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Research Article

ANTIOXIDANT ACTIVITIES OF EXTRACTS FROM *ACACIA MELANOXYLON, ACACIA DEALBATA* AND *OLEA EUROPAEA* AND ALKALOIDS ESTIMATION

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ABSTRACT

The purpose of this work was to determine the phenolic, flavonoid and alkaloid contents of the ethanol, methanol, acetone and hydroalcoholic crude extracts of *Olea europaea*, *Acacia melanoxylon* and *Acacia dealbata.* The Folin-Ciocalteu's method was used for the determination of total phenols, a colorimetric method with aluminum chloride was used in the determination of flavonoids and Dragendorff's reagent method was used to alkaloids estimation. The methods of DPPH (2,2-diphenyl-1-picrylhidrazil) and β-carotene bleaching test were used to assess the antioxidant activity of extracts. Moreover, the phenolic compounds present in the extracts were analyzed by RP-HPLC.

Keywords: Phenolics, Flavonoids, Antioxidant activity

INTRODUCTION

Some plants may become invasive in certain areas, leading to profound changes in ecosystem processes, community structure, and displacing native species. In most cases, the first noticeable change in invaded areas is the loss of the plant biodiversity, due to the establishment of monocultures of invasive species [1]. Different *Acacia* species are aggressive invaders that affect ecosystem integrity worldwide. *Acacia dealbata* Fabales (Fabaceae) and *Acacia melanoxylon* Fabales (Fabaceae), the most invasive Australian acacias in southern Europe, particularly in Portugal, invade farmland, and autochthonous forest, establishing monocultures and modifying the ecosystem structure. This invasive species has become a serious environmental problem because they displace the indigenous plant species [1].

The olive tree [*Olea europaea* Lamiales (Oleacea)] is one of the most important trees in Mediterranean countries, were they cover ~8 million ha, accounting for almost 98% of the world harvest [2]. This demonstrates the great economic and social importance of this tree and the possible benefits derived from the utilization of any of their by-products [3]. The raw material from olive trees pruning has low cost and high availability because it is concentrated in the olive oil production centers, but at present a very limited productivity is obtained from it, because the main interest is concentrated in the olive oil production and not in the use of their by-products. Some studies dealing with the chemical composition of the olive fruits and their oil have been carried out [2,3]; however, only a few works have been focused on the isolation and identification of some compounds found on olive extracts [2,3]. Because of its nutritional and biological characteristics, virgin olive oil is one of the most important components of the Mediterranean diet and local agriculture. The traditional Mediterranean diet, which consists of fruits, vegetables, cereals, legumes and fish, is thought to represent a healthy lifestyle; especially the incidence of several cancers, including colorectal cancer, is lower in Mediterranean countries compared to Northern Europe [4]. Olives and olive derived are an important part of this diet and are recognized as a valuable source of natural phenolic antioxidants. In fact, an increasing number of epidemiologic and experimental studies report that the olive oil may have a role in the prevention of coronary heart disease, cognitive impairment, e.g., Alzheimer's disease, protective effects against of the cancer of the colon, breast and ovary, diabetes accompanied by hypertryglyceridemia and inflammatory and autoimmune diseases, such as rheumatoid arthritis [4]. Also, olive oil has been shown to reduce low-density lipoprotein (LDL) oxidisability in the post prandial state. These beneficial health effects of olive oil are ascribable to monounsaturated, and low unsaturated fatty acids and a number of phenolic compounds [4].

