UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

Dissertação de Mestrado em Engenharia Biológica

Evaluation of the *in vitro* biological activities of extracts from Carob tree and Mediterranean oaks

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Evaluation of the *in vitro* biological activities of extracts from Carob tree and Mediterranean oaks

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(João Daniel Domingues Patarra)

Faro, 2009

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Abbreviations

A.I. - Amylase inhibition

ABTS - 2, 2'- azino-bis (3-ethylbenzthiazoline-6-sulphonic) acid

ACh - Acetylcholine

AChE - Acetylcholinesterase

AD - Alzheimer's disease

$AlCl_3$ - Aluminium chloride

ALD - Adrenoleucodystrophy

ANOVA - Analysis of variance

BChE - Butyrylcholinesterase

BHT - Butylated hydroxytoluene

CC - Column chromatography

CE - Catechin equivalents

COMT - Catechol O-methyltransferase

CNS - Central nervous system

DAD - Diode array detection

dH_2O - Distilled water

DMSO - Dimethylsulfoxide

DPPH - 1, 1 - diphenyl-2-picrylhydrazyl

DTNB - 5, 5 - dithio-bis (2-nitrobenzoic) acid

DW - Dry weight

ET - Electron transfer

FRAP - Ferric reducing antioxidant power

GAE - Gallic acid equivalent

GC - Gas chromatography

GC-MS - Gas chromatography-Mass spectrometry

H_2SO_4 - Sulfuric acid

HAT - Hydrogen atom transfer

HPLC - High pressure liquid chromatography

$K_2S_2O_8$ - Potassium persulfate

LBG - Locust bean gum

MeOH - Methanol

$MgSO_4$ - Magnesium sulfate

Na_2CO_3 - Sodium carbonate

NaCl - Sodium chloride

NaOH - Sodium hydroxide

NO - Nitric oxide

ORAC - Oxygen Absorbance Radical Capacity

PLC - Phytochemoluminescence

PPM - parts per million

RE - Rutin equivalents

ROS - Reactive oxygen species

RSA - Radical scavenging activity

RT - Room temperature

SD - Standard deviation

SPSS - Statistical package for social sciences

TCT - Total condensed tannins content

TEAC - Trolox Equivalent Antioxidant Capacity

TFC - Total flavonoids content

TLC - Thin layer chromatography

TPC - Total phenolic content

UV - Ultra violet

Abstract:

In this work it was determined the antioxidant, neuroprotective and anti-hyperglycemic activity of methanol (MeOH), hexane and water extracts made from two species of Mediterranean oaks, *Quercus suber* L. (cork oak) and *Quercus ilex* L. (holm oak), and *Ceratonia siliqua* L. (carob tree). Additionally, a phytochemical evaluation was made. The extracts were made from different organs or products namely leaves, fruits, pulps, germ flour, gum and stem bark from carob tree and leaves and acorns from *Quercus* species. Samples were collected from mature trees growing in the Algarve. Acorns from cork oak were also sampled in Alentejo, to check for the influence of geographic origin on the biological activities of the extracts. Moreover, a thermal treatment ('dry roasting') at 200 ºC was applied to a part of the acorn samples, and compared to the results obtained in samples dried at 50 ºC. The chemical profile varied in all the parameters, the MeOH extracts were richer in phenolic compounds, which were present in higher amounts in leaves (*Quercus* species), and in leaves and stem bark (carob tree). Acorns had a considerable amount of phenolics and lipids. There was a great variation in the major compound between samples. Methanol extracts from leaves, acorns and stem bark exhibited an interesting antioxidant activity. The leaf extracts from the two species had a high inhibitory activity against acetylcholinesterase and butyrylcholinesterase. The extracts had no relevant activity against α-amylase, but a potent inhibitory activity on α-glucosidase from baker's yeast. Altogether, these results indicate that leaves and stem bark from carob tree, and acorns from cork oak and holm oak have bioactive compounds useful in the prevention/treatment of the above mentioned disorders. Further research is needed in order to identify the bioactive compounds, trough a bio-guided fractioning of the extracts.

Keywords: Mediterranean oaks, carob tree, antioxidant, anti-cholinesterase, anti-hyperglycemic

Resumo

Neste trabalho foi avaliada a actividade biológica de extractos feitos a partir de órgãos de *Quercus suber* L., (sobreiro), *Quercus ilex* L., (azinheira) e de órgãos e produtos de *Ceratonia siliqua* L., (alfarrobeira). Para além disso, foi estudada a composição química das fracções fenólica e lipídica dos extractos. A extracção foi feita em Soxhlet com solventes de polaridade crescente, nomeadamente hexano, metanol e água. Foram feitos extractos de folhas e bolotas de sobreiro de duas origens geográficas, Algarve e Alentejo, e de azinheira proveniente do Alentejo. Relativamente à alfarrobeira foram feitos extractos de folhas, polpa de frutos, farinha de germe e goma (obtidas em laboratório e de origem comercial), e casca. No caso das bolotas foi também estudada a influência de um tratamento térmico, que consistiu em submeter as amostras já secas e reduzidas a pó a uma temperatura de 200 ºC durante 10 min, para comparação com a secagem normal a 50 ºC. A avaliação da actividade biológica consistiu na determinação das actividades antioxidante, neuroprotectora e anti-hiperglicémica. A caracterização química da fracção fenólica dos extractos foi feita através da determinação por espectrofotometria dos compostos fenólicos totais, taninos condensados e flavonóides, e da identificação e quantificação de alguns compostos através de cromatografia líquida de alta precisão com detector 'Diode Array' (HPLC-DAD). A fracção lipídica foi avaliada por cromatografia gasosa com espectrometria de massa (GC/MS). O teor mais elevado em compostos fenólicos totais foi detectado nos extractos metanólicos, especialmente no extracto de folhas do sobreiro do Algarve (211.0 ± 19.8 mg AGE/g extracto), e de folhas e casca de alfarrobeira (310.7 ± 25.2 e 238.2 ± 15.9 AGE/g extracto), respectivamente. Nesses extractos os compostos maioritários foram o ácido gentísico, (+)-catequina, (-)-epicatequina e ácido clorogénico. O ácido oleico foi identificado como constituinte maioritário da fracção lipídica dos extractos de *n*-hexano de bolotas, seguido dos ácidos palmítico, linoleico e esteárico. A actividade antioxidante foi determinada por três métodos complementares: acção de 'scavenging' dos radicais DPPH e ABTS, e capacidade redutora no ião ferroso Fe^{3+}. Os extractos metanólicos revelaram uma elevada actividade antioxidante (> 50%), sendo de destacar os extractos de folhas e casca da alfarrobeira, com percentagens de inibição do radical DPPH de 88.5 ± 0.8% e 95.6 ± 0.4%, respectivamente. Estas amostras foram, também as que apresentaram a maior capacidade redutora do ião ferroso. Relativamente à e na inibição do radical ABTS os melhores resultados foram obtidos com as amostras de

bolotas de sobreiro do Algarve (78.2 ± 15.3%) e Azinheira (75.1 ± 11.6%). O estudo da actividade neuroprotectora foi realizado através da determinação da actividade inibitória dos extractos nas enzimas acetilcolinesterase (AChE) e butirilcolinesterase (BChE) através do método de Ellman. Estas enzimas são responsáveis pela degradação do neurotransmissor acetilcolina, o qual participa no processo de transmissão de sinais cerebrais ao nível das sinapses. Os extractos metanólicos e de hexano demonstraram maior capacidade inibitória do que os aquosos. Relativamente às espécies de *Quercus*, não foi observada influência significativa da espécie ou da zona geográfica de origem, assim como do tratamento térmico. Quando foi testada a concentração de 1 mg/mL todos os extractos de sobreiro e alfarrobeira exibiram activada moderada (30-50% de inibição) ou muito elevada (> 50 % de inibição) na AChE. Os melhores resultados relativamente às espécies de Quercus foram obtidos com a aplicação de extractos de sobreiro do Algarve nomeadamente do extracto metanólico de folhas (79.1 ± 7.6%), e do extracto de hexano de bolotas submetidas ao tratamento térmico (77.5 ± 6.4%). Os extractos de folhas e casca de alfarrobeira permitiram obter resultados elevados de actividade inibitória da AChE, com valores de 73.3 ± 1.5% e 71.7 ± 7.3%, nos extractos aquosos e metanólicos, respectivamente. Com a concentração de 10 mg/mL a inibição foi de cerca de 88%, semelhante à obtida com a aplicação do controlo positivo (94.3 ± 2.3%). Os resultados obtidos na BChE foram semelhantes aos observados com a AChE onde, geralmente, os melhores resultados foram obtidos nos extractos metanólicos, seguidos dos de hexano e aquosos. As amostras de folhas do Algarve, assim como as folhas, polpa e casca da alfarrobeira foram as únicas com actividade muito elevada (> 50% de inibição). A aplicação do extracto aquoso das folhas de alfarrobeira resultou numa inibição de 93.0 ± 1.7%, superior ao obtido com o controlo positivo (81.3 ± 3.1%).

A actividade anti-hiperglicémica foi avaliada através da determinação da actividade inibitória dos diferentes extractos nas enzimas α-amilase e α-glucosidase (origem animal e microbiana), as quais são consideradas enzimas chave envolvidas no processo de degradação do amido e de absorção intestinal, respectivamente. No geral, foram obtidos melhores resultados com a aplicação de extractos de sobreiro e azinheira, não se observando uma influência significativa da espécie e da zona geográfica. Os extractos de sobreiro e azinheira revelaram uma baixa actividade inibitória na α-amilase (< 50% de inibição), mesmo com a concentração de 10 mg/mL, enquanto que com a aplicação de extractos metanólicos de folhas e casca de alfarrobeira se observaram taxas de

inibição de 86.9 ± 11.5% e 79.9 ± 7.8%, respectivamente. Geralmente, os extractos apresentaram uma menor capacidade de inibição da actividade da α-glucosidase de origem animal, comparativamente com a de origem microbiana. Nesta última, os melhores resultados obtidos para as espécies de *Quercus* foram observados após a aplicação do extracto aquoso das folhas do sobreiro (Algarve), na concentração de 10 mg/mL (97.4 ± 0.1% inibição). No caso da alfarrobeira a aplicação do extracto aquoso das folhas na concentração de 10 mg/mL resultou numa inibição de 99.1 ± 0.7%. Quanto à α-glucosidase de origem animal, a actividades de inibição obtidas com os extractos de sobreiro e azinheira foram geralmente baixas (< 50% de inibição), tendo-se apenas obtido bons resultados (> 50% inibição) nos extractos metanólicos na concentração de 10 mg/mL, especialmente nas bolotas do sobreiro do Algarve (59.0 ± 1.1 %). A aplicação dos extractos de alfarrobeira resultou geralmente numa menor inibição, comparativamente com as espécies de *Quercus*, apesar da obtenção de resultados mais elevados, nos extractos aquosos das folhas (68.8 ± 16.5%) e metanólicos da farinha de germe (73.6 ± 27.6%), na concentração de 10 mg/mL.

Os resultados indicam que as espécies incluídas neste trabalho possuem compostos bioactivos com potencial interesse para o tratamento de Alzheimer, diabetes e outros distúrbios associados a stress oxidativo. De particular interesse são os extractos que evidenciaram uma elevada actividade antioxidante associada a uma elevada actividade neuroprotectora e anti-diabética, tais como os extractos metabólicos de folhas e casca. De referir também que embora apresentando menor actividade biológica, os extractos de bolotas de sobreiro e azinheira, e frutos de alfarrobeira (polpa e farinha de germe) são particularmente interessante uma vez que podem ser incluídos de uma forma fácil na dieta, quer de uma forma directa (ingestão), ou indirecta, através da incorporação em produtos alimentares.

Palavras-chave: Sobreiro, Alfarrobeira, Antioxidante, Anti-colinérgica, Anti-diabetes

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1 – GENERAL INTRODUCTION

The history of drug discovery showed that plants are highly rich sources in the search for new active compounds and they have become a challenge to modern pharmaceutical industry (Heitzman et al., 2005). In fact, the characterization and study of the toxicology of bioactive substances from plants and food ingredients has continued to gather momentum over the last years. There are many ingredients from plants that are now associated with possible beneficial effects. Terms as nutraceuticals or herbal extracts have become widely used, and the market is being increasingly flooded with functional foods and dietary supplements.

Many synthetic drugs owe their origin to plant-based complementary medicine. Plant products, whether volatile or non-volatile, are valuable sources of novel bioactive components useful in the treatment of various diseases such as cancer, inflammation, viral infection, allergic responses, as well as in the provision of primary healthcare in most developed countries (Heitzman et al., 2005).

1.1 - OVERVIEW OF SOME BIOLOGICAL DISORDERS AND/OR DISEASES

1.1.1 - Oxidative stress

Reactive oxygen species (ROS) are chemical entities that include oxygen free radicals, such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-), nitric oxide (NO), peroxinitrite and also non-radical species, such as H_2O_2 and singlet oxygen ($^1O_2$). Over production of ROS in humans, by endogenous or external sources such as tobacco smoke, pollutants, organic solvents or pesticides, leads to oxidative stress (Gülçin et al., 2003b), which has been implicated in the pathogenesis of several disease processes including ischemia/reperfusion injury, Alzheimer's Disease (AD), Parkinson's and *diabetes mellitus* (Kannan and Jain, 2000).

1.1.2 - Diabetes

There are several herbal extracts which are effective in lowering blood glucose level with minimal or no side effects, being used as antidiabetic remedies like the Nepalese

herb Pakhan bhed (Bhandari et al., 2008) and Devil tree leaves (Jong-Anurakkun et al., 2007). Many compounds isolated from these plants are used in combinational therapy for diabetes (Bath et al., 2009), since oral antidiabetic drugs used today fail to give a long-term glycemic control. The oxidative stress may have a common pathway linking to diverse mechanisms for the diabetes complications such as vascular dysfunctions, nephropathy, neuropathy and retinopathy (Baynes, 1991). Moreover, *diabetes mellitus* is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin (Jong-Anurakkun et al., 2007), resulting in a reduction of endogenous antioxidants and an increase in oxidative stress in the human body.

Antioxidants have been shown to reduce the risk of diabetes onset (Montonen et al., 2004), improve glucose disposal (Ylonen et al., 2003) and improve some of the associated implications (Young et al., 2004). Phenolic phytochemicals from plants are reported to play an important role in modulating amylase and glucosidase activities, that are key enzymes involved in starch breakdown and intestinal absorption, respectively, and therefore contribute to the management of type 2 diabetes (Matsuura et al., 2004; McCue and Shetty, 2004).

1.1.3 - Neurodegenerative disorders

The brain is an organ particularly vulnerable to oxidative stress, and the hypothesis that this process is involved in neurodegenerative events and neuronal cell death has emerged (Behl, 1997).

Alzheimer's (AD) is a chronic neurological disease frequent in elderly people, as a result of malfunctioning of biochemical alterations (Ferreira et al., 2006) namely the reduction of the acetylcholine (ACh) levels in the hippocampus and cortex of the brain (Jaen et al., 1996). Acetylcholine is inhibited primarily by acetylcholinesterase (AChE) and secondly by butyrylcholinesterase (BChE) (Hebert et al., 1995). There are many reports on the biological effect of plants traditionally used either in infusions or in traditional remedies as acetylcholinesterase inhibitors *in vitro* and also as memory enhancers *in vivo* (Perry et al., 2000; Ingkaninan et al., 2003; Tildesley et al., 2003; Heinrich and Teoh, 2004). Some of the drugs approved for therapeutic use show

hepatotoxicity (Knapp et al., 1994), and thus there has been a continuous search for new drugs.

1.2 - OVERVIEW OF THE SPECIES USED IN THIS STUDY

In this work it was used samples from two Mediterranean species of the genus *Quercus*, *Quercus suber* L. (cork oak) and *Quercus ilex* (holm oak), and from carob tree (*Ceratonia siliqua* L.).

1.2.1 - Mediterranean oaks

The genus *Quercus* (Fagaceae) includes more than 300 species growing in temperate ecosystems, extending from Portugal to Turkey and from France to the north of Africa, (Fig. 1) (Camacho et al., 2004; Petrovic et al., 2004*)*.

Quercus suber (cork oak), (Figs. 2 and 3) and *Quercus ilex* (holm oak), (Figs. 4 and 5) are among the most important trees from an economical and ecological point of view in the Western Mediterranean basin, and dominate formations in extended areas along the region (Soto et al., 2007). They are known to be good sources of phenolic compounds and fatty acids which may participate in the control of the lipid oxidation phenomena (Cantos et al., 2003). Oak acorns are a rich source in carbohydrates, amino acids, proteins, lipids and various sterols (Rakić et al., 2007). Shelled out oak acorns have high energy value and are highly degradable (Saricicek and Kilic, 2004). Kernels of other species of the genus *Quercus* contain carbohydrates, proteins, fats, and tannins, and are known to exhibit important biological activities. Kernel oils of *Quercus robur* (English Oak) and *Quercus cerris* (Turkish Oak) contain α-linolenic acid, a ω-3 polyunsaturated fatty acid, which is important in eicosanoid synthesis and in the prevention of cardiovascular diseases (Rakić et al., 2007). Moreover, the acorns from English oak contain various biologically active compounds (tannins, gallic and ellagic acid, and different galloyl and hexahydroxydiphenoyl derivatives) which possess antioxidant activity (Rakić et al., 2007). Roasted oak kernels of *Quercus semen tostum (Glandes Quercus tostae)*, are used in traditional medicine as astringent, antidiarrheal, antidote, and in menstrual disorders. Roasted kernels can also be used as a substitute for coffee (Petrovic et al., 2004).

Besides its applications in human feed, *Quercus* species have a high importance in pig alimentation, particularly in Iberian pig. Due to its high starch and fat content (Charef et al., 2008), and thus, the production of the Iberian pig is very strongly connected with the survival of *Quercus* trees (Figs. 3 and 5). The meat obtained from Iberian pigs is richer in oleic acid, and consequently, a higher nutritional value. The feasibility of producing commercial feeds from acorns was explored previously, but oxidation phenomena occurred owing to their high fat content (Camacho et al., 2004).

Figure 1 - *Quercus suber* distribution areas (Bozzano and Turok, 2002).

1.2.1.1 - Cork oak

The economical importance of this species is due to its outer bark (cork). Cork production and its processing industry (mainly for the production of cork stoppers and thermal and/or acoustic insulation materials) exist mainly in the Mediterranean region (Silva et al., 2005). In Portugal, about 185.000 ton/year of cork are produced, representing more than 50 % of the world production (APCOR, 2008).

The use of cork oak acorns (Fig. 3) in the human diet has been reported since the end of the 19th century in Serbia, with recommendations about its application and beneficial action. The preparation of drinks based on thermally treated acorns (dry roasting) was especially recommended for children. During the roasting of kernels, starch transforms into dextrin and tannin content decreases, as well as the astringency. The *Quercus* fruit

is poor in protein but rich in starch and fat (Charef et al., 2008). The sweet acorn is considered an edible fruit in some Mediterranean countries, including Spain, where it is used in ice-creams and other desserts and liqueurs (Camacho et al., 2004).

There are reports on the antioxidative action of some acorn components like the skin and the endosperm that can contribute to the antioxidant ability (Cantos et al., 2003). The potential use of acorn oil appears to be promising, as indicated by a chemical composition that is rich in phytochemicals, especially sterols, tocopherols, and terpenic alcohols, suggesting possible applications in the pharmaceutical industry (Camacho et al., 2004). α-tocopherol, a chain-breaking antioxidant that traps peroxyl free radicals, is the principal and most potent lipid-soluble antioxidant in plasma and low-density lipoprotein (Kaul et al., 2001) and is frequently present in *Quercus* species (Cantos et al., 2003). The potential of acorn oil as antioxidant supplement could add value to an underutilized agricultural product (Camacho et al., 2004).

