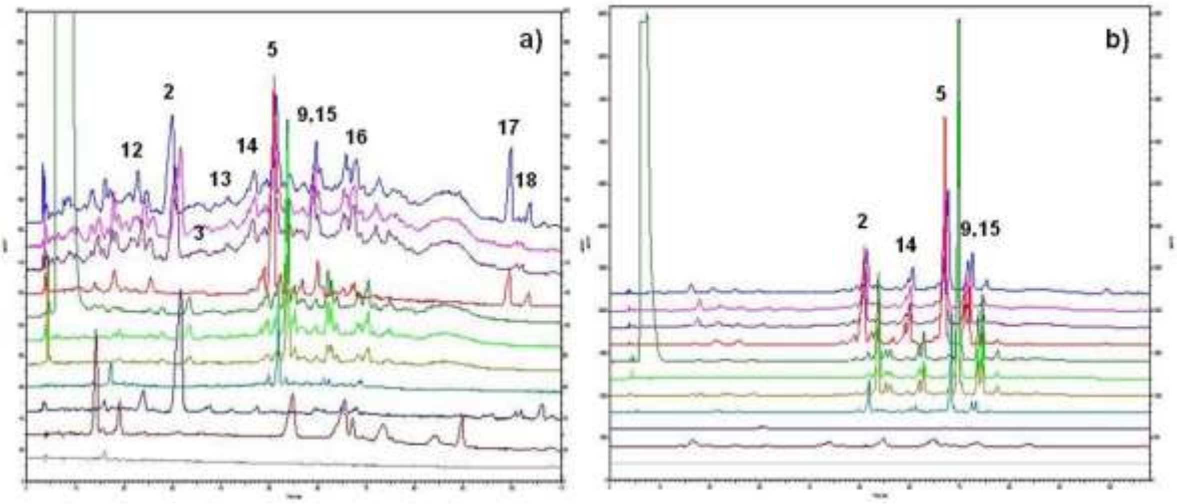

Figure 10 - Chromatographic profiles of olive stone extracts obtained by different procedures. Extracts from olive stones obtained at 280 nm (a) and 240 nm wavelength (b): refined olive oil, standard olive oil, and extraction with refined olive oil, conventional extracts with water, MeOH, EtOH, acetone, zambujeiro olive seeds, and extraction with MeOH at reflux with Et$_2$O, CH$_2$Cl$_2$, only at reflux. Peaks identification is present in table 2 **(section 5.1.1.2., page 29)**.

As can be seen in Figure 10, there is a higher abundance of peaks in extracts using MeOH at reflux than in the other profiles, which is also supported by the TCA values **(table 3, section 5.1.2.1., page 31)**. Thus, it's possible to conclude that a more aggressive method, such as MeOH at reflux and 65°C, allows obtaining a higher number of compounds than when using the other methods tested. These results are in agreement with the experiments of López et al (2008). Between the three procedures with MeOH at reflux there aren't significant differences among the obtained values. Both the extraction with water and the extraction with refined olive oil had the lowest TCA values in both wavelengths. Due to the large amount of peaks, the chromatographic resolution, and the possible low concentration of some metabolites, the sole use DAD was considered not sufficient to ascertain the presence of lignans. A MS analysis was carried out for the detection of these compounds. A 280 nm wavelenght is usually used to detect lignans, therefore, in the obtained chromatographic profiles, it is possible to observe the different peak intensities at 240 nm and 280 nm. The peaks identified are present in table 2 **(section 5.1.1.2, page 29)**.

Among the extracted compounds, were identified lignan compounds, but only in the reflux extracts. The lignan compounds have an absorbance maximum between 240 and 280 nm, and as we can see in Figure 10, their chromatographic peaks (14, 15) appeared in both wavelengths. Considering that lignans were only found in reflux extracts, maybe there is a positive effect of temperature in lignan removal from olive stones, since the extracts were performed above 60°C. The lignan identification was carried out through HPLC-DAD/ESI/MSMS analysis. Since we did not have standard solutions, were prepared extracts with refined olive oil and with MeOH at reflux, from sesame seeds. These seeds are a rich source of lignan compounds; hence, their extracts have been used in order to compare the peaks of sesame seeds with the peaks of the extract prepared from olive stones.

In Figure 11 it’s possible to compare the peaks obtained with extracts from sesame seeds and from olive stones. Some of the peaks obtained in sesame seeds extracts were also found in the extracts from zambujeiro olive stones (*Olea europaea* var. *sylvestris*). The identified peaks are described in table 2 **(section 5.1.1.2., page 29)**. Two main ions were found but not identified in both matrices (323 and 357 m/z). It will be necessary to apply a higher energy of collision, in order to break the ions and get their respective fragments, and to compare them with those described in literature.

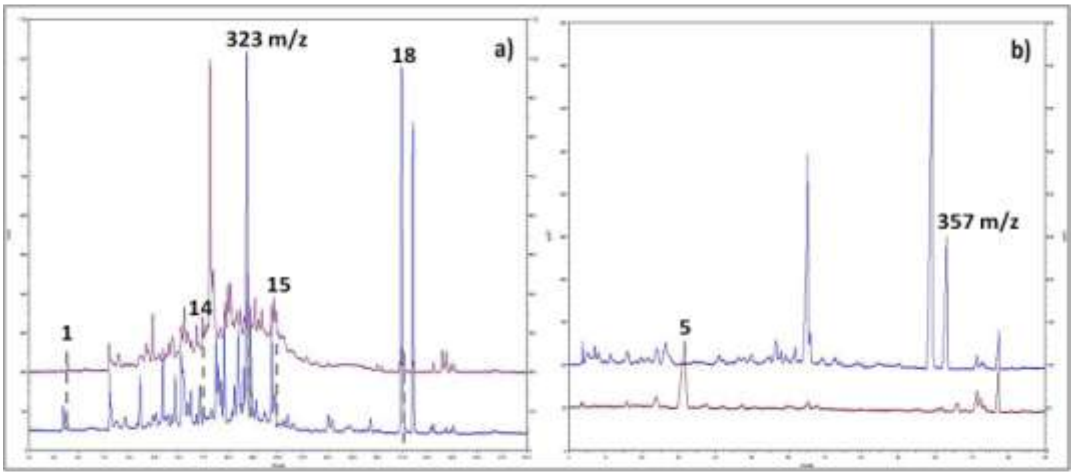

Figure 11 – Chromatographic profiles of extracts from **sesame seeds** and **zambujeiro** olive stones **a)** reflux procedure and **b)** refined olive oil extraction. The identification is in the table 2 **(section 5.1.1.2., page 29)**.

The obtained electronic ion (EI) profiles allowed the detection of Ac-Pr (at 415 m/z) and OH-Pr (at 373 m/z), as represented in figure 10 and table 2, by the peaks number 14 and 15, respectively. It is possible that OH-Pr mainly formed from its percursos Ac-Pr. The detection of both may be explained by a partial hydrolysis of the ester bond during the extraction with hot methanol at reflux. The quantification of lignans was not possible due to absence of an appropriate standard solution.

Other phenol compounds were also detected in olive stones, such as glucosids of tyrosol and hydroxytyrosol. The first one was only detected in reflux extracts, whereas hydroxytyrosol glucosid was present in all the extracts. Compounds previously identified in olive seed of zambujeiro, such as nuzhenide, elenolic acid glucoside, 11-methyl oleoside and di (11-methyl oleoside) of nuzhenide, were also detected in stone extracts. Two compounds in olive stones extracts, represented by the peaks number 17 and 18, were not identified. The peak number 18 represents an ion at 375 m/z, which may possibly correspond to the ion reported in wood ash matrix, as (+) - cycloolivil (Sanz et al., 2012). However, since we were unable to obtain the respective fragments in this experiment, it wasn’t possible to confirm this identification.

In summary, the conventional extraction with acetone was the more adequate extraction procedure for obtaining a high content of total phenols, from zambujeiro olive stones. In the other and, the extraction at reflux allowed to extract a larger number of compounds, as revealed by the abundance of chromatographic peaks, and was the only procedure that allowed the identifiable extraction of lignans. Hence, in future studies would be interesting to somehow combine both methods, developing an extraction procedure with acetone at reflux, and to determine its efficiency.

