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Study of the best technique to extract compounds from *Olea europaea* and *Acacia dealbata* with bioactivity

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Helena Manuel de Azevedo Ferreira José

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Supervisors: Prof. Manuel Simões and Prof. Vera Homem

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Supervisors

Manuel Simões

Vera Homem

‘‘The future is an opportunity.’’

J. F. Ware

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Aos meus pais, à minha irmã e aos meus avós.

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ABSTRACT

Plants are used with several purposes, like in medicine, decoration, food and as sustainable resource of chemical products. Medicine is increasingly receptive to their use. The interaction between antibiotic exposure and the transmission of resistance within and between individuals results in a worrying antibacterial resistance. The compounds extracted from plants have different mechanisms of action from antibiotics and can have an important role in the treatment of resistant microbial strains.

The main bioactive compounds from plants are phenolics and polyphenols, which involve simple phenols, phenolic acids, quinones, flavones, flavonoids, flavonols, tannins and coumarins, terpenoids and essential oils, alkaloids, lectins and polypeptides. To obtain these compounds there are some methods that are usually used, like solid-liquid extraction, Soxhlet, microwave assisted extraction and superfluid critical extraction.

The main purpose of this study was to investigate the methods of extraction of two plants in order to obtain the best extracts with higher bioactive properties, particularly, antimicrobial and antioxidant. Extracts from *Olea europaea* (olive) and *Acacia dealbata* (mimosa), obtained by solid-liquid extraction, ultrasounds extraction, Soxhlet and micro-wave extraction, were tested in their antimicrobial and antioxidant activity. Different solvents (methanol, ethanol, acetone, dichloromethane, hexane and water) were tested in solid-liquid and ultrasounds extraction.

In relation to the extraction yield, methanol and water were considered the best solvents to solid-liquid and ultrasound extractions. Comparing all the techniques used in the current study, Soxhlet and micro-wave were the methods that were able to achieve the best extraction efficiency. For olive leaves the efficiency was 12.3 ± 1.2% and 10.7 ± 0.8% and for mimosa leaves it was 13.4 ± 1.4 %and 11.9 ± 1.8%, respectively.

It was concluded that mimosa and olive can produce extracts with similar antimicrobial and antioxidant activities. Ethanol proved to be the best solvent to extract compounds with antimicrobial activity, whereas to extract compounds with antioxidant properties acetone proved to be the best solvent. Soxhlet and micro-wave extractions were the best techniques to extract compounds with antimicrobial activity, whereas to extract compounds with antioxidant activity any method was highlighted.

In most cases, extracts were more efficient against *S. aureus* than *E. coli*, suggesting the susceptibility of *S. aureus* due to the permeability of the cell wall. It was also proved that with the decreasing of extract concentration the antimicrobial activity also decreases, being interesting to find what is the minimal concentration at which the extract is efficient. The combination of extracts of olive and mimosa with tetracycline and erythromycin against *E. coli* and *S. aureus* was not benefic to improve the antimicrobial action, and the leave extracts and the leaves themselves were not efficient on quorum-sensing inhibition.

RESUMO

As plantas são usadas em diversos fins, como na medicina, decoração, alimentação e, ainda, como recurso sustentável de produtos químicos. A medicina está cada vez mais recetiva ao seu uso. A interação entre a exposição a antibióticos e a transmissão da resistência entre indivíduos resulta numa preocupante resistência antibacteriana. Os compostos extraídos das plantas têm diferentes mecanismos de acção dos antibióticos e podem ter um papel importante no tratamento de estirpes de microrganismos resistentes.

Os principais compostos bioactivos das plantas são os alcalóides, compostos fenólicos e polifenóis, que envolvem os fenóis simples, ácidos fenólicos, quinonas, flavonas, flavonóides, taninos e cumarinas, lectinas e polipéptidos e terpenenóides e óleos essenciais. Para obter estes compostos existe algumas técnicas que são normalmente usadas, como a extração sólido-líquido, Soxhlet, extração com micro-ondas e extração por fluido supercrítico.

O principal objetivo deste estudo foi investigar os métodos de extração de duas plantas para obter os extratos com propriedades bioativas mais revelantes, particularmente propriedades antimicrobianas e antioxidantes. Extratos de *Olea europaea* (oliveira) e *Acacia dealbata* (mimosa), obtidos por extração sólido-líquido, extração com ultra-sons, Soxhlet e extração com micro-ondas, foram testados nas suas atividades antimicrobianas e antioxidantes. Diferentes solventes (metanol, etanol, acetona, diclorometano, hexano e água) foram testados na extração sólido-líquido e com ultra-sons.

Em relação ao rendimento de extração, o metanol e a água foram considerados os melhores solventes para extração sólido-líquido e com ultra-sons. Comparando todas as técnicas usadas neste estudo, Soxhlet e extração com micro-ondas foram os métodos que promoveram a melhor eficácia de extração. Nestas técnicas, para as folhas de oliveira a eficiência foi de 12.3 ± 1.2% e 10.7 ± 0.8% e para as folhas de mimosa foi de 13.4 ± 1.4% e 11.9 ± 1.8%, respetivamente.

Concluiu-se que as folhas de mimosa e oliveira podem produzir extratos com atividade antimicrobiana e antioxidante similar. Etanol provou ser o melhor solvente para extrair compostos com atividade antimicrobiana, enquanto que, para extrair compostos com propriedades antioxidantes, a acetona provou ser o melhor solvente.

Extrações com Soxhlet e micro-ondas foram as melhores técnicas para extrair compostos com atividade antimicrobiana. Para extrair compostos com atividade antioxidante nenhuma técnica se sobressaiu.

Na maioria dos casos, os extratos foram mais eficientes contra *S. aureus* do que *E. coli*, sugerindo a suscetibilidade de *S. aureus* devido à permeabilidade da parede celular. Foi provado que, com a diminuição da concentração do extrato a atividade antimicrobiana também diminui, sendo interessante descobrir qual é a concentração mínima em que o extracto é eficiente. A combinação dos extratos de oliveira e mimosa com tetraciclina e eritromicina contra *E. coli* e *S. aureus* não foi benéfica na melhoria da atividade antimicrobiana dos antibióticos e os extratos das folhas e as próprias folhas não foram eficientes na inibição do *quorum-sensing*.

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GLOSSARY

ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
CFU	Colony-forming unit
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
HIV	Human immunodeficiency virus
LBA	Luria-Bertani Agar
MAE	Micro-wave assisted extraction
MHA	Mueller-Hinton Agar
MIC	Minimum inhibitory concentration
QS	Quorum-sensing
QSI	Quorum-sensing inhibition
RSV	Respiratory syncytial virus
SFE	Supercritical fluid extraction
SPSS	Statistical Package for the Social Sciences
TE	Trolox Equivalent

CHAPTER 1 - Work Outline

1.1 Background

Plants have been used with several purposes, like in medicine, decoration, food and as sustainable resource of chemical products. Medicine is one of the areas where plants are becoming a great subject of study, being increasingly receptive to their use. Actually, the evolution of bacterial resistance to antibiotics is becoming more and more an issue of concern. A promising alternative could be the antimicrobial compounds extracted from plants, which have different mechanisms of action from antibiotics and can have an important role in the treatment of resistant microbial strains (Abreu et al., 2012). The search for new anti-infective agents (including vaccines) could also be answered by plant extracts.

There are studies that have been reported that extracts from plants are effective in treating febrile illnesses, sleeping sickness, wounds, diarrhea, reproductive and liver problems, circulatory and respiratory problems and parasitic infections. Therefore, they have a great power in health care (Chah et al., 2006; Obi et al., 2006). Their main compounds are alkaloids, lectins, polypeptides, phenolics, polyphenols, terpenoids and essential oils, which have recognized antimicrobial and antioxidant properties (Cowan, 1999; Karou, 2005; Gallo, 2010).

To obtain the extracts from plants there are several techniques that can be used. Solid-liquid, Soxhlet, supercritical fluid and micro-wave assisted extractions are some examples (Aziz, 2006; Tatke and Jaiswal, 2011).

1.2 Objectives

The main purpose of this study was to investigate the methods of extraction of two plants in order to obtain the best extracts with higher bioactive properties, particularly, antimicrobial and antioxidant. Extracts from *Olea europaea* (olive) and *Acacia dealbata* (mimosa), obtained by solid-liquid extraction, ultrasounds extraction, Soxhlet and micro-wave extraction were tested for their antimicrobial and antioxidant activities. Different solvents (methanol, ethanol, acetone, dichloromethane, hexane and water) were tested in solid-liquid and ultrasounds extraction. Many studies about *Olea europaea* demonstrate its antimicrobial and antioxidant properties, suggesting that it can

be used in diverse applications. Therefore, the results obtained with the extracts from this plant were taken as reference values. About *Acacia dealbata*, an invasive plant, information on the extracts bioactivity is lacking.

The antimicrobial activity of the extracts was assessed against two bacteria. *S. aureus*, a Gram-positive bacterium, and *E. coli*, a Gram-negative bacterium, were chosen for this study since they are considered two of the most clinical significant bacteria involved in drug-resistant infections (Simões et al., 2008). Antimicrobial properties were evaluated with the extracts alone and together with two antibiotics, erythromycin and tetracycline. Thereby, it was possible to observe if these combinations are benefic or not. Moreover, the leaves and extracts of plants were also tested for their capacity of inhibiting quorum-sensing (QS).

The antioxidant activity was studied by two methods: using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS). The goal of both tests was to evaluate the capacity of the redox molecules from the plants extracts to scavenge the free radicals.

The main objective of this work was to verify what was the type of extraction which allowed to obtain extracts with the highest antimicrobial and antioxidant activities, in order to maximize the extraction yield of the bioactive products with potential medicinal application.

1.3 Thesis Organization

This thesis is divided in 6 Chapters and respective subchapters.

It starts with a background of the theme in Chapter 1, where the main goals of the study are presented.

Chapter 2 is dedicated to a literature review about the plants and their relevance in diverse applications, focusing on the use of plant extracts in the clinical setting. The main bioactive compounds from plants are briefly described, as well as the main methods to extract them and the relation between the extraction solvent and the compounds extracted.

Chapter 3 provides information on the assessment of the extraction efficiency of *Olea europaea* and *Acacia dealbata* extracts, using different techniques and extraction solvents. The goal of this work was to find the type of extraction and the solvent which caused the higher efficiency.

Chapter 4 shows the data from the bioactivity assessment of *Olea europaea* and *Acacia dealbata* extracts. Extracts were tested for their antimicrobial potential, alone and together with antibiotics, and for their antioxidant activities. This chapter aims to evaluate the antimicrobial and antioxidant activities of the plant extracts and to conclude about the best extraction method that produces an extract with high bioactive properties.

Finally, Chapter 5 presents the main conclusions about this study and gives suggestions for future research.

CHAPTER 2 - Literature Review

2.1 Introduction

It is known that there are 250000 to 500000 species of plants on Earth (Borris, 1996) and they are used with several purposes, in areas such as medicine, decoration, food and as sustainable resources of chemical products. Plants produce diverse secondary metabolites and part of this chemical diversity helps to protect plants against pathogenic microorganisms (Dixon, 2001).

Medicine is increasingly receptive to the use of plants. The interaction between antibiotic exposure and the transmission of resistance within and between individuals results in a worrying antibacterial resistance (Guillemot, 1999). The relationship between antibiotic use and bacterial resistance is most evident when resistance is due to mutations selected during therapy, which can result in clinical failure of the therapeutic strategy (Guillemot, 1999). Plants have different mechanisms of action of current antibiotics (Abreu et al., 2012) and can be a promising alternative and/or complement.

Several plants have shown to have medicinal applications. Giving some examples, Webster et al. (2006) reported the antiviral, antifungal and antibacterial effects of *Heracleum maximum* and Chiang et al. (2003) reported the efficiency of *Plantago major* in the treatment of infectious diseases. Traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetical drugs use medicinal plants as the main bio-resource of drugs (Ncube et al., 2008).