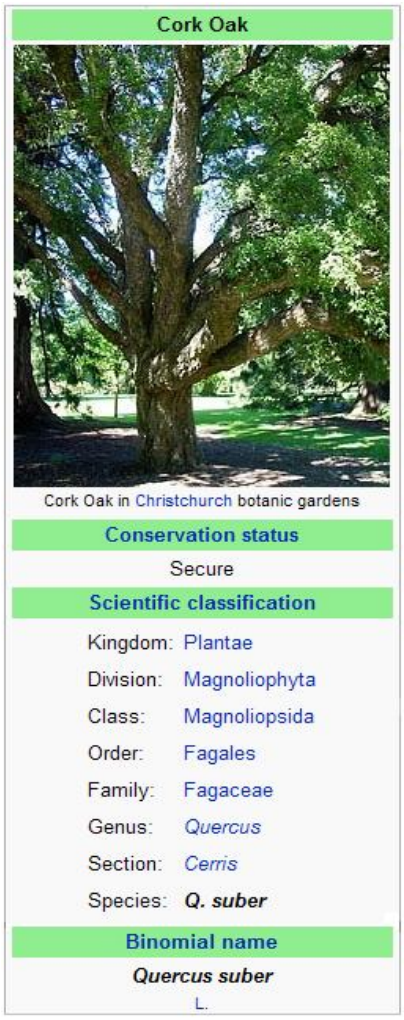

Figure 2 - Cork oak taxonomy (www.wikipedia.org).

Figure 3 - *Quercus suber* L. tree (www.arquivonatural.naturlink.pt) and acorns (commons.wikimedia.org), respectively.

1.2.1.2 - Holm oak

The holm oak is a deep-rooted dominant evergreen species in Mediterranean forests (Fig. 5). The foliage of holm-oak (Fig. 4) has a low crude protein level, high level of parietal compounds and condensed tannins and low organic matter digestibility (Dentinho et al., 2005/2006). Pfeifhofer (1998) determined the volatile composition of the holm oak leaves by steam distillation and showed that they are mostly constituted by terpenoides, followed by alkanes and free fatty acids.

Several phenolic compounds have been identified in acorns from holm oak (Cantos et al., 2003) and in the leaves (Skaltsa et al., 1994). The anthocyanin cyanidin-3-O-glucoside was present in young reddish developing leaves and epicatechin was the most abundant flavonol found (Brossa et al., 2009).

The lipidic fraction of acorns of this species is dominated by oleic acid, followed by palmitic and linoleic acids at similar concentrations (Cantos et al., 2003).

Figure 4 - Holm oak taxonomy (www.wikipedia.org).

Figure 5 - *Quercus ilex* tree (www.ubcbotanicalgarden.org) and acorns (www.flickr.com), respectively.

1.2.2 - Carob tree

The carob tree (Fig. 8) is an evergreen species which grows throughout the Mediterranean region, mainly in Spain, Italy, Portugal and Morocco (Batlle and Tous, 1997, Fig. 7). The genus *Ceratonia* belongs to the family *Leguminosae* (Makris and Kefalas, 2004) (syn. *Fabaceae*) of the order *Rosales* (syn. *Fabales*). Carob fruits consist of long pods (Figs. 6 and 8) with a sweet pulp and hard seeds, at the age of 5-7 years, yielding ~10 kg/tree, when mature they will yield 250-500 kg/tree and will continue to produce for up to 200 years (Batlle and Tous, 1997). The carob cultivars can be female, characterized by the flowers with abortive staminodes and fully developed pistils, hermaphrodite, flowers having fully developed stamens and pistils and male, were the flowers can have long or short filaments and abortive pistils (Meikle, 1977).

Female trees have always been selected in preference to the hermaphrodites, since generally they are better pod bearers (Batlle and Tous, 1997). Thus, the most common cultivars in commercial orchards are female and only few hermaphrodites have sufficient agronomic desirable attributes (Batlle and Tous, 1997). The information regarding the cultivars of carob tree in the Algarve is very scarce, and the existing refers only to the female ones. In the south of Portugal the most important female cultivars in terms of fruit productivity are *Mulata, Galhosa, Aida, Costela/Canela, Gasparinha,* and *Preta de Lagos.*

The economical importance of this species is due to the locust bean gum (LBG, E410) obtained from its seeds, used in food and non-food industries for its ability to form a very viscous solution at relatively low concentration. It is also exploited for its synergy property with carrageenan, agar and xanthan to form stronger and more elastic gels (Goycoolea et al., 1995). Furthermore, LBG has proven efficient in adjunct dietary treatment of diabetes (Tsai and Peng, 1981), and has found increased applications as a component of water-soluble dietary fiber, in programs of cholesterol management (Jensen et al., 1997).

The world production of commercial carob seeds is about 32.000 tons/year, and the production of pods is 310.000 tons/year (Batlle and Tous, 1997). The processing of the seeds to yield the corresponding endosperm involves the removal of the husks and of the germ fractions, either by chemical or by thermo-mechanical treatment (Ensminger et al., 1994). The germs recovered as by-products of the seed processing are mainly used, after milling and heat treatment, as dietetic food or as animal feed (Batlle and Tous, 1997). They are also a potential ingredient in cereal-derived foods for celiac people (Feillet and Roulland, 1998).

The chemical composition of carob pods varies among the different cultivars and is characterized by a high content of different types of phenolic compounds (Avallone et al., 1997; Kumazawa et al., 2002; Owen et al., 2003; Makris and Kefalas, 2004; Papagiannopoulos et al., 2004), comparable to other Mediterranean foods, such as olives, exhibiting high antioxidative potential, superior to many other products rich in polyphenols, such as red wine (Owen et al., 2003). They contain high level of sugars (40-60 %), but low protein (3-4 %) and lipids (0.4-0.8 %). Apart from non-structural carbohydrates, the pods contain high amounts of dietary fiber and polyphenols especially highly condensed tannins (Marakis, 1996; Avallone et al., 1997).

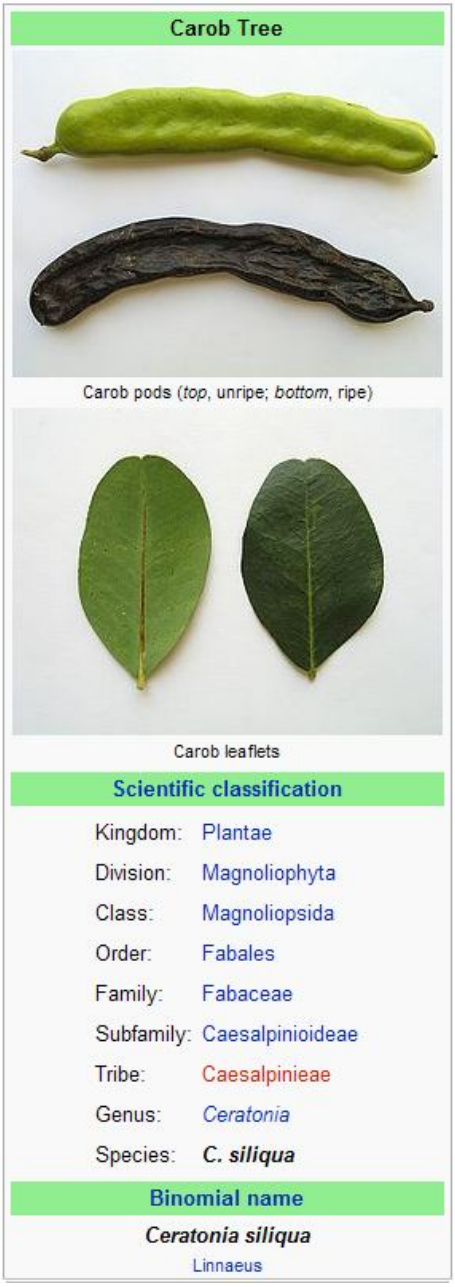

Figure 6 - Carob tree taxonomy (www.wikipedia.org).

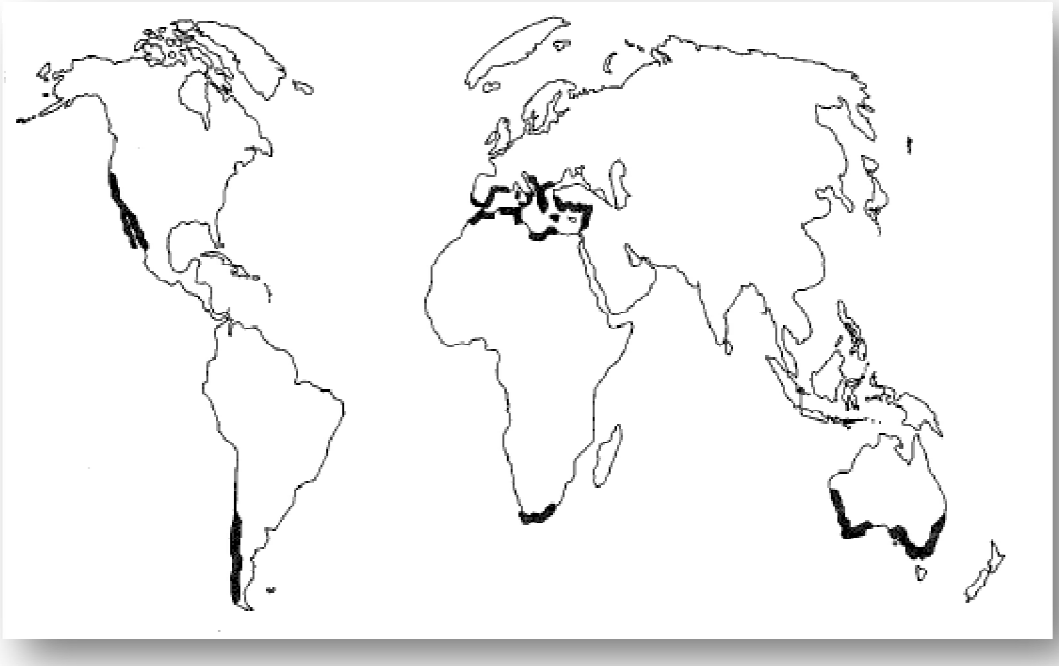

Figure 7 - World carob distribution and centers of origin (Batlle and Tous, 1997).

The two main constituents of carob fruits are (by weight): pulp (90 %) and seed (10 %). The pulps are used in infants for the treatment of diarrhea of bacterial and viral origin (Loeb et al., 1989). The seeds mainly composed principally of gallactomannans, which is a polysaccharide composed of mannose and galactose sugar units (ratio 4:1) (Batlle and Tous, 1997). The main property of this natural polysaccharide is the high viscosity of the solution in water, over a wide range of temperature and pH (Ochoa and Casas, 1992). Two other important properties of carob bean gum are its high water-binding capacity to form very viscous stable solutions in high dilution (1 % and lower) and its potential interaction with other polysaccharides, having a synergistic effect (Puhan and Weilinga, 1996). They are used as a growth medium for microorganisms and as a food stabilizer, and they have other applications in the textile, food, cosmetic, and pharmaceutical industries (Marakis, 1996; Avallone et al., 1997).

The leaves and pulps have antioxidant (Kumazawa et al., 2002; Custódio et al., 2009) antiproliferative (Corsi et al., 2002; Custódio et al., 2009) and antimicrobial activities (Kivçak and Ozturk, 2002). Extracts from carob's pulps and leaves exhibited *in vitro* antiproliferative activity in different human cancer cell types (Custódio et al., 2009), and it was demonstrated the presence, in extracts from leaves, of natural compounds

with ability to bind to both central and peripheral benzodiazepines (BDZ) receptor sites, making possible to use those extracts to obtain anxiolytic and sedative effects (Avallone et al., 2002). Methanol (MeOH) leaf extracts from Portuguese carob trees strongly inhibited the proliferation of human cervical cancer cells (HeLa line) (Custódio et al., 2009). Previously isolated constituents includes gallic acid, hydrolysable and condensed tannins, flavonolglycosides, flavonoids (Owen et al., 2003; Papagiannopoulos et al., 2004) and pinitol (Baumgartner et al., 1986).

The chemical composition of carob germ flour has been studied by several authors (Calixto and Canellas, 1982; Avallone et al., 1997; Yousif and Alghzawi, 2000; Dakia et al., 2007; Bengoechea et al., 2008), and a particular attention has been paid to the lipidic (Dakia et al., 2007) and proteic fraction (Bengoechea et al., 2008). It is also known that germ meal contains a significant amount of polyphenols (Avallone et al. 1997), which are potent antioxidants and free radical scavengers.

Figure 8 - *Ceratonia siliqua* tree (www.metrotrees.com) and pods (www.i4at.org), respectively.

1.3 - OBJECTIVES

The main goal of this work was to determine the *in vitro* biological activities of different extracts made from different organs of cork oak, holm oak and carob tree. Within this main goal, several objectives were addressed, namely:

1) Phytochemical evaluation of the extracts trough spectrophotometric and chromatographic methods.

2) Determination of the antioxidant ability of the extracts using DPPH, ABTS and Fe^{3+}/Fe^{2+} power reducing methods.

3) Evaluation of the neuroprotective potential of the extracts trough the determination of their anticholinesterase activity.

4) Assessment the anti-hyperglycemic activity of the extracts, namely their inhibitory activity on α-amylase and α-glucosidase enzymes.

2 - PREPARATION OF THE EXTRACTS AND PHYTOCHEMICAL EVALUATION

2.1 - INTRODUCTION

2.1.1 - Phenolic compounds

Phenolic compounds are important components of many fruits, vegetables, and beverages, to which they contribute to flavor, color, and sensory properties such as bitterness and astringency. They are plant secondary metabolites which play important roles in disease resistance (Antolovich et al., 2000; Servili and Montedoro, 2002), protection against pests and species dissemination. The interest on these compounds is related with their antioxidant activity and promotion of health benefits (Ryan et al., 2002). Recent interest in functional foods and the medicinal use of phenolic compounds have also stimulated interest in their separation (Nollet and Lee, 2000).

Chemically, phenolics can be classified into three major categories: simple phenols, polyphenols and a miscellaneous group (Fig. 9), (Luthria, 2006). Simple phenols primarily consist of phenolic acids (cinnamic acid and benzoic acid derivates). However the polyphenols are subdivided into two main classes: tannins and flavonoids. Tannins are classified in condensed tannins or proanthocyanidins, where the fundamental structural unit is the phenolic flavan-3-ol (catechin) nucleus (Haslam, 1998), and in hydrolysable tannins that are polymers of gallic acid (3,4,5- trihydroxyl benzoic acid) or ellagic acid (gallo- and ellagitannins) esterified with a core molecule, commonly glucose or a polyphenol such as catechin (Avallone et al., 1997). All the flavonoids have a flavone nucleus (2-phenyl-benzo-γ pyrane) as a structural basis and depending of classification method, the flavonoid group can be divided into several categories (Kühnau, 1976).

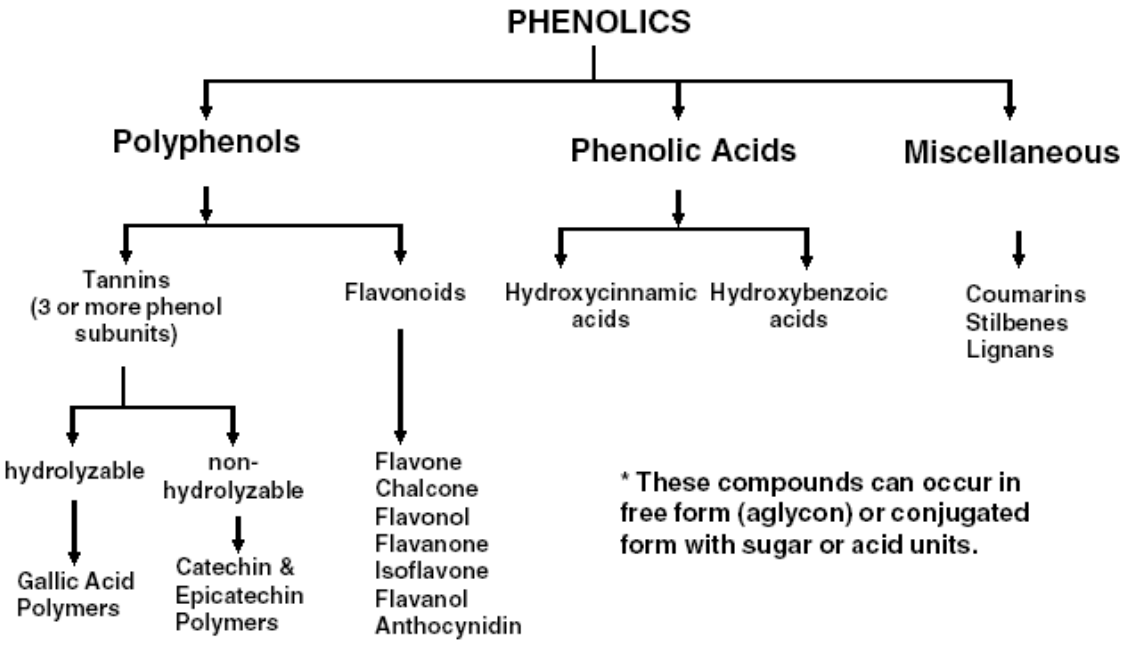

Figure 9 - Classification of phenolic compounds (Luthria, 2006).

Due to the large number of structural variations in closely related food phenolic compounds, analytical procedures for the analysis of individual phenolics have been relatively difficult and complicated (Nollet and Lee, 2000). The analysis of phenolic compounds in natural products can vary from simple colorimetric test for detection to the use of sophisticated instrumentation for separation, quantification, and characterization of individual components like high performance liquid chromatography (HPLC), thin layer liquid chromatography (TLC) and gas chromatography (GC), more specific to analyze them in oil solutions. In contrast to other liquid chromatographic methods (paper, thin layer, and column), HPLC approaches can provide a rapid response offering both high sensitivity and separation efficiencies through the use of tightly packed columns with small particles (Nollet and Lee, 2000).

2.1.2 - Lipids

Lipids are chemically diverse compounds which can be extracted from animal, plant and microbial sources with a variety of methods. Lipids are usually described, broadly, as those compounds which are insoluble in water and soluble in selected

organic solvents such as chloroform, hexane, benzene, diethyl ether, or methanol (Perkins, 1991).

Based on their structure, lipids can be classified as derived, simple and complex. The derived lipids include fatty acids and alcohols, which are the building blocks for the simple and complex being the fatty acids the obvious starting point in lipid structures. Their two main classes are the saturated, as palmitic, stearic, lauric and myristic acids and unsaturated, were the most common monounsaturated fatty acid is oleic acid, although more than 100 monounsaturated fatty acids have been identified in nature (Akoh and Min, 2002).

Lipids in nature are associated with other molecules, therefore, to separate and isolate them from a complex cellular matrix, different chemical and physical treatments must be made. The existing procedures for their extraction from animal or plant tissues usually include several steps: (1) pre-treatment of the sample, which includes drying, size reduction or hydrolysis; (2) homogenization of the tissue in the presence of a solvent; (3) separation of the liquid (organic and aqueous) and solid phases; (4) removal of non-lipid contaminants; (5) removal of the solvent and drying of the extract (Akoh and Min, 2008). After the extraction of the lipids their chemical characterization is usually made by chromatographic procedures, must including column chromatography (CC), gas-chromatography (GC), high performance liquid chromatography (HPLC) and thin layer chromatography (TLC). The GC analysis of lipids as been much studied in literature (Akoh and Min, 2008).