5.2. Bioactivity

In order to determine the bioactivity of olive seed and stone extracts, two effects were evaluated. First, the antioxidant activity was evaluated for olive seed extracts from zambujeiro and from the 5 varieties referred before, through the application of ORAC, HORAC and HOSC assays. Afterwards, the antioxidant activity of olive stones from zambujeiro was also assessed through these assays and compared regarding the extraction method used **(section 5.2.1.)**. In a second part, the antiproliferative effect on HT29 cell lines was evaluated for ethanol extracts of olive seeds from zambujeiro and from olive cultivars **(section 5.2.2.)**.

5.2.1. Antioxidant activity evaluation

In order to evaluate the antioxidant activity of olive seed and stones from zambujeiro, three different assays were performed: ORAC, HORAC and HOSC. The ORAC assay analyzes the potential to scavenge harmful oxygen reactive species, namely peroxyl, the HORAC assay measures the efficacy of the sample to prevent hydroxyl radical formation, and the HOSC assay measures the scavenging capacity of antioxidant samples against hydroxyl radicals.

5.2.1.1. Olive seeds

The antioxidant activity values can be seen in Figure 12 and 13.

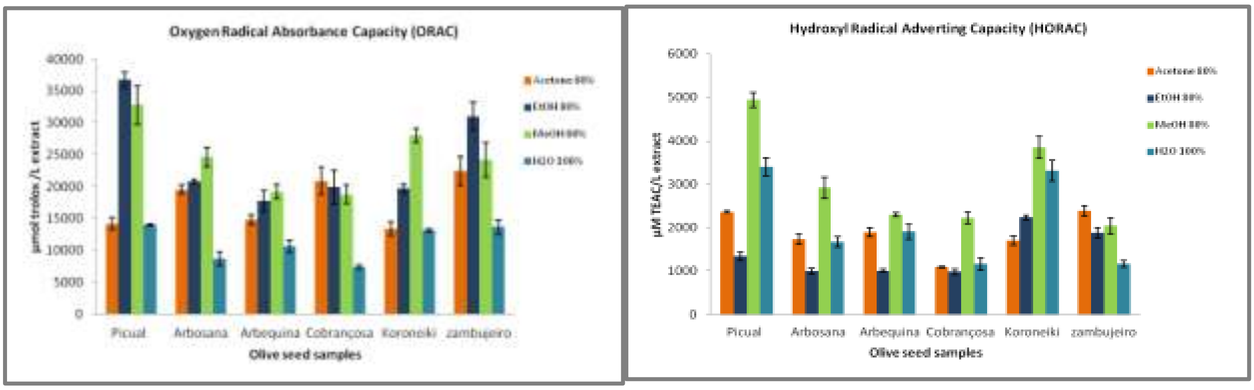

Figure 12 - Antioxidant capacity (ORAC, HORAC values) of olive seeds extracts from zambujeiro and 5 cultivars.

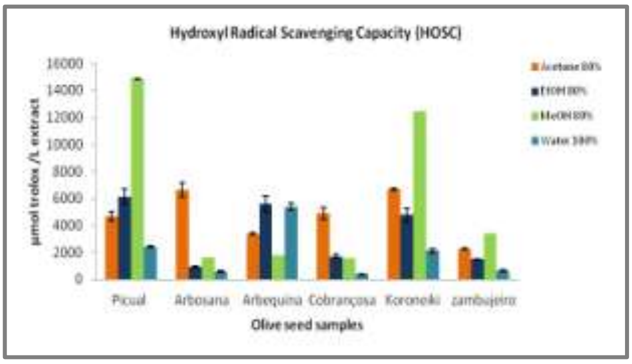

Figure 13 - Antioxidant capacity (HOSC values) of olive seeds extracts from zambujeiro and 5 cultivars.

In olive seeds extracts, the obtained ORAC, HORAC and HOSC values ranged from 7372.6 ± 282.7 to 36735.5 ± 1223.7 µmol TE/L, 974.8 ± 62.1 to 4941.2 ± 167.9 µmol CAE/L, and 390.4 ± 3.0 to 6684.9 ± 86.2 µmol TE/L, respectively.

Picual and Koroneiki olive seeds were the varieties that presented the highest ORAC, HORAC and HOSC values. Olive seeds of zambujeiro were also one of the varieties with the highest ORAC and HORAC values, while in HOSC assay zambujeiro together with Arbosana variety showed the lowest value. In general, the extracts have high scavenging peroxyl radical capacity, and the ability to prevent the formation of HO'. The extracts of olive seeds also appear to be capable of scavenging OH', but less effectively.

Concerning ORAC, HORAC and HOSC results, to your knowledge, this is the first report where these assays were performed to evaluate the antioxidant activity of olive seeds extracts. Usually, the antioxidant capacity is mainly related with the phenolic content in the extracts. In fact, there seems to be a correlation between phenolic composition and the results from ORAC ($r^2 = 0.6814$), HORAC ($r^2 = 0.4446$) and HOSC ($r^2 = 0.4611$). In this report, we observed that there is also an apparent correlation between nuzhenide, the major compound in olive seeds, and ORAC ($r^2 = 0.5026$). This is in aggrement with the known high antioxidant capacity of seicoiridoids (Scognamiglio et al., 2012).

5.2.1.2. Olive stones

In olive stone extracts from zambujeiro, the obtained ORAC, HORAC and HOSC values ranged from 3872.3 ± 282.7 to 25755.2 ± 179.9 µmol TE/L, from 1030.3 ± 42.3 to 3704.0 ± 100.4 µmol CAE/L, and 641.6 ± 57.7 to 4468.9 ± 394.5 µmol TE/L, respectively **(Figure 14 and table 3, section 5.1.2.1., page 31)**.

The ORAC results are higher when compared with HORAC and HOSC activity in extracts from olive stones, prepared using different procedures. It means that the extracts are more efficient in scavenging the peroxyl radical than in scavenging or preventing the hydroxyl radical production. These results were highest in the reflux extracts, which present much more compounds in the chromatographic profile.

When we compared the ORAC values between zambujeiro seed extracts and stone extracts, the values of seeds' extracts were three times higher than the values of stone extracts. However, the HORAC and HOSC values of zambujeiro seed extracts were lowest than the values obtained from zambujeiro stone extracts. These results can be explained by the presence of a family of compounds in olive stones but not in olive seeds, such as lignans, which are also known to have a high antioxidant capacity.

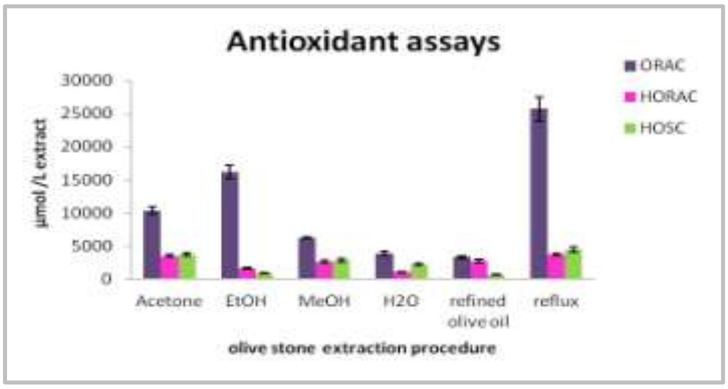

Figure 14 - Antioxidant capacity (ORAC, HORAC and HOSC values) of different extracts of olive stone extracts from zambujeiro.

5.2.2. Antiproliferative effect evaluation

Several compounds usually found in olives and olive oil have been reported to have a potential anticancer effect, specifically in breast cancer (Bulotta et al., 2011; Siriani et al., 2010), colon cancer (Ghichard et al., 2006), prostate cancer (Quiles et al., 2002), colorectal cancer and leukemia (Casaburi et al., 2013).

In order to evaluate the anticancer potential of olive seeds extracts from zambujeiro and 5 cultivars, a cellular proliferative assay was performed **(see appendix 1)** using a human colon cancer cell line (HT29). Cells were exposed to ethanolic extracts of olive seeds from zambujeiro and 5 cultibar varieties, which were previous described in **section 5.1.1**., and cell viability was evaluated using Presto assay. Results are represented in figure 15, and as can be seen, the olive seed extract from zambujeiro was the only one that had an antiproliferative effect on human colon cancer cell line.