Apart from their medicinal use, plants also have a great potential as an alternative resource for the production of polymeric materials. Characteristics such as renewability, world-wide availability and low price make them industrially attractive. Plants products have another advantage: with some chemical modifications it is possible to obtain suitable monomers for many different applications, including these medicinal (Espinosa and Meier, 2011).

2.2 The main compounds from plants

Many of the compounds of the plants act as defense mechanisms against predation by microorganisms, insects and herbivores. Furthermore, they have other functions, for instance some terpenoids are responsible for plant favour (e.g., terpenoid capsaicin) and quinones and tannins are responsible for plant pigment (Cowan, 1999). The major groups of bioactive compounds from plants are summarily presented in this section.

2.2.1 Alkaloids

Alkaloids are a diverse group of low molecular-weight, heterocyclic nitrogen compounds derived mostly from amino acids. As secondary metabolites found in approximately 20% of plant species, these compounds are purported to play a defensive role against herbivores and pathogens (Ziegler and Facchini, 2008). Owing to their potent biological activity, many of the approximately 12000 known alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poison (Ziegler and Facchini, 2008).

In most cases, the mechanism of action of alkaloids is attributed to their ability to intercalate with DNA (Phillipson and O'Neill, 1997). Antimicrobial activity is also due to an activity depending upon the chemical composition of the extracts and the membrane permeability of the microbes (Savoia, 2012). Alkaloids also possess antioxidant effects. They reduce nitrate generation which is useful for proteins synthesis (Tiwari et al., 2011).

There are many studies involving the properties of the alkaloids, for example, Karou et al. (2005) investigated the antimicrobial activity of alkaloids from *Sida acuta* against Gram-positive and Gram-negative bacteria. The alkaloids showed good antimicrobial activity against several microorganisms tested.

2.2.2 Lectins and polypeptides

Peptides which are inhibitory to microorganisms are often positively charged and contain disulfide bonds (Zhang and Lewis, 1997). The mechanism of antimicrobial action of these compounds is thought to be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Terras et al., 1993). Important examples of peptides are the

thionins (47 amino acid residues), which are toxic to yeasts and both Gram-negative and Gram-positive bacteria (Caleya et al., 1972).

Lectins are proteins/glycoproteins commonly found in seed, bark, stem and leaves (Varki et al., 1999). They have at least one non-catalytic domain that exhibits reversible binding to specific monosaccharides or oligosaccharides. They can bind to the carbohydrate moieties on the surface of erythrocytes and agglutinate the erythrocytes, without altering the properties of the carbohydrates (Lam and Ng, 2011). These compounds have been used for blood typing due to their ability to distinguish carbohydrate determinants in human blood cells. They are also efficient in defense against fungal and bacterial pathogens (Varki et al., 1999). Also, lectins and polypeptides have mainly antiviral activity. They block viral fusion or adsorption and forms disulfide bridges (Tiwari et al., 2011).

2.2.3 Phenolic and polyphenols

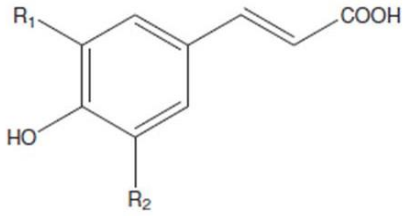
2.2.3.1 Simple phenols and phenolic acids

Some of the simplest bioactive compounds consist of a single substituted phenolic ring, like cinnamic and caffeic acids. These are representatives of a wide group of phenyl-propane-derived compounds (Cowan, 1999). The caffeic acid is effective against viruses, bacteria and fungi (Brantner et al., 1996).

Catechol and pyrogallol are hydroxilated phenols and the site and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, wherein an increased hydroxylation results in increased toxicity (Geissman, 1963). Catechol has two –OH groups and pyrogallol has three. There are phenolic compounds that contain a C_3 side chain at a lower level oxidation and no oxygen; they are classified as essential oils and often cited as antimicrobial as well. An example is eugenol, which is considered microbialstatic against both fungi (Duke et al., 2002) and bacteria (Cowan, 1999).

Phenolic acids are aromatic secondary plant metabolites (Stalikas, 2007). Predominant phenolic acids include hydroxibenzoic acids and hydroxicinnamic acids (Cai et al., 2006). Many of the phenolic acids like cinnamic and benzoic acid derivatives exist in all plants and plant-derived foods (like fruits, vegetables and grains) (Shahidi and Nacsk, 1995). Only a small percentage exists in the free acid form, since the major fraction is linked through ester, ether or acetal bonds to cellulose, proteins, lignin, flavonoids or glucose (Stalikas, 2007). Then, the phenolic acids have a great diversity.

Figure 1 shows the structure of three typical phenolic acids, namely the *p*-coumaric acid, the caffeic acid and the ferulic acid.



p-coumaric acid: R_1 = H; R_2 = H

caffeic acid: R_1 = H; R_2 = OH

ferulic acid: R_1 = H; R_2 = OCH_3

Figure 1 - Structure of three typical phenolic acids (adapted from Huang et al., 2009).

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. Furthermore, they have a metal chelation potential (Gallo et al., 2010).

The radical scavenging ability of phenolic acids depends on the number and position of hydroxyl groups and methoxy substituents in the molecules (Cai et al., 2006). Furthermore, these compounds and analogs can inhibit tumor cells and induce apoptosis by inducing cell cycle arrest; regulate signal transduction pathways; induce or inhibit some enzymes and enhance detoxification. Finally, some phenolic acids and analogs also exhibit antibacterial, antifungal, antiviral, antimutagenic and anti-inflammatory activities (Silici et al., 2007; Chaubal et al., 2005; Larrosa et al., 2006).

2.2.3.2 Quinones

Quinones are aromatic rings with two ketone substitutions and they are characteristically highly reactive. The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions (Cowan, 1999).

These compounds are a source of stable free radicals and they complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function (Stern et al., 1996). Then, the potential range of quinone antimicrobial effects is great.

There are four types of quinones, namely anthraquinones, phenanthraquinones, naphthoquinones and benzoquinones (Cai et al., 2004). The largest class of natural quinones is the first, anthraquinones. They occur more widely in the medicinal and dietary plants than other natural quinones (Cai et al., 2006). One of the types of the

anthraquinones is the hydroxyanthraquinones and they usually have one to three hydroxyl groups on the anthraquinone structure (Cai et al., 2004).

Huang et al. (2009) described several medicinal herbs where they found quinones, for example *Polygalaceae*, *Rubiaceae*, *Boraginaceae*, *Labiatae*, *Leguminosae* and *Myrsinaceae*.

Figure 2 illustrates the prototypical member of this class, cyclohexadienedione.

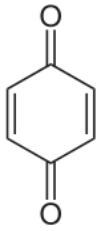


Figure 2. Structure of 1,4-benzoquinone or cyclohexadienedione, the prototypical member of the quinones.

2.2.3.3 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group (Figure 3 (a)). Flavonols are phenolic structures containing one carbonyl group plus a 3-hydroxyl group (Cowan, 1999). Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 until linked to an aromatic ring (Figure 3 (b)). These compounds are planar molecules ubiquitous in plants, formed from the aromatic amino acids phenylalaine, tyrosine and malonate (Stalikas, 2007). One example of flavonoids is the catechins (Cowan, 1999).

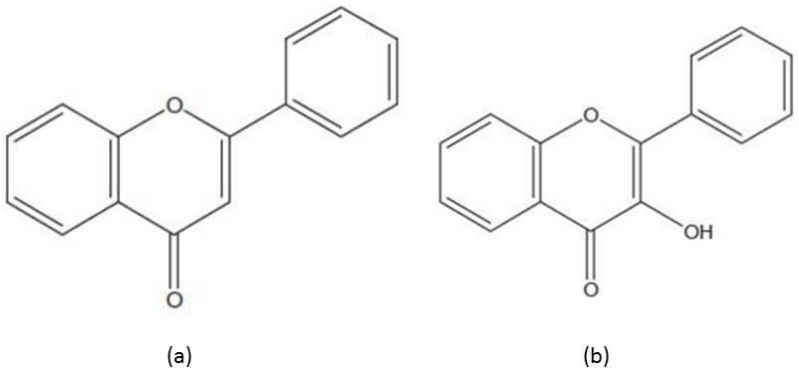


Figure 3. Structures of (a) flavones and (b) flavonoids (adapted from Huang et al., 2009).

The antimicrobial activity of flavonoids is probably due to their ability to complex with bacterial cell walls and soluble proteins, as well as with bacterial membrane (Cowan, 1999). These compounds have also been linked to reducing the risk of major chronic diseases including cancer, because they have powerful antioxidant

activities *in vitro*, being able to scavenge a wide range of reactive species (Hollman and Katan, 2000).

Flavonoid compounds exhibit inhibitory effects against multiple viruses, like human immunodeficiency virus (HIV) (Critchfield et al., 1996) and respiratory syncytial virus (RSV) (Barnard et al., 1993).

Although there are several studies about the topic, there is no clear predictability for the degree of hydroxylation and toxicity to microorganisms relatively to the flavonoids and flavones.

Flavonoids are the most common pigments, together with chlorophyll and carotenoids, and they generally occur in plants as glycosylated derivatives. Two of the most important functions of the flavonoids are the catalytic action in the light phase of photosynthesis and the regulation of the ion channels involved in phosphorylation (Pietta et al., 1999).

2.2.3.4 Tannins

Tannins are natural and water-soluble compounds with molecular weights ranging from 500 to 4000 g/mol (Cai et al., 2004) and they are present in many plant foods (Chung et al., 1998).

Tannins are a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution (astringency). They are found in almost every plant part, like bark, wood, leaves, fruits and roots (Scalbert, 1991). There are two groups of tannins. One is the hydrolysable tannins; in this group, tannins are based on gallic acid. The other group is the condensed tannins, also called proanthocyanidins, which are derived from flavonoids monomers. Tannins are commonly found combined with alkaloids, polysaccharides and, particularly, proteins (Han et al., 2007).

The antimicrobial action of tannins is probably due to their ability to inactivate microbial adhesins, enzymes and cell envelop transport proteins (Cowan, 1999). Tannins also have antimutagenic, anticarcinogenic and antioxidant activities (Chung et al., 1998).

Oligomeric proanthocyanidins (Figure 4) are considered to be the most potent antioxidants in tannins and they are frequently used in health care and cancer treatments (Huh et al., 2004).

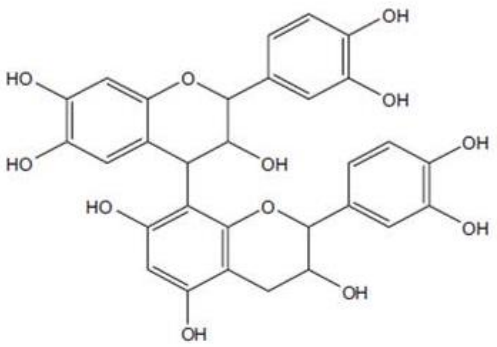


Figure 4 - Structure of the oligomeric proanthocyanidins (adapted from Huang et al., 2009).

2.1.3.5 Coumarins

Coumarins (Figure 5) are phenolic substances made of fused benzene and α-pyrone rings (Kennedy and Thornes, 1997) and they are responsible for the characteristic odor of hay (Hoult and Payá, 1996). Warfarin is an important coumarin which has antimicrobial properties (Rice et al., 2003).

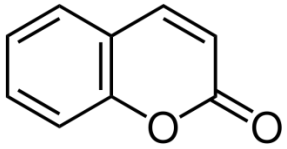


Figure 5. Structure of a simple coumarin.

These compounds are obtained by the cyclization of *cis-ortho*-hydroxicinnamic, belonging to the phenolics with the basic skeleton of $C_6 + C_3$, and this cyclization forms lactones (Cai et al., 2004).