Plant-derived natural chemicals, known as secondary metabolites, are effective in their roles of protection, adaptation and pollination. Secondary metabolites are mainly used in food, pharmaceutical, chemical, cosmetic industries and agriculture. Plant-derived natural products are abundant in nature and many of them exhibit numerous biological activities and some can be employed as food additives. Synthetic antioxidants have been used in the food industry since the 1940s, but trends in many health-related industries tend to shift preferences to natural sources. Therefore, investigation of natural antioxidants has been a major research interest for the past two decades as many research groups and institutions have been screening plant materials for possible antioxidant properties [5-7]. Antioxidants also play important role preventing oxidative deterioration of food and indirectly eliminating radicals from it. Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tertiary butylhydroquinone are often used in foods to prevent oxidative degradation. Application of synthethic antioxidants in foods is negatively perceived by consumers due to safety and health effect. Moreover, the need to understand how different antioxidant compounds work and what components may form an antioxidant system, stimulate the search for antioxidants, particularly from plant sources. It has also been proposed that antioxidant activity of plant origin components can be mainly ascribed to the presence of phenolic compound [6-8]. No single chemical component is responsible for the medicinal properties of plant-based drugs, and their synergic action or bioenhancement is due to the presence of other chemical substances in the plant material. Therefore, the determination of the total amount of different classes of components is essential for the standardization of the plants [9]. Alkaloids are low molecular weight nitrogen-containing substances with characteristic toxicity and pharmacological activity. These properties, which have traditionally been exploited by humans for hunting, execution and warfare, have also been used for the treatment of disease [10]. As alkaloids have therapeutic efficacy and bioenhancing properties, the estimation of total alkaloids in plants bearing alkaloids and formulations that contain them as therapeutic agents becomes essential [9].

The purpose of this work was to determine the phenolic, flavonoid and alkaloid contents of the ethanol, methanol, acetone and hydroalcoholic crude extracts of *Olea europaea*, *Acacia melanoxylon,*

and *Acacia dealbata,* and then trying to correlate it with antioxidant activity of corresponding extracts. Then the phenolic compounds present in the extracts were analyzed by RP-HPLC. The Folin-Ciocalteu's method was used for the determination of total phenols, a colorimetric method with aluminum chloride was used in the determination of total flavonoids and Dragendorff's reagent method was used to alkaloids estimation. The methods of DPPH (2,2-diphenyl-1-picrylhidrazil) and β-carotene bleaching test were used to assess the antioxidant activity of extracts.

MATERIALS AND METHODS

Plant material

Aerial parts (wood, bark and leaves) of the trees (*Olea europaea*, *Acacia melanoxylon* and *Acacia dealbata*) were collected in November 2009 in Serra da Estrela and were identified by a botanist. Plant materials were air-dried at room temperature during 3 months and reduced to coarse powder (< 1 mm) using a laboratory cutting mill. The composition of raw material was analyzed following the Standard Biomass Analytical Procedures of National Renewable Energy Laboratory [11], with respect to sugars, extractives, acid insoluble lignin, acid soluble lignin and ashes. A voucher specimen of all species of trees has been deposited in the Biology Laboratory of Health Sciences Research Centre, University of Beira Interior.

Extraction process

Ethanol, methanol and acetone extracts were carried out with Soxhlet apparatus until the solvent became colorless, using 100 g of raw material and 1000 mL of solvent. Hydroalcoholic extractions, water/ethanol 50/50 (v/v), were performed by refluxing, using 100 g of plant samples during 45 min with 1000 mL of solvent. The extract solutions were filtered under vacuum using a crucible of porosity #2 and then distilled under vacuum to remove the solvents to a final volume of 150 mL. Then, 5 mL of each extract was diluted in 45 mL of methanol. Aliquots (5 mL) of the extracts were removed for subsequent evaporation to dryness for the calculation of extraction yield and extracts concentration.

Total phenolic compounds determination

The phenols were determined by Folin-Ciocalteu's colorimetric method. The methanol solutions of each extract (50 µL) or gallic acid (standard phenolic compound) were mixed with 450 µL of distilled water and then 2.5 mL of Folin-Ciocalteu's reagent 0.2 N (diluted with distilled water) was added. The mixtures were allowed to stand for 5 min, and then 2 mL of aqueous Na_2CO_3 (75 g/L) was added. After incubation of these reaction mixtures (90 min/30°C) the total phenols were determined by colorimetry at 765 nm. The standard curve was prepared using 500, 400, 350, 325, 300, 250, 225, 200, 150, 125, 100 and 50 mg/L solutions of gallic acid in methanol ($y = 0.0009x$; $R^2 = 0.9875$). Total phenol values were expressed as gallic acid equivalents (mg GAE/g of dry mass), which is a common reference compound for phenolic compounds [12-14]. The tests were conducted in triplicate.