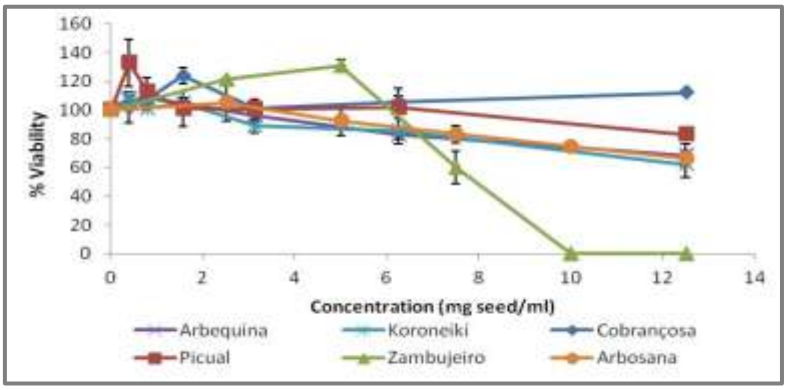

Figure 15 – Antiproliferative effect of olive seeds extracts on human colon cancer cells (HT29) (incubation time 1 week).

This result could be related to the higher content in polyphenols and antioxidant activity. However, other varieties of olive seeds, such as Picual, also had high phenol content and antioxidant activity. This suggests that there are some specific compounds in zambujeiro extract that contributed towards this effect, such as nuzhenide, which was higher in zambujeiro olive seed extracts, when compared with the other varieties' seed extracts **(see section 5.1.1.2, page 29)**. There are reports that suggest an anticancer effect by seicoiridoids, mainly oleuropein, which was shown to reduce the viability and proliferation of the human breast cancer cell lines (MCF-7) (Han et al., 2009). Moreover, it is important to note that the olive seed' extracts didn't display cytotoxicity.

To our knowledge, this is the first report concerning the antiproliferative effect of zambujeiro olive seed extracts on HT29 cells. Olive oil and some of their phenolic compounds, such as hydroxytyrosol and oleuropein, have already been reported to inhibit the growth and promote apoptosis in several tumor cell lines, such as leukemia tumor cells (HL60), colorectal carcinoma cells, and in two breast cancer cell lines (MDA-MB-231 and MCF-7) (Casaburi et al., 2013). The results in this experiment results are very interesting, since only the wild variety and not the cultivar seed' extracts demonstrated to have antiproliferative effect. However, further experimental studies will be necessary, in order to, for example, nuzhenide and the observed effect.

5.3. Development of a GRAS process to recover bioactive compounds from zambujeiro olive fruit

Clean technologies including SFE and PLE were used for the extraction of phenolic compounds from olive fruits (*Olea europaea* var. *sylvestris*). There are many reports that refer the application of clean technologies in the recovery of phenolic compounds from olives. Authors have used different combinations of pressure, temperatures and ethanol concentration as variables, in order to find the optimal conditions to recover phenolic compounds and antioxidants. As far as we know, there are currently no published reports on such extraction methods from zambujeiro fruits. Considering this, the aim of this section was to develop a new process of extraction, using PLE and SFE, to recover bioactive compounds from zambujeiro olive fruits.

The methodology employed – high pressure extraction – comprised a first part with a static period (15 minutes) plus a continuous batch of sc-CO_2, performed at 50ºC and 250 bar. This step allowed removes all nonpolar and lipophilic part of the matrix due to the characteristics of CO_2, and investigate extraction duration. For that, ~150 g of olive fruits of zambujeiro were dried and further submitted to a high pressure extraction methodology.

In order to investigate the best conditions for the recovery of previously identified compounds in the conventional extracts of seeds and stones from wild olive, the delipidated olive fruits (30 g each), were submitted to high pressure extraction methodologies. For that, firstly high pressure extracts were obtained with different sc-CO_2 plus EtOH different concentrations. Afterwards, different mixtures of CO_2+EtOH+H_2O were chosen and applied in accordance with the phase diagram tested by Seabra et al. (2012), which contains the mole fractions of each of the solvent, so that all three are in a single phase. The content of the extracts from zambujeiro olive fruits, obtained by using high pressure technologies, was identified and quantified through total phenolic content and HPLC analysis. Afterwards, the extracts were submitted to antioxidant activity evaluation through ORAC and HORAC assays, in order to evaluate their bioactivity.

5.3.1. Kinetics of SCF-CO_2 extraction

A continuous batch of sc-CO_2 was applied in order to remove the nonpolar fraction from olive fruits during 6 hours with and without a static-period (~15-20 min). Figure 16 represents the mass yields from lipophilic and nonpolar fraction along the time, during sc-CO_2 extraction, with and without a static-period.

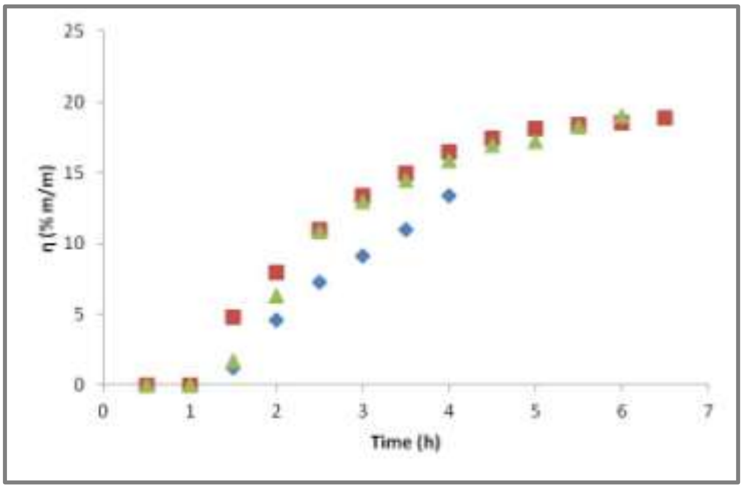

Figure 16 – Efficiency of sc-CO_2 extraction from olive fruits, with or without a static period. Red points correspond to the extraction with static-period, green points are the duplicate, and blue points represent the extraction with sc-CO_2 without static-period.

These results show that an initial incubation period favors the extraction, possibly because during that period the sc-CO_2 had enough time to contact with the matrix and to diffuse into it, therefore increasing the area of contact. It is possible to observe (red and green datasets) that the mass yield is fairly constant after 3 to 4 hours of extraction, which may indicate that this time would be enough to retrieve the maximum extract concentration.

The chromatographic profiles obtained by HPLC/DAD/MSMS analysis didn't allow to identify those compounds, in these extracts. Considering the kinetic curves and the chromatographic profiles, it's possible to conclude that a 2 to 3 hours extraction with CO_2 is needed to delipidated the matrix.

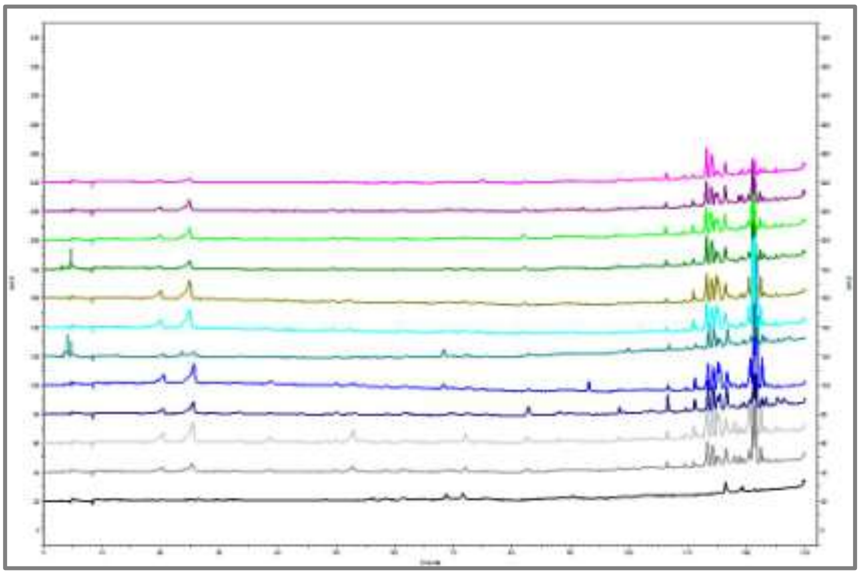

Figure 17 – Chromatographic profiles of each 30 min of the extraction with supercritical CO_2 during 6 hours. Legend: **30min**, **1h**, **1h30min**, **2h**, **2h30mi**n, **3h**, **3h30min**, **4h**, **4h30min**, **5h**, **5h30min** and **6h**, at 240nm.