Coumarins are present in plants in the free form and as glycosides (Fresco et al., 2006). Cai et al. (2006) characterized these compounds with a great chemical diversity, mainly differing in the degree of oxygenation of their benzopyrane moiety. Major coumarin constituents include simple hydroxilcoumarins, furocoumarins, isofurocoumarins, pyranocoumarins, bicoumarins and di-hydro-isocoumarins (Surveswaran et al., 2007; Cai et al., 2003). Studies reported that these compounds have antimicrobial, anti-inflammatory, antioxidant, anticoagulation, antiestrogenic and sedative activity (Paramjeet et al., 2012).

Huang et al. (2009) described the medicinal herbs where they found coumarins, specifically *Umbelliferae*, *Asteraceae*, *Convolvulaceae*, *Leguminosae*, *Magnoliaceae*, *Oleaceae*, *Rutaceae*, and *Ranunculaceae*, such as simple coumarins from *Artemisia*

annua, furocoumarins from *Angelica sinensis*, pyranocoumarins from *Citrus aurantium* and isocoumarins from *Agrimonia pilosa*.

2.2.4 Terpenoids and essential oils

Essential oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure (Cowan, 1999). The general chemical structure of essential oils is $C_{10}H_6$ and they are called terpenes; they occur as diterpenes, triterpenes, tetraterpenes, hemiterpenes and sesquiterpenes. Terpenoids are these compounds containing additional elements, usually oxygen (Cowan, 1999). Camphor is a monoterpene and farnesol and artemisn are sesquiterpenoids.

Terpenoids are active against bacteria, fungi, viruses and protozoa (Cowan, 1999).

It is speculated that the mechanism of antimicrobial action of terpenes involves membrane disruption by the lipophilic compounds (Cowan, 1999). Terpenoids and essential oils have antidiarrhoeal activity. They inhibit release of autocoids and prostaglandins (Tiwari et al., 2011).

Terpenes are the most numerous and structurally diverse plant natural products. Then, these compounds offer much potential in an array of industrial and medicinal applications (Zwenger and Basu, 2008). They have a complex nomenclature. The single isoprene unit (five carbon molecule) represents the most basic class of terpenes, the hemiterpenes. A terpene is an isoprene unit bonded with a second isoprene and it is also called a monoterpene (C_{10}) (Zwenger and Basu, 2008).

Some important terpenes are menthol and pyrethrins (insecticides), limonene and digitoxigenin (Croteau et al., 2000), besides the most known, the rubber. Rubber is a polyterpene, composed of repeating subunits of isoprene (Zwenger and Basu, 2008).

2.3 Extraction of the phytochemicals compounds

There are several methods of extraction that can be used to obtain the plants compounds. Solid-liquid, Soxhlet, supercritical fluid and micro-wave assisted extractions are some examples (Aziz, 2006; Tatke and Jaiswal, 2011; Patil and Shettigar, 2010).

2.3.1 Solid-liquid extraction

The first step of this type of extraction is the contact of an appropriate solvent with the product to be treated for a certain period of time. This enables the transfer of the soluble constituent or solute to the solvent. The second step is the separation of the solid phase from the liquid. The recovery of the solute and solvent are also included in the complete process. This is done by another operation such as evaporation or distillation (Aziz, 2006). To increase the extraction efficiency of the compounds it is possible to vary some parameters, such as the solvent, the temperature and the agitation.

Simeonov and Koleva (2012) published a study wherein by solid-liquid extraction they extracted tannins (with 70% ethanol and water) from *Geranium sanguineum L*. and studied the extraction kinetics, the influence of solid phase particle size and the liquid-solid ratio on extraction rate. Also, using a solid-liquid extraction, Simeonov et al. (2011) extracted from *Tribulus terrestris* furostanal saponins. They used methanol as the extraction solvent. Wongkittipong et al. (2004) used the same type of extraction for leaves and stems of *Andrographis paniculata* in ethanol-water solvent (0, 60, 70 e 80% in ethanol) in order to obtain andrographolide, a diterpenoid lactone. Mirela et al. (2007) extracted the terpenic and phenolic compounds by solid-liquid extraction from *Olea europaea*, using ethanol as solvent.

2.3.2 Soxhlet

The sample is placed in a thimble-holder (usually a filter paper inside the main chamber of the apparatus) that is gradually filled with condensed fresh extraction solvent from a distillation flask. When the solvent reaches the overflow level, a siphon aspirates the solute from the thimble-holder and unloads it back to the distillation flask. Therefore, the extracted analytes are carried into the bulk liquid (Luque de Castro and Priego-Capote, 2010). After extraction the solvent is removed, typically by means of a rotary evaporator, yielding the extracted compound. The non-soluble portion of the extracted solid remains in the thimble, and is usually discarded (Jensen, 2007). Figure 6 represents a conventional Soxhlet extractor, where is indicated the position of the heat source, the distillation flask, the sample, the siphon, the extractor and the condenser.

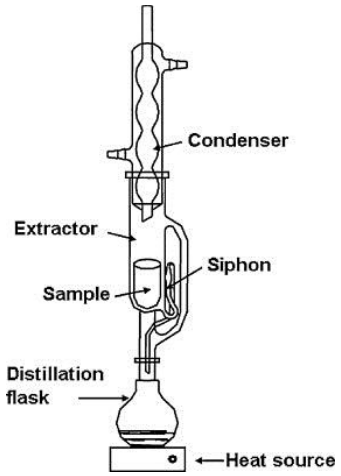


Figure 6. Conventional Soxhlet extractor (Luque de Castro and Priego-Capote, 2010).

The main advantages of this method are to be a very simple methodology that requires little training and can extract more sample mass than most of the latest techniques (such as supercritical-fluid extraction). Besides that, the sample is repeatedly in contact with fresh portions of the extractant, which facilitates the displacement of the transfer equilibrium. Also, the sample throughput can be increased by performing several simultaneous extractions in parallel, which is facilitated by the low cost of the basic equipment (Luque de Castro and Priego-Capote, 2010). Lastly, no filtration is required after the process.

Ahmad et al. (2009) described the Soxhlet extraction with methanol and n-hexane from *Elephantopus scaber L.* and the study shows that methanol is better in terms of extraction yield. The different yields of extracts might be influenced by the polarities of solvents (Romdhane and Gourdon, 2001). This happens for all parts of the plant.

2.3.3 Microwave assisted extraction (MAE)

A microwave device is composed of four major components. The microwave generator, also called magnetrom, is responsible for generation of microwaves. The gave wide is used to direct the propagation of microwave from the source to the microwave cavity. The applicator is where the sample holder and the sample are placed. The last component is the circulator, which regulates the movement of microwaves only in the forward direction (Tatke and Jaiswal, 2011).

There are two microwave systems for extraction: closed extraction vessels/multi-mode microwave ovens and focused microwave ovens. The first is brought by controlled pressure and temperature. As the name suggests, in the focused microwave

ovens only the part of the extraction vessel containing the sample is focused for irradiation with microwave. This process can also be named as microwave assisted Soxhlet or solvent extraction (Mandal et al., 2007). This process is based on exposing the analytes to the solvent through cell rupture (Jyothi et al., 2010).

With this method, the plants can contain microscopic traces of moisture that serves as the target for the microwave heating. Due to microwave heating, the moisture is heated up inside the plant cell, evaporates and generates tremendous pressure on the cell, because of the swelling of the plant cell. The pressure pushes the cell wall from inside, stretching and ultimately rupturing it (Mandal et al., 2007), thus the exudation of active constituents from the ruptures cells occurs, hence increasing the yield of phytoconstituents (Tatke and Jaiswal, 2011). There are some factors which affect MAE, namely the solvent nature and the volume, extraction time, microwave power, temperature and matrix characteristics (Mandal et al., 2007). MAE methods require shorter time, less solvent and provide higher extraction rates and with lower cost (Gallo et al., 2010).

Gallo et al. (2010) described the MAE of phenolic compounds from *Cinnamomum zeylanicum*, *Coriandrum sativum*, *Cuminum cyminum* and *Crocus sativus*. In this experiment, the efficiency of extraction of bioactive compounds obtained with the microwave extraction process was in general about four times higher than that resulting from sonication extraction. Earlier, Pan et al. (2003) had already shown that MAE has more efficiency than conventional techniques in the extraction of tea polyphenols and tea caffeine. On the other hand, Waksmundzka-Hajnos et al. (2004) concluded the MAE is not an appropriate method for furanocoumarin recovery. They studied the optimal conditions for the extraction of furanocoumarins from fruits of *Archangelica officinalis* and determined that some compounds, like imperatorin and phellopterin, may be transformed during pressurized MAE.

2.3.4 Supercritical fluid extraction (SFE)

In this method a gas, usually CO_2, is compressed into a dense liquid and it is pumped through a cylinder containing the material to be extracted. The extract-laden liquid is pumped into a separation chamber where the extract is separated from the gas and the gas is recovered for re-use. It is very easy to recover the supercritical solvent after extraction, it is only necessary to adjust the pressure and/or the temperature

(Doughari, 2012). One advantage of this process is the fact that the final extract has virtually no solvent residues left in it (gas evaporates completely) (Patil, 2010).

SFE has been increasingly used in the extraction of plant volatile components and essential oils (Pourmortazavi and Hajimirsadeghi, 2007). There are several studies with supercritical fluids to extracts compounds from plants. Fadel et al. (1999) made a research performed with eucalyptus which demonstrates the differences that exist between the composition and functional properties of extracts obtained with SFE and hydrodistillation. Supercritical fluid extracts had a higher content of sesquiterpenes and oxygenated compounds (Fadel et al., 1999).

2.3.5 Ultrasounds extraction

This type of extraction utilizes acoustic cavitation to cause molecular movement of solvent and sample (Jerman et al., 2010). Cavitation forces are the result of the propagation of ultrasound pressure (Knorr et al., 2002). In the case of plants, bubbles can explosively collapse and generate localized pressure causing plant tissue rupture and improving the release of intracellular substances into the solvent (Knorr et al., 2002).

There are two commons devices for ultrasounds extraction. The more widely used is the bath system but the one which offers more advantages is the probe system. This provides directly cavitation in the solution, being more efficient (Priego-Capote and Luque de Castro, 2004).

Jerman et al. (2010) studied the extraction of phenolic compounds of olive fruit (*Olea europaea*) with ultrasounds extraction. The authors used a high intensity probe sonication. They studied different parameters (sonication time, temperature, solvent composition and extraction steps) in order to optimize the extraction. It was concluded that the method used was more efficient in comparison to ultrasound bath and agitation.

2.4 Extraction solvent

For all of these techniques a solvent extraction is needed. The extraction solvents can be various and the choice is influenced by what is pretended with the extract and what are the targeted compounds to be extracted (Das et al., 2010). The low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract and inability to cause the extract to complex or disassociate are the main characteristics

to be a good solvent. The factors that affect the choice of the solvent are, briefly, the quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds existing in the initial matrix, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the extractants (Eloff, 1998).

Then, the type of solvent is essential on the successful determination of biologically active compounds from plant material (Tiwari et al., 2011). Table 1 shows the main solvents used to extract the different compounds, according to Cowan (1999).

Table 1. Main solvents used for active compounds extraction (Cowan, 1999).