Gas chromatography (GC) is a powerful separation technique for resolving and quantifying lipids. Methyl ester derivatives of fatty acids are almost exclusively used by GC analysts. However, other derivatives can often provide improved peak resolution, like oleic (*cis*-9 18:1) acid. Complete separation of common fatty acids is usually achieved by using capillary columns with highly polar liquid phases. These columns can readily separate fatty acids according to chain length and degree of saturation (Firestone et al., 1997). Mass spectroscopy has been described as a technology that as revolutionized every discipline in its wake since it is inception. The combination of capillary gas chromatography with mass spectrometry (GC-MS) is recognized as the “gold standard” analytical method for identification and quantitative analysis of organic compounds, and mass spectrometry has been essential in revealing much of the lipid biochemistry knowledge. This can be attributed to improved

detection limits, the richness of structural information obtained, unrivaled quantitative accuracy, and the proliferation of low-cost benchtop mass spectrometers (Jones and Shibamoto, 1994).

2.1.3 - Factors with influence in the phytochemical evaluation

The extraction yield, the composition and thus the biological activity of the extracts highly depend on the solvent polarity, which determines both qualitatively and quantitatively the extracted compounds (Franco et al., 2008). In the particular case of phenolic compounds, the highest yields are usually achieved with ethanol and methanol and their mixtures with water, although other solvents have been widely used in the extraction of polyphenols from plants, as ethyl acetate or acetone (Franco et al., 2008). Water and ethanol are the most widely used because of their low toxicity and high extraction yield, with the advantage of modulating the polarity of the solvent by using ethanol/water mixtures at different ratios. The main drawback of the aqueous extraction is the low yield in antioxidants with low polarity or liposoluble antioxidants as, for example, the carotenoids (Franco et al., 2008).

Geographical origin is often used by coopers as a so-called indication of quality of the oak. The influence of geographic origin has not been correctly established from a scientific point of view, because evaluated samples have usually been mono-specific. It is therefore difficult to separate the influence of species from ecological circumstances when comparing two sites with different species ratios. Oak being characterized by a wide variation in the quality of the volatile and phenolic compounds, it is only possible to understand the influence of origin by using large sample sizes. The influence of a tree's age and the distribution of the ellagic tannins in the tissue of the wood may also complicate conclusions and lead to the wrong interpretations (Prida and Peuch, 2008). It is well-known that cork quality is very sensitive to the growth conditions of the trees, mainly in connection with the overall maintenance of the stands where they grow, but an intraspecies genotypic component might be important as well, which can be exploited for breeding purposes (Oliveira et al., 2003).

Rakić et al. (2007) studied the influence of thermal treatment in the antioxidant activity of two *Quercus* species, consisting in drying the acorns at 200 ºC during 10

min. The first conclusion was that they exhibited higher antioxidant activity than the extracts with no treatment. As expected, these activities were correlated with phenolics content in investigated extracts. The samples were subjected to critical temperature conditions that led to more severe hydrolytic reactions, the degradation of existing, and the formation of new, compounds is within a broader research program of studying the changes in chemical composition of acorn subjected to thermal treatment (dry roasting), hydrothermal treatment (preparation of aqueous extract), as well as drying of solution (Rakić et al., 2006).

2.2 - MATERIALS AND METHODS

2.2.1 - Plant material

2.2.1.1 - Mediterranean oaks

Acorns samples were taken from cork oak growing in two geographical origins: Algarve and Alentejo, while acorns were sampled from holm oak, growing in Alentejo. From the Algarve cork oak leaves were also sampled. Healthy, ripe acorns that had fallen to the ground, without mechanical or other damage, were collected in October 2007 and stored at -20 ºC. Leaf samples were collected on February 2008. Acorns were shelled out, the kernels dried at 50 ºC for 2-3 days, crushed and milled in a laboratory-scale hammer mill and stored in the dark at -20 ºC. These acorns were designated as native acorns. One part of the acorns were thermally treated, which consisted of 'dry roasting' of the samples (previously dried at 50 ºC), at 200 ºC for 10-15 min (Rakić et al., 2006, 2007). These acorns were designated thermal treated acorns.

2.2.1.2 - Carob tree

The sampling of carob tree was done in August and September of 2005. Fully expanded leaves and stem bark were randomly collected from the middle third at the branches and in the trunk, in all canopy orientations. Mature fruits, corresponding to stage III of development (Fig. 10) were also collected, manually deseeded and the

seeds used by DANISCO Portugal-Industrias de Alfarroba, LDA for the production of carob germ flour and locust bean gum (LBG). Commercial germ flour and LBG were obtained from Industrial Farense. Samples were dried at 50 ºC for 2 days (except carob germ flour, which was already dried), crushed and milled and stored in the dark at -20 ºC until extraction. The germ flour is prepared by a chemical treatment of the seeds with sulphuric acid, to remove the husk, followed by a chemical treatment to separate the endosperm from the germ.

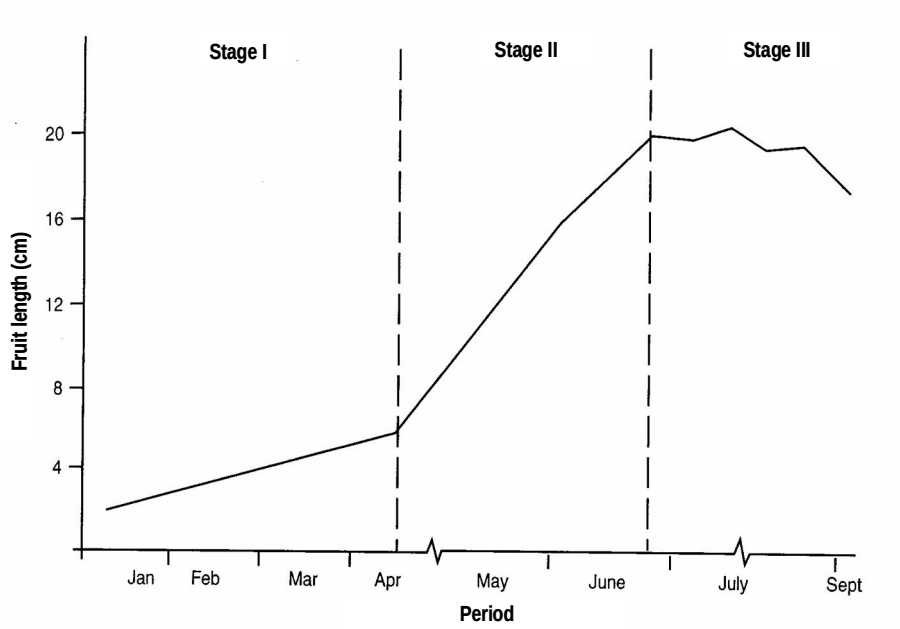

Figure 10 - Stages of carob fruit development (Batlle and Tous, 1997). The fruit growth follows a typical sigmoidal curve, with an initial period of slow growth (stage I), a middle period of linear growth (stage II) and a final period of declining growth (stage III).

2.2.2 - Preparation of the extracts

2.2.2.1 - Mediterranean oaks

Methanol (MeOH) extracts were prepared by soxhlet extraction according to Owen et al. (2003) and Custódio et al. (2009). The different samples (10 g) were extracted first with hexane (2 × 3h), to remove lipids, and then with MeOH (1 × 5h) using 200 mL of the extracting agent. Aqueous extracts were prepared as described by Corsi et al. (2002): 1 g of the different samples was boiled in 100 mL of distilled water for 15

minutes and then filtered. The MeOH and hexane extracts were concentrated in vacuum and resuspended in dimethylsulfoxide (DMSO), while the aqueous extracts were used without further treatment. Every extraction was performed at least two times and the MeOH and hexane extracts were kept at -20 °C in the dark until necessary.

2.2.2.2 - Carob tree

The carob tree extracts were prepared as described in section 2.2.2.1. In this work we used samples from female cv. *Mulata* because it is the most representative female cultivar in the Algarve, in terms of area of production. Additionally, the samples of LBG and germ flour were suspended in the buffer solution used in the different assays and concentrations, and utilized in the assays for biological activity.

2.3 - PHYTOCHEMICAL EVALUATION

2.3.1 - Phenolic fraction

2.3.1.1 - Total phenolic content (TPC)

For the quantification of the total contents of phenolic compounds a derived method of Folin-Ciocalteau colorimetric method (Tiito, 1985) was used: aliquots of the extracts (0.1 mL) were added to 5 mL of distilled water (dH_2O) and 0.5 mL of Folin-Ciocalteau reagent and vigorously shaken. After 3 min, 1 mL of saturated solution of sodium carbonate (Na_2CO_3) was added, and the volume made up to 10 mL with dH_2O. The mixtures were allowed to stand for 60 min at room temperature (RT) for complete reaction, and the total phenols determined by colorimetry, measuring the absorbance at 720 nm (Shimadzu UV-160A) against a reagent blank. The amount of total polyphenols was calculated as gallic acid equivalent (GAE) from the calibration curve of the gallic acid standard solutions, and expressed as mg GAE/g of dry plant material.

2.3.1.2 - Total condensed tannins content (TCT)

Catechins and proanthocyanidins were analyzed by the vanillin method as described by Broadhurst and Jones (1978). In short, samples (0.1 mL) were added to 2 mL of dH_2O and 4 mL of the vanillin reagent (1 % vanillin in 7 M H_2SO_4), shaken, and cooled on ice for 5 min. After 10 min at RT, the absorbance was measured at 500 nm (Shimadzu UV-160A). The amount of total condensed tannins content was calculated as (+)-catechin equivalent (CE) from the calibration curve of the (+)-catechin standard solutions, and expressed as mg CE/g of dry plant material.

2.3.1.3 - Total flavonoids content (TFC)

The flavonoid content was estimated in the extracts by the aluminium chloride ($AlCl_3$) method (Lamaison and Carnat, 1990). Briefly, the extracts (0.2 mL) were mixed with 1 mL of 2 % methanolic $AlCl_3.6H_2O$, and the absorbance was measured 10 min later at 430 nm (Shimadzu UV-160A). The amount of total flavonoids content was calculated as rutin equivalent (RE) from the calibration curve of the rutin standard solutions, and expressed as mg RE/g of dry plant material.

2.3.2 - High performance liquid chromatography (HPLC)

The extracts at the concentration of 10 mg/mL were analyzed by high performance liquid chromatography with diode array detection (HPLC-DAD, Agilent 1100 Series LC system, Germany). The results were expressed in mg/g extract, dry weight (DW).

Commercial standards of phenolic acids (chlorogenic, gallic, syringic, caffeic, cinnamic, ferulic, protocatchuic and gentisic), flavonoids [(+)-catechin, (-)-epicatechin, myricetin, rutin, kaempferol and quercetin], phenols (methyl gallate, vanillin and catechol) and theophylline were prepared in MeOH with desired concentration. Analyses were performed on a Tracer excel 120 ODS-A column, 150 mm x 4.0 mm, 5 µm particle size (Teknokroma, Spain). The mobile phase consisted on a mixture of 2.5 % acetic acid in water (A) and MeOH (B), the applied gradient was 0-50 min: 30-80 % B, 50-55 min: 80-30 % B and hold for 5 min and the flow rate was 1.0 mL/min. The analyses were performed at 25 ºC and the injection volume was

40 µL. For identification, the retention parameters of each assay were compared with the standard controls and the peak purity with the UV-visible spectral reference data. For quantification purposes, the external standard methodology was performed, using calibration standard solutions having concentrations ranging from 1.0 to 1000.0 mg/L for chlorogenic acid, (+)-catechin, gallic acid and gentisic acid, and 1.0 to 100.0 mg/L for the remaining compounds. The samples were filtered through 0.45µm nylon filters (Corning Inc., U.S.A.) before injection. The results were expressed in mg/g dry weight (DW).

2.3.3 - Lipidic fraction

The hexane extracts from oak acorns were analyzed by Gas Chromatography-Mass Spectrometry (GC-MS), after methylation as follows:

The extracts (40 mg) were mixed with 3 mL of MeOH solution with sodium hydroxide (NaOH) (0.5 %), and boiled until a homogeneous solution was obtained. Then, 5 mL of BF_3/MeOH solution was added, and this mixture was boiled for 2-3 min. The solution was allowed to cool and transferred to a separating funnel with 20 mL of hexane and 20 mL of a saturated solution of sodium chloride (NaCl). After mixing the organic phase was taken and dried with 1 g of anhydrous magnesium sulfate ($MgSO_4$) and then filtered. The resulting solution was used to analysis by GC-MS.

Analyses were performed on an Agilent 6890 Series gas chromatograph coupled to an Agilent 5973N mass selective detector (Agilent Technologies, Little Falls, DE, U.S.A.). The split injection mode (split ratio 1:50; pressure 9.78 psi) was performed, for which the inlet temperature was set at 220 ºC and the injection volume was 1 µL. Capillary GC analyses were performed on a TRB-5MS (27.5 m x 0.25 mm I.D.; 0.30 µm film thickness) capillary column (5% diphenyl, 95% dimethylpolysiloxane; Teknokroma) and helium as carrier gas maintained in the constant pressure mode was used. The oven temperature was programmed from 200 ºC (3 min) with 20 ºC min^{-1} to 220 ºC (held during 5 min). The transfer line, ion source and quadrupole analyzer temperatures were maintained at 280, 230 and 150 ºC, respectively, and a solvent delay of 2.50 min were selected. In the full-scan mode, electron ionization mass spectra in the range 35-400 Da were recorded at 70 eV electron energy with an

ionization current of 34.6 μA. Data recording and instrument control were performed by the MSD ChemStation software (G1701CA; version C.00.00; Agilent Technologies). The standards used were, palmitic acid, palmitoleat, behenic acid, stearic acid, myristate, linoleate, laurate, arachidate, heneicosanoate and oleic. Heptadecanoic acid was used as the internal standard, and all the compounds were in the methylated form. The results were expressed in *parts per million* (ppm).

2.4 - STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard deviation (SD). All the experiments were conducted at least in triplicate, and all the testes and measurements were repeated at least three times. The data were analyzed using the analysis of variance (ANOVA) method to assess differences using the SPSS statistical package for Windows (release 15.0, SPSS Inc.), and significance between means was tested by Duncan's New Multiple Range Test ($p = 0.05$).

2.5 - RESULTS

2.5.1 - Phenolic fraction

2.5.1.1 - Mediterranean oaks

Results of TPC, TCT and TFC are presented in Table 1. The MeOH extracts exhibited the highest contents of total phenolics, followed by the aqueous ones, and the higher amounts were observed in the leaf extracts (211.0 ± 19.8 mg GAE/g extract, DW). When comparing cork oak and holm oak, we observe that generally the samples from cork oak are better sources of TPC, more specifically from the Algarve region. When evaluating the effect of dry roasting (thermal treatment at 200 ºC), we observe that generally it resulted in an increase of the TPC amount in the MeOH extracts. In the acorn samples of cork oak (Alentejo), thermal treated acorns a lower content in TPC, while in the holm oak thermal treatment produced an increase in the TPC of the aqueous extracts.

The amount of total condensed tannins content was higher in the aqueous extracts, except in the cork oak (Alentejo). The highest amounts were detected in the native acorns of holm oak, (91.6 ± 0.1 mg RE/g extract, DW). Usually, the 'dry roasting' resulted in a significant increase in the tannins content of the aqueous extracts.

The highest value of flavonoid content was observed in the leaf extract (MeOH: 8.2 ± 0.6 mg RE/g extract, DW) and, in the acorns, the aqueous exhibited the highest TFC.

Table 1 - Total phenolic content (TPC), total condensed tannins content (TCT) and total flavonoids content (TFC) from Mediterranean oaks. G.O. - Geographical origin, D.T. - Drying temperature

Species/G.O.	Material	D.T. (°C)	Extract	TPC[a]	TCT[b]	TFC[c]
	Acorns	**50**	Hexane	tr	tr	tr
			MeOH	49.3±2.6aA	3.1±0.1bB	tr
			H_2O	17.0±0.2bA	87.8±0.1aB	0.6±0.1aB
		200	Hexane	tr	tr	tr
Cork oak			MeOH	71.4±10.2aA*	7.5±0.1bB***	tr
(Algarve)			H_2O	16.2±0.2bA	41.5±0.1aA***	2.0±0.0aB
	Leaves	**50**	Hexane	2.2±0.8c	0.1±0.0c	4.6±0.1b
			MeOH	211.0±19.8a	59.9±0.2b	8.2±0.6a
			H_2O	61.3±1.5b	63.6±0.1a	2.7±0.0c
	Acorns	**50**	Hexane	0.7±0.1cB	0.2±0.0b	0.5±0.0c
			MeOH	25.2±1.7aB	0.4±0.0aC	0.8±0.0bA
Cork oak			H_2O	10.2±0.2bB	tr	1.7±0.0aA
(Alentejo)		**200**	Hexane	1.0±0.0cA	tr	0.9±0.3b***
			MeOH	27.8±0.4aB*	1.3±0.0aC***	0.8±0.0bA
			H_2O	9.4±0.0bB**	tr	1.5±0.0aB
	Acorns	**50**	Hexane	1.3±0.2cA	tr	tr
			MeOH	13.6±0.1aC	10.2±0.3bA	0.2±0.0bB
Holm oak			H_2O	6.7±0.1bC	91.6±0.1aA	0.4±0.0aC
		200	Hexane	0.6±0.0cB**	0.2±0.0c	tr
			MeOH	19.0±0.3aB**	8.4±0.2bA***	0.4±0.0bB
			H_2O	16.2±0.2bA***	40.5±0.4aB***	5.4±0.9aA***

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same species, plant material and drying temperature (small caps), between species for the same plant material, drying temperature and extract (all caps) and between drying temperatures for the same extract, plant material and species (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] mg GAE/g extract. GAE, gallic acid equivalents; [b] mg CE/g extract. CE, catechin equivalents; [c] mg RE/g extract. RE, rutin equivalents; tr - trace amounts; MeOH - methanol; H_2O – aqueous

2.5.1.2 - Carob tree

Similar to the observed in extracts from *Quercus* species, in carob tree, the MeOH extracts exhibited the highest levels of total phenolic compounds ($p < 0.05$, Table 2).

The MeOH leaf and stem bark extracts had the highest content of TPC, with levels of 310.7 ± 25.2 mg GAE/g extract, DW and 238.2 ± 15.9 GAE/g extract, DW, respectively (Table 2).

The results on TCT varied greatly among samples and extracts (Table 2). The highest tannins content (95.3 ± 1.2 mg CE/g extract, DW) was observed in aqueous pulp extract, followed by the MeOH extract from stem bark (25.8 ± 4.2 mg CE/g extract, DW) (Table 2).

Regarding the TFC, we observed that generally the MeOH extracts exhibited the highest content of that class of compounds, which was highest in the leaf extracts (44.2 ± 2.8 mg CE/g extract, DW). Only trace amounts of the three classes of compounds were detected on samples from LBG and commercial LBG (Table 2).

Table 2 - Total phenolic content (TPC), total condensed tannins content (TCT) and total flavonoids content (TFC) from carob tree.