5.3.2. Extraction using CO_2 + co-solvents enhancers or PLE (CO_2:EtOH:H_2O)

In order to isolate functional ingredients from zambujeiro olive fruits, extractions were carried out at 250 bar and 50°C during 15 min (incubation) plus 2 hours, according to previous works developed in a similar matrix (Almeida, 2009)

30 g samples of delipidated matrix were extracted using either: 1) supercritical fluid extraction with CO2 plus three different percentages of EtOH (25, 50 and 75%); or 2) PLE extraction through the use of different CO_2/EtOH/H_2O mixtures, namely, 25:65:10 and 40:50:10. These mixtures were chosen according to the phase diagram displayed by Seabra et al. (2012), for the extraction of phenolic compounds, allowing to work with solvents in a homogeneous stage (Table 4).

Table 4

Mixtures of CO_2/EtOH/H_2O used to obtain high pressure extracts designed by, A, B, C, D and E.

Extract	Molar fractions		
(30g of matrix)	CO_2	EtOH	H_2O
A	**0.75**	**0.25**	**0.00**
B	**0.50**	**0.50**	**0.00**
C	**0.25**	**0.75**	**0.00**
D	**0.25**	**0.65**	**0.10**
E	**0.40**	**0.50**	**0.10**

The extraction kinetics was followed and the comparative study of the extraction yields (g extract/g matrix) over the time, for the different extracts. In figure 18, as expected, EtOH concentration in the solvent mixture affected positively the extract yields. It seems that zambujeiro olive fruit contains substances which present high solubility towards EtOH.

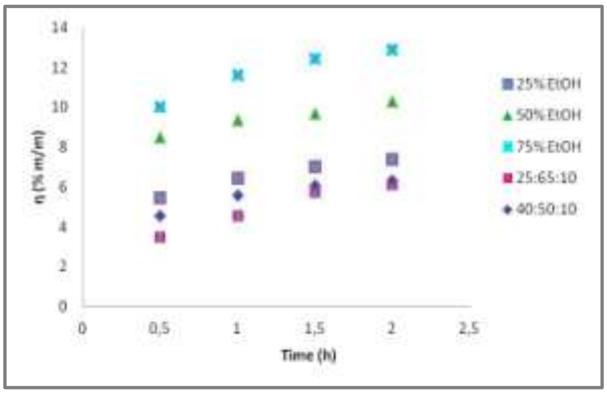

Figure 18 – High pressure extraction kinetics of extracts obtained from zambujeiro olive fruits, according to the mixture used.

The yields obtained during two hours of extraction were: 3.5 – 10.0% (30 min), 4.6 – 11.6% (30min - 1 h), 5.7 – 12.5% (1h – 1h30min), and 6.1 – 12.9% (1h30min – 2h). The lowest yield values were achieved with the solvent mixtures using water, followed by the extraction with lower percentage of EtOH (25%). The presence of EtOH was directly and positively related with phenols extraction efficiency. This may be related with more hydrogen bonding and dipole-dipole interactions in ethanol, which increases the solubility of phenolic compounds, since the solubility of polyphenols depends on their polarity (Cháfer et al., 2004).

In summary, we observed that the different mixtures tested had the same kinetic profile. Therefore, it's possible to conclude that, after the first 30 minutes of extraction, the target compounds may be being diluted in the extract due to the parallel extraction of other compounds after that time.

5.3.3. Characterization of extracts obtained from wild olives

The phytochemical characterization of the extracts was carried out, first, by performing a general screen to determine their total phenolic content, and second, by making a more specific analysis using HPLC-DAD.

Figure 19 shows that in the extracts performed using CO_2 and EtOH, in both 25% and 75% EtOH percentages, the phenolic compounds are more abundant after one hour of extraction, and their concentrations decrease along the time.

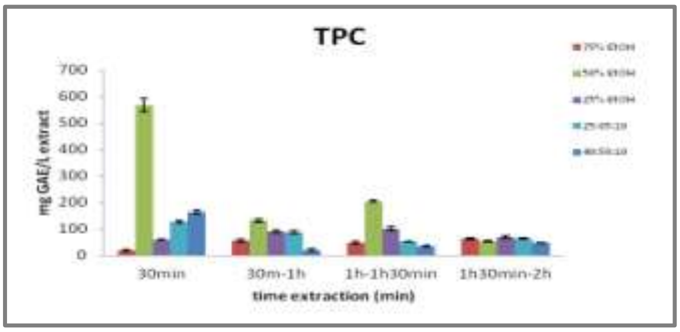

Figure 19 – Total phenolic content from high pressure extracts of olive fruits along the extraction time.

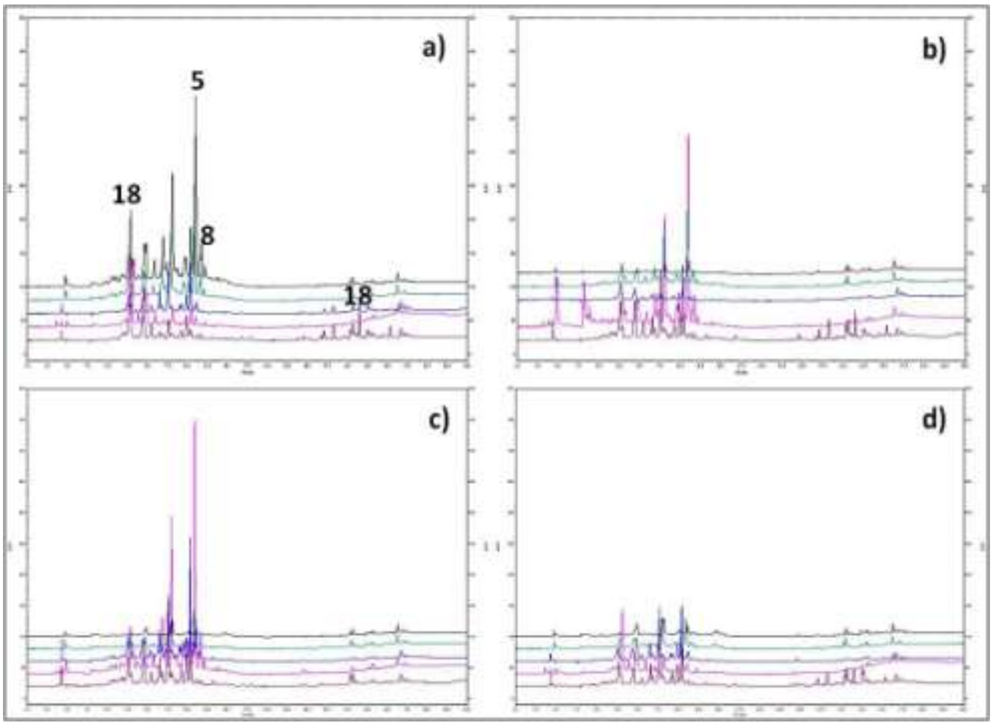

Figure 20 – Chromatographic profiles of high pressure extracts from zambujeiro olive fruits (**25%**, **50%** **75%, 25:65:10** and **40:50:10**) during extraction time **a)** 30min, **b)** 1h, **c)** 1h30min and **d)** 2h, at 240nm. The peaks identification is on table 2 **(section 5.1.1.2., page 29)**.

The total chromatographic areas and the compounds' values of the extracts obtained, were determined by HPLC-DAD (Figure 20 and table 5). The TCA values of ternary and 75% of EtOH extracts are higher at 30 min than in the rest of the extraction time. Figure 20 shows the chromatographic profiles of all the extracts (5 mg/L), at 240 nm (at 280 nm wavelength, the peaks had low intensities, which didn't allow their identification).