Water	Ethanol	Methanol	Ether	Acetone	Chloroform
Anthocyanins	Tannins	Anthocyanins	Alkaloids	Phenols	Terpenoids
Starches	Polyphenols	Terpenoids	Terpenoids	Flavonols	Flavonoids
Tannins	Polyacetylenes	Saponins	Coumarins		
Saponins	Flavonols	Tannins	Fatty acids		
Terpenoids	Terpenoids	Xanthoxyllines			
Polypeptides	Sterols	Totarol			
Lectins	Alkaloids	Quassinoids			
		Lactones			
		Flavones			
		Phenones			
		Polyphenols			

Water is an universal solvent and it is mainly used to extract compounds with antimicrobial activity. However, organic solvents extracts give more consistent antimicrobial activity than water extracts. Water soluble flavonoids, such as anthocyanins, have no antimicrobial activity and water soluble phenolics are only important as antioxidant compound (Dask et al., 2010). Acetone dissolves many hydrophilic and lipophilic components from plants, is volatile and has low toxicity, which makes it an useful extractant. It is very used for antimicrobial studies, where more phenolic compounds are required to be extracted (Tiwari et al., 2011; Dask et al., 2010). Ethanolic extracts have high amounts of polyphenols, which means they are more efficient in cell wall and seeds degradation which have nonpolar character and cause polyphenols to be released from cells (Dask et al., 2010). Methanol is more polar

than ethanol but it has a cytotoxic nature, which makes it unsuitable for extraction in certain studies (Tiwari et al., 2011).

The composition of the plant extract is influenced by the temperature, solvent extracting power, extraction time and method adapted for the extraction (Ksouri et al., 2009). This variability is due to the different affinities of these compounds for solvent extraction and specialty to the polarity of the molecules constituting of the solvent (Hayouni et al., 2007). Therefore, it is necessary to optimize the solvent/concentration to utilize in order to obtain the highest possible yield of the desired compounds.

CHAPTER 3 – Evaluation of Extraction Efficiency of *Olea europaea* and *Acacia dealbata*

3.1 Introduction

Man has used plants since the start of humankind. In the course of time, plants became a useful source of disease cure and health improvement across various human communities (Vinatoru, 2001). Compounds of plants, such as alkaloids, phenolic and terpenoids, have currently recognized antimicrobial and antioxidant properties (Cowan, 1999; Karou, 2005; Gallo, 2010).

The selection of the proper extraction method is very important for the qualitative and quantitative studies of bioactive compounds from plants. The different techniques of extraction have all the same goals in the process: to extract targeted bioactive compounds from complex plant samples, to increase sensitivity of bioassay by increasing the concentration of targeted compounds, to convert the bioactive compounds into a more suitable form for detection and separation and to provide a strong and reproducible method that is independent of variations in the sample matrix (Smith, 2003). The extracting power of different solvents in use and the application of heat and/or mixing are the basis of most techniques (Azmir et al., 2013).

Soxhlet and solid-liquid extraction belong to the conventional or classic techniques of extraction. Nowadays, the major challenges of conventional extractions are longer extraction time, requirement of costly and high purity solvents, evaporation of the huge amount of solvent, low extraction selectivity and thermal decomposition of thermo labile compounds (Luque de Castro and Garcia-Ayuso, 1998). To overcome these limitations, new techniques are been introduced, as micro-wave and ultrasound extractions (Azmir et al., 2013).

This chapter aims to select the solvent and extractions conditions in order to achieve the maximum recovery of bioactive compounds from selective plants, *Olea europaea* and *Acacia dealbata*.

3.2 Material & Methods

3.2.1 Plants

Acacia dealbata and *Olea europaea* were selected for this study.

3.2.1.1 *Acacia dealbata* (mimosa)

Leaves of *Acacia dealbata* (Figure 7) were studied.

Figure 7. Leaves and flower of *Acacia dealbata*.

3.2.1.2 *Olea europaea* (olive)

Leaves of *Olea europaea* (Figure 8) were studied.

Figure 8. Leaves of *Olea europaea*.

3.2.2 Collection of the leaves

Olive leaves were collected in the middle of February and mimosa leaves were collected in the beginning of March, in the year of 2013. The collection area was Braga (Portugal).

3.2.3 Extraction of leaves

Several extraction methods were used for both plants. For every extraction leaves were cut in small pieces with approximately 5 mm of length and 4 mm of width and the amount used was 5 g of wet weight.

3.2.3.1 Solid-Liquid Extraction

Leaves were placed in a flask (capacity of 250 mL) with 50 mL of the solvent at 20 ºC (Lovibond) with magnetic shaking (Velp Scientific) for 1 h according to the method suggested by Tomsone et al. (2012). Water, acetone, ethanol, dichloromethane, methanol and hexane (Sigma-Aldrich) were the solvents used for the extraction.

3.2.3.2 Ultrasounds Extraction

Leaves were placed in a lidded flask (capacity of 220 mL) with 50 mL of the solvent and then put in a sonicator (P Selecta) for 1 h. Water, acetone, ethanol, dichloromethane, methanol and hexane (Sigma-Aldrich) were the solvents used.

3.2.3.3 Soxhlet Extraction

Leaves were placed on a filter paper inside the main chamber of the Soxhlet (P Selecta) and the solvent (approximately 300 mL of water) was placed on the top of the Soxhlet apparatus. Extraction time was 16 h.

3.2.3.4 Micro-wave extraction

Microwave-assisted extractions were performed with a modified version of the domestic Electric Co. WP700P17-3 oven (2450 MHz, China), with an input power of 1200 W and frequency of 2450 MHz.

A round bottom flask with a capacity of 100 mL was introduced into the microwave and coupled to an external condenser. Cooling liquid was water at 5 ºC from a thermostatic bath. The top of the condenser was attached to an L-shaped tube containing activated carbon in order to adsorb any compound that could be released. The condenser was used to prevent sample evaporation, minimizing the volume variation. All experiments were performed in a fume hood, using a microwave radiation detector (MSM128, Meet Int., Hong Kong) to check for leaks during operation.

Leaves and 50 mL of water were placed in the flask with some glass beads to prevent superheating. The micro-wave was operated at a nominative output power of 397 W, which corresponds to an effective output power of 162 W (Homem et al., 2013).

After all the extractions the extracts were evaporated in a rotary evaporator (Büchi Rotovapor R-114), except the samples with water which were lyophilized, in order to remove the solvent. Dry extracts are then taken in dimethyl sulfoxide (DMSO) or methanol, depending on the test to perform.

3.2.4 Extraction Efficiency

Extraction efficiency was calculated by the Equation [1].

$$Extraction\ efficiency\ (\%) = \frac{m_e}{m_l}\ x\ 100 \qquad \text{Equation [1],}$$

In which m_e is the mass of the extract and m_l is the initial mass of the leaves, before the extraction (wet weight).

3.2.5 Statistical Analysis

Data were analyzed using the statistical program SPSS 21.0 (Statistical Package for the Social Sciences). The data was analyzed using One-Way Anova. Significance level for the difference was set at $p < 0.05$.

3.3 Results and Discussion

Mimosa is an invasive tree and it is widely spread in several parts of the world, namely in Chile, Madagascar, South Africa, France, Spain and Portugal (Richardson and Rejmánek, 2011). Although there are several studies in America and Africa about the impact of mimosa in biodiversity (Lorenzo et al., 2010), in Europe little is known, despite the large areas currently invaded. In Portugal, mimosa is considered one of the most aggressive species and it is found in all Portuguese provinces (Ferreira et al., 2011). Ferreira et al (2011) said that one way to control the distribution of this invasive plant should be its use as raw material, and they proved that it may be a promising renewable raw-material for bioethanol production. The high content in polysaccharides of mimosa gives it the ability to be fractionated by chemical methods to yield products suitable for the chemical, food and pharmaceutical industries (Yánez et al., 2009). Therefore, the potential biotechnological harnessing of this plant deserves to be explored.

In relation to olive, this is one of the oldest known cultivated trees in the world (Zamora et al., 2001). It contains oleuropein (a polyphenolic iridoid glycoside), oleacein and oleanolic acid as active substances (Susalit et al., 2011). It has a fruit, usually named olive fruit, which is a green drupe, becoming generally blackish-purple when fully ripe (Pinheiro and Silva, 2005). It is known that this species have been used since ancient times in order to combat high blood pressure, atherosclerosis and diabetes (Jänicke et al., 2003). Nowadays, studies show that the olive leaf extract has an effective

activity against various diseases, such as coronary artery disease, high cholesterol level, arrhythmia, cancer, overweight, osteoporosis, herpes, flu and colds and some bacterial, fungus and yeast infections. Furthermore, it is an allergen free product (Ritchason, 2000). Lee and Lee (2010), as well as Sudjana et al. (2009), showed in their studies that *Olea europaea* has antioxidant and antimicrobial activity.

The extraction efficiency for every extraction and solvent for both plants was determined and it can be seen in Table 2.

Table 2. Extraction efficiency (%) per extraction technique and solvent used with both olive and mimosa leaves.

	Olive				Mimosa			
	Extraction Technique				Extraction Technique			
Solvent	Solid-Liquid	Ultrasounds	Soxhlet	Micro-wave	Solid-Liquid	Ultrasounds	Soxhlet	Micro-wave
Water	5.7±0.9	6.2±0.9	12.3±1.2	10.7±0.8	8.1±0.8	6.4±1.2	13.4±1.4	11.9±1.8
Methanol	7.2±0.3	6.0±1.1	-	-	7.9±0.4	5.7±1.1	-	-
Ethanol	4.1±1.3	4.6±0.7	-	-	6.0±2.2	4.2±1.3	-	-
Acetone	6.8±1.1	7.4±0.3	-	-	5.1±0.8	2.8±0.0	-	-
Dichloromethane	6.2±1.6	2.8±0.3	-	-	4.1±1.5	3.8±2.1	-	-
Hexane	0.9±0.1	3.2±1.3	-	-	7.8±2.1	3.7±0.1	-	-

According to Altıok et al. (2008) the choice of the solvent is the most important factor affecting the efficiency of solid-liquid extraction. In relation to olive leaves, this type of technique/solvent caused extraction efficiencies from 0.9 ± 0.1% to 7.2 ± 0.3%. Hexane was the weakest solvent. Methanol was the best solvent and for the others there was not found significant differences ($p>0.05$). For mimosa leaves, solid-liquid extraction was from 4.1 ± 1.5% to 8.1 ± 0.8%. Here, the weakest solvent was dichloromethane and the best was water ($p<0.05$).

Methanol and water were the best solvents for ultrasounds extraction ($p<0.05$). Jerman et al. (2010) also considered methanol as the best solvent to extract phenols by ultrasounds, pure or combined with water (80/20, v/v). This solvent did not degrade the phenols present, since no hydrogen peroxide neither large proportion of free radicals are formed due to cavitation when exposed to sonication (Paniwnyk et al., 2011). Ahmad *(*2009) reported that the different yields might be influenced by the polarities of the solvents, which can explain the variation in the results. It was also reported by Ahmad in 2009 in the extraction of chemical ingredients of *Elephantopus scaba L*. and Klejdus

et al. (2005) in the extraction of isoflavones soybeans samples that methanol is a better solvent for extraction than hexane, which supports the results for this experience. According to Cowan (1999), methanol and water are the solvents that can extract more diversity of compounds, which can explain the higher extraction efficiency.

Ultrasounds extraction offers numerous advantages comparing to conventional extraction techniques, like solid-liquid extraction. An improved efficiency, reduced extraction time and low solvent consumption make this technique one of the most used (Chen et al., 2007). However, although in the current study it have been used the same extraction time and the same quantity of solvent, the ultrasounds extraction did not obtained an higher extraction efficiency than solid-liquid extraction. Comparing all the solvents, for olive leaves the average extraction yield for solid-liquid extraction was 4.7 ± 2.1% and for ultrasounds extraction was 5.0 ± 1.8%, and this difference is not significant ($p>0.05$). For mimosa leaves the values were 6.5 ± 1.7% and 4.4 ± 1.4%, respectively, and this difference is also not significant ($p>0.05$).