Plant Material	Extract	TPC[a]	TCT[b]	TFC[c]
Leaves	Hexane	3.6±0.5cB	2.6±2.3bB	6.3±0.1cA
	MeOH	310.7±25.2aA	15.3±1.4aB	44.2±2.8aA
	H_2O	65.5±1.9bA	0.3±0.0cC	8.3±0.1bA
Pulp	Hexane	1.2±0.7bD	tr	tr
	MeOH	13.7±0.8aC	tr	0.9±0.0E
	H_2O	8.2±0.1cD	95.3±1.2A	tr
Germ Flour	Hexane	2.4±0.2cC	4.2±0.5aA	0.5±0.0bB
	MeOH	21.9±0.5aC	2.6±2.4bC	11.9±0.1aB
	H_2O	6.1±0.1bE	tr	0.3±0.1cC
Commercial Germ Flour	Hexane	3.9±1.3cA	tr	1.0±0.3bB
	MeOH	16.9±0.5aC	1.2±0.1D	4.6±0.0aD
	H_2O	9.3±0.2bC	tr	5.1±0.0aB
Stem Bark	Hexane	tr	tr	tr
	MeOH	238.2±15.9aB	25.8±4.2aA	5.3±0.1aC
	H_2O	48.7±1.7bB	12.9±0.8bB	0.3±0.0bC
LBG	Buffer	tr	tr	tr
Commercial LBG	Buffer	tr	tr	tr

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material (small caps) and between plant materials for the same extracts (all caps). [a] mg GAE/g extract. GAE, gallic acid equivalents; [b] mg CE/g extract. CE, catechin equivalents; [c] mg RE/g extract. RE, rutin equivalents; tr - trace amounts; MeOH - methanol; H_2O - aqueous

2.5.2 - HPLC analysis

2.5.2.1 - Mediterranean oaks

Table 3 presents the phytochemical content of cork oaks extracts determined by HLPC-DAD. Generally the highest content and number of phenolic compounds was detected in MeOH extracts, followed by the aqueous and hexane ones (Table 3).

The MeOH leaf extract exhibited the highest diversity and total mean yield of identified phenolic compounds (53.6 mg/g extract, DW). In this sample the most abundant compounds was gentisic acid (24.3 mg/g extract, DW) followed by (+)-catechin (9.8 mg/g extract, DW) and chlorogenic acid (7.3 mg/g extract, DW).

It was observed that the thermal treatment resulted in an increase of phenolic compounds identified, especially in the MeOH extractions, but decreases their total amount in the MeOH extractions. Between the cork oaks, that treatment causes an increase in the total amount of phenolics in the Algarve samples and a decrease in the Alentejo samples.

In general, the most common phenolic compound in the oaks samples is the gallic acid, being the principal compound in the thermally treated samples of acorns from Algarve (MeOH). The flavonoids (+)-catechin, well present in the leaves (9.8 mg/g extract, DW), and (-)-epicatechin (29.6 mg/g extract DW), that was the most abundant compound in the holm oak MeOH acorns also had remarkable presence in the different samples. Teophylline, in spite of their smaller amounts in each sample are generally present in the acorns, especially in the MeOH and aqueous samples.

Table 3 - Phytochemical evaluation of cork oak extracts by HPLC-DAD (mg/g extract, DW).

Origin		**Algarve**									**Alentejo**					
Organ		**Acorns**						**Leaves**			**Acorns**					
Drying Temperature		**50 ºC**			**200 ºC**			**50 ºC**			**50 ºC**			**200 ºC**		
Extract		Hex	MeOH	H_2O	Hex	MeOH	H_2O	Hex	MeOH	H_2O	Hex	MeOH	H_2O	Hex	MeOH	H_2O
Compounds	**(-)-Epicatechin**								3.3			4.0			1.6	
	(+)-Catechin						0.3		9.8			5.0				
	Caffeic acid	0.1														
	Catechol			0.1									1.4			
	Chlorogenic acid						0.3		7.3							
	Cinnamic acid								4.5							
	Ferulic acid					0.1						0.1				
	Gallic acid	0.1	0.1	0.1		12.1		0.1	4.0	3.3	0.1	5.2	0.6	0.3	2.6	0.4
	Gentisic acid						1.9		24.3							
	Kaempferol															
	Methyl Gallate											0.1				
	Myricetin														0.1	
	Protocatchuic acid															
	Quercetin								0.2							
	Rutin															
	Syringic acid									0.1						
	Teophylline			0.1		0.4						0.4			0.3	
	Vannilin								0.2							
Total		0.2	0.1	0.3		12.6	2.5	0.1	53.6	3.4	0.1	14.8	2.0	0.3	4.6	0.4

Hex - hexane; MeOH - methanol; H_2O - aqueous

In the holm oaks samples, the MeOH extract from native acorns are similar to the cork oaks leaves samples, in diversity and total mean yield of identified phenolics (49 .9 mg/g extract, DW, Table 4) . That sample had like major constituents the catechol (16.2 mg/g extract, DW), (-)-epicatechin (29.6 mg/g extract, DW), gallic acid (3.3 mg/g extract, DW) and an alkaloid (theophylline, 0.6 mg/g extract, DW).

Table 4 - Phytochemical evaluation of holm oak extracts by HPLC-DAD (mg/g extract, DW).

Origin		**Alentejo**					
Component		**Acorns**					
Drying Temperature		**50 ºC**			**200 ºC**		
Extract		Hex	MeOH	H_2O	Hex	MeOH	H_2O
Compounds	**(-)-Epicatechin**		29.6				
	(+)-Catechin				0.1		0.3
	Caffeic acid						
	Catechol		16.2			4.7	
	Chlorogenic acid						
	Cinnamic acid						
	Ferulic acid					0.2	
	Gallic acid	0.4	3.3	0.6	0.3	2.1	1.1
	Gentisic acid						
	Kaempferol					0.1	
	Methyl Gallate					0.2	
	Myrcetin		0.2				
	Protocatchuic acid						
	Quercetin						
	Rutin						
	Syringic acid					0.2	
	Teophylline		0.6	0.1			
	Vannilin						
Total		0.4	49.9	0.7	0.4	7.5	1.4

Hex - hexane; MeOH - methanol; H_2O - aqueous

2.5.2.2 - Carob tree

On the carob tree, the extraction with MeOH afforded the highest number and total mean yield of identified compounds, except for the pulp extracts (Table 5).

The MeOH samples of stem bark extract had the highest amounts of compounds (131.4 mg/g extract, DW), followed by leaf (54.0 mg/g extract, DW), commercial germ flour (7.4 mg/g extract, DW) and germ flour (4.1 mg/g extract, DW). Different major compounds were associated with different samples (Table 5). In the stem bark extracts high amounts of gentisic acid (65.3 mg/g extract, DW), (-)-epicatechin (45.1 mg/g extract, DW) and (+)-catechin (10.3 mg/g extract, DW) were observed. In the leaf samples the major compounds was gentisic acid (24.4 mg/g extract, DW), followed by (+)-catechin (9.8 mg/g extract, DW) and chlorogenic acid (7.4 mg/g extract, DW). The commercial germ flour had higher amounts and number of identified phenolics in the MeOH and hexane extracts than the laboratorial germ flour, only better in the aqueous extracts. The commercial germ flour had syringic acid (2.2 mg/g extract, DW), catechol (2.0 mg/g extract, DW) and teophylline (1.3 mg/g extract, DW) as major compounds, while the laboratorial germ flour had chlorogenic acid (1.7 mg/g extract, DW) and also teophylline (1.6 mg/g extract, DW).

Table 5 - HPLC-DAD analysis of phenolic compound content (mg/g extract, DW) of extracts from carob tree.

Component		Leaves			Pulp			Germ Flour			Commercial Germ Flour			Stem Bark		
Extract		Hex	MeOH	H_2O	Hex	MeOH	H_2O	Hex	MeOH	H_2O	Hex	MeOH	H_2O	Hex	MeOH	H_2O
Compounds	**(-)-Epicatechin**		3.4					0.3							45.1	
	(+)-Catechin		9.8								0.4	0.5			10.3	0.4
	Caffeic Acid				0.2											
	Catechol						0.2	0.2			0.6	2.0				
	Chlorogenic Acid	tr	7.4						1.7						6.2	0.3
	Cinnamic Acid		4.5													
	Ferulic Acid							0.2	0.3	0.2						
	Gallic Acid	0.1	4.1	3.3	0.1	0.2	0.2	0.2	0.3	0.1	0.4	0.4			3.0	
	Gentisic Acid		24.4												65.3	1.9
	Kaempferol														0.2	
	Methyl Gallate										0.2	0.4				
	Myricetin											0.2				
	Protocatchuic Acid															
	Quercetin		0.2									0.2				
	Rutin											0.2			0.2	
	Syringic Acid			0.2						0.2	0.8	2.2	0.3		0.9	
	Theophylline						0.2		1.6	0.2	0.4	1.3	0.2			
	Vannilin		0.2						0.2						0.2	
Total		0.1	54.0	3.5	0.3	0.2	0.6	0.9	4.1	0.7	2.8	7.4	0.5	n.r	131.4	2.6

Hex - hexane; MeOH - methanol; H_2O - aqueous; n.r. - not realized; tr - trace amounts

2.5.3 - Lipidic fraction

Table 6 presents the results of the phytochemical evaluation of the lipidic fraction of acorns from acorn extracts, determined by GC-MS.

The cork oaks from Algarve had the highest lipidic content (23.33 ppm), followed by the cork oak from Alentejo (18.10 ppm) and holm oak (11.38 ppm). The major compound was oleic acid, followed by palmitic, linoleic and stearic acids. The Algarve cork oaks were composed by oleic acid (17.89 ppm), palmitic acid (2.67 ppm), linoleic acid (1.86 ppm) and stearic acid (0.81 ppm).

Table 6 – GC/MS analysis of the lipidic fraction of the Mediterranean oaks.

Species/G.O.	D.T. (ºC)	Palmitic Acid (ppm)	Linoleic Acid (ppm)	Stearic Acid (ppm)	Oleic Acid (ppm)	Total (ppm)
Cork oak (Algarve)	**50**	2.67	1.86	0.91	17.89	23.33
	200	0.46	0.24	0.61	1.15	2.46
Cork oak (Alentejo)	**50**	2.22	1.83	0.45	13.60	18.10
	200	1.80	1.06	0.57	11.37	14.80
Holm oak	**50**	1.22	0.69	0.59	8.88	11.38
	200	0.68	0.54	0.42	4.62	6.26

G.O. - Geographical origin, D.T. - Drying temperature

2.6 - DISCUSSION

Phytochemicals have health promoting effects as antioxidants, blood pressure or blood sugar influencing substances, or agents with anticarcinogenic, immunity-supporting, antibacterial, antifungal, antiviral, cholesterol-lowering, antithrombotic or anti-inflammatory effects. Some of these phytochemicals, such as phenolic compounds, are marked by a broad spectrum of health-promoting functions (Luthria, 2006). On the other hand, lipids play numerous important functions in biology, for example as the basic building block of all biomembranes or as essential signaling molecules in both health and disease. The signaling roles of lipids are now widely recognized as being of major importance in regulating such diverse physiological processes as cell division, apoptosis, brain development, fertilization and vascular physiology (O `Donnell, 2005).

2.6.1 - Mediterranean oaks

In this work, a phytochemical evaluation of the phenolic fraction of MeOH, hexane and aqueous leaf and acorn extracts of cork oak and holm oak was made, by spectrophotometric and chromatographic methods. The MeOH extracts had a higher content of total phenolic compounds, while the aqueous extracts exhibited a higher concentration in tannins and flavonoids. Moreover, the leaf extracts were richer in phenols than acorns. It is known that leaves of cork oak have a high content in total condensed tannins which is consistent with our findings, were the content in total tannins was higher than the total flavonoids content. The amount of total phenols in leaf extracts from cork oak was higher than the observed in *Salvia spinosa*, a plant species known to be a rich source of phenolic compounds (Alali et al., 2007). Working with *Quercus robur*, Almeida et al. (2008) obtained a concentration of 346.0 ± 19.8 mg GAE/g extract on leaf extracts, which is higher than the value obtained in this work in MeOH leaf extract from cork oak (211.0 ± 19.8 mg GAE/g extract, DW).

Oak acorns are a rich source of carbohydrates, aminoacids, proteins, lipids and sterols (Hopkins and Chisholm, 1953; Taleb et al., 1989; Mamedova et al., 1993; Camacho et al., 2004; Lopes and Bernardo-Gil, 2005). Shelled out oak acorns have high energy value and are high degradable (Saricicek and Kilic, 2004). Furthermore, it is known that acorns from cork oak and holm oak have a high content in phenolic compounds (Cantos et al., 2003; Dentinho et al., 2005/2006). Cross-varietal screening tests have repeatedly shown that some genotypes within a plant species can exhibit different levels of antioxidant compounds (Lila, 2006). In this work both the species and the geographic origin of the samples influenced the phenolic content of acorns. Acorns from cork oak were generally richer in total phenolics, with the samples collected in Algarve exhibiting the highest phenolic content. However, when analyzing the HPLC results, we can observe that generally acorn samples from holm oak were richer in phenolic compounds. This is similar to the results reported by Cantos et al. (2003), who determined the phenolic content of acorns from *Quercus ilex, Quercus rotundifolia* and *Quercus suber* growing in Spain by HLPC, and concluded that the first had the highest levels of phenolics, with an amount of more than 2 mg of phenolics/g of acorn, while *Q. suber* had the lowest concentration of those compounds (< 0.5 mg of phenolics/g of acorns).

Rakić et al. (2007) evaluated the influence of thermal treatment on the phenolic profile of acorns from *Q. robur* and *Q. cerris*. The thermal treatment consisted of heating the acorn samples at 200 ºC for 10 min. Those authors observed that thermally treated acorns had a

higher content in non tannin phenolics, such as gallic acid, but a lower concentration in tannin content. Our results indicate that the application of a similar thermal treatment influenced the content of phenolic compounds, especially the TPC of Algarve cork oak acorns. However, the thermal treatment causes a significant decrease in the TCT of the aqueous extracts, also as in the holm oak acorns. This may be explained by the presence of thermally degradable hydrolysable tannins in native samples, which are degradable at high temperatures, causing an increase of non-tannin phenolics and gallic acid contents (Rakić et al., 2007).The 'dry roasting' caused a significant variation in the TFC of the holm oak aqueous samples, providing higher results. When evaluating the HPLC results, we observe than thermally treated acorns from cork oak collected in the Algarve exhibits a higher content and number of identified compounds, and a higher amount of gallic acid, which is similar to the results of Rakić et al. (2007). However, in the similar samples of the Alentejo origin and in the holm oak acorns that was not observed. The most frequent identified compound in acorn extracts from the oaks was gallic acid which is consistent with earlier findings by other authors (Cantos et al., 2003).

The phytochemical profile determined by HPLC differed between cork oak and holm oak samples. On MeOH extracts from native samples (-)-epicatechin was the most abundant compound. (-)-epicatechin and (+)-catechin are epimers being most common optical isomers found in nature, being the principal constituents of total condensed tannins. Epicatechin is reported to have insulin mimetic action with protective effects on erythrocytes in a manner similar to insulin, also offering antioxidant protection against lipid peroxidation and inhibiting platelet aggregation (Ertan and Vural, 2009).There are studies suggesting the beneficial aspects of (-)-epicatechin intake to the human plasma, and that the increase of their pure content in human plasma results in the increase of antioxidant capacity and in a decrease in the concentration of plasma oxidation (Rein et al., 2000).

The main composition of the lipidic fraction of acorn samples was evaluated by GC/MS. Native acorns had a higher content of lipids than the thermal treated ones, and oleic acid was identified as the major compound, representing near 75% of the total fatty acid content. Our results are in accordance with earlier findings by other authors (Cantos et al., 2003), were oleic acid was accounted of more than 63% of the total fatty acid content on acorns from different species of *Quercus*. The same authors observed that linoleic and palmitic acids were in the same proportions, and in the range of 12–20% of the total fatty acids, while in this work the mean value found for linoleic and palmitic acid was 8.3%, and 12.7% respectively.

Monounsaturated fatty acids such as oleic acid have great importance because of their nutritional implication and effect on oxidative stability of oils (Aguilera et al., 2000). The cardiovascular protective effect of oleic acid is widely recognized (Massaro et al., 1999). Oleic acid has also been shown to slow the progression of adrenoleucodystrophy (ALD), a fatal disease that affects the brain and adrenal glands (Rizzo et al., 1986). The relatively large quantities of both C18:1n-9 (oleic acid) and C18:2n-6 (linoleic acid) acids in the oak acorns could provide beneficial amounts of these important fatty acids in the diets of people who live in the Mediterranean region, due to its relatively low content of saturated fatty acids and much higher content of unsaturated fatty acids.

2.6.2 - Carob tree

Similarly to the Mediterranean oaks, the MeOH samples had the higher phenolic contents. Between the materials, the leaves and stem bark shows the highest concentrations of total phenolics. The idea of using leaves of carob as a source of polyphenols, was raised after the evidence that carob leaves contain a high amount of those compounds (Corsi et al., 2002). Our results support those findings, in this study the leaf extracts displayed remarkable high levels of total phenolic content, with GAE values well above > 20 mg/g dry weight (Kähkönen et al., 1999). On the other hand, the amount of total phenols in leaf extracts from the carob tree were higher than those observed in *Salvia spinosa* L. (Lamiaceae), a plant species that is well-known as a rich source of polyphenols (Alali et al., 2007). Tawaha et al. (2007) studied Jordanian plants species and many showed remarkably high total phenolic content (GAE > 20 mg/g DW). For methanolic extracts, *Chrysanthemum coronarium* and *A. andrachne* were the richest on such compounds, with 59.6 and 57.6 mg GAE/g extract, respectively, above the content of 310.7 ± 25.2 and 238.2 ± 15.9 mg GAE/g extract in leaves and stem bark samples, respectively.

The total condensed tannin content was higher in the aqueous pulp extracts, followed by the MeOH extracts from stem bark and leaves. The tannin content obtained in this study was higher than the values obtained by Kumazawa et al. (2002) and Avallone et al. (1997). The carob pods tannin content was described by Batlle and Tous (1997) affirming that 18-20% of the pulp constitution was tannins, higher than the obtained in this study that was 9.5%. Chemical studies have reported that the phenolic fraction of carob fruit pulps consists of water

soluble and insoluble tannins (Papagiannopoulos et al., 2004). In the flavonoids content the leaves samples had the highest values in all the extracts.

The higher phenolic content of the leaves and stem bark extracts was confirmed by the HPLC analysis, were they exhibited the highest number and amount of identified compounds in the MeOH extracts. According to the literature the main phenolic compound found in leaves of carob tree is gallic acid (Corsi et al., 2002). In our study, the most abundant compound was the gentísic acid, followed by (+)-catechin and (-)-epicatechin. Gentísic acid (2, 5-dihydroxybenzoic acid) is an aspirin metabolite, has a potent antioxidant capacity and inhibits oxidation of low-density lipoprotein and the formation of cholesterol ester hydroperoxides in human plasma, although its precise mechanism remains elusive (Ashidate et al., 2005). There is an increasing amount of evidence indicating that gentísic acid has a broad spectrum of biological activities such as anti-inflammatory, anti-rheumatic and antioxidant properties, independent of the action of salicylic acid (Liu et al., 1995; Glinkowska et al., 1997).

The difference between our results and earlier findings in the same materials may be explained by the fact that phenolic compounds are secondary metabolites produced and accumulated in plant tissues, and changes in phytopathogenesis, among other factors, may result in different concentrations of these compounds in plant organs (Ferguson, 2001). The high amounts of both (+)-catechin and (-)-epicatechin, in leaf and stem bark samples reveals that this species also are a good source of flavonoids. Flavonoids are a class of natural products of importance as constituents of the human diet and for their pharmacological activities (Havsteen, 1983). (+)-Catechin is one of the most known flavonoids and had been shown *in vitro* to inhibit catechol O-methyltransferase (COMT), the enzyme that degrades norepinephrine. Given the important role of the sympathetic nervous system and its neurotransmitter norepinephrine in the control of thermogenesis and fat oxidation, it is conceivable that these catechins, by inhibiting COMT, result in an increase in or a more prolonged effect of norepinephrine on thermogenesis and fat metabolism or both, leading to lose body weight (Dulloo et al, 1999). Catechin is a more potent antioxidant than ascorbate or α-tocopherol in certain *in vitro* assays of lipid peroxidation (Zhao et al., 1999).