In the chromatographic areas corresponding to 25% and 50% EtOH, the values were higher after one hour of extraction. This observation may be due to the fact that the presence of one or two co-solvents enhances the extraction of the compounds, therefore decreasing the extraction time. These results are in accordance with those found by other authors, who have proposed the use of co-solvents to enhance the solubility of the target compounds (Serra et al., 2010; Cháfer et al., 2004).

The identification of chromatographic peaks was made by HPLC-DAD/MS. Table 1 shows the results of the identification of compounds **(section 5.1.1.2., page 29)**.

The seicoiridoid nuzhenide (peak 5) was, as it had been in the seed and stone extracts, the main phenolic compound present in the extracts of olive fruits. Oleuropein, another seicoiridoid, was also identified (peak 16). In this extract, it was not possible to identify the lignans acetoxypinoresinol, pinoresinol and hydroxypinoresinol. However, a fourth lignan was determined, (+) - cycloolivil.

Among all the extracts, 50% of EtOH at 30min contained the highest phenolic concentrations, 566.2 ± 25.0 mg GAE/L extract, followed by the ternary extracts at 30 min. Furthermore, also in ternary extracts, when the portion of CO_2 was decreased (25:65:10) and water was added, the TPC increased. Hence, the compounds appear to have water affinity, which might be explained by their high polarity.

Table 5

Characterization of high pressure extracts obtained using PLE and SFE processes, and analyzed using TPC determination and HPLC/DAD analysis.

Extract	Time extraction (min)	Compound			TCA of the extract at 240nm	TPC mg GAE/L extract[1]
		Nuz	Oleuro	Lignan		
75% EtOH	30min	593074	87271	346310	9729574	18.72222
	1h	245786	33018	419455	6040195	55.66667
	1h30min	970383	82293	315565	14509771	47.32144
	2h	594199	79615	249606	9121649	62.33333
50% EtOH	30min	257770	138852	1473690	924618	566.2222
	1h	1689666	125880	458341	6794750	131.5
	1h30min	2661072	3881625	642913	24337805	203.7222
	2h	1156858	47911	301373	24259171	52.6111
25% EtOH	30min	261040	120024	1805877	13342037	58.4444
	1h	933905	106296	647710	15320778	90.3888
	1h30min	1308254	95213	535143	11581273	100.944
	2h	1119160	92982	271095	12701200	69.5556
25:65:10 CO_2:EtOH:H_2O	30min	1609533	56913	851660	15903258	126.778
	1h	1123341	33893	384086	10888878	87.6111
	1h30min	590974	47584	168108	6535736	52.3333
	2h	526341	36116	224340	6535736	63.4444
40:50:10 CO_2:EtOH:H_2O	30min	2650066	94499	1260085	24792575	163.722
	1h	193207	14097	38485	486631	19.5555
	1h30min	135049	41379	1605	4387902	34.2777
	2h	185663	52708	3981	1802684	46.7777

Seicoiridoids, nuzhenide and oleuropein **(Figure 22)**, have higher molecular weights (MW>500 g/mol), while lignans have smaller ones (MW<500 g/mol). The TCA values of the extracts suggest that when the EtOH concentration increased (75% EtOH) and/or when water was added, the concentration of extracted nuzhenide and oleuropein increased too. However, those compounds appear to increase in the 75% EtOH extract, but to decrease in the ternary mixture extracts, along the time. The added water and/or EtOH enhanced the solubility of the target compounds and increased the extraction selectivity. Nuzhenide and oleuropein have many hydroxyl groups, so they are highly polar compounds.

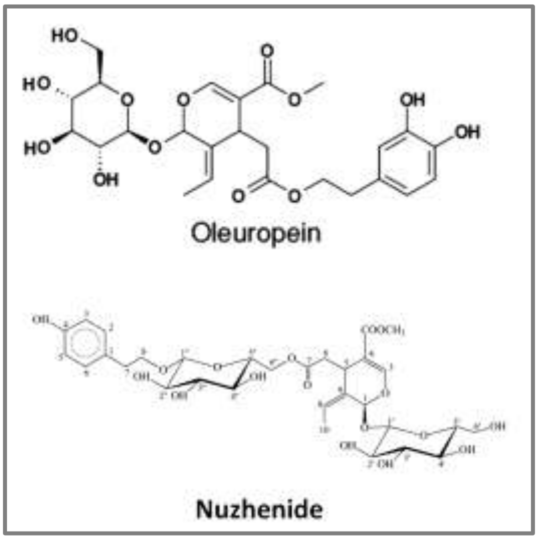

Figure 21 – Molecular structure of the seicoiridoids oleuropein and nuzhenide.

As shown in figure 20, in all the extracts, the peak 18, corresponding to (+)-cycloolivil **(table 2, section 5.1.1.2., page 29),** was identified in the first 30 min of extraction, but its concentration appears to decrease along the time. It is a compound with low molecular weight and with less hydroxyl groups than seicoiridoids. Its concentration appears decreased in the presence of water, but increased with the presence of higher EtOH concentrations (extract of 75%). These results may be explained by the fact that, in order to remove lignans from lignocellulosic matrix, such as the olive stone, the production of low molar mass lignan fragments and their further dissolution in the solvent mixture are required. In high pressure extraction the lignan was mostly removed during the first 30 min of reaction due to the use of solvent mixture, possibly because the addition of water promoted the delignification reactions (i.e., separation of lignans from the matrix), and the EtOH increased the ability to dissolve the lignan particles generated in the process.

According to Pasquini et al. (2005), the best solvent mixture ratio to separate lignans is 1:1 (v/v) ethanol-water mixture. In this work, the best mixture to separate lignan compound at 30 min, was the mixture of CO_2 plus 25% of EtOH, followed by a ternary mixture 40:50:10 (CO_2:EtOH:H_2O). However, the mixture composition with CO_2 and 50% of EtOH was better at longer times.

The extracts obtained from PLE using the mixture of 25:65:10 (CO_2: EtOH: H_2O), were those that allowed to obtain the compounds of interest, nuzhenide, oleuropein and lignans, in the first 30 minutes of extraction. Extraction by SFE with sc-CO_2 plus 75% of EtOH also allowed the recovery of seicoiridoids in the first 30 minutes of extraction. But, to extract lignans using this combination or other of EtOH + CO_2, it took at least one hour of extraction.

Considering all the results presented in this section, it’s possible to conclude that in case this method is integrated as a compound recovery system from wild olives, it would require an initial 2-3 hour extraction with sc-CO_2, in order to remove nonpolar fraction from the matrix. The delipidated matix must then be extracted with PLE plus the mixture 25:65:10 (CO_2: EtOH: H_2O), during 15 min (incubation) plus 30 minutes.

5.3.4. Antioxidant Activity

In order to evaluate the antioxidant activity from high pressure extracts of zambujeiro olive fruits two assays were performed – ORAC and HORAC. The previously prepared conventional extracts form zambujeiro seed and stones didn’t show high activity in the hydroxyl radical scavenging assay (HOSC). Thus, in this part of the work, we chose to use only the other two antioxidant capacity assays, in which the conventional extracts of zambujeiro displayed higher activity.

Results from both assays are represented in **Figure 22**. The ORAC values ranged from 607.3 ± 81.2 to 8789.5 ± 638.9 µmol trolox/L extract, 1615.9 ± 146.4 to 6569.9 ± 360.2 µmol trolox/L extract, 1502.8 ± 81.2 to 4537.4 ± 320.3 µmol trolox/L extract, and from 1418.9 ± 132.5 to 4418.2 ± 406.4 µmol trolox/L extract, at 30min, 1h, 1h30min and 2 hours of extraction, respectively. The highest value of peroxyl scavenging capacity was observed at 30 min with the ternary extract 25:65:10, and the lowest value was observed with the 75% EtOH extract, at 30 min. Along the time, the ORAC values decreased in both ternary extracts until they reached a plateau, whereas in the extracts with binary mixtures, the values increased after 1 hour of extraction until also reaching a plateau.