The extraction techniques which provide a more efficient extraction were the Soxhlet and the micro-wave extraction, and the differences between them were not significant ($p>0.05$). This happened for both plants. Soxhlet has a great advantage in relation to the others methods used: no filtration is required after the process. Furthermore, it is a very simple method and can extract more sample mass than most of latest alternatives, such as micro-wave assisted extraction. Nevertheless, a long time is required for extraction and a large amount of extractant is wasted (Luque de Castro and Priego-Capote, 2010). The superiority in the extraction yield of micro-wave extraction was also showed by Gallo et al. (2010). The authors extracted phenolic compounds from *Cinnamomum zeylanicum, Coriandrum sativum*, *Cuminum cyminum* and *Crocus sativus* and the efficiency of extraction of bioactive compounds obtained with micro-wave extraction was in general about four times higher than that resulting from ultrasounds extraction.

3.4 Conclusions

Water and methanol proved to be the solvents that caused a higher efficiency for solid-liquid and ultrasounds extractions for olive and mimosa extracts. The polarity of the solvents can explain the differences in the extraction yield.

Comparing all the solvents, the differences were not significant between solid-liquid and ultrasounds extraction.

Soxhlet and micro-wave extractions showed to be the methods with higher efficiency to extract compounds of olive and mimosa.

CHAPTER 4 – Analysis of the Bioactivity of *Olea europaea* and *Acacia dealbata* Extracts

4.1 Introduction

Plants extracts can be a promising alternative to current antimicrobials, particularly antibiotics, taking in account the evolution of bacterial resistance (Abreu et al., 2012). Many compounds of plants have recognized antimicrobial and antioxidant properties. Giving some examples, alkaloids have been exploited as pharmaceuticals, stimulants, narcotics and poison due to their potent biological activity (Ziegler and Facchini, 2008) and they have good antimicrobial activity against several microorganisms (Karou et al., 2005). Phenolic compounds have antioxidant activity mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Gallo et al., 2010) and some phenolic acids and analogs also exhibit antibacterial, antifungal, antiviral, antimutagenic and anti-inflammatory activities (Silici et al., 2007; Chaubal et al., 2005; Larrosa et al., 2006). Tannins have antimicrobial (Cowan, 1999), antimutagenic, anticarcinogenic and antioxidant activity (Chung et al., 1998). Studies also reported that coumarins have antimicrobial, anti-inflammatory, antioxidant, anticoagulation, antiestrogenic and sedative activity (Paramjeet et al., 2012).

The analysis of the bioactivity of the phytochemicals compounds is very important to understand their potential applications in clinical settings. The antioxidant activity of the natural compounds is related to the three major groups: vitamins, phenolics and carotenoids (Halliwell, 1996). Temple (2000) reported that the frequent consumption of natural oxidants is associated with a lower risk of cardiovascular disease and cancer, which indicates their potential application. Also, the efficacy of antimicrobial effects of antibiotics can be improved by combining them with extracts of plants against several pathogens, such as *S. aureus* and *E. coli* (Adwan and Mhanna, 2008). Besides that, the combination between antibiotics and phytochemical compounds can help in the prevention of the development of microbial resistance (Sakharkar et al., 2009).

This chapter aims to evaluate the antimicrobial and antioxidant activities of *Olea europaea* and *Acacia dealbata* extracts, complementing the previous chapter on the role of selection of an extraction method and solvent to maximize the biological effects of the plant extracts.

4.2 Material & Methods

4.2.1 Extracts

The extracts from *Olea europaea* and *Acacia dealbata* previously obtained were used in this chapter.

4.2.2 Bacterial Strains

The bacteria used in this study were obtained from the Spanish Type Culture Collection (CECT): the Gram-negative bacterium *Escherichia coli* (CECT 434), the Gram-positive bacterium *Staphylococcus aureus* (CECT 976) and the Gram-negative *Chromobacterium violaceum (*ATCC 12472). *E. coli* and *S. aureus* were distributed over the surface of Mueller-Hinton Agar (MHA, Merck) and incubated for 24 h at 27 ± 3 ºC and *C. violaceum* was distributes over the surface of Luria-Bertani Agar (LBA, Merck) and incubated for 24 h at 27 ± 3 ºC.

4.2.3 Antimicrobial Activity Assessment

Antimicrobial activity was tested using a modification of the disc diffusion method originally described by Bauer et al. (1966). Bacteria were grown overnight and the turbidity was adjusted to match 0.5 in McFarland standards with sterile saline (Spectrometer VWR V-1200). Petri dishes with 90 mm of diameter were prepared with approximately 20 mL of MHA. Sterile filter paper discs (6 mm of diameter) impregnated with 10 µL of the samples were placed on the agar plate seeded with the respective bacteria. The plates were incubated at 37 ˚C for 24 h. Discs impregnated with DMSO were used as negative control. After incubation, the diameter in mm of the inhibitory zones around the discs was recorded (Saavedra et al., 2010).

4.2.4 Antioxidant Activity Assessment

Assays used to estimate the antioxidant capacity of the plants mostly include ABTS (Leong and Shui, 2002) and DPPH (Gil et al., 2002).

4.2.4.1 ABTS Assay

A stock solution included 7.4 mM ABTS solution (Sigma-Aldrich) and 2.6 mM potassium persulfate solution (Sigma-Aldrich). Then these two stock solutions were mixed in equal quantities and allowed to react for 12 h at room temperature in the dark. The resulting solution was diluted by mixing 1 mL of this solution with 60 mL of methanol to obtain an absorbance of 1.10 ± 0.02 units at 734 nm. The samples are then mixed with the prepared solution (1:20 V/V) and allowed to react for 2 h in a dark condition. The absorbance was taken at 734 nm (Spectrometer VWR V-1200). The standard curve was linear between 25 and 800 μM Trolox (Sigma-Aldrich) and the results are expressed in μM Trolox equivalents (TE)/g fresh mass (Thaipong et al., 2006).

4.2.4.2 DPPH Assay

A stock solution included 0.024 mg/mL DPPH solution (Sigma-Aldrich) in methanol. The working solution was prepared by mixing 10 mL of the stock solution with 45 mL of methanol to obtain an absorbance of 1.10 ± 0.02 units at 515 nm. The samples (150 μL) are then mixed with the working solution (2850 μL) and allowed to react for 24 h in a dark condition. The absorbance was taken at 515 nm (Spectrometer VWR V-1200). The standard curve was linear between 25 and 800 μM Trolox and the results are expressed in μM TE/g fresh mass (Thaipong et al., 2006).

4.2.5 Antibiotics-Extracts Dual Combination Assay

To study the antimicrobial effects of the extracts with antibiotics, the extract (dissolved in DMSO) was inserted in MHA medium (at a final concentration of 5000 μg/mL) after autoclaved and cooled. Suspensions were prepared mixing bacteria from an overnight culture with sterile saline solution in order to obtain a final cell turbidity matching 0.5 McFarland standards (Spectrometer VWR V-1200). The cellular suspension was poured over hardened MHA/extracts plates using a sterilized cotton swab and allowed to set. Antibiotic discs containing erythromycin (15 μg/disc) and tetracycline (30 μg/disc) (Sigma-Aldrich), according to the Clinical Laboratory Standards Institute standards (CLSI, 2005), were placed on the surface of the plates and then the plates were incubated at 37 ˚C for 48 h (Saavedra et al., 2010). The zones of grown inhibition were measured after 24 and 48 h (CLSI, 2005).

4.2.6 Antibiotics-Extracts Dual Combination Classification

The effect of dual combinations of antibiotics and extracts was classified according to Saavedra et al. (2010):

• antagonism (-)

if [inhibition halo – (antibiotic inhibition halo + extract inhibition halo)/2] < 0;

• indifference (+)

if 0 ≤ [inhibition halo – (antibiotic inhibition halo + extract inhibition halo)/2] < antibiotic inhibition halo or extract inhibition halo;

• additive (++)

if antibiotic inhibition halo < [inhibition halo – (antibiotic inhibition halo + extract inhibition halo)/2] < 2 × antibiotic inhibition halo or extract inhibition halo;

• synergy (+++)

if inhibition halo > 3 × antibiotic inhibition halo or extract inhibition halo.

For the classification was selected the highest inhibition halos caused by the antibiotic or phytochemical application for each condition tested.

4.2.7 Quorum-sensing Inhibition (QSI)

Chromobacterium. violaceum was grown overnight in LB broth at 30 ˚C with shaking. To test the plant leaves, they were placed in LB agar plates and overlain with 5 mL of LBA soft (tempered at 45 ˚C) containing 10^6 CFU/mL of the *C. violaceum*. Then the plates were incubated at 30 ºC for 24 h and, after this, the color was examined. To test the extracts of the plants the cell suspension was prepared in order to obtain 10^6 CFU/mL of the *C. violaceum* (by mixing with LB broth). Sterile filter paper discs (6 mm of diameter) impregnated with 10 µL of the extracts were placed on LB agar plates seeded with 100 µL of the respective bacterium. Then the plates were incubated at 30 ºC for 24 h and, after this, the color was examined (McLean et al., 2004).

4.2.8 Statistical Analysis

The statistical analysis of the results obtained was performed as described in section 3.2.5.

4.3 Results and Discussion

4.3.1 Antimicrobial Activity Assessment

Olive leaves – solid-liquid and ultrasounds extractions

Regarding to olive leaves extracted with solid-liquid extraction, activity for almost all solvents was found, as indicated in Table 3. Concentrations of the plant extracts were not standardized, ranging from 48.9 mg/mL for hexane to 254.1 mg/mL for acetone. Concentrations depend on the volume of DMSO used to dissolve the dry extracts.

Table 3. Antibacterial activity of olive leaves extracts for solid-liquid extraction and for the selected solvents against *S. aureus* and *E. coli* – diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
Extract concentration (mg/mL)	150.3	100.5	84.9	254.1	253.2	48.9
S. aureus	0.0±0.0	16.7±1.2	25.3±2.3	22.7±1.2	8.7±0.6	11.3±1.2
E. coli	0.0±0.0	16.7±1.2	16.7±1.2	14.7±1.2	0.0±0.0	12.0±2.0

The extraction with water did not show activity against any microorganism. Several studies support that the olive leaves in the aqueous extracts have antimicrobial activity against pathogenic bacteria (Aliabadi et al., 2012; Keskin et al., 2012). Therefore, maybe, if some conditions were different, for example if the extraction time was superior, the results would have been different for these extracts.

The extraction with dichloromethane only showed activity against *S. aureus*. All the other extracts presented activity for both bacteria. Ethanol and acetone were the solvents that caused the highest inhibition halo ($p<0.05$) against *S. aureus* and the differences between these solvents were not significant ($p>0.05$). For these solvents the activity was superior against *S. aureus* ($p<0.05$). According to Cowan (1999), ethanol and acetone are highly efficient in the extraction of phenolic compounds. Concerning the effects of the extracts obtained against *E. coli*, there were no significant differences between the antimicrobial activity of methanol, ethanol, acetone and hexane ($p>0.05$).

The fact that the antimicrobial activity is higher against *S. aureus* is supported by Simões et al. (2008). This species is Gram-positive and the permeability of the cell wall can make it more susceptible and, for that reason, does not restrict the penetration

of antimicrobials. However, this only happens with the extracts obtained with ethanol, acetone and dichloromethane. For methanol and hexane extracts there was no significant differences on the effects caused by the plant extracts on both Gram-positive and Gram-negative bacteria ($p>0.05$).

In order to have more consistent results on the relation between the solvent used and antimicrobial effects, the concentrations of the extracts were standardized and tested at 5 mg/mL. This concentration is also more appropriated to use the extracts in medicine. The results are shown in Table 4.

Table 4. Antibacterial activity of olive leaves extracts at 5 mg/mL for solid-liquid extraction and for the selected solvents against *S. aureus and E. coli* - diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
S. aureus	0.0±0.0	0.0±0.0	10.7±0.6	0.0±0.0	0.0±0.0	0.0±0.0
E. coli	0.0±0.0	0.0±0.0	11.0±0.0	0.0±0.0	0.0±0.0	0,0±0.0

With lower concentrations the results were very different. All the extracts lost their antimicrobial activity, except the sample extracted with ethanol. However, this extract also lost some activity against both species, since the inhibition halo decreased 58% against *S. aureus* ($p<0.05$) and 52% against *E. coli* ($p<0.05$). However, with a concentration of 5 mg/mL the activity of this extract did not change depending on the Gram-type of the bacteria.