Theophylline was one of the most abundant compounds in germ flour MeOH extracts, and was been previously detected in aqueous extracts of carob pods (Corsi et al., 2002). Between those two samples, the hexane and MeOH samples of the commercial germ flour provide a higher amount of phenolics, and the highest number of identified compounds. Teophylline is

an alkaloid, and one of the caffeine metabolites known as xanthine compounds, widely found in the human diet. These compounds naturally occur in food products such as tea, coffee, and cocoa beans (Thomas et al., 2004). In recent years, xanthine derivatives have received increased attention in the food and nutrition industry because they can cause various physiological effects. Theophylline is widely used as smooth muscle relaxant, and has found application in the treatment of asthma as a bronchodilator. It has been shown that low-dose theophylline is an effective add-on therapy to corticosteroids in controlling asthma, due to its anti-inflammatory action (Ito et al., 2002). Due to the stimulatory influence of theophylline and other methylxanthines in the central nervous system (CNS) (Pleuvry, 2006), future work is needed to evaluate the CNS effects of carob germ flour.

2.7 - CONCLUSION

The MeOH extracts from leaves of both species and stem bark of carob tree had the highest content in phenolic compounds. The thermal treatment applied to the Mediterranean oaks acorns samples did not result in consistent variations in the phytochemical content. Both genotype and geographic origin of samples influenced the chemical composition of acorns, and generally, the Algarve cork oak exhibited the higher amounts of total phenolics, cork oak from Alentejo the highest tannins content and the holm oak the highest content in flavonoids. In carob tree the highest values of total phenolics and flavonoids were observed in the leaf samples, and the highest levels of tannins in the pulps aqueous extracts. In the HPLC analysis, different major compounds seemed to be associated with different samples. Generally, the gallic acid was the most common compound in both thermal treated and native acorns. In leaf samples, phenolic acids like gentisic, chlorogenic, cinnamic and gallic, and (+)-catechin were the major compounds. The holm oak, with a phenolic profile different of the cork oaks, exhibited (-)-epicatechin, catechol and gallic acid as the major compounds. From the carob tree materials, the stem bark, with the higher amount in phenolics was essentially composed by gentisic acid, (-)-epicatechin, (+)-catechin and chlorogenic and gallic acids. It was followed by leaves, commercial germ flour, germ flour and pulp when regard the total amount and number of phenolics identified.

3 – ANTIOXIDANT ACTIVITY

3.1 - INTRODUCTION

The importance of free radicals and reactive oxygen species (ROS) has attracted increasing attention over the past decade. ROS, which include free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals (OH^-) and non free-radical species such as H_2O_2 and singlet oxygen ($_1O^2$), are various forms of activated oxygen. These molecules are exacerbating factors in cellular injury and aging process (Gülçin et al., 2002a, 2002b).

In living organisms, various ROS can form in different ways and are considered a potential double-edged sword in disease prevention and promotion. Whereas generation of ROS once was viewed as detrimental to the overall health of the organism, advances in research have shown that ROS play crucial roles in normal physiological processes including response to growth factors, the immune response, and apoptotic elimination of damaged cells (Seifried et al., 2006). Notwithstanding these beneficial functions, aberrant production or regulation of ROS activity has been demonstrated to contribute to the development of some prevalent diseases and conditions, including cancer and cardiovascular disease (Seifried et al., 2006). Normal aerobic respiration stimulates polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells.

The formation of ROS is a natural consequence of aerobic metabolism and is integral for maintaining tissue oxygen homeostasis (Sohal and Weindruch, 1996). Oxygen homeostasis - the balance between constitutive oxidants and antioxidants - is maintained through a natural series of reduction-oxidation (redox) reactions involving the transfer of electrons between two chemical species: compounds that lose electrons (oxidized) and those that gain electrons (reduced). When oxygen homeostasis is not maintained, the cellular environment becomes oxidatively stressed. Approximately 1-3% of oxygen consumed by the body is converted into ROS (Seifried et al., 2006). Exogenous sources of ROS include tobacco smoke, certain pollutants, organic solvents and pesticides. ROS induce oxidative damage to biomolecules such as lipids, nucleic acids, proteins and carbohydrates, and have been implicated in more than 100 diseases; including malaria,

acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer and gastric ulcer (Gülçin et al., 2005b).

ROS are continuously produced during normal physiological events and are removed by antioxidant defense mechanisms. There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules (Gülçin et al., 2005a). Transient fluctuations of ROS levels influence activity of signal transduction pathways leading to cell proliferation, or to apoptosis or necrosis, depending on the dosage and duration of ROS and also on cell type. Typically, low doses of ROS can be mitogenic, whereas medium doses lead to temporary or permanent growth arrest (replicative senescence), and high doses usually result in cell death either by apoptosis or necrosis (Holbrook and Ikeyama, 2002). Many antioxidant compounds, naturally occurring in plant sources have been identified as free radical or active oxygen scavengers.

Recently, interest has increased considerably in finding naturally occurring antioxidants for use in foods or medicinal materials to replace synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are restricted due to their side effects such as carcinogenicity (Gülçin, 2005; Gülçin et al., 2006b). Most antioxidants isolated from higher plants are polyphenols. In vascular plants, more than 4000 phenolic and polyphenolic compounds have been identified (e.g. phenolic acids, tannins, coumarins, anthraquinones, flavonoids) (Trease and Evans, 1989; Middleton and Kandaswami, 1994). A wide range of low and high molecular weight plant polyphenols with antioxidant properties has been studied (Hagerman et al., 1998). The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In addition, they have metal-chelating potential (Evans et al., 1995).

Several analytical methods have been developed to determine the antioxidant capacity of natural substances *in vitro*. They can be categorized into two groups (Wangensteen et al., 2004): (i) assays for radical-scavenging ability and (ii) assays for lipid oxidation inhibitory effect. Or, more specifically, into another two types: assays based on hydrogen atom transfer (HAT) and assays based on electron transfer (ET). HAT-based assays, apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals and include methods like

Oxygen Absorbance Radical Capacity (ORAC), Phytochemoluminescence (PLC) and Trolox Equivalent Antioxidant Capacity (TEAC). ET-based assays measure the capacity of an antioxidant to reduce an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentration. ET-based assays include the total phenols assay by DPPH and ABTS radical scavenging capacity assays and the ferric reducing antioxidant power (FRAP) assay (Huang et al., 2005). However, the total antioxidant activities of plant extracts cannot be evaluated by using one single method, due to the complex composition of phytochemicals as well as of oxidative processes.

In this work, the antioxidant activity of the extracts was determined by 3 complementary methods: 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and Fe^{3+}/Fe^{2+} reducing power method.

3.2 - MATERIALS AND METHODS

3.2.1 - DPPH method

The DPPH method is representative of the methods employing model radicals in the evaluation of radical scavengers, witch have gained high popularity over the last decade because of their rapidity and sensitivity (Koleva et al., 2002). Antioxidants react with DPPH, which is a stable free radical, and convert it to 1, 1-diphenyl-2-picryl hydrazine. The degree of discoloration indicates the radical scavenging potential of the antioxidant (Singh et al., 2002).

Aliquots (0.1 mL) of the extracts at the concentration of 1 mg/mL were reacted with 2.5 mL of DPPH solution in MeOH (100 μM) for 30 min in the dark, and the decrease in absorbance at 517 nm was measured spectrophotometrically (Infinite M200 spectrophotometer, Tecan). Lower absorbance of the reaction mixture indicates higher free radical scavenging activity (Gülçin et al., 2007). Control samples contained DMSO or water in the place of the extracts. The radical scavenging activity (RSA) of the extracts was calculated using the following equation:

$$\%\,RSA = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Were $A_{control}$ is the control absorbance and A_{sample} is the absorbance of the samples. Butylated hydroxytoluene (BHT, 1 mg/mL) was used as reference.

3.2.2 - ABTS method

In this work it was used the method described by Robert et al. (1999) and modified by Wang et al. (2008). In this assay $ABTS^+$ radical is generated directly in a stable form prior to reaction with putative antioxidants. The $ABTS^+$ cation radical was produced by reacting 7.4 mM ABTS in dH_2O and 2.6 mM potassium persulfate ($K_2S_2O_8$). The reaction mixture was stored in the dark at RT for 12 -16 h. Before use, the $ABTS^+$ solution was diluted with ethanol to get an absorbance of 1.4 units at 414 nm. The $ABTS^+$ antioxidant reaction mixture contained 200 µL of $ABTS^+$ solution, and 50 µL of the extracts at the concentration of 1 mg/mL and DMSO or water for the control. The absorbance at 420 nm of the resulting solution was measured after 6 min by a 96-well plate reader (Infinite M200 spectrophotometer, Tecan). RSA was calculated using the same formula presented for DPPH. BHT was used as reference at the concentration of 1 mg/ml.

3.2.3 - Fe^{3+}/Fe^{2+} reducing power method

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The presence of reductants such as antioxidants substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (Chung et al, 2002; Gülçin, 2006c). Reducing power was determined according to the method of Oyaizu (1986), and modified by Choi et al. (2007). Samples (250 µL at the concentration of 1 mg/mL), sodium phosphate buffer (250 µL, pH 6.6) and 1% potassium ferricyanide (250 µL) were mixed and incubated in a water bath at 50 ºC for 20 min. Then, 250 µL of 10% trichloroacetic acid (w/v) were added to the mixture and centrifuged at 1000 rpm for 10 min. The supernatant (500 µL) was then mixed with equal volume of dH_2O and ferric chloride solution (0.1%, w/v). Absorbances were measured at 700 nm (Infinite M200 spectrophotometer, Tecan). Increased absorbance of the reaction mixture indicates

increased reducing power. BHT was used as a positive control at the concentration of 1 mg/ml.

3.3 - STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard deviation (SD). All the experiments were conducted at least in triplicate, and all the testes and measurements were repeated at least three times. The data were analyzed using the analysis of variance (ANOVA) method assess differences using the SPSS statistical package for Windows (release 15.0, SPSS Inc.), and significance between means was tested by Duncan's New Multiple Range Test (p = 0. 05).

3.4 - RESULTS

3.4.1 - Mediterranean oaks

The extracts revealed antioxidant activity, which varied according to the samples (Table 7). Generally, the extracts exhibited a higher capacity to scavenge the $ABTS^{+}$ radical, than the stable DPPH, and the highest RSA was usually detected in MeOH extracts.

The highest RSA on $ABTS^{+}$ radical was detected on acorn from the cork oak growing in the Algarve, namely the MeOH (78.2 ± 15.3%) and aqueous extracts (60.6 ± 2.3%) of thermal treated acorns, and on MeOH extracts from native acorns (63.3 ± 1.8%), leaf MeOH (67.0 ± 6.7%) and aqueous (71.0 ± 0.0%) extracts. Significant RSA on $ABTS^{+}$ radical was also observed on MeOH extracts from thermal treated (75.1 ± 11.6%) and native acorns (63.8 ± 6.9%) from holm oak (Table 7). Noteworthy is the fact that a high number of extracts displayed a higher RSA on $ABTS^{+}$ than the synthetic antioxidant BHT (Table 7). On cork oak samples, the geographical origin influenced the RSA on $ABTS^{+}$, and the samples from the trees growing in the Algarve usually exhibited the best results (Table 7). On acorn sampled in cork oak from Alentejo, the thermal treatment significantly enhanced the RSA of the water acorn extracts (Table 7).

The highest RSA against the stable DPPH radical was observed for the MeOH extracts of all samples, but they do not achieved 50% inhibition and were lower than the

value observed for BHT (Table 7). When using this method, no influence was observed on RSA by either species or geographical origin, whilst dry roasting, generally resulted in a promotion of RSA on cork oak samples (Table 7).

The reducing activity was higher on MeOH extracts, followed by the water ones. The best results were obtained in leaf MeOH (2.2 ± 0.0) and aqueous (0.7 ± 0.0) extracts.

Table 7 - Antioxidant activity measured by the DPPH and ABTS assays (% inhibition), and Fe^{3+}/Fe^{2+} reducing power assay (absorbance at 700 nm) of extracts from Mediterranean oaks. G.O. - Geographical origin, D.T. - Drying temperature

Species/G.O.	Material	D.T. (ºC)	Extract	RSA %		Ferro (abs)
				ABTS	DPPH	
Cork Oak (Algarve)	Acorns	50	Hexane	5.8±0.2cA	n.a.	n.a.
			MeOH	63.3±1.8aA	49.3±1.4aA	0.7±0.0aA
			H_2O	57.8±0.0bA	14.3±3.7bA	0.1±0.0b
		200	Hexane	n.a.	n.a.	n.a.
			MeOH	78.2±15.3aA	49.2±0.3aA	0.7±0.0A
			H_2O	60.6±2.3aA	30.1±2.3bA**	n.a.
	Leaves	50	Hexane	25.8±5.7b	n.a.	0.1±0.0c
			MeOH	67.0±6.7a	46.7±0.3a	2.2±0.0a
			H_2O	71.0±0.0a	41.2±2.9b	0.7±0.0b
Cork Oak (Alentejo)	Acorns	50	Hexane	4.7±0.5cA	n.a.	n.a.
			MeOH	51.9±3.4aB	41.8±4.0aB	0.2±0.0C
			H_2O	34.5±3.5bC	10.5±0.6bAB	n.a.
		200	Hexane	n.a.	n.a.	n.a.
			MeOH	46.8±3.5bB	48.9±0.2aA*	0.3±0.0aB*
			H_2O	54.3±0.9aA*	31.7±2.9bA**	0.2±0.0bA
Holm Oak	Acorns	50	Hexane	8.0±3.2cA	n.a.	n.a.
			MeOH	63.8±6.9aA	49.7±2.0aA	0.4±0.0B
			H_2O	41.1±0.8bB	4.2±0.3bC	n.a.
		200	Hexane	6.0±1.7c	n.a.	n.a.
			MeOH	75.1±11.6aA	49.8±1.6aA	0.3±0.0aB*
			H_2O	34.5±6.2Bb	3.7±1.5bB	0.1±0.0bB
BHT[a]				37.9±1.3	60.4±3.3	3.0±0.0

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same species, plant material and drying temperature (small caps), between species for the same plant material, drying temperature and extract (all caps) and between drying temperatures for the same extract, plant material and species (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] - positive control; n.a. - no activity; MeOH - methanol; H_2O - aqueous

3.4.2 - Carob tree

The results on the antioxidant activity of carob tree samples are summarized on Table 8. Similar to the observed for extracts from Mediterranean oaks, the best

antioxidant activity was generally displayed by the MeOH extracts. However, in leaf extracts, the RSA on $ABTS^{+}$ radical was highest in the hexane extracts (39.7 ± 2.2%), corresponding to the best result obtained in this assay, but lower than the RSA of BHT (56.3 ± 0.5%).

In the DPPH method the best results were obtained by the MeOH extracts of stem bark (95.6 ± 0.4%), followed by the leaves (88.5 ± 0.8%), highest than the value obtained with BHT (77.7 ± 2.5%).

In the Fe^{3+}/Fe^{2+} reducing power assay we obtained similar results to the DPPH method, with leaves and stem bark exhibiting the best results in the MeOH extractions, with values of 2.9 ± 0.0 and 2.6 ± 0.0, respectively, similar to the obtained with BHT (3.0 ± 0.0).

Table 8 - Antioxidant activity measured by the DPPH and ABTS assays (% inhibition), and Fe^{3+}/Fe^{2} reducing power assay (absorbance at 700 nm) of extracts from carob tree.

Material	Extract	RSA %		Ferro (abs)
		ABTS	DPPH	
Leaves	Hexane	39.7±2.2aA	23.2±1.8c	0.1±0.0cA
	MeOH	29.9±1.0bA	88.5±0.8Ab	2.9±0.0aA
	H_2O	24.2±4.8bB	64.2±5.8Ba	0.6±0.0bA
Pulp	Hexane	9.1±3.2cB	n.a.	0.1±0.0aA
	MeOH	32.7±0.4aA	10.1±0.9Ac	0.1±0.0aC
	H_2O	15.9±0.1bC	1.5±0.1bC	n.a.
Germ Flour	Hexane	n.a.	n.a.	n.a.
	MeOH	5.0±4.2B	5.2±0.3D	0.1±0.0C
	H_2O	n.a.	n.a.	n.a.
Commercial Germ Flour	Hexane	n.a.	n.a.	n.a.
	MeOH	4.3±0.6B	n.a.	0.1±0.0C
	H_2O	n.a.	n.a.	n.a.
Stem Bark	Hexane	n.r.	n.r.	n.r.
	MeOH	32.7±1.5aA	95.6±0.4aA	2.6±0.0aB
	H_2O	27.7±1.4bA	49.4±2.6bB	0.3±0.0bB
BHT[a]		56.3±0.5	77.7±2.5	3.0±0.0

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material (small caps) and between plant materials for the same extracts (all caps). [a] - positive control; n.a. - no activity; n.r. - not realized; MeOH - methanol; H_2O - aqueous

3.5 - DISCUSSION

In this work, the antioxidant activity of the extracts was determined by 3 complementary methods: 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and Fe^{3+}/Fe^{2+} reducing power.

The Fe^{3+}/Fe^{2+} reducing assay measures the ability of a sample to reduce metals, while ABTS and DPPH measure a sample free radical scavenging capacity. From a mechanical standpoint, in the Fe^{3+}/Fe^{2+} reducing power and ABTS methods there is a single electron transfer reaction, while in DPPH is combined with a hydrogen atom transfer reaction (Foti et al., 2004; Prior et al., 2005). Each method gives accurate,

repeatable values, but antioxidant capacity may differ substantially between one method and another (Jiménez et al., 2008).

The involvement of free radicals, especially their increased production, appears to be a feature of most, if not all human diseases, including cardiovascular disorders and cancer. The hydrogen-donating ability is an index of the primary antioxidants, which donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation (Ordoñez et al., 2006). Moreover, compounds with reducing power indicate that they are electron donors, and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen and Chen, 1995).

3.5.1 - Mediterranean oaks

The MeOH extracts exhibited a higher antioxidant activity and the best results were obtained with MeOH extracts from thermally treated acorns of Algarve origin and holm oak. Those samples showed a significant RSA, suggesting that they are capable of scavenging free radicals, and thus, may be able to prevent the initiation of free radical-mediated chain reactions by preventing the abstraction of hydrogen from susceptible polyunsaturated fatty acids.

Cantos et al. (2003) evaluated the antioxidant ability of *Q. ilex*, *rotundifolia*, and *suber*, and found a correlation between the total phenolic content of the samples and their antioxidant ability. Regarding the results obtained in this study a similar situation was observed since the samples with the highest phenolic content also exhibited the best antioxidant activities. Antioxidant properties of phenolic compounds are directly linked to their structure. Phenolics are composed of one (or more) aromatic rings bearing one or more hydroxyl groups and are therefore potentially able to quench free radicals by forming resonance-stabilized phenoxyl radicals (Evans et al, 1996; Bors and Michel, 2002). Both MeOH and aqueous samples had a higher RSA on ABTS radical than the synthetic antioxidant used, BHT, while the RSA on DPPH radical was lowest than the control. The thermally treated samples of acorns from Algarve cork oaks was the sample with the highest scavenge ability, because had highest values to both ABTS and DPPH assays. The reducing ability of the samples was always below the control in the Fe^{3+}/Fe^{2+} power reducing method, suggesting a better capacity of the extracts to

scavenge free radicals than to reduce the Fe^{3+}. The results indicate that the extracts were good electron and hydrogen donors and could finish the radical chain reaction, converting free radicals in stable products.