The obtained HORAC values were similar among all the extracts tested. The HORAC values ranged from 918.9 ± 77.1 to 2053.5 ± 154.5 µmol CAE/L extract. The ternary extracts showed a slight increase in their hydroxyl radical prevention (HORAC) capacity. The correlation value between the TPC and each of the assays was low – 0.1662 and 0.2731, for ORAC and HORAC, respectively. However, there is a possible correlation between HORAC results and lignan content (r^2 = 0.5841).

Summarizing, the extracts from zambujeiro olives obtained with a mixture of 25:65:10 (CO_2:EtOH:H_2O) in the first 30 min of extraction, were the ones that showed the highest antioxidant activity in both assays performed. The antioxidant activity of those extracts is in agreement with the behaviour of the compounds referred in the previous section **(section 5.3.2.)**, in which, the target compounds were all determined during the first 30 minutes of extraction. The previously obtained total phenollic content was also higher during the first 30 minutes of extraction, in all the tested solvent mixtures.

In future works, it will be interesting to isolate the target compounds from the extracts, in order to determine their specific bioactivity.

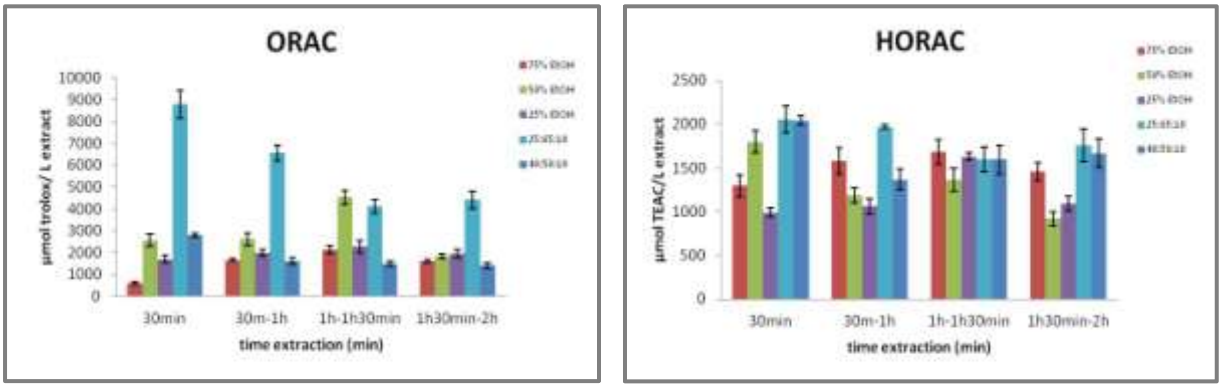

Figure 22 – Antioxidant assays of olive fruits high pressure extracts from zambujeiro.

6. Conclusions

This study described the characterization of zambujeiro olive seeds' and stones.

Conventional extracts of olive seeds from zambujeiro were prepared with different solvents, and compared with extracts of olive seed's from cultivars. The methanolic extracts were those that permitted a better extraction of phenolic compounds from the matrices analyzed. The extracts of zambujeiro seeds showed high phenolic content, higher than some cultivares and similar to others.

In order to characterize zambujeiro olive stones, three different procedures were used. Among the extracts obtained, those from extraction with MeOH at reflux showed highest phenolic content, more peaks detected in HPLC-analysis and, it was the only one where lignans were detected.

The bioactivity from the obtained extracts was evaluated through the antioxidant activity. The extracts from olive seeds showed high peroxyl radical scavenge capacity, whereas the extracts obtained from olive stones showed antioxidant activity against hydroxyl radical. Those differences may be due to the different composition of seeds and stones. The major compounds found in olive seeds were seicoiridoids, mainly nuzhenide, while in stones the main compounds were lignans. Upon a cellular proliferation assay, the extracts from zambujeiro seeds were the only ones that had antiproliferative effect in HT29 cells line, which might be related to their nuzhenide content.

In order to recover phenolic compounds from zambujeiro olive fruits, a GRAS process was developed, through the use of SFE with sc-CO_2 plus EtOH (25, 50 and 75%), and PLE with different mixtures of CO_2:EtOH:H_2O (25:65:10 and 40:50:10). The obtained extracts from PLE with the mixture 25:45:10 were those with the highest antioxidant activity and TPC values. This mixture permitted the extraction of target compounds, such as nuzhenide and lignans, in the first 30 minutes of the extraction. These results can be explained by the utilization of EtOH which increases the solubility of nuzhenide and enhances the separation of lignans from the matrix. Considering all the results presented in this section, it's possible to conclude that in case this method is integrated as a compound recovery system from wild olives, it would require an initial 2-3 hour extraction with sc-CO_2, in order to remove nonpolar fraction from the matrix. The delipidated matix must then be extracted with PLE plus the mixture 25:65:10 (CO_2: EtOH: H_2O), during 15 min (incubation) plus 30 minutes.

More experimental studies are necessary in order to valorize these olive fruits from zambujeiro. Next studies should be focused on the isolation of target compounds from the matrix, in order to determine the direct relation of them with the bioactivity showed by the obtained extracts.

7. References

Alagna, F., Mariotti, R., Panara, F., Caporali, S., Urbani, S., Veneziani, G., Esposto, S., Taticchi, A., Rosati, A., Rao, R., Perrota, G., Servilli, M., Baldoni, L. (2012). Olive phenolic compounds: metabolic and transcriptional profiling during fruit development.BMC Plant Biology, 12, 1 – 19.

Alam, M. N., Bristi, N. J., Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharmaceutical Journal, 21, 143 – 152.

Almeida, Ana Patricia Correia. (2009). Utilização da Tecnologia Supercritical no Isolamento de Ingredientes Biofuncionais – aplicação ao caroço de azeitona e refugo de cereja. IBET/ITQB-UNL Nutraceuticals and Controlled Delivery Laboratory.

Alu'datt MH., Alli I., Ereifej K., Alhamad MN., Alsaad A., Rababeh T. 2011. Optimisation and characterization of various extraction conditions of phenolic compounds and antioxidant activity in olive seeds. *Natural Prod Researc*, 25 (9): 876 – 889.

Andjelkovic M., Camp JV., Meulenaer B., Depaemelaere G., Socaciu C., Verloo M., Verhe R. (2006). Iron-chelation properties of phenolic acids bearing catechol and galloyl groups. *Food Chem*, 98: 23 – 31.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N., Omar, A. K. M. (2013). Techniques for extraction of bioactive compounds from plant materials: a review. Journal of Food Engineering, 117, 426 – 436.

Brahimi, F., Mechri, B., Dabbou, S., Dhibi, M., Hammami, M. (2012). The efficacy of phenolics compounds with different polarities as antioxidants from olive leaves depending on seasonal variations. Industrial Crops and Products, 38, 146 – 152.

Brenes, M., Hidalgo, F. J., García, A., Rios, J. J., García, P., Zamora, R., Garrido, A. (2000). Pinoresinol and 1 – acetoxypinoresinol, two new phenolic compounds identified in olive oil. Journal of American Oil Chemists' Society, 77, 715 – 720.

Brower, V. (1998). Nutraceuticals: poised for a healthy slice of the healthcare market?.Nature Biotechnology, 16, 728 – 731.

Bubonja-Sonje, M., Giacometti, J., Abram, M. (2011). Antioxidant and antilisterial activity of olive oil, cocoa and rosemary extract polyphenols. Food Chemistry, 127, 1821 – 1827.

Bulotta, S., Corradino, R., Celano, M., D'Agostinho, M., Maiuolo, J., Oliverio, M. (2011). Antiproliferative and antioxidant effects on breast cancer cells of oleuropein and its semisynthetic peracetylated derivatives. Food chemistry, 127, 1609 – 1614.

Cardoso SM., Guyot S., Marnet N., Lopes-da-Silva JA., Renard CMGC. And Coimbra MA. (2005). Characterization of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J Scie Food and Agricult*. 85: 21 – 32.

Casaburi, I., Puoci, F., Chimento, A., Sirianni, R., Ruggiero, C., Avena, P., Pezzi, V. (2013). Potential of olive oil phenols as chemopreventive and therapeutic agents agains cancer: A review of in vitro studies. Molecular Nutrition and Food Research, 57, 71 – 83.

Cháfer, A., Fornari, T., Berna, A., Esteva, R. P. (2004). Solubility of quercetin in supercritical CO2 + ethanol as a modifier: measurements and thermodynamic modelling. Journal of Supercritical Fluids, 32, 89 – 96.