These results indicate that it is necessary a concentration relatively high of olive leaves extract to have antimicrobial activity. It would be interesting to discover the minimal concentration of the extract to obtain activity

The results on the antimicrobial activity of olive leaves extract obtained by ultrasounds extraction are shown in Table 5. The concentrations of the extracts range from 59.6 mg/mL (with methanol) to 239.3 mg/mL (with acetone).

Table 5. Antibacterial activity of olive leaves extracts for ultrasounds extraction and for the selected solvents against *S. aureus* and *E. coli* – diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
Concentration (mg/mL)	123.4	59.6	168.6	239.3	93.4	161.3
S. aureus	11.3±1.2	18.7±1.2	27.3±2.3	18.7±1.2	0.0±0.0	15.0±1.4
E. coli	9.3±0.6	16.7±1.2	16.7±1.2	14.0±2.0	0.0±0.0	11.3±1.2

Ethanol, with its ability to extract phenolic compounds (Cowan, 1999), seems to be the best solvent to extract olive leaves, since its activity causes the higher inhibition halo against *S. aureus* ($p<0.05$). The sample extracted with dichloromethane did not show activity against both *S. aureus* and *E. coli*.

For water, methanol, acetone and hexane the results did not significantly differ for both bacteria ($p>0.05$). Once again, for ethanol, *S. aureus* is the bacterium more susceptible to the antimicrobials ($p<0.05$). The exterior membrane of *E. coli*, with a set of outer membrane proteins, which are powerful barriers to the antimicrobials, can explain the lower inhibition halos (Simões et al., 2008).

As for the solid-liquid extraction, the concentrations of the extracts were standardized to 5 mg/mL and the results of the antimicrobial activity assessment are presented in Table 6.

Table 6. Antibacterial activity of olive leaves extracts at 5 mg/mL for ultrasounds extraction and for the selected solvents against S. aureus and E. coli - diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
S. aureus	9.3±0.0	0.0±0.0	10.7±0.6	9.3±0.6	0.0±0.0	0.0±0.0
E. coli	0.0±0.0	0.0±0.0	10.7±0.6	10.7±0.6	0.0±0.0	0.0±0.0

In this case, extracts with methanol and hexane did not show antimicrobial activity. Extracts with water and dichloromethane only demonstrate activity against *S. aureus* and, as already said, this is the bacterium more susceptible to the plant extracts. For extracts with ethanol and acetone the results were similar for both bacteria and there were no significant differences ($p>0.05$). No solvent extraction excelled in this assay: results are similar for water, ethanol, acetone and dichloromethane against *S. aureus* ($p>0.05$) and for ethanol and acetone against *E. coli* ($p>0.05$).

Comparing to higher concentrations, extracts performed with methanol and hexane lost their activity with the decrease of concentration. The same happened with the extract obtained with water which lost activity against *E. coli*. Differences were not observed by decreasing the concentration for extracts with water against *S. aureus* and with acetone against *E. coli* ($p>0.05$). Ethanol was more efficient in extract antimicrobial compounds with a high concentration ($p<0.05$) and the same happened with extracts performed with acetone, but only against *S. aureus*.

Mimosa leaves – solid-liquid and ultrasounds extractions

The results for mimosa leaves extract from solid extraction are presented in this section. The concentrations of the extracts ranged from 100.2 mg/mL (with dichloromethane) and 253.3 mg/mL (with ethanol) on a first approach (Table 7), having been later standardized to 5 mg/mL (Table 8).

Table 7. Antibacterial activity of mimosa leaves extracts for solid-liquid extraction and for the selected solvents against *S. aureus* and *E. coli* – diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
Concentration (mg/mL)	106.3	140.5	253.3	190.4	100.2	145.7
S. aureus	0.0±0.0	16.7±1.2	28.0±2.0	12.0±0.0	0.0±0.0	11.3±1.2
E. coli	0.0±0.0	16.7±1.2	13.3±1.2	10.0±0.0	0.0±0.0	10.0±0.0

Table 8. Antibacterial activity of mimosa leaves extracts at 5 mg/mL for solid-liquid extraction and for the selected solvents against *S. aureus* and *E. coli* - diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
S. aureus	0.0±0.0	0.0±0.0	10.0±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E. coli	0.0±0.0	0.0±0.0	9.3±0.6	0.0±0.0	0.0±0.0	0.0±0.0

With the extracts at higher concentrations the results were similar to olive leaves extract. Ethanol was the only solvent that caused different activities depending on the bacteria ($p<0.05$). It also seems that ethanol is the best solvent to extract antimicrobial compounds against *S. aureus* ($p<0.05$). With extracts of water and dichloromethane no activity was found against the microorganisms. Extracts with methanol, acetone and hexane did not express differences between both bacteria ($p>0.05$).

For the test with the extracts at 5 mg/mL only the ethanol showed activity against the bacteria, which proves its good ability to extract the antimicrobials compounds from plants. However, its activity was less efficient with the decreased concentration against both bacteria ($p<0.05$).

Relatively to the ultrasounds extraction, the results are shown in Table 9 (concentrations of the extracts range from 19.0 mg/L with hexane to 181.5 with methanol) and Table 10 (concentrations at 5 mg/mL).

Table 9. Antibacterial activity of mimosa leaves extracts for ultrasounds extraction and for the selected solvents against *S. aureus* and *E. coli* – diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
Concentration (mg/mL)	93.4	181.5	129.1	27.5	24.4	19.0
S. aureus	10.0±1.8	9.3±0.6	25.3±2.3	15.0±0.0	0.0±0.0	12.0±0.0
E. coli	0.0±0.0	16.7±1.2	16.7±1.2	16.7±1.2	0.0±0.0	12.0±0.0

Table 10. Antibacterial activity of mimosa leaves extracts at 5 mg/mL for ultrasounds extraction and for the selected solvents used against *S. aureus* and *E. coli* - diameter of inhibition (mm).

Solvents	Water	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
S. aureus	10.0±1.8	0.0±0.0	10.0±1.0	0.0±0.0	0.0±0.0	0.0±0.0
E. coli	0.0±0.0	0.0±0.0	9.7±0.6	0.0±0.0	0.0±0.0	0.0±0.0

Once again the extract with dichloromethane did not show activity against the bacteria for any concentration. In relation to the extract with water the decrease of the concentrations did not cause changes in the antimicrobial activity: antimicrobial activity was found against *S. aureus* but not against *E. coli*.

Ethanol demonstrated to be the best solvent and, once again, its activity decreased with the decrease of the concentration ($p<0.05$). The extract performed with ethanol was again more efficient against *S. aureus* than against *E. coli* ($p<0.05$), but only for the higher concentration. Extracts with acetone and hexane at higher concentrations did not present differences against the Gram-positive and Gram-negative bacteria ($p>0.05$).

With the extracts at 5 mg/mL, the extracts with methanol, acetone, dichloromethane and hexane did not show antimicrobial activity. Water and ethanol presented similar results against *S. aureus* ($p>0.05$).

Regarding to the sample extracted with methanol at 181.5 mg/mL, a higher antimicrobial activity was found against *E. coli* than against *S. aureus* ($p<0.05$), which contradicts what Simões et al. (2008) reported. However, this result can be supported by the previous findings of Taguri et al. (2006). The authors said that there is no relation between the Gram-type and the antimicrobial activity and that it depends on bacterial species, mode of action of the phytochemicals and its physico-chemical properties.

Olive and mimosa leaves – Soxhlet and micro-wave extractions

The extractions with Soxhlet and micro-wave used water as solvent and the results are presented in Table 11 (concentrations between 82.3 mg/mL and 123.5 mg/mL) and Table 12 (standardized concentrations at 5 mg/mL).

Table 11. Antibacterial activity of olive and mimosa leaves extracts for Soxhlet and micro-wave extraction against *S. aureus* and *E. coli* - diameter of inhibition (mm).

Water				
Plant	Olive		Mimosa	
Concentration (mg/mL)	89.3	82.3	123.5	100.2
Extractions	Soxhlet	Micro-wave	Soxhlet	Micro-wave
S. aureus	14.0 ± 0.8	14.3 ± 1.2	14.7 ± 0.5	14.3 ± 0.5
E. coli	11.0 ± 0.0	11.7 ± 0.5	0.0 ± 0.0	0.0 ± 0.0

Table 12. Antibacterial activity of olive and mimosa leaves extracts at 5 mg/mL for Soxhlet and micro-wave extraction against *S. aureus* and *E. coli* - diameter of inhibition (mm).

Water – 5 mg/mL				
Plant	Olive		Mimosa	
Extractions	Soxhlet	Micro-wave	Soxhlet	Micro-wave
S. aureus	11.0 ± 0.0	10.7 ± 0.5	10.3 ± 0.5	9.3 ± 1.2
E. coli	10.0 ± 1.4	9.7 ± 0.9	0.0 ± 0.0	0.0 ± 0.0

For higher concentrations there are no significant differences in the antibacterial activity between the type of extraction for both plants, and the same happened with the extract at 5 mg/mL ($p>0.05$). Once again, the activity is higher against *S. aureus* ($p<0.05$) for higher concentrations. Decreasing the concentration made the extracts to lose some activity against *S. aureus*.

Extracts obtained from mimosa leaves did not show activity against *E. coli*. Soxhlet and micro-wave extractions proved to be ineffective in the extraction of antimicrobial compounds from mimosa. This probably happened because the extracts do not have a sufficient antibacterial power to pass the exterior membrane of the Gram-negative bacterium (Simões et al., 2008). Maybe if the conditions of extraction were different, like a higher time of extraction, the activity would be found.

When comparing the samples with water for all the techniques, it is possible to conclude that there is a distinct advantage in the antimicrobial activity of the extracts from Soxhlet and micro-wave extractions in relation to those from solid-liquid and

ultrasounds extraction. This confirms the advantages of these techniques already described by Gallo et al. (2010) and Luque de Castro and Priego-Capote (2010).

To resume the study of the antimicrobial activity of the plants it is possible to say that olive and mimosa leaves proved to have antimicrobial properties. Antimicrobial activity of oleuropein, a constituent of olive, has been extensively studied and reported (Bisignano et al., 1999; Aziz et al., 1998), but the available information is reduced about the entire extract. Studies report that the antimicrobial efficiency of the olive leaves extracts are directly related with their polyphenols, which already proved that can inhibit the sporulation of *Bacillus cereus* and growth of *E. coli*, *Klebsiella pneumoniae*, *Salmomella typhimurium*, *Vibrio parahaemolyticus* and *S. aureus* (Korukluoglu et al., 2010; Sudjana et al., 2008). About mimosa leaves the information on the antimicrobial activity of its extracts are scarce.

For both plants, ethanol seemed to be the best solvent for solid-liquid and ultrasounds extraction to extract the compounds that confer antimicrobial activity. Ethanol is highly efficient in the extraction of tannins, flavonols, polyphenols, polyacetylenes, terpenoids, sterols and alkaloids and many of these compounds have recognized for their antimicrobial properties (Cowan, 1999; Karou et al., 2005). Burt (2004) reported that terpenoids and phenolic compounds give to essential oils the antimicrobial power and Omulokoli et al. (1997) reported that there are common diterpenoids alkaloids with antimicrobial properties. Cushnie and Lamb (2005) referenced the antimicrobial properties of flavonoids. Korukluoglu et al. (2010) studied the effect of the extraction solvent on the antimicrobial activity of some bacteria, including *E. coli* and *S. aureus*. They reported that the choice of the solvent influences the phenolic distribution and concentration of the extracts, which affects the antimicrobial activity. In that study, acetone was considered the best solvent to extract antimicrobial (phenolic compounds) from olive leaves.