3.5.2 - Carob tree

Similar to the observed for extracts from *Quercus* the extracts from carob tree significantly scavenged DPPH and ABTS free radicals, but also had a significant reductive activity measured by the Fe^{3+}-reducing power assays. The higher RSA was obtained with the application of stem bark MeOH extracts. That antioxidant activity was, usually, in concordance with the content of phenolic compounds of the samples and varied according to the extract, probably due to the different phytochemical profile. Extracts with a higher phenol or flavonoid content generally show higher antioxidant activity, and good correlations have been found among these parameters (Alali et al., 2007). This indicates that the phenolic compounds detected in carob tree extracts, especially in the stem bark and leaves, could act as antiradical or antioxidant molecules. This affirmation obtains fundament with the good phenolic profile of that samples and their superior ability to scavenge hydrogen radicals (DPPH), higher than the BHT. A positive correlation between phenolics and antioxidant activity has been previously demonstrated to other plant materials (Zubia et al., 2009). The antioxidant activity of phenolic compounds involve the ability to donate a phenolic hydrogen as well as the stabilization of the resulting antioxidant radical through electron delocalization and/or intramolecular hydrogen bonding or further oxidation (Frankel and Meyer, 2000). Our observations suggest that both the combination of biocomponents and the synergistic mechanisms between them may be responsible for the antioxidant activity of the extracts.

3.6 - CONCLUSION

The MeOH extracts from acorn and leaves from cork oak from the Algarve and the leaves and stem bark from carob tree, had the highest antioxidant capacity. In the $ABTS^{+}$ radical assay all the MeOH and aqueous extracts from Mediterranean oaks had higher antioxidant activity than the positive control. In the DPPH assay, all the MeOH

samples had similar results but below the control. In the Fe^{2+}/Fe^{3+} reducing power method no result was higher than the control and the closest was from the Algarve cork oak leaves. In general no influence was observed by the thermal treatment or by the the geographical origin of the cork oaks trees.

The carob tree leaves and stem bark extracts exhibited a high antioxidant activity, with higher results than the positive control in the DPPH method. The best RSA and reductive activity were obtained in the MeOH samples of stem bark. Further experiments are necessary to verify the relation between chemical composition and antioxidant activity.

4 – EVALUATION OF THE CHOLINESTERASE INHIBITORY ACTIVITY OF THE EXTRACTS

4.1 - INTRODUCTION

AD is a chronic neurological disorder characterized by memory impairment, cognitive dysfunction, behavioral disturbances, and deficits in activities of daily living (Adams et al., 1984; Aisen and Davis, 1997; Jann, 1998). The most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain (Jaen et al., 1996). Acetylcholine is a neurotransmitter inhibited primarily by acetylcholinesterase (AChE, Fig. 10) and secondly by butyrylcholinesterase (BChE), considered to play a role in the pathology of AD (Hebert et al., 1995). Both enzymes are present in the brain and are detected among neurofibrillary tangles and neuritic plaques (Beard et al., 1995). The AChE has long been an attractive target for rational drug design and discovery of mechanism-based inhibitors. Because of its role in the hydrolysis of the neurotransmitter acetylcholine the inhibition of the enzyme is considered as a promising approach for the treatment of Alzheimer's disease (AD) and for other therapeutic applications in the treatment of Parkinson's disease, ageing and myasthenia gravis and glaucoma (Ahmad et al., 2007). Several AChE inhibitors such as tacrine, donzepil and galanthamine have been approved for the treatment of AD (Racchi et al., 2004).

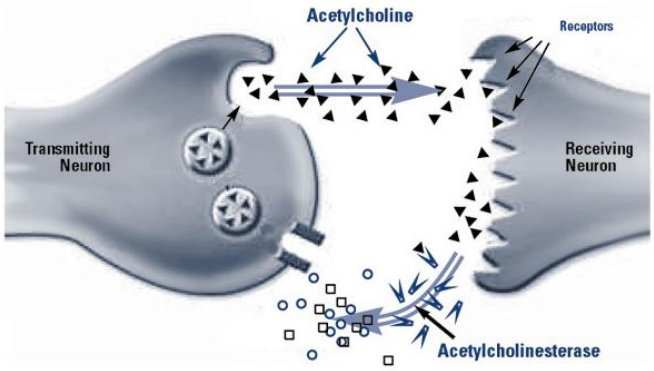

Figure 10 – Actuation of acetylcholinesterase in a neurotransmission signaled by acetylcholine (www.vrp.com)**.**

The inhibition of BChE is also important as it has been observed that the inhibition of this enzyme may be an effective tool for the treatment of AD and related dementia, although the role of BChE in the normal ageing and diseased brain is still unknown (Yu et al., 1999; Ahmad et al., 2007). Furthermore, a standard drug tacrine has increased the acetylcholine level in mouse brain through selective inhibition of BChE (Liston et al., 2004). This indicates the importance of BChE as therapeutic target in AD. BChE is

produced in the liver and enriched in the circulation. The exact physiological role of this enzyme is still elusive, but it is generally viewed as a back up for the homologous AChE. Like AChE, BChE inactivates the neurotransmitter acetylcholine (ACh). Greig et al. (2005) showed that selective BChE inhibition elevates brain acetylcholine, and lowers Alzheimer β-amyloid peptide. In addition, they mentioned that BChE activity progressively increases in patients with AD, while AChE activity remains unchanged or declines. This suggests that the development of specific BChE inhibitors will improve understanding of AD and may lead to a wider variety of potent treatment options (Aboudi et al., 2009).

The inhibition of AChE is currently the most established approach for treating AD (Schneider et al., 1996; Tariot et al., 2000). In fact, AChE and BChE inhibitors have become the remarkable alternatives in treatment of AD. However, some of the drugs approved for therapeutic use show hepatotoxicity (Knapp et al., 1994), consequently there have been a continuous search for new drugs, especially from natural sources.

4.2 - MATERIALS AND METHODS

4.2.1 - Anti-cholinesterase determination

AChE and BChE inhibitory activities were assessed according to Orhan et al. (2007, 2008). Briefly, 140 µL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 µL of extracts at the concentrations of 1 mg/mL and 10 mg/mL (the last concentration was only used for the aqueous extracts) and 20 µL of AChE or BChE (0.28 U/mL) solution were dispensed in a 96 well microplate and incubated for 15 min at 25 °C. After that time a solution of 10 µL of acetylthiocholine iodide or butyrylthiocholine chloride (4 mg/mL) was added to initiate the reaction and a solution of 20 µL of 5, 5-Dithio-bis (2-nitrobenzoic) acid (DTNB) at 1.2 mg/mL was added. The hydrolysis of acetylthiocholine iodide or butyrylthiocholine chloride was monitored by the formation of the yellow 5-thio-2-nitrobenzoato anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm using a 96-well micro-plate reader (Infinite M200 spectrophotometer, Tecan).

The percentage of inhibition of AChE or BChE was determined by using the following formula:

$$\text{AChE or BChE inhibitory capacity (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Were $A_{control}$ is the activity of the enzyme without test sample and A_{sample} is the activity of the enzyme with test sample. All the experiments were done in triplicate. Galanthamine was used as the positive control at the concentration of 1 mg/mL.

4.3 - STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard deviation (SD). All the experiments were conducted at least in triplicate, and all the testes and measurements were repeated at least three times. The data were analyzed using the analysis of variance (ANOVA) method assess differences using the SPSS statistical package for Windows (release 15.0, SPSS Inc.), and significance between means was tested by Duncan's New Multiple Range Test (p = 0. 05).

4.4 - RESULTS

4.4.1 - Mediterranean oaks

4.4.1.1 - AChE inhibitory activity

The extracts showed inhibition effects against AChE with various intensities and the results are shown in Table 9. The inhibitory activity (%) was classified as potent (> 50% inhibition), moderate (30-50% inhibition), low (< 30% inhibition) or null (< 5% inhibition) (Vinutha et al., 2007).

When tested at the concentration of 1 mg/mL all the extracts showed moderate (30-50% inhibition) or potent (> 50% inhibition) inhibitory activity on AChE, except the aqueous extracts. The best results were obtained with the application of MeOH leaf extracts (79.1 ± 7.6%), and with hexane extracts from the thermal treated acorns from cork oak from the Alentejo (77.5 ± 6.4%).

In general, there was no significant influence of the species or geographical origin on AChE inhibitory activity. Galanthamine was used as AChE inhibitor in this study, and exhibited a 94.3 ± 2.3% inhibition at 1 mg/mL. At the concentration of 10 mg/mL, galanthamine had completely inhibited the activity of the enzyme.

4.4.1.2 - BChE inhibitory activity

Similar to the observed to AChE, the hexane and MeOH extracts were more active against BChE than the aqueous ones, showing moderated (30-50% inhibition) to potent (> 50% inhibition) inhibitory activity.

The best results were observed after treatment with MeOH (80.1 ± 12.1%) and hexane leaf samples (61.3 ± 5.9%). The thermal treatment did not influence the results, and between the acorns from the different species no significant differences were observed.

Galanthamine was used as a standard drug, and exhibited an 81.3 ± 3.1% inhibition at 1 mg/mL (Table 9). At the concentration of 10 mg/mL, it had completely inhibited the activity of the enzyme.

Table 9 - Anti-cholinesterase activity of the Mediterranean oaks extracts. G.O. - Geographical origin, D.T. - Drying temperature

Species/G.O.	Material	D.T. (ºC)	Extract	AChE (1 mg/mL)	AChE (10 mg/mL)	BChE (1 mg/mL)	BChE (10 mg/mL)
Cork oak (Algarve)	**Acorns**	**50**	Hexane	60.8±1.7aA	n.r.	47.3±6.3aA	n.r.
			MeOH	67.1±4.2aAB	n.r.	48.4±2.4aA	n.r.
			H_2O	21.9±6.5bA	27.6±9.9A	26.1±8.1bA	24.1±9.8A
		200	Hexane	55.6±9.6aB	n.r.	29.6±8.5aA*	n.r.
			MeOH	36.2±3.8bB**	n.r.	43.3±6.7aA	n.r.
			H_2O	22.5±9.9bA	35.6±8.6A	33.5±16.4aA	28.6±4.4A
	Leaves	**50**	Hexane	59.7±2.9b	n.r.	61.3±5.9a	n.r.
			MeOH	79.1±7.6a	n.r.	80.1±12.1a	n.r.
			H_2O	22.3±7.8c	74.2±5.8	36.8±9.5b	69.8±14.1
Cork oak (Alentejo)	**Acorns**	**50**	Hexane	58.1±14.1aA	n.r.	42.4±12.5aA	n.r.
			MeOH	69.4±6.4aA	n.r	39.1±10.3aA	n.r
			H_2O	5.3±4.2bB	19.7±4.2A	24.0±7.4aA	15.1±6.3A
		200	Hexane	77.5±6.4aA	n.r.	39.0±5.5aA	n.r.
			MeOH	36.9±3.3bAB**	n.r.	38.9±3.0aA	n.r.
			H_2O	17.0±11.8cA	15.7±1.5B	27.1±4.2bA	30.0±11.8A
Holm oak	**Acorns**	**50**	Hexane	35.2±2.4bB	n.r	33.1±11.8aA	n.r.
			MeOH	55.1±8.6aB	n.r.	46.0±8.8aA	n.r.
			H_2O	18.5±4.4cA	26.5±3.2A	19.4±12.7aA	18.7±4.9A
		200	Hexane	30.4±7.9bC	n.r.	23.3±10.5bA	n.r.
			MeOH	50.1±10.3aA	n.r.	47.8±10.1aA	n.r.
			H_2O	14.6±4.9cA	35.0±8.1A	22.0±4.6bA	36.3±6.9A
Galanthamine[a]				94.3±2.3	n.r.	81.3±3.1	n.r.

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same species, plant material and drying temperature (small caps), between species for the same plant material, drying temperature and extract (all caps), and between drying temperatures for the same extract, plant material and species (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] - positive control, at the concentration of 10 mg/mL, Galanthamine completely inhibited the activity of both enzymes; n.r. - not realized, MeOH - methanol, H_2O - aqueous

4.4.2 - Carob tree

4.4.2.1 - AChE inhibitory activity

The percent inhibition data relative to the extracts from carob tree extract is presented on Table 10. All the MeOH and hexane extracts exhibited moderate (30-50% inhibition) to potent (> 50% inhibition) inhibitory activities. With the exception of leaves and stem bark, the aqueous and buffer samples had low (< 30% inhibition) inhibitory activity. When the extracts were applied at the concentration of 1 mg/mL, the best results were obtained in the aqueous leaf extract (73.3 ± 1.5%) and in the MeOH stem bark extract (71.7 ± 7.3%). In this samples, treatment with aqueous extracts at the concentration of 10 mg/mL resulted in an inhibition of 88.4 ± 6.0% and 87.7 ± 4.0%, for leaves and stem bark, respectively.

4.4.2.2 - BChE inhibitory activity

The BChE inhibition by the carob tree extracts is presented on Table 10. MeOH and aqueous extracts from leaves and stem bark, and the MeOH and hexane pulp extracts exhibited potent (> 50%) inhibitory activities. The germ flour, commercial germ flour, LBG and commercial LBG had moderate (30-50% inhibition) to low (< 30% inhibition) activities. Similarly to the AChE inhibition the best result were obtained in the aqueous extracts of leaf (93.0 ± 1.7%) (highest than the control) and in the stem bark, MeOH and aqueous extractions (71.4 ± 17.5% and 71.5 ± 9.3%, respectively).

Table 10 - Anti-cholinesterase activity of the carob tree extracts.

Plant material	Extract	AChE (1 mg/mL)	AChE (10 mg/mL)	BChE (1 mg/mL)	BChE (10 mg/mL)
Leaves	Hexane	37.1±6.21bB	n.r.	28.1±19.0cAB	n.r.
	MeOH	44.2±13.5bC	n.r.	61.4±6.5bA	n.r.
	H_2O	73.3±1.5aA	88.4±6.0A	93.0±1.7aA	81.3±6.5A
Pulp	Hexane	63.1±9.3aA	n.r.	53.2±18.3aA	n.r.
	MeOH	66.2±7.1aAB	n.r.	56.7±13.4aA	n.r.
	H_2O	27.7±7.4bC	27.1±12.0B	36.1±18.9aC	31.7±12.6B
Germ Flour	Hexane	36.3±10.3abB	n.r.	32.2±15.3aAB	n.r.
	MeOH	45.7±7.0aC	n.r.	31.0±1.9aB	n.r.
	H_2O	n.i.	n.i.	15.1±8.1aD	29.1±12.6B
	Buffer	27.9±11.7bA	n.r.	14.1±12.0aA	n.r.
Commercial Germ Flour	Hexane	41.1±13.3abB	n.r.	13.8±9.0aB	n.r.
	MeOH	52.2±8.7aBC	n.r.	22.5±17.8aB	n.r.
	H_2O	n.i.	8.1±6.6C	16.3±9.9aD	n.i.
	Buffer	26.3±10.1bA	n.r.	30.6±16.9aA	n.r.
Stem Bark	Hexane	n.r.	n.r.	n.r.	n.r.
	MeOH	71.7±7.3aA	n.r.	71.4±17.5aA	n.r.
	H_2O	52.4±7.1bB	87.7±4.0A	71.5±9.3aB	80.6±8.4A
LBG	Buffer	15.0±1.2A	n.r.	26.6±9.2A	n.r.
Commercial LBG	Buffer	22.4±9.0A	n.r.	17.6±2.9A	n.r.
Galanthamine[a]		94.3±2.3	n.r.	81.3±3.1	n.r.

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material (small caps) and between plant materials for the same extracts (all caps). [a] - positive control, at the concentration of 10 mg/mL, Galanthamine completely inhibited the activity of both enzymes; n.i. - no inhibition (< 5 %); n.r. - not realized

4.5 - DISCUSSION

Despite the unknown etiology of AD, elevation of acetylcholine amount through AChE enzyme inhibition has been accepted as the most effective treatment strategy against AD (Arnold and Kumar, 1993). Therefore, AChE and BChE inhibitors have become the remarkable alternatives in treatment of AD. However, the present drugs (tacrin, rivastigmin and donepezil) with AChE inhibitory activity possess some side effects and are effective only against the mild type of AD and there has been no drug

available with BChE inhibitory activity to present, yet (Schneider, 2001). Consequently, it is compulsory to develop new drugs in order to combat AD.

4.5.1 - Mediterranean oaks

There are many studies on the anticholinergic activity of plant extracts but they are, usually, made with herbal species (Mata et al., 2006; Ferreira et al., 2007; Orhan et al., 2008). To the best of our knowledge, there are no such studies on species of the genus *Quercus*.

Generally, the samples exhibited both AChE and BChE inhibitory activities specially in the MeOH extracts. The inhibitory activity of MeOH and hexane extracts against AChE was moderate (30-50% inhibition) or potent (> 50% inhibition), while it was low (< 30% inhibition) in the aqueous. Ferreira et al. (2006) studied the anticholinergic activity of *Melissa officinalis* and *Salvia officinalis* extracts, which are medicinal herbs with memory improving properties (Perry et al., 1999). Those authors reported AChE inhibitory values of 12.8 ± 1.2% (*M. officinalis*) and 6.0 ± 8.1% (*S. officinalis*) after treatment with 1 mg/mL of aqueous extracts, which were lower than the best result obtained with oaks, which was 22.5 ± 9.9% for the thermal treated acorns of cork oak from Algarve. Geographical origin of cork oak acorns had no significant influence on the results.

The AChE inhibitory potential of the extracts from holm oak was smaller than the one observed for cork oaks, and the thermal treatment caused a reduction on the inhibitory in all the extracts. Orhan et al. (2007) in their study with Turkish *Salvia* species obtained a value of 46.1 ± 3.5% of AChE inhibition in a MeOH extract at 0.2 mg/mL of *Salvia frigida* species that is considerable regarding the concentration, the same sample does not exhibit any inhibition against BChE.

The acorn samples had a moderate (30-50% inhibition) to low (< 30% inhibition) inhibitory activity on BChE, while leaf extracts exhibited potent (> 50% inhibition) activity. Usually, the highest results were obtained with the MeOH extracts, between the species, also as in the acorns from different geographical cork oaks, the results are analogous. The thermal treatment resulted in a small lost of activity in the MeOH and hexane extracts but in an increase in the aqueous ones. In general, the samples had more affinity to inhibit AChE than to BChE and to both no results were obtained above the

positive control. In that way, our best regards goes to the cork oak Algarve leaves (1 mg/mL), in the MeOH extraction, with very similar results, rounding 80% of inhibition to both enzymes.

4.5.2 - Carob tree

The MeOH and hexane extracts from carob tree had moderate (30-50% inhibition) to potent (> 50% inhibition) inhibitory activities against AChE, while the aqueous extracts exhibited no (< 5% inhibition) or low (< 30% inhibition) to potent (> 50% inhibition) activities. The Germ flour and LBG had low inhibitory affects.