Charoenprasert S. and Mitchell A. 2012. Factors influencing compounds in table olives (*Olea europaea*). *J Agric Food Chem*, 60: 7081 – 7095.

Ciftci, O. N. (2012). Supercritical fluid technology: application to food processing. Journal of Food Processing & Technology, 3, 1 – 2.

Cioffi, G., Pesca, M. S., de Caprariis, P., Branca, A., Severino, L., de Tommasi, N. (2010). Phenolic compounds in olive oil and olive pomace from Cilento (Campania, Italy) and their antioxidant activity. Food Chemistry, 121, 105 – 111.

Chiappetta, A. and Muzzalupo, I. (2012). Botanical Description: Olive Germplasm – The Olive Cultivation, Table Olive and Olive Oil Industry in Italy. Available in http://cdn.intechopen.com/pdfs/41350/InTech-Botanical_description.pdf .

Choi, Y. H., Kim J., Jeon, S., H., Yoo, K. P., Lee, H. K. (1998). Optimum SFE condition for lignin of Schisandra chinensis fruits. Chromatographia, 48, 695 – 699.

Conde, E., Cara, C., Moure, A., Ruiz, E., Castro, E., Domínguez, H. (2009). Antioxidant activity of phenolic compounds released by hydrothermal treatments of olive tree prunning. Food Chemistry, 114, 806 – 812.

Covas, M. I. (2007). Olive oil and the cardiovascular system. Pharmacological Research, 55, 175 – 186.

Davis, J. P., Dean, L. L., Price, K. M., Sanders, T. H. (2010). Roast effects on the hydrophilic and lipophilic antioxidant capacities of peanut flours, blanched peanut seed and peanut skins. Food Chemistry, 119, 539 – 547.

Espín, J . C., Garcia-Conesa, M. T., Tomas-Barberan, F. A. (2007). Nutraceuticals: facts and fiction. Phytochemistry, 68, 2986 – 3008.

Esteve, C., D'Amato, A., Marina, M. L., García, M. C., Citterio, A., Righetti, P. G. (2012). Identification of olive (Olea europaea) seed and pulp proteins by nLC-MS/MS via combinatorial peptide ligand libraries. Journal of Proteomics, 75, 2396 – 2403.

Fabiani R., Bartolomeo AD., Rosignoli P., Servili M., Selvaggini R., Montedoro GF., Di Saverio C., Morozzi G. (2006). Virgin olive oil phenols inhibit proliferation of human promyelocytic leukemia cells (HL60) by inducing apoptosis and differentiation. *J Nutr*, 136: 614 – 619.

Fink, M. P. (2002). Role of reactive oxygen and nitrogen species in acute respiratory distress syndrome. *Current Opinion in Critical Care*, 8, 6 -11.

Gomes, A., Fernandes, E., Lima, J. L. F. C. (2005). Fluorescense probes used for detection of reactive oxygen species.Journal of Biochemical and Biophysical Methods, 65, 45 -80.

Hayes, J. E., Allen, P., Brunton, N., O'Grady, O., Kerry, J. P. (2011). Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (*Olea europaea* L.), lutein, sesamol and ellagic acid. Food Chemistry, 126, 948 – 955.

Herrero, M., Castro-Puyana, M., Mendiola, J., A., Ibañez, E. (2013). Compressed fluids for the extraction of bioactive compounds. Trends in Analytical Chemistry, 43, 67 – 83.

Herrero, M., Cifuentes, A., Ibañez, E. (2006). Sub- and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae. A review. Food Chemistry, 98, 136 – 148.

Huang, D., Ou, **B.,** Hampsch-Woodill, M., Flanagan, J. A., Prior, R. L.. (2002). High Throughput Assay of Oxygen Radical Absorbance Capacity (ORAC) using a multichannel liquid handling system coupled with a Microplate Fluorescence Reader in 96-well format, Journal of Agricultural and Food Chemistry, 50, 4437-44.

Ignat, I., Volf, I., Popa, V. I. (2011). A critical review of methods for characterization of polyphenolic compounds in fruits and vegetables. Food Chemistry, 126, 1821 – 1835.

Kostas, K., Kontominas, M. G., Kontogiorgis, C., Hadjipavlou-Litina, D., Moustakas, A., Kiritsakis, A. (2009). Composition and antioxidant activity of olive leaf extracts from Greek olive cultivars. Journal of American Oil Chemistry Society, 87, 369 – 376.

Kwak, N. S., & Jukes, D. J. - (2001). Functional foods. Part 1. The development of a regulatory concept. Food Control, 12, 99 – 107.

Lafka, T. I., Lazou, A. E., Sinanoglou, V. J. (2013). Phenolic extracts from wild olive leaves and their potential as edible oils antioxidants. Foods, 2, 18 – 31.

Lee, H. J. and Kim, C. Y. (2010). Simultaneous determination of nine lignans using pressurized liquid extraction and HPLC-DAD in the fruits of Schisandra chinensis. Food Chemistry, 120, 1224 – 1228.

Le Floch, F., Tena, M. T., Ríos, A., Valcárcel, M. (1998). Supercritical fluid extraction of phenol compounds from olive leaves. Talanta, 46, 1123 – 1130.

López, M. J. O., Innocenti, M., Ieri, F., Giaccherini, C., Romani, A., Mulinacci, N. (2008). HPLC/DAD/ESI/MS detection of lignans from spanish and italian Olea europaea L. fruits. Journal of Food composition and Analysis, 21, 62 – 70.

Lowcock, E. C., Cotterchio, M., Boucher, B. A. (2013). Consumption of flaxseed, a rich source of lignans, is associated with reduced breast cancer risk. Cancer Causes Control, 24, 813 – 816.

Mahabir, S. (2013). Methodological challenges conducting epidemiological research on nutraceuticals in health and disease. PharmaNutrition, 23, 1 – 6.

Martinez-Dominguez E., De la Puerta R., Ruiz-Gutierrez V. (2001). Protective effects upon experimental inflammation models of a polyphenol-supplemented virgin olive oil diet. *Inflammation Res*, 50: 102 – 106.

Matos, M., Barreiro, M. F., Gandini, A. (2010). Olive stone as a renewable source of biopolyols. Industrial Crops and Products, 32, 7 – 12.

Ministério da Agricultura, do Desenvolvimento Rural e das Pescas (MADRP). (2007). Olivicultura – Diagnóstico Sectorial. Gabinete de Planeamento e Políticas. Lisboa, Portugal. Disponível em http://www.gpp.pt/pbl/diagnosticos/azeite__diagnostico_sectorial.pdf [Accessed data 10 March de 2013].

Motilva, M. J., Serra, A., Macià, A. (2013). Analysis of food polyphenols by ultra high-performance liquid chromatography coupled to mass spectrometry: An overview. Journal of Chromatography A, 1292, 66 – 82.

Moure, A., Cruz, J. M., Franco, D., Domínguez, J. M., Sineiro, J., Domínguez, H., Núñez, M. J., Parajo, J. C. (2001). Natural antioxidants from residual sources. Food Chemistry, 72, 145 – 171.

Naczk, M., and Shahidi, F. (2004). Extraction and analysis of phenolics in food. Journal of chromatography A, 1054, 95 – 111.

Obied, H. K., Bedgood, D. R., Jr., Prenzler, P. D., Robards, K. (2007). Bioscreening of Australian olive mill waste extracts: biophenol content, antioxidant, antimicrobial and molluscicidal activities. Food and Chemical Toxicology, 45, 1238 – 1248.

Othman, N. B., Roblain, D., Thonart, P., Hamdi, M. (2008). Tunisian table olive phenolic compounds and their antioxidant capacity. Journal of Food Science, 73, 235 – 240.

Ou, B., Hampsch-Woodill, M., Flanagan, J., Deemer, E. K., Prior, R. L., Huang, D. (2002). Novel fluorometric assay for hydroxyl radical prevention capacity using fluorescein as the probe. Journal of Agriculture and Food Chemistry, 50, 2772 – 2777.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Spiegelhalder, B., Bartsch, H. (2000). The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. European journal of Cancer, 36, 1235 – 1247.