It was observed that the reduction of the concentration of the extracts caused loss of activity, being this complete in some cases. Korukluoglu et al. (2010) also reported that the inhibitory effects of the olive extracts increased with increasing concentration, which corroborates these results.

It was common to observe a higher antimicrobial activity against *S. aureus*, principally for the extracts performed with ethanol, which is apparently due to the absence of an outer membrane layer, typical of Gram negative bacteria. This result is contradictory to the study of Markin et al. (2003), which found evidences that *E. coli* is

more susceptible to the olive leaves extract than *S. aureus*. However, Taguri et al. (2006) reported that there is no relationship between the Gram-type and the antimicrobial susceptibility.

There was no method of extraction that excelled in the extraction of antimicrobial compounds. Comparing the extractions performed with water the antimicrobial activity is similar for all samples ($p>0.05$) and this happened for olive and mimosa leave extracts.

4.3.2 Antibiotic-Extract Dual Combination Assessment

The antimicrobial activity of the extracts was also tested in combination with two antibiotics, tetracycline and erythromycin, in order to maximize the positive effects on bacterial inactivation and killing. The phytochemicals present in extracts plants have usually a minimum inhibitory concentration (MIC) higher than antibiotics and sometimes they cannot be used in medicine as sole agents. The effect of the combination of antibiotics and extracts is an important assessment, since some compounds of plants are known to modulate or modify resistance mechanisms in bacteria (Tegos et al., 2005).

Methanol, water and hexane were the chosen extraction solvents, with the goal to test solvents with different polarities. The chosen strains, *S. aureus* and *E. coli*, are considered susceptible to tetracycline and erythromycin (results no shown).

Tables 13 and 14 show the results of this test and its classification as antagonism (-), indifference (+), additive (++) and synergy (+++), as explained in section 4.2.6.

Table 13. Classification of the antimicrobial potential of antibiotic-extract dual combination against *S. aureus* as antagonism (-), indifference (+), additive (++) and synergy (+++).

	Olive (Methanol)	Olive (Water)	Olive (Hexane)	Mimosa (Methanol)	Mimosa (Water)	Mimosa (Hexane)
Tetracycline	(+)	(-)	(+)	(+)	(+)	(+)
Erythromycin	(-)	(-)	(-)	(+)	(-)	(-)

Table 14. Classification of the antimicrobial potential of antibiotic-extract dual combination against *E. coli* as antagonism (-), indifference (+), additive (++) and synergy (+++).

	Olive (Methanol)	Olive (Water)	Olive (Hexane)	Mimosa (Methanol)	Mimosa (Water)	Mimosa (Hexane)
Tetracycline	(+)	(+)	(+)	(+)	(+)	(+)
Erythromycin	(+)	(+)	(+)	(+)	(+)	(+)

Regarding to the activity against *S. aureus* the combination between the extracts and tetracycline caused an insignificant antimicrobial activity, except for the olive leaves extract with water. With this extract the effect is antagonistic. However, the combination between the extracts and erythromycin caused an antagonism effect in almost all extracts, except the mimosa leaves extract with methanol (the effect is indifferent). This antagonistic effect found in some combinations occurred probably because some compounds of the plants extracts inhibited completely the biological activity of the antibiotics. Their biological activity was inhibited probably by reducing their stability or bioavailability or by increasing their metabolism, which causes a reduced effect comparatively to the effect of antibiotics alone (Lila and Raskin, 2005).

About the activity against *E. coli* the results are similar for all extracts and both antibiotics: the effect of the combination is indifferent. Therefore, there are no advantages in using the extracts of olive and mimosa in combination with tetracycline and erythromycin against this bacterium.

4.3.3 Quorum-Sensing Inhibition Assessment

In this study the leaves of the both plants were tested, as well as the extracts obtained with water, methanol and hexane. The pigmentation of the indicator microorganism, purple-colored, provides a naturally occurring and the color was observed without additional substrates. The results of the QSI are presented in Table 15 (olive) and Table 16 (mimosa). The results are presented as the effects of the extracts in *C. violaceum* growth (inhibition halo) and also the effects of extracts in QSI, through the detection of pigment inhibition (QS halo).

Table 15. Quorum-sensing inhibition for olive leaves and olive leaves extracts.

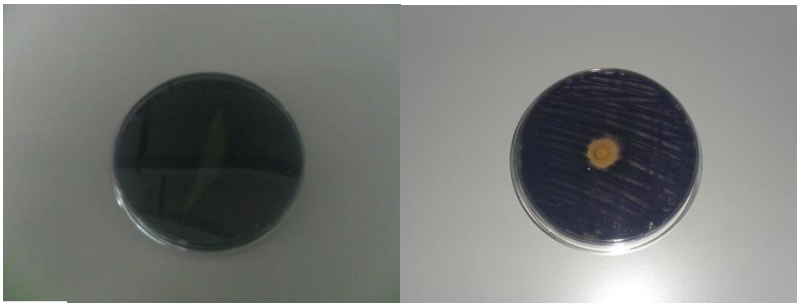
	Olive Leaves	Olive leaves extract (methanol)	Olive leaves extract (water)	Olive leaves extract (hexane)
QSI pigment	Yes	Yes	Yes	Yes
Inhibition halo (mm)	0	0	15.0±1.4	11.5±2.1
QS halo (mm)	0	0	0	0

Table 16. Quorum-sensing inhibition for mimosa leaves and mimosa leaves extracts.

	Mimosa Leaves	Mimosa leaves extract (methanol)	Mimosa leaves extract (water)	Mimosa leaves extract (hexane)
QSI pigment	Yes	Yes	Yes	Yes
Inhibition halo (mm)	0	0	20.0±1.4	12.0±1.4
QS halo (mm)	0	0	0	0

The quorum-sensing modulate the expression of genes involved in processes related with the survival, virulence and pathogenicity of bacteria. Functions like swarming, biofilm formation, secretion of virulence factors and acquiring competency represent an important role in bacterial infections in living systems pathogenicity and are related with QS (Vattem et al., 2007). QSI represents a natural strategy used by plants and other organisms with an important impact in the formation of biofilms and is now recognized as a global regulatory mechanism in bacteria (McLean et al., 2004).

Tests with the leaves of the plants did not present inhibition of the bacterium growth, as well as it did not present quorum-sensing inhibition. Figure 9 (a) shows the result of the QS test for olive leaves, where is possible to see the pigment but not the inhibition. The same happened for the extracts performed with methanol, for both plants. This means that the samples tested did not show antimicrobial activity against *C. violaceum*. Extracts performed with water and methanol demonstrated antimicrobial activity but they were not efficient in QSI. Figure 9 (b) shows the result of the QS test for methanol extract of olive leaves, where is possible to see the inhibition of the bacterium growth. Activity of the water extracts was significantly higher for the hexane extracts ($p<0.05$).



(a) (b)

Figure 9. QSI assessment in (a) olive leave and (b) methanol extract of olive leaves.

Diverse studies showed the effectiveness of several plants in QSI with *C. violaceum*, as *Tecoma capensis*, *Laurus nobilis* and *Lavandula angustifolia* (Al-Hussaini and Mahasneh, 2009). Al-Hussaini and Mahasneh (2009) used extract concentrations higher than those used in the present study (5 mg/mL). So, maybe if higher concentrations had been tested, the results would be different and maybe, the extracts from *Olea europaea* and *Acacia dealbata* would successfully inhibit QS.

4.3.4 Antioxidant Activity Assessment

In this test the approach is based on an electron transfer and involves reduction of a colored oxidant. ABTS test is based in formation of ABTS blue/green which can be reduced by antioxidants. DPPH test is based on the reduction of purple DPPH to 1,1-diphenyl-2-picryl hidrazina (Floegel et al., 2011). Results of antioxidant activity assessment are expressed in Trolox equivalent/g fresh mass, wherein Trolox equivalent is a synthetic vitamin E analogue (Martysiak-Zurowska and Wenta, 2012).

Olive leaves

Results for olive leaves extracts from solid-liquid and ultrasound extractions with methanol, ethanol, acetone, dichloromethane and hexane at 5 mg/mL are presented in Table 17 and Table 18, respectively.

Table 17. Antioxidant activity estimated by DPPH and ABTS of olive leaves extracts at 5 mg/mL for solid-liquid extraction and for the selected solvents (expressed in TE/g fresh mass).

Solvents	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
DPPH (TE/g fresh mass)	166.5±2.8	402.9±5.7	734.6±7.9	560.8±10.9	322.9±2.9
ABTS (TE/g fresh mass)	46.1±6.9	210.4±6.8	607.9±4.4	517.9±5.1	382.9±2.9

Table 18. Antioxidant activity estimated by DPPH and ABTS of olive leaves extracts at 5 mg/mL for ultrasounds extraction and for the selected solvents (expressed in TE/g fresh mass).

	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
DPPH (TE/g fresh mass)	107.3±7.9	372.3±10.8	539.2±12.9	449.8±3.4	309.8±6.9
ABTS (TE/g fresh mass)	184.3±12.2	208.6±10.7	719.4±5.9	526.5±4.9	569.4±10.7

For both techniques, the extract obtained with acetone is the one with the highest antioxidant ability, for DPPH and ABTS ($p<0.05$). This confirms the studies of Cowan (1999) and Gallo et al. (2010). These authors stated that acetone is one of the main solvents used to extract phenolic compounds from plants and these compounds have a high antioxidant activity. Acetone dissolves many hydrophilic and lipophilic compounds from plants, which makes it a very useful solvent (Eloff, 1998).

For both extractions, in relation to the extract with lower antioxidant power, methanol is the solvent which was less efficient to extract compounds with antioxidant properties, as assessed by both DPPH as ABTS assays. Relatively to the ultrasounds extraction, methanol and ethanol originated the extracts with less antioxidant activity ($p>0.05$) with the method of ABTS.

The antioxidant activity of plant extracts is strongly dependent on the nature of the extracting solvent, due to the presence of several antioxidant compounds of diverse chemical characteristics and polarities. Polar solvents are most frequently employed for the recovery of polyphenols, which are one of the main groups of compounds more responsible for antioxidant activity (Peschel et al., 2006). Actually, ethanol, acetone, ethyl acetate, methanol and aqueous mixtures of them have been extensively used to extract compounds from plants and plant-based foods with antioxidant properties, such as broccoli and rosemary (Peschel et al., 2006: Abdille et al., 2005). By this way, it was not expected that methanol and ethanol provided the extracts with lower antioxidant activities.

Table 19 presents the results of antioxidant activity for olive leaves extracts with water for every types of extraction.

Table 19. Antioxidant activity estimated by DPPH and ABTS of olive leaves extracts at 5 mg/mL for extraction techniques with water (expressed in TE/g fresh mass).

Water				
Extractions	Solid-Liquid	Ultrasounds	Soxhlet	Micro-wave
DPPH (TE/g fresh mass)	686.5±6.9	677.9±4.0	738.6±5.5	740.8±10.8
ABTS (TE/g fresh mass)	414.8±5.9	459.2±11.9	450.4±6.9	482.3±9.7

With the method of DPPH, differences between the types of extraction were not found: all the extracts had similar antioxidant potential ($p>0.05$). The same did not happened with the ABTS method; the solid-liquid extraction proved to be less efficient in extracting antioxidant compounds.

Mimosa leaves

The acetone extracts proved to be those with higher antioxidant activity, as indicated in Table 20 and Table 21. This fact is probably due to the presence of phenolic compounds.

Table 20. Antioxidant activity estimated by DPPH and ABTS of olive leaves extracts at 5 mg/mL for solid-liquid extraction and for the selected solvents (expressed in TE/g fresh mass).