Flavonoids determination

Aluminum chloride colorimetric method was used for flavonoids determination according to Pourmorad, *et al.* (2006) [13]. Each extract (500 µL) in methanol was separately mixed with 1.5 mL of methanol, 0.1 mL of 10 % aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. This solution remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm using a spectrophotometer. The calibration curve was constructed by preparing eight quercetin solutions at concentrations ranging from 12.5 to 200 µg/mL in methanol ($y = 0.0071x$; $R^2 = 0.9979$). Total flavonoid values were expressed as quercetin equivalents (mg QE/g of dry mass), which is a common reference compound for flavonoids [13]. These determinations were made in duplicate.

Alkaloids estimation

The alkaloids estimation was performed by spectrophotometric method of Dragendorff's reagent as it was described by Sreevidya and Mehrotra (2003) [9]. Briefly, 10 mL amount of each crude extract was centrifuged over 10 min (3000 rpm) to remove residual suspended particles and then 5 mL of the supernatant were mixed with 1 mL of HCl 0.1 N. Then, 2.5 mL of Dragendorff's reagent was added to the previous mixture for precipitation and the precipitate was centrifuged over 5 min (3000 rpm). This precipitate was further washed with 2.5 mL of ethanol. The filtrate was discarded and the residue was then treated with 2.5 mL of disodium sulfide solution (1% w/v). The brownish black precipitate formed was then centrifuged (5 min, 3000 rpm). This residue was dissolved in 2 mL of concentrated nitric acid, with warming if necessary; this solution was diluted to 10 mL in a standard flask with distilled water and 1 mL was then pipetted out and mixed with 5 mL of thiourea solution (3% w/v). The absorbance of this solution was measured at 435 nm against a blank containing 1 mL of concentrated nitric acid and 2.5 mL of thiourea solution (3% w/v). The standard curve was prepared using 750, 500, 400, 250, 200, 150 and 100 mg/L solutions of pilocarpine nitrate in HCl 0.1N ($y = 0.0012x-0.1044$; $R^2 = 0.9851$). Alkaloid contents were expressed as pilocarpine nitrate equivalents (mg PNE/g of dry mass) [9]. These tests were performed in duplicate.

Evaluation of antioxidant activity

DPPH scavenging assay

The antioxidant activity of the extracts and standards (gallic acid, quercetin, rutin and trolox) was determined by the radical scavenging activity method using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as it was described by Scherer and Godoy (2009) [15]. Briefly, about 0.1 mL aliquots of methanol solutions of the extracts or standards at different concentrations were added to 3.9 mL of a DPPH methanol solution. Three DPPH solutions were tested, 0.2000, 0.1242 and 0.0800 mM, prepared by dissolving 39.4, 24.5 and 15.8 mg in 500 mL of methanol, respectively. These concentrations were selected due to the linearity range of DPPH solutions: above 0.2 mM the concentration is very high, and below 0.5 mM the color is very weak having a limited range of absorbance reading. The control sample consisted in a solution of 0.1 mL of methanol mixed with 3.9 mL of DPPH. After a 90 min incubation period at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: $I\ \% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the control and Abs_1 was the absorbance in the presence of the test sample at different concentrations. The IC_{50} (concentration providing 50% inhibition of DPPH radicals) was calculated graphically using a calibration curve in the linear range by plotting the extract concentration *vs.* the corresponding scavenging effect. The antioxidant activity was expressed as the antioxidant activity index (AAI), calculated as follows: AAI = (final concentration of DPPH in the control sample- $\mu g.mL^{-1}$)/(IC_{50}-$\mu g.mL^{-1}$) [15]. Thus, the AAI was calculated considering the mass of DPPH and the mass of the tested sample in the reaction, resulting in a constant for each sample, independent of the concentration of DPPH and sample used. In this work it was considered that shrub extracts showed poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when AAI between 0.5 and 1.0, strong antioxidant activity when AAI between 1.0 and 2.0, and very strong when AAI > 2.0 [15]. Assays were carried out in duplicate and all the samples and standard solutions, as well as the DPPH solutions, were prepared daily [15].