Pasquini, D., Pimenta, M. T. B., Ferreira, L. H., Curvelo, A. A. S. (2005). Extraction of lignin from sugar cane bagasse and Pinus taeda wood chips using ethanol-water mixtures and carbon dioxide at high pressures. The Journal of Supercritical Fluids, 36, 31 – 39.

Peralbo-Molina, A., de Castro, M., D., L. (2013). Potential of residues from the Mediterranean agriculture and agrifood industry. Trends in Food Science & Technology, 32, 16 – 24.

Petti, S., Scully, C. (2009). Polyphenols, oral health and disease: a review. Journal of Clinical Dentistry, 37, 413 – 423.

Plaza, M., Herrero, M., Cifuentes, A., Ibáñez, E. (2009). Innovative natural functional ingredients from microalgae. Journal of Agricultural and Food Chemistry, 57, 7159 – 7170.

Poejo, Joana Margarida de Andrade. (2011). Evaluation of Opuntia spp. Bioactive products as promising natural chemotherapeutical agents – an in vitro approach", IBET/ITQB-UNL Nutraceuticals and Controlled Delivery Laboratory.

Price, J. A., Sanny, C. G., Shevlin, D. (2006). Application of manual assessment of oxygen radical absorbent capacity (ORAC) for use in high throughput assay of "total" antioxidant activity of drugs and natural products. Journal of Pharmacological and Toxicological Methods, 54, 56 – 61.

Quiles, J. L., Farquharson, A. J., Simpson, D. K., Grant, I., Wahle, K. W. J. (2002). Olive oil phenolics: effectson DNA oxidation and redox enzyme mRNA in prostate cells. British Journal of Nutrition, 88, 225 – 234.

Robards K., Prenzler PD., Tucke G., Swatsitang P. and Glover W. (1999). Phenolic compounds and their role in oxidative processes in fruits. *Food Chem*, 66: 401 – 436.

Ryan D. and Robards K.(1998). Phenolic compounds in olives. *Analysis*, 123: 31R – 44R.

Ryan, D., Antolovich, M., Prenzler, P., Robards, K., Lavee, S. (2002). Biotransformation of phenolic compounds in Olea europaea L. Scientia Horticulture, 92, 147 – 176.

Şahin, S. and Bilgin, M. (2012). Study on oleuropein extraction from olive tree (Olea europaea) leaves by means of SFE: comparison of water and ethanol as co-solvent. Separation Science and Technology. 47, 2391 – 2398.

Sánchez, C. S., González, A. M. T., García-Parrilla, M. C., Granados, J. J. Q., de la Serrana, H. L. G., Martínez, M. C. L. (2007). Different radical scavenging tests in virgin olive oil and their relation to the total phenol content. Analytica Chimica Acta, 593, 103 – 107.

Scognamiglio, M., D'Abrosca, B., Pacifico, S., Fiumano, V., De Luca, P. F., Monaco, P., Fiorentino, A. (2012). Polyphenol characterization and antioxidant evaluation of Olea europaea varieties cultivated in Cilento National Park (Italy). Food Research International, 46, 294 – 303.

Seabra, I. J., Braga, M. E. M., de Sousa, H. C. (2012). Statistical mixture design investigation of CO_2 – Ethanol – H_2O pressurized solvent extractions from tara seed coat. The Journal of Supercritical Fluids, 64, 9 - 18.

Serra, Ana Teresa. (2009). "Valorization of Traditional Portuguese Apples and Cherries. Biochemical Characterization and Development of Functional Ingredients", IBET/ITQB-UNL Nutraceuticals and Controlled Delivery Laboratory.

Serra, A. T., Seabra, I. J., Braga, M. E. M., Bronze, M. R., Sousa, H. C., Duarte, C. M. M. (2010). Processing cherries (Prunus avium) using supercritical fluid technology. Part 1: Recovery of extract fractions rich in bioactive compounds. The Journal of Supercritical Fluids, 55, 184 – 191.

Shilpi, A., Shivhare, U. S., Basu, S. (2013). Supercritical CO_2 extraction of compounds with antioxidant activity from fruits and vegetables waste – a review. Journal of Food and Modern Industry, 2, 43 – 62.

Silva S., Gomes L., Leitão F., Coelho, A.V., Vilas Boas, L. (2006). Phenolic compounds and antioxidant activity of *Olea europaea* L. fruits and leaves. *Food Science and Technology Int*, 12:385-396.

Silva S., Gomes L., Leitão F., Bronze M., Coelho, A.V., Vilas Boas, L. 2010. Secoiridoids in olive seeds: characterization of nüzenide and 11-methyl oleosides by liquid chromatography with diode array and mass spectrometry. *Grasas Y Aceites*, 61:157-164.

Siriani, R., Chimento, A., De Luca, A., Casaburi, I., Rizza, P., Onofrio, A. (2010). Oleuropein and hydroxytyrosol inhibit MCF-7 breast cancer cell proliferation interfering with ERK1/2 activation. Molecular Nutrition & Food Research, 54, 833 – 840.

Soler-Rivas, C., Espín, J. C., Wichers, J. (2000). Review oleuropein and related compounds. Journal of Science of Food and Agriculture, 80, 1013 – 1023.

Torres de Pinedo, A., Peñalver, P., Morales, J. C. (2007). Synthesis aand evaluation of new phenolic-based antioxidants: structure-activity relationship. Food Chemistry, 103, 55 – 61.

Vinha, A. F., Ferreres, F., Silva, B. M., Valentão, P., Gonçalves, A. , Pereira, J. A., Oliveira, M. B., Seabra, R. M., Andrade, P. B. (2005). Phenolic profiles of Portuguese olive fruits (*Olea europaea* L.): influences of cultivar and geographical origin. *Food Chem*, 89: 561 – 568.

Appendix 1 – Cell viability assays

Cell culture

Human colon cancer cell lines, HT-29 and Caco-2 provided by American type Culture Collection (ATCC, USA) Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ, Germany), respectively, were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine. Both cell lines were kept at 37 ^{0}C in a humidified incubator with 5% CO_2 and routinely grown in 75 cm^2 culture flasks. Cell culture medium and supplements were obtained from Invitrogene (Gibco, Invitrogene Corporation, Paisley, UK). The cell lines were split once or twice a week and morphology and growth of cells were monitored daily.

Proliferation assay

To evaluate anticancer potential and effective dose (ED50-amout of sample necessary to decrease 50% of viability) of natural extracts, antiproliferative assay was performed on human colon adenocarcionoma. HT29 cell line growth as monolayer was the *in vitro* model used.

The assay was carried out in 96-well microplates at a cellular density of $1x10^4$ cell/well. Cells remained 24 hours in 5% CO_2 humidified atmosphere at 37 ^{O}C. After this period medium was removed and cells were incubated with natural extracts samples previews diluted in RPMI medium supplemented with 0,5% FBS. After 24h of exposure to the extracts, the medium was removed, once again, and cell viability assay was performed using commercial PrestoBlue® Viability Reagent (Molecular Probes, Invitrogen, US). In addition, the results were expressed as a percentage of growth inhibition and determinate by comparing the cell density of extracts-treated cells with the untreated in the same period of incubation. The experiment was performed in triplicate and ED50 were obtained from dose-response curves non-linear curve fitting using software GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

Cytotoxicity assay

In vitro cytotoxicity assay was accomplished using differentiated Caco-2 cells. Despite were obtained from a human colon adenocarcionoma, this cells can be used as intestinal barrier model when cultured under special conditions. When Caco-2 becomes differentiated and polarized shows a resemblance morphological and functional to enterocytes from small intestine. Their differentiation occurs spontaneously when cells grow in monolayer in a long term culture.

Cells are seeded into 96-well culture plates at a density of $2x10^4$ cells/well, and are allowed to growth for 8 days, with medium exchange every 48h. Extracts samples previously diluted with culture medium (RPMI with 0.5% FBS and 2mM glutamine) was added except to control samples. Incubation with natural extracts was carried out during 24 hours and cytotoxicity evaluation was performed with PrestoBlue® Viability Reagent (Molecular Probes, Invitrogen, US). Viability, expressed as percentage of living cells, was determinate relatively to the control and IC50 were obtained from dose-response curves using software GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) fit.