Working with extracts from medicinal plants from Portugal, Ferreira et al. (2006) and Mata et al. (2007) observed that the highest AChE inhibition (59.8 ± 5.8%) was achieved after treatment with aqueous extracts of *Hypericum undulatum*, at the concentration of 1 mg/mL. That value was lower than the obtained in this work with the application of aqueous leaf extracts, at the same concentration (73.3 ± 1.5% of inhibition), which indicated that carob tree leaves are endowed with biocompounds with a significant cholinesterase inhibitory activity. Moreover, it was detected the presence of substances with central benzodiazepine activity in MeOH extracts from pulps and leaves from Italian cultivars of carob tree, pointing the possibility to use carob extract as potential natural products with anxiolytic-sedative effects (Avallone et al., 2002).

Regarding the results concerning BChE, we observe that both MeOH and aqueous extracts from leaves and stem bark, had potent (> 50%) inhibitory activity also as the hexane and MeOH extracts from pulps. The other samples had, usually, low (< 30% inhibition) inhibitory activity. Orhan et al. (2008) obtained a BChE inhibition of 83.9 ± 1.0% from a MeOH extraction (1 mg/mL) of *Rosmarinus officinalis* L. That species, also known as rosemary, is an aromatic evergreen shrub widely distributed throughout the Mediterranean region. *R. officinalis* L. has a very old reputation for improving memory and has been used as a symbol of remembrance in Europe (Moss et al., 2003). In similar assay conditions, a BChE inhibition of 71.4 ± 17.5% was obtained by the stem bark samples also suggesting memory improvement abilities.

In general, the MeOH and hexane extracts had more capacity to inhibit the AChE, while the aqueous extracts were more effective against BChE. In that way, the results obtained by a polar extract agent contradicts the studies were natural compounds had

more selectivity to inhibit BChE than AChE (Ahmad et al., 2003; Choudhary et al., 2004; Khan et al., 2006).

4.6 - CONCLUSION

In this chapter, the extracts from Mediterranean oaks and carob tree were analyzed for their activity towards AChE and BChE enzymes. Almost all of the extracts showed inhibitory activity on both enzymes, which was more pronounced on the MeOH and hexane extracts. Among the samples from *Quercus* species, the MeOH leaf extract showed the best inhibition rates on both enzymes at the concentration of 1 mg/mL. There was no apparent influence of the species and geographic origin of the oaks samples, on the inhibitory activity. On carob tree the highest inhibitions at the lowest concentration was observed for aqueous leaf extracts, on both enzymes, followed by the stem bark and pulps. Our results indicate that the studied species are endowed with bioactive compounds that may help in preventing or alleviating patients suffering from AD.

5 - ANTI-HYPERGLYCEMIC ACTIVITY

5.1 - INTRODUCTION

Diabetes mellitus is a serious disease characterized by hyperglycemia, with a rising incidence both in developed and developing countries (Mai and Chuyen, 2007). It is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secret insulin. Such a deficiency results in increased blood glucose level, which in turn can damage many of the body systems, including blood vessels and nerves (Matsui et al., 2007).

Alpha-amylase and α-glucosidase are key enzymes involved in starch breakdown and intestinal absorption, respectively. The α-amylases are a group of enzymes widely distributed in microorganisms, plants and animal secretions which catalyzes the hydrolysis of the (α - 1, 4) glycosidic linkages in starch and various oligosaccharides (Ball et al., 2008). It is now believed that inhibition of these enzymes involved in the digestion and uptake of carbohydrates can significantly decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet and therefore can be an important strategy in the management of hyperglycemia linked to type-2 diabetes (Kwon et al., 2007). Those enzymes delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the post-prandial plasma glucose rise (Lhoret and Chiasson, 2004). Examples of such inhibitors which are in clinical use are acarbose, miglitol and voglibose (Bailey, 2003).

Although powerful synthetic α-glucosidase inhibitors (i.e. voglibose) are available, they usually can cause hepatic disorders and other negative gastrointestinal symptoms (Murai et al., 2002). A main drawback of currently used α-glucosidase and α-amylase inhibitors such as acarbose are side effects such as abdominal distention, flatulence, meteorism and possibly diarrhea (Bischoff et al., 1985). It has been suggested that such adverse effects might be caused by the excessive inhibition of pancreatic α-amylase resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon (Horii, 1987; Bischoff, 1994). Therefore, natural inhibitors from dietary plants have shown to have lower inhibitory effect against α-amylase activity and a stronger

inhibitory activity against α-glucosidase and can be used as effective therapy for postprandial hyperglycemia with minimal side effects (Kwon et al., 2006).

5.2 - MATERIALS AND METHODS

5.2.1 - α-Amylase inhibitory assay

The α-Amylase inhibitory activity was determined according to the method described by Kwon et al. (2007). The extracts (500 µL) at the concentrations of 1 and 10 mg/mL, were mixed with 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/mL). This mixture was pre-incubated at 25 °C for 10 min. After pre-incubation, 500 µl of a 1 % starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at 5 s intervals. The reaction mixtures were then incubated at 25 °C for 10 min. The reaction was stopped with 1.0 mL of dinitrosalicylic acid color reagent. The test tubes were incubated in a boiling water bath for 5 min and cooled to RT. The reaction mixture was diluted with 10 mL of distilled water and absorbance was measured at 540 nm. The positive control used in this experiment was a α-amylase inhibitor from *Triticum aestivum* (wheat seed). The negative control, consisted by a sample mixture containing 500 µL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride). The results are expressed in two ways, percentage of inhibition (%) and amylase inhibition (A.I.). The α-amylase percentage of inhibition was measured by the following formula:

$$\%\ \text{Inhibition} = \left(\frac{A_{540,\text{control}} - A_{540,\text{sample}}}{A_{540,\text{control}}}\right) \times 100$$

Were $A_{540,\ control}$ is the absorbance of the negative control and $A_{540,\ sample}$ is the absorbance measured in the samples with extract or positive control.

The A.I. is defined as the ratio of the amylase activity of the control (enzyme alone) to that of the enzyme extract mixture. Values above the unit suggest amylase inhibitory activity and below suggest amylase stimulatory activity (Correia et al., 2004).

5.2.2 - Baker's yeast α-Glucosidase inhibitory assay

The α-Glucosidase inhibitory activity was determined in 96 well plates according to the method described by Kwon et al. (2007). This enzyme was obtained from the yeast, *Saccharomyces cerevisiae*, as an example of microbial origin. The extracts (50 µL) at the concentrations of 1 and 10 mg/mL, were mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/mL), and pre-incubated at 25 °C for 10 min. Then, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at 5 s intervals. The reaction mixtures were incubated at 25 °C for 5 min, and the absorbance readings were recorded at 405 nm by micro-plate reader (Infinite M200 spectrophotometer, Tecan) and compared to a control which had 50 µL of buffer solution in place of the extract. Glucobay® and acarbose were used as positive controls at the concentrations of 1 and 10 mg/mL. The α-glucosidase inhibitory activity was expressed as inhibition (%) and was calculated as follows:

$$\%\ \text{Inhibition} = \left(\frac{\Delta A_{405,\text{control}} - \Delta A_{405,\text{sample}}}{\Delta A_{405,\text{control}}}\right) \times 100$$

Were $\Delta A_{405,\ control}$ is the variation of absorbance before and after incubation of the control and $\Delta A_{405,\ sample}$ is the variation of absorbance before and after incubation of the samples.

5.2.3 - Rat's intestinal α-Glucosidase inhibitory activity

This assay was done according to Kwon et al. (2007). Rat's intestine acetone powder was used as a crude enzyme extract as an example of enzyme mammalian origin. The rat intestinal acetone powder (250 mg) was carefully homogenized with a 0.1 M phosphate buffer at pH 6.9 (10 mL) and then centrifuged at 5000 g for 20 min at 4ºC, the supernatant was used as a crude enzyme solution. The extracts (50 µL) at the concentrations of 1 and 10 mg/mL, were mixed with 100 µL of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (25 mg/mL), and pre-incubated at 25 °C for 10 min. After pre-incubation, 50 µL of 5 mM p-nitrophenyl-α-D-glucopyranoside

solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at 5 s intervals. The reaction mixtures were incubated at 37 °C for 30 min. The absorbance's were recorded at 405 nm by micro-plate reader (Infinite M200 spectrophotometer, Tecan) and compared to a control which had 50 μL of buffer solution in place of the extract. Glucobay® and acarbose were drugs used as positive controls at the concentrations of 1 and 10 mg/mL. The percentage of inhibition was calculated as described for the baker's yeast α-glucosidase inhibitory assay.

5.3 - STATISTICAL ANALYSIS

The experimental results were expressed as mean ± standard deviation (SD). All the experiments were conducted in triplicate, and all the testes and measurements were repeated at least three times. The data were analyzed using the analysis of variance (ANOVA) method assess differences using the SPSS statistical package for Windows (release 15.0, SPSS Inc.), and significance between means was tested by Duncan's New Multiple Range Test (p = 0. 05).

5.4 - RESULTS

In this assays, the thermally treated samples were not used since they did not exhibited significant influences in the previous assays.

5.4.1 - α-Amylase inhibitory assays

5.4.1.1 - Mediterranean oaks

When applying the extracts at the concentration of 1 mg/mL, almost all exhibited low (< 30% inhibition) to none (< 5% inhibition) inhibitory activity. Even when applied at 10 mg/mL, only the MeOH extract from cork oak acorns (Alentejo) and the hexane and water extracts from holm oak showed moderate (30-50%) inhibitory activities (Table 11). When looking at the results expressed in A.I., we can observe that generally the extracts had no inhibitory activity or even a promotion effect on amylase activity (Table 11).

Table 11 - α-amylase inhibition activity of extract from Mediterranean oaks. G.O. - Geographical origin

Species/G.O.	Plant material	Extract	Inhibition % (1 mg/mL)	Inhibition % (10 mg/mL)	A.I. (1 mg/mL)	A.I. (10 mg/mL)
Cork oak (Algarve)	**Acorns**	Hexane	n.i.	11.7±13.7aA	0.9±0.2bA	1.1±0.2aA
		MeOH	13.7±0.0bA	27.0±26.6aA	1.0±0.2bA	1.4±0.6aA
		H_2O	31.8±4.2aA	n.i.	1.5±0.1a	0.8±0.1aA***
	Leaves	Hexane	16.5±9.9a	n.i.	1.0±0.2a	0.9±0.1a
		MeOH	20.2±18.7a	12.2±7.4a	1.1±0.3a	1.1±0.1a
		H_2O	n.i.	n.i.	1.0±0.1a	0.9±0.1a
Cork oak (Alentejo)	**Acorns**	Hexane	15.3±6.3aA	n.i.	1.1±0.1aA	1.0±0.0a
		MeOH	15.2±8.9aA	37.8±35.2aA	1.0±0.2aA	1.1±0.3aA
		H_2O	20.6±3.6aB	n.i.	1.3±0.1a	1.0±0.2aA***
Holm oak	**Acorns**	Hexane	18.0±16.2aA	41.3±32.2aA	1.2±0.3aA	1.3±0.4aA
		MeOH	13.2±12.8aA	39.4±24.2aA	1.0±0.2aA	1.2±0.2aA
		H_2O	22.2±5.9aB	n.i.	1.3±0.1a	0.8±0.1aA***
Amylase inhibitor[a]			n.r.	51.5±5.6	n.r.	2.1±0.2

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material and species (small caps) and between plant materials for the same extracts and different species (all caps). To each row, statistical analysis was made between different concentrations for the same extract, plant material and species (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] – positive control, under experimental conditions, the standard used did not exhibited activity at the concentration of 10 mg/mL; n.i. - no inhibition (< 5 %); n.r. - not realized

5.4.1.2 – Carob tree

The amylase inhibitory activities of carob tree extracts are summarized on Table 12. Generally, the extracts had little inhibitory effect, except to the aqueous extracts from leaves (1 mg/mL: 74.7 ± 8.1%; 10 mg/mL: 86.9 ± 11.1%) and stem bark (1 mg/mL: 65.8 ± 5.4%; 10 mg/mL: 79.9 ± 7.8%). Those extracts also exhibited the highest A.I., comparatively to the positive controls used.

Table 12 - α-amylase inhibition activity of extracts from Carob tree.

Plant material	**Extract**	**Inhibition % (1 mg/mL)**	**Inhibition % (10 mg/mL)**	**A.I. (1 mg/mL)**	**A.I. (10 mg/mL)**
Leaves	Hexane	12.5±6.0bB	n.r.	1.1±0.2bA	n.r.
	MeOH	13.7±1.8bBC	n.i.	1.1±0.2bA	0.9±0.0bBC
	H_2O	74.7±8.1aA	86.9±11.5aA	4.3±1.6aA	6.1±2.3aA
Pulp	Hexane	n.r.	n.r.	n.r.	n.r.
	MeOH	23.6±6.1aAB	n.i.	1.2±0.3aA	0.7±0.1aC***
	H_2O	5.7±2.5bC	n.i.	1.0±0.1aC	0.9±0.2aB
Germ Flour	Hexane	28.9±8.9aA	28.3±14.4aA	1.3±0.3abA	1.2±0.1aA
	MeOH	30.7±11.6aA	23.5±11.7aA	1.3±0.3abA	1.2±0.2aA
	H_2O	40.5±13.7aB	n.i.	1.7±0.4aBC	1.0±0.1aB***
	Buffer	7.0±0.6bAB	n.i.	1.1±0.3Ba	0.8±0.1bA
Industrial Seed Germ	Hexane	19.7±1.0aAB	11.6±8.9aA	1.1±0.2bA	1.1±0.1aA
	MeOH	n.i.	14.0±7.1aA	1.0±0.1bA	1.1±0.2abAB
	H_2O	41.0±17.0aB	n.i.	1.9±0.6aBC	1.0±0.3abAB
	Buffer	n.i.	n.i.	0.9±0.3bA	0.8±0.1bA
Stem Bark	Hexane	n.r.	n.r.	n.r.	n.r.
	MeOH	22.4±3.5bAB	n.i.	1.1±0.3aA	0.8±0.1bBC
	H_2O	65.8±5.4aA	79.9±7.8aA***	3.0±0.5aAB	5.4±1.8aA***
Gum	Buffer	18.9±9.1A	n.i.	1.1±0.3A	0.7±0.1A
Industrial Gum	Buffer	17.7±13.7A	n.i.	1.1±0.3A	0.7±0.0A
Amylase inhibitor[a]		n.i.	51.5±5.6	n.i.	2.1±0.2

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material (small caps) and between plant materials for the same extracts (all caps). To each row, statistical analysis was made between different concentrations for the same extract, and plant material (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] - positive control; n.i. - no inhibition (< 5%); n.r. - not realized

5.4.2 - Baker's yeast α-glucosidase assays

5.4.2.1 - Mediterranean oaks

The application of the extracts at the lowest dose resulted in a low or no inhibitory activity, except to the aqueous extracts from acorns (36. 0 ± 6.3%) and leaves (44.6 ± 4.4%) from cork oak (Algarve) (Table 13).

When applying the extracts at the concentration of 10 mg/mL, higher inhibitory values were achieved, and generally the water extracts lead to the best results, followed by the MeOH and the hexane (Table 13). The results observed after treatment with the aqueous extracts were similar or higher than the ones observed after application of the positive controls, with the application of leaf extracts resulting in a 97.4 ± 0.1% of inhibition (Table 13). Generally, the Algarve cork oaks acorns exhibited better bakers yeast α-glucosidase inhibition, with the best results observed after treatment with the concentration of 10 mg/mL, for all the extracts.

Table 13 - Inhibitory activity of extracts from Mediterranean oaks on baker's yeast α-glucosidase. G.O. - Geographical origin

Species/G.O.	**Plant material**	**Extract**	**Inhibition % (1 mg/mL)**	**Inhibition % (10 mg/mL)**
Cork oak (Algarve)	**Acorns**	Hexane	7.7±0.6bB	31.8±1.5cA***
		MeOH	8.5±1.6bA	50.9±16.0bA***
		H_2O	36.0±6.3aA	86.0±2.5Aa***
	Leaves	Hexane	n.i.	20.0±6.3c
		MeOH	32.8±3.9b	89.4±0.6b***
		H_2O	44.6±4.4a	97.4±0.1a***
Cork oak (Alentejo)	**Acorns**	Hexane	11.9±1.4bB	22.8±3.7cAB***
		MeOH	9.0±2.8bA	46.1±2.4bA***
		H_2O	22.3±5.6aB	75.8±3.4aB***
Holm oak	**Acorns**	Hexane	19.4±5.9aA	20.3±6.8cB
		MeOH	7.2±2.8bA	50.9±2.9bA***
		H_2O	n.i.	80.8±3.1aAB***
Acarbose			35.5±5.8	85.7±0.6***
Glucobay[a]			51.9±5.2	95.6±1.1***

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material and species (small caps), and between plant materials for the same extracts and different species (all caps). To each row, statistical analysis was made between different concentrations for the same extract, plant material and species (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] - positive control; n.i. - no inhibition (< 5%)

5.4.2.2 - Carob tree

Generally, the aqueous and the MeOH extracts exhibited the highest inhibitory activities (Table 14). After the application of carob tree extracts at the concentration of 1 mg/mL the best results were observed with the aqueous extracts from and stem bark with inhibitions of 97.6 ± 0.5% and 65.2 ± 1.5%, respectively (Table 14). When using the highest concentration, the best results were obtained with the application of aqueous and MeOH extracts from leaves and stem bark, similar or higher than the values obtained with the positive controls.

Table 14 - Inhibitory activity of extracts from carob tree on baker's yeast α-glucosidase.

Plant material	Extract	Inhibition % (1 mg/mL)	Inhibition % (10 mg/mL)
Leaves	Hexane	7.6±2.3cA	n.r.
	MeOH	57.0±2.2bA	96.4±1.1bA***
	H_2O	97.6±0.5aA	99.1±0.7aA***
Pulp	Hexane	8.4±0.9abA	n.r.
	MeOH	9.9±3.6aC	16.1±1.6bE***
	H_2O	n.i.	22.2±2.9aB***
Germ Flour	Hexane	7.7±4.9aA	49.7±7.8aA***
	MeOH	5.0±2.7aC	22.8±5.1bD***
	H_2O	8.2±4.0aD	15.0±0.8bcC**
	Buffer	n.i.	11.8±3.1cB
Commercial Germ Flour	Hexane	5.3±0.4cA	21.0±11.8bB*
	MeOH	9.2±4.1bC	52.9±2.8aC***
	H_2O	47.6±2.7aC	6.7±0.5cD***
	Buffer	n.i.	31.1±9.1bA
Stem Bark	Hexane	n.r.	n.r.
	MeOH	17.9±3.9bB	90.2±2.7bB***
	H_2O	65.2±1.5aB	99.7±0.7aA***
LBG	Buffer	n.i.	13.3±8.7B
Commercial LBG	Buffer	n.i.	30.4±11.3A
Acarbose		35.5±5.8	85.7±0.6***
Glucobay[a]		51.9±5.2	95.6±1.1***

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material (small caps) and between plant materials for the same extracts (all caps). To each row, statistical analysis was made between different concentrations for the same extract, and plant material (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] - positive control; n.i. - no inhibition (< 5%); n.r. - not realized

5.4.3 - Rat's intestinal α-glucosidase assays

5.4.3.1 - Mediterranean oaks

The oaks exhibited some activity against the enzyme and the results are summarized on Table 15. Generally, the MeOH extracts had the highest results in all plant materials, followed by the hexane and aqueous extracts. The extracts from the holm oak generally

exhibits worst results than the extracts from cork oaks. The application of the acorns collected from cork oak from the Algarve (59.0 ± 1.1%) resulted in a higher inhibition than the obtained within the positive control (57.5 ± 10.9%).