	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
DPPH (TE/g fresh mass)	739.8±6.9	312.2±8.8	782.9±4.7	225.8±11.8	84.4±6.0
ABTS (TE/g fresh mass)	474.8±4.3	514.2±6.9	607.9±5.9	539.2±12.9	392.9±7.5

Table 21. Antioxidant activity estimated by DPPH and ABTS of mimosa leaves extracts at 5 mg/mL for ultrasounds extraction and for the selected solvents (expressed in TE/g fresh mass).

	Methanol	Ethanol	Acetone	Dichloromethane	Hexane
DPPH (TE/g fresh mass)	730.8±4.8	477.21±12.8	770.1±6.6	44.4±4.5	134.4±8.7
ABTS (TE/g fresh mass)	451.1±6.6	534.2±14.9	607.9±7.9	502.3±10.8	464.8±5.9

Acetone and methanol were the best solvents to extract antioxidant compounds with the DPPH method, for both extractions. In several studies, methanol is considered the best solvent to extract antioxidant compounds (Sultana et al., 2009). With the ABTS method, dichloromethane joined to acetone as the best solvents, in solid-liquid extraction, ($p<0.05$). With ultrasounds extractions only acetone was highlighted as the best solvent.

For extracts with water, the highest values of antioxidant ability for DPPH were found for solid-liquid, ultrasounds and Soxhlet extraction ($p<0.05$). Assays with ABTS did not show significant differences between the types of extraction ($p>0.05$). Results are presented in Table 22.

Table 22. Antioxidant activity estimated by DPPH and ABTS of mimosa leaves extracts at 5 mg/mL for extraction techniques with water (expressed in TE/g fresh mass).

Water				
Extractions	Solid-Liquid	Ultrasounds	Soxhlet	Micro-wave
DPPH (TE/g fresh mass)	765.8±8.9	756.5±4.2	698.6±12.0	595.1±2.1
ABTS (TE/g fresh mass)	356.7±5.9	387.3±5.5	338.6±9.1	387.3±9.8

To finalize the study about the antioxidant properties of the studied plants, it is possible to conclude that both of them can produce extracts with antioxidant activity. Several studies have already reported the antioxidant activity of olive leaves extract, such as Fitó et al. (2007), who reported the antioxidant properties of the extracts and the corresponding health benefits such as cardioprotective and chemopreventive effects. The presence of oleuropein and phenolic compounds is an important factor for antioxidant capacity of olive leaf extracts (Lee et al., 2009).

The magnitude of the antioxidant power depends on the extraction solvent used. For both plants, acetone was the solvent that originated the extracts with higher antioxidant potential as assessed by both methods (DPPH and ABTS), which is probably due to its ability to extract phenolic compounds. Phenolic compounds have antioxidant activity mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Gallo et al., 2010).

According to Martysiak-Zurowska and Wenta (2012), DPPH method has lower sensitivity than ABTS, probably because the DPPH method has more limitations. ABTS radical is reactive towards most antioxidants and it is soluble in both aqueous and organic solvents (Cano et al., 2000), while DPPH is more restrictive. However, in the current study, DPPH assay obtained in most cases the highest values. For that reason, DPPH method was considered a more useful method in the assessment of antioxidant activity witholive and mimosa leaves extracts.

Despite the observed differences between the values of DPPH and ABTS methods, the most important information to retain is that, almost always, both assays ranked the extracts in a similar order. Both gave acetone extracts as the most effective free radical scavenger and this evidence is actually more relevant than knowing the exact chemical reactivity of each sample (Wooton-Beard et al., 2011).

Comparing the plants used in the current study, differences were not observed between the antioxidant activities for the extracts with higher antioxidant properties (extracts of acetone) ($p>0.05$). Then, olive and mimosa extracts have similar ability to scavenge free radicals.

Lou et al. (2012) studied the antioxidant properties of *Morus alba L.* (mulberry fruits) and they found values between 75 μM TE/g fresh mulberry fruits and 240 μM TE/g fresh mulberry fruits for ABTS method and 95 μM TE/g fresh mulberry fruits and 180 μM TE/g fresh mulberry fruits for DPPH method, which indicates that olive and mimosa have higher antioxidant activity than mulberry fruits. In fact, the values of antioxidant activity in the current study are extremely high comparing with other studies. Wang et al. (1996) studied the antioxidant activity of 12 fruits and they obtained from 1 μM TE/g for melon to 15 μM TE/g for strawberry.

4.4 Conclusions

This work allowed to conclude that *Olea euopaea* and *Acacia dealbata* extracts have antimicrobial and antioxidant activities.

Typically, ethanol was the solvent that could extract compounds with more antimicrobial properties. This means that it was apparently very efficient in the extraction of phenolic compounds (Cowan, 1999). On the contrary, extracts with dichloromethane were rarely efficient against the bacteria, which allow to conclude that this solvent was not a good option.

In most cases, antimicrobial activity of the extracts was superior against *S. aureus*, which suggest the susceptibility of the bacterium due to the permeability of the cell wall (Simões et al., 2008). With the decreasing of the extracts concentration, extracts lost some activity, and, in some cases, they suffered complete loss of activity.

Comparing all the extracts performed with water, it was found that Soxhlet and micro-wave extractions are those for which the extracts have higher antimicrobial activity.

The combination between the plant extracts and antibiotics did not prove to be beneficial. No plant showed positive effects in bacterial killing when combined with tetracycline and erythromycin.

Extracts with water and hexane from both plants had antimicrobial activity against *C. violaceum* but they were not efficient in QSI. Antimicrobial activity of water extracts was superior than that of hexane extracts. Leaves and methanol extracts did not obtained antimicrobial activity against *C. violaceum* and they were not efficient in QSI.

In relation to antioxidant activity, it was concluded that acetone was the best solvent to extract compounds with antioxidant properties.

DPPH method achieved the highest values in most cases, whereby it was considered the method more useful in determination of antioxidant activity of olive and mimosa extracts. However, both methods ranked the extracts almost always in the same order.

Comparing with previous studies, it was found that olive and mimosa leave extracts have much higher antioxidant activity than some fruits, like melon and strawberry.

Any method excelled in the extraction of antioxidant compounds.

CHAPTER 5 – Conclusions and Perspectives for Future Work

Differences found between the results of bioactivity of *Olea europaea* extracts in previous studies and the present study can be explained by the preparation of the extracts. Usually, the extracts come from powdered leaves and are boiled or autoclaved and in this study the extracts were obtained from fresh leaves. Besides that, the choice of extraction solvent, crop origin, harvesting time and climate may influence the leaf composition, which can influence the bioactivity of extracts (Sudjana et al., 2008).

In relation to the extraction yield, methanol and water were considered the best solvents to solid-liquid and ultrasound extractions. Despite the advantages reported in the literature about ultrasound extraction, solid-liquid extraction allowed to obtain extracts with similar extraction efficiency. Comparing all the techniques used in the current study, Soxhlet and micro-wave were the methods that were able to achieve the best extraction efficiency.

It can be concluded that mimosa and olive leaves can provide extracts with antimicrobial and antioxidant activities. Ethanol proved to be the best solvent to extract compounds with antimicrobial activity, whereas for extract compounds with antioxidant properties acetone proved to be the best solvent. Dichloromethane was considered a weakest solvent to extract antimicrobial compounds. This happened for both mimosa and olive leaves. It was also concluded that olive and mimosa extracts have similar bioactivity. Soxhlet and micro-wave extractions were the best techniques to extract compounds with antimicrobial activity, whereas to extract compounds with antioxidant activity no method was highlighted.

In most cases, extracts were more efficient against *S. aureus* than *E. coli*, suggesting the higher susceptibility of *S. aureus* due to the permeability of the outer layer of the cell. It was also demonstrated that, by decreasing the extract concentration,, the antimicrobial activity also decreases (in some cases the extract lost completely its activity at 5 mg/mL). As so, it would be interesting to find what is the minimal inhibitory concentration for each extract. The combination of extracts of olive and mimosa with tetracycline and erythromycin against *E. coli* and *S. aureus* was not benefic and the extract leaves and the leaves themselves were not efficient on QSI.

To conclude, no extraction solvent was the best in all of the parameters tested.

It would be interesting in a future work to make a more detailed study about the leaves extracts, particularly on the identification of the molecules present in extracts, using chromatographic techniques. The analysis of the cytotoxic activity of the extracts against selected cell lines would provide relevant information on the potential therapeutic potential of the plant products.

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Appendix

A. Antioxidant Activity – Linear regression

A.1 ABTS method

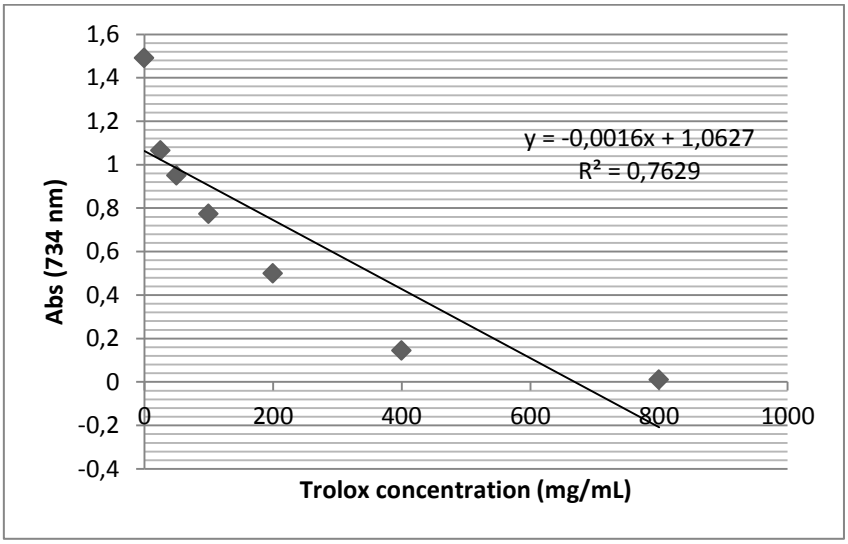

Figure A.1. Linear regression for antioxidant activity estimated by ABTS method.

A.2 DPPH method

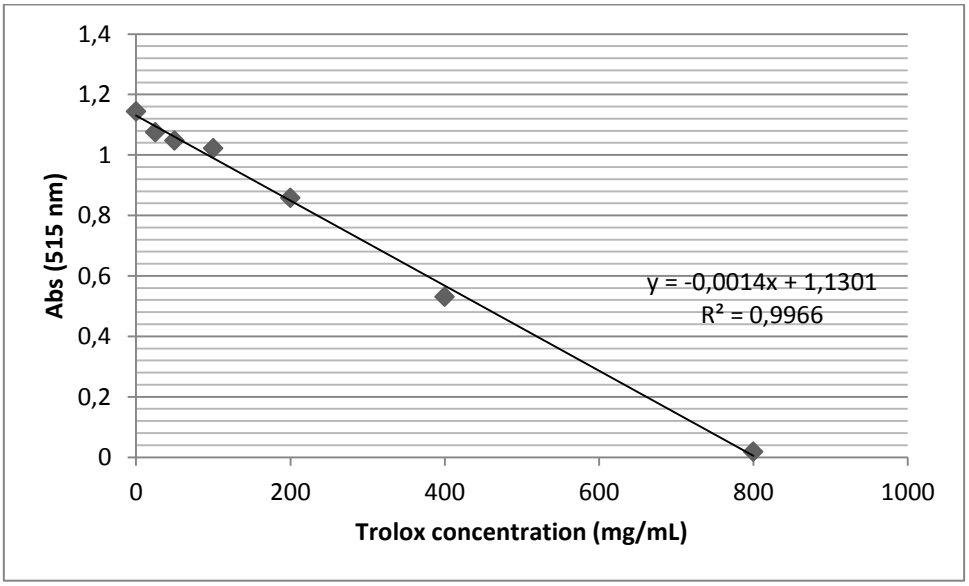

Figure A.2. Linear regression for antioxidant activity estimated by DPPH method.