β-carotene bleaching test

This test was also used to evaluate the antioxidant activity of the extracts. After preparation of β-carotene solution (20 mg/mL in chloroform), 20 µL of it was added to 40 µL of linoleic acid, 400 mg of Tween 40 and 1 mL of chloroform. This mixture was then evaporated at 45°C for 5 min by using a rotary vacuum evaporator to remove chloroform and immediately diluted with 100 mL of oxygenated distilled water. The water was added slowly to the

mixture and vigorously agitated to form an emulsion. About 5 mL of the emulsion were transferred into test tubes containing 300 µL of extracts in methanol at different concentrations. About 5 mL of the emulsion and 300 µL of samples in methanol were used as control. Standard butylated hydroxytoluene (BHT) in methanol, at the same concentration as samples, was used as reference. The tubes were then gently shaken and placed at 50°C in a water bath for 2 h. The absorbances of the extracts, standard and control were measured at 470 nm, using a spectrophotometer, against a blank consisting of an emulsion without β-carotene. The measurements were carried out at initial time (t = 0) and at final time (t = 2). All samples were assayed in duplicate. The antioxidant activity was measured in terms of percentage of inhibition of β-carotene's oxidation by: % Inhibition = $(Abs^{t=2}{}_{sample} - Abs^{t=2}{}_{control})/(Abs^{t=0}{}_{control} - Abs^{t=2}{}_{control})$. Where $Abs^{t=2}$ was the absorbance of the sample or control at final time of incubation and $Abs^{t=0}$ was the absorbance in the control at initial time of incubation [6].

RP-HPLC analysis of phenolics

A simple and quick reversed-phase-HPLC (RP-HPLC) method for determination of phenolic compounds was developed. Chromatographic analysis was performed with the use of liquid chromatographic system, which consisted of Perkin Elmer Binary LC Pump 250, Perkin Elmer UV-visible Spectrophotometric Detector LC 290 and Perkin Elmer LC Oven 101 with loop of 50 µL. Chromatographic systems was connected through the Data Apex U-PAD2 USB Acquisition Device to the PC computer. Software used for data acquisition and evaluation was Clarity Lite Data Apex. The separation was carried out on 150×4.60 mm, Phenomenex Kinetex Luna 2.6 µ PFP 100 A reversed phase column equipped with Phenomenex KrudKatcher Ultra HPLC In-Line Filter with 0.5 µm porosity. Samples were filtered through a 0.22 µm pore size membrane filter before injection. The injection volume for all samples was 50 µL. The mobile phase consisted of two solvents: Solvent A, water with acetic acid (pH = 3) and Solvent B, acetonitrile/solvent A (60:40; v/v). The mobile phase was filtered through a 0.22 µm pore size membrane filter and degasified with nitrogen. Phenolic compounds were eluted under the following conditions: 1 mL/min flow rate and the column temperature was set at 35°C, isocratic conditions from 0 to 10 min with 0% B, linear gradient conditions from 0% to 5% B in 30 min, from 5% to 15% B in 18 min, from 15% to 25% B in 14 min, from 25% to 50% B in 31 min, from 50% to 100% B in 3 min, followed by washing and reconditioning the column. For detection, chromatograms were monitored by the ultra-violet-visible spectra (280 nm) which were recorded for all peaks. Duplicate analyses were performed for each sample. The identification of phenolic compounds were obtained by using authentic standards and by comparing the retention times, while quantification was performed using the external standard method. Stock solution of standard compounds at concentration 1 mg/mL each was prepared in methanol, and several dilutions with methanol were made. Standards compounds used were: gallic acid, vanillic acid, cafeic acid, chlorogenic acid, syringic acid, *p*-coumaric acid, ferulic acid, ellagic acid and quercetin. The solutions of standards at various concentrations (1.00, 0.75, 0.50, 0.25, 0.15 mg/mL) were injected into the HPLC system and the calibration curves were established for each standard compound. The concentration of the compound was calculated from peak area according to calibration curves. The amount of each phenolic compound was expressed as milligram per gram of dry mass (mg/g of dry mass) [16-18].

RESULTS AND DISCUSSION

Chemical composition and extraction yields

Analysis of chemical composition of *Olea europaea*, *Acacia melanoxylon* and *Acacia dealbata* was carried out and it was determinate its main chemical components. The composition of the trees used in this study is presented in Table 1, expressed on a dry matter basis. It is possible to verify that *Acacia dealbata* is the specie that has minor quantity of extractives but in other hand, is the tree that has