Table 15 - Inhibitory activity of extracts from Mediterranean oaks on rat's intestinal α-glucosidase. G.O. - Geographical origin

Species/G.O.	**Plant material**	**Extract**	**Inhibition % (1 mg/mL)**	**Inhibition % (10 mg/mL)**
Cork oak (Algarve)	**Acorns**	Hexane	23.0±4.4aC	32.6±5.5aA**
		MeOH	22.1±8.4aC	59.0±1.1aA***
		H_2O	33.3±13.0aA	37.5±28.6aA
	Leaves	Hexane	42.8±4.6a	33.5±17.9ab
		MeOH	21.1±4.1b	53.1±14.0a***
		H_2O	18.8±8.8b	20.8±3.6b
Cork oak (Alentejo)	**Acorns**	Hexane	33.2±14.3aAB	14.3±0.7bB
		MeOH	41.8±3.7aA	52.6±4.6aAB***
		H_2O	33.3±19.1aA	50.0±17.6aA
Holm oak	**Acorns**	Hexane	50.5±15.9aA	19.9±4.0aB**
		MeOH	37.6±11.0aAB	41.3±9.8aB
		H_2O	28.1±30.9aA	22.9±8.8aA
Acarbose			55.0±14.7	54.5±1.4
Glucobay[a]			39.1±9.4	57.5±10.9**

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material and species (small caps), and between plant materials for the same extracts and different species (all caps). To each row, statistical analysis was made between different concentrations for the same extract, plant material and specie (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). [a] - positive control

5.4.3.2 - Carob tree

The extracts from carob tree showed some inhibitory effect on rat's intestinal α-glucosidase, with various intensities according to the extract, organ and concentration used (Table 16).

When the lowest concentration was used (1 mg/mL), the best results (47.9 ± 3.6%) were obtained with the aqueous extracts for stem bark and leaves (43.8 ± 17.7%), significantly higher than the obtained with the positive control (39.1 ± 9.4%). When the extracts were applied at the 10 mg/mL dose, the highest activities were observed in the

germ flour (MeOH: 73.6 ± 27.6%) and leaves (aqueous: 68.8 ± 16.5%), higher than positive control (57.5 ± 10.9%, Table 16).

Table 16 - Inhibitory activity of extracts from carob tree on rat's intestinal α-glucosidase.

Plant material	**Extract**	**Inhibition % (1 mg/mL)**	**Inhibition % (10 mg/mL)**
Leaves	Hexane	21.9±13.6aA	22.9±7.5bA
	MeOH	24.1±4.5aA	12.6±6.7bB
	H_2O	43.8±17.7aA	68.8±16.5aA
Pulp	Hexane	25.7±11.0aA	24.7±16.4aA
	MeOH	n.i.	35.9±19.9aB
	H_2O	31.3±22.5aA	37.5±3.0aAB
Germ Flour	Hexane	18.3±2.4aA	22.9±2.6bA
	MeOH	11.2±5.9aB	73.6±27.6aA*
	H_2O	33.3±28.2aA	31.3±10.8bC
	Buffer	17.4±7.5aAB	38.6±21.9bA
Commercial Germ Flour	Hexane	27.1±10.7aA	22.6±17.7aA
	MeOH	n.i.	31.0±7.5aB
	H_2O	25.0±22.5aA	27.1±9.6aC
	Buffer	33.8±16.2aA	35.2±11.2aA
Stem Bark	Hexane	n.r	n.r.
	MeOH	13.3±4.4bB	24.1±9.2aB
	H_2O	47.9±3.6aA	45.8±20.1aAB
LBG	Buffer	19.1±14.2AB	39.2±7.3A
Commercial LBG	Buffer	5.3±1.3C	40.7±13.2A*
Acarbose		55.0±14.7	54.5±1.4
Glucobay[a]		39.1±9.4	57.5±10.9**

Values represent means ± SD of 3 assessments. For each column, statistical analysis was made between extracts of the same plant material (small caps), and between plant materials for the same extracts (all caps). To each row, statistical analysis was made between different concentrations for the same extract, and plant material (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).[a] - positive control; n.i. - no inhibition; n.r. - not realized

5.5 - DISCUSSION

One therapeutic approach for treating diabetes is to decrease the post-prandial hyperglycemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolyzing enzymes α-glucosidase and α-amylase in the digestive tract. Inhibitors of these enzyme delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Lhoret and Chiasson, 2004).

5.5.1 - Mediterranean oaks

In this work we observed that generally the extracts had a higher inhibitory activity on α-glucosidase, than on α-amylase, which support the finding that the natural α-amylase and α-glucosidase inhibitors from plants have lower inhibitory effect against α-amylase and a stronger inhibitory activity against α-glucosidase (Kwon et al., 2006). Moreover, stronger inhibitions of the baker's yeast α-glucosidase were noted at the concentration of 10 mg/mL, while in the lower concentration, the extracts had more selectivity to inhibit the α-glucosidase from mammalian origin. An effective strategy for the type 2 diabetes management was suggested to be a mild inhibition of α-amylase and strong inhibition of intestinal α-glucosidase activity (Krentz and Bailey, 2005).Various α-glucosidase inhibitors for mammalian species have been reported, and most of these inhibitors showed either a low effect or no effect on α-glucosidase from microbial origins (Kim et al., 2004). As reported previously, α-glucosidase broadly consists of type I from yeast *S. cerevisiae*, and type II from the mammalian source, and there are structural differences between the two types (Kim et al., 2004).

The results obtained after treatment with Glucobay® and its pure active compound (Acarbose) as positive controls shows that the Glucobay hava high selectivity to inhibit the baker's yeast α-glucosidase, especially at the concentration of 10 mg/mL with significantly higher results than the pure compound. To the mammalian α-glucosidase, acarbose shows highest inhibition than Glucobay® at 1 mg/mL and similar inhibition at 10 mg/mL. The acarbose results at 10 mg/mL obtained in this study contradict Kim et

al. (2004) that report Acarbose with high inhibitory effects on mammalian α-glucosidase, but no inhibitory activity for yeast *S. cerevisiae.*

5.5.2 - Carob tree

Some of the carob tree extracts used in this work significantly inhibited the activity of α-amylase, even at the lower concentration, and usually the best results were obtained with the application of the aqueous extracts from leaves and stem bark, being highest than the positive control. Those samples at 10 mg/mL had 86.9 ± 11.5 % and 79.9 ± 7.8 %, respectively, against only 51.5 ± 5.6% on the control. Those extracts also had a potent inhibitory activity (leaves: 99.1 ± 0.7%; stem bark: 99.7 ± 0.7%) than both acarbose (85.7 ± 0.6%) and Glucobay (95.6 ± 1.1%) on baker's yeast α-glucosidase. Regarding the rat's intestinal α-glucosidase inhibitory activity, the best inhibition was obtained in the MeOH sample of germ flour (73.6 ± 27.6%) followed by the aqueous extracts of leaves and stem bark. That germ flour result can be related to the amount of teophylline (1.6 mg/g extract, DW), with reported effects to protect against diabetes (Rabinovitch and Sumoski, 1990). This alkaloid it is included in the methylxanthines group, also as caffeine and theobromine. That group of alkaloids are known to be composed by similar molecules with similar effects (Roper, 2006) and the caffeine consumption was related to a lower risk of develop diabetes (Dam et al., 2006).

The *leguminosae* family, to which carob tree belongs, is one of the most studied family in relation to the anti-hyperglycemic potential (Bnouham et al., 2006). However, there are no reports of the potential anti-diabetic activity of extracts from carob tree. The Indian remedy fenugreek is derived from the defatted seed *Trigonella foenum graecum* and contains gallactomannan, nicotin, coumarin, and the alkaloid trigonelline. In insulin-dependent diabetic patients, the fenugreek diet significantly reduced fasting blood glucose and improved the glucose tolerance test (Davis et al., 1998). The seeds of that species are found to remarkably suppress the clinical symptoms of diabetes such as polyurea, polydypsia, weakness and weight losses (Sharma, 1986). McCue and Shetty (2004) studied water soluble extracts with optimized phenolic content of selected Asian foods for inhibitory activity against porcine pancreatic α-amylase and yeast α-glucosidase, and included the fenugreek species at the concentration of 100 mg/mL. Their results, expressed in terms of A.I. and α-glucosidase inhibitory activity were 1.2

and 8.0% respectively, which were lower than the results obtained with the application of leaf aqueous extracts (A.I. value of 4.3 ± 1.3 and 97.6 ± 0.5% of inhibition on baker's yeast α-glucosidase), indicating that this extract have potential as source of antidiabetic compounds.

5.6 - CONCLUSION

The inhibitory activity of the extracts from Mediterranean oaks was low on α-amylase, and higher on the α-glucosidase from the baker's yeast. The aqueous extracts had the best inhibitory activities, followed by the MeOH and hexane. They generally produced moderate results at the lower concentration tested, and high at the highest dose (10 mg/mL). The best results were usually obtained with the application of aqueous leaf extracts from cork oak from the Algarve, and no significant differences were observed between species and geographical origins. In comparison to the Mediterranean oaks, the carob tree had higher anti-hyperglycemic activity. The samples with the highest inhibitory activities were the leaves, stem bark, and the germ flour (the last one in the α-glucosidase rat's intestinal assay). This species also had more affinity to inhibit the α-glucosidase from the baker's yeast than from the rat's intestine and moderate α-amylase inhibitions. Because some of the results are similar or higher than the positive control, the leaves from the Algarve cork oak, and the leaves and stem bark from the carob tree are interesting materials to future works.

6 – GENERAL DISCUSSION

Plants have been used for the prevention and treatment of various human diseases trhougouth history, and some natural foods (vegetables and fruits such as tea, grape, bean, garlic, etc.) are considered to possesses beneficial physiological effects such as antioxidant, anticancer, anti-aging, and anti-inflammatory effects, with the phenolics being indicated as the major compounds with antioxidant activities (Yokozawa and Nakagawa, 2004).

Oxidative stress has been implicated in the pathogenesis of several disease processes including, ischemia/reperfusion injury, Alzheimer's, Parkinson's and *diabetes mellitus* (Kannan and Jain, 2000). ROS has been shown to play both beneficial and deleterious roles. At very low concentration, ROS may act as a second messenger in some of the signal transduction pathways (Suzuki et al., 1997). However, when produced in excess, they can cause oxidative damage to many vital components of the cell.

Especially in the past few years, there has been increasing interest in finding natural antioxidants because they can protect the human body from free radicals and ROS related effects and retard the progress of many chronic diseases (Pryor, 1991; Kinsella et al., 1993, Gülçin et al., 2003a). That is the reason because the samples with the highest antioxidant activity, related to the anti-cholinesterase and anti-hyperglycemic activities were the most important findings of this study, also as the inexistent data reporting this study on these species.

Several therapeutic strategies have been developed to treat AD, including anti-inflammatory, antioxidant, and antiamyloid approaches. Oxidative stress is a major factor associated with the development and progression of AD and other forms of dementia. Compared to other organs, the brain is more vulnerable to oxidative stress due to its high lipid content, its relatively high oxygen metabolism, and its low level of antioxidant defenses (Olanow, 1992; Varadarajan et al., 2000; Butterfield et al., 2001). In the literature on AD, the terms "oxidative stress" or "oxidative damage" are commonly used to explain the balance between the production of oxidants and the endogenous antioxidant defenses in neuronal cells. In general, cells undergo apoptotic death when there is an imbalance between oxidants and antioxidants (more oxidants than antioxidant defenses) (Reddy, 2006). In the past few years, some studies were been applied to know the impact of the antioxidants in the AD and, in general, their found a

direct relationship (Singh et al., 2004; Ferreira et al., 2006; Orhan et al., 2007). In Ferreira et al. (2006), the plants expresses both the activities, but the special regards goes to *Hypericum undulatum*, *Melissa officinalis* and *Laurus nobilis*. The first species were the most homogeneous in the results, with 59.8 ± 0.9% of AChE inhibition and 96% of DPPH scavenging ability in an aqueous extract at the concentration of 1 mg/ml. The leaves of *Laurus nobilis* had a moderate AChE inhibition, 36.2 ± 2.4% and 61% to DPPH assay, in the same conditions. *Melissa officinalis* in spite of their high DPPH result (96%), have a weak AChE inhibition (12.8 ± 1.2%). Regarding those assay conditions, similar results were obtained in this study, but normally with weakest DPPH results. The most relevant was those obtained by the carob tree leaves, with an AChE inhibition of 73.3 ± 1.5% and a DPPH scavenging activity of 64.2 ± 5.8%, followed by the stem bark (52.4 ± 7.1% to AChE and 49.4 ± 2.6% to DPPH). In the Mediterranean oaks the leaves of cork oak were the samples with most uniform results, giving 22.3 ± 7.8% inhibition to AChE and 41.2 ± 2.9% to the DPPH assay. Orhan et al. (2007) made a similar studie, but with *Salvia* species (*lamiaceae*) that have been recorded to be used against memory loss in European folk medicine. Here, differences between AChE and BChE inhibitions were also noted and attributed to the different phytochemical contents of the plants. The sample with most regular inhibitions was from the *S. verticillata* subsp. *amasiaca* species, given 39.1 ± 3.1% to AChE and 72.0 ± 3.0% to BChE. In spite of the moderated result to AChE, to BChE it was the best obtained in the study also has they DPPH scavenging ability (93.5 ± 1.2%), the sample was extracted with MeOH and tested in the concentration of 1 mg/ml. At the same conditions, *S. multicaulis* species had the second best result to the DPPH assay (92.6 ± 0.4%). With these species, the AChE inhibition was the best obtained in the study (47.7 ± 3.6%) and the BChE it is moderated (36.2 ± 0.9%). In this study some variations in the results are noted, but some species gives similar inhibitions to both enzymes including good DPPH results, this situation happens with the Algarve cork oak leaves and the stem bark from carob tree. The first gives 79.1 ± 7.6% of inhibition to AChE and 80.1 ± 12.1% to BChE, and a DPPH scavenging activity of 41.2 ± 2.9%, revealing a good neuroprotective utility. The stem bark, with the inhibitions of 71.8 ± 7.3% to AChE and 71.4 ± 17.5% to BChE, included the DPPH activity of 49.4 ± 2.6%, it is the most interesting sample of the carob tree in this assays, followed by the pulp. Because of these reasons and making a review of the studies made by Ferreira et al. (2006) and Orhan et al. (2007) witch conclude the

good relationship between the antioxidant and anticholinesterase activities of their samples, the same affirmation can be applied to this study.

In *diabetes mellitus*, hyperglycemia increases the oxidative stress, and excess free radicals cause injury to many organs in the body (Allen et al., 2003). Moreover, phenolic compounds are reported to play an important role in modulating glucosidase and amylase activities and therefore contribute to the management of type 2 diabetes (Matsuura et al., 2004; McCue and Shetty, 2004). The biological, pharmaceutical, and medicinal properties of flavonoids and proanthocianidins have been focused on extensively because they are natural antioxidant products with low citotoxicity. In *diabetes mellitus*, uncontrolled hyperglycemia is a high risk factor for diabetic complication development and progression, including diabetic nephropathy, because hyperglycemia increases oxidative stress such as ROS, witch leads to cellular dysfunction and induces apoptosis. In regard to this point it is important not only to regulate hyperglycemia but also to inhibit hyperglycemia mediated-oxidative stress, since oxidative stress is considered to be the major contributing factor in cellular dysfunction, including abnormalities in cell cycling and delayed replication (Fugii et al, 2006).

Similarly to the anticholinesterase, the antioxidant ability of some foods and plant materials can help in the prevention or attenuation of the diabetes disease. Studies made with that purpose also indicates good relations between the antioxidant and anti-hyperglycemic ability of natural compounds (Matsuura et al., 2004; McCue and Shetty, 2004; Ani and Naidu, 2007; Mai and Chuyen, 2007). Ani and Naidu (2007) describes a possible synergistic effect of the catechin, a polyphenol inhibitor of α-glucosidase and other polyphenols such as, gallic acid, protocatchuic acid, caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, quercetin and kaempferol to augment their inhibition effect. In this study, that situation can be fundament, because the samples with the highest contents in catechin, and some of those other phenolic compounds, also represent the best α-glucosidase inhibitors. However, studies support the finding that the natural α-amylase and α-glucosidase inhibitors from plants have lower inhibitory effect against α-amylase and a stronger inhibitory activity against α-glucosidase (Kwon et al., 2006), also reported in this study. An effective strategy for the type 2 diabetes management was suggested to be a mild inhibition of α-amylase and strong inhibition of intestinal α-glucosidase activity (Krentz and Bailey, 2005). Mai and Chuyen (2007) screening five

plant extracts with influence in anti-hyperglycemic assays, but without know the possible effect of the polyphenolic content, and found that the samples with the highest phenolic content also was the better α-glucosidase inhibitor. They study aqueous extracts with a concentration of 20 mg/ml and the best result found was to *Cleistocalyx operculatus* leaves, with 68.2 ± 3.4% of inhibition of rat's intestinal α-glucosidase and 122.5 ± 1.0 mg CE/g extract of total phenols. In this study, no sample induced that inhibition, but a smaller concentration was used, at 1 mg/ml the best obtained was 33.3 ± 13.0 % to the α-glucosidase enzyme, in the Algarve cork oak acorn (50 ºC) with 87.8 ± 0.1 mg CE/g extract. Regarding the different concentrations used, our result it is relevant and the influence of the phenolic content can be accurate. Mai et al. (2007), found positive relationships among α-glucosidase inhibitory activities, antioxidant activities and polyphenol contents of 28 edible plants in both aqueous and methanolic extracts.

All these studies demonstrate the influence of the antioxidant ability of natural resources to obtain functional foods, providing health benefits to humans and animals.

7 – GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The different test conditions applied to the samples resulted in the observation that the MeOH extractions of the leaves samples from Algarve cork oak and carob tree had the highest antioxidant, anti-cholinesterase and anti-hyperglycemic activities. In the extraction of the phenolic compounds the thermal treatment had no significant influence, but the opposite was observed in the quantification of lipids. Between the different species used, the best results were obtained in the carob tree and in the cork oak of Algarve origin that had generally best results than the Alentejo cork oak, being the holm oak the species with weakest results. In the chemical characterization the Algarve cork oak exhibited the higher amounts of total phenolics, cork oak from Alentejo the highest tannins content and the holm oak the highest content in flavonoids. In carob tree the highest values of total phenolics and flavonoids were observed in the leaf samples, and the highest levels of tannins in the pulps aqueous extracts. In the HPLC analysis, the gallic acid was the most frequent compound among the samples but the most abundant were, generally, the gentisic acid, (+)-catechin and (-)-epicatechin. In the antioxidant activity, the MeOH leaves samples of Algarve cork oak had the best RSA against ABTS and DPPH, and ferric reduction capacity, similarly to the stem bark samples of carob tree, followed by the leaves of the same species. The best AChE and BChE inhibitory capacities were provided by the MeOH samples of the Algarve cork oak leaves, and in the carob tree the aqueous samples of leaves and stem bark presented higher inhibitory activity. From the analysis of the results obtained in this work it can be concluded that the leaves of Algarve cork oak, and the leaves and stem bark of carob tree are plant materials with singular interest to future studies.

Therefore, more research is needed in order to identify the biocompounds responsible for the biological activities detected, particularly in the case of extracts exhibiting multiple pharmacological actions. The selected extracts should be submitted to a fractioning process, and the resulting fractions submitted to the *in vitro* biological testing according to the methods applied to the crude extracts. Finally, it should be made the chemical identification of the compounds in the bioactive fractions, by chromatographic methods such as HPLC or GC/MS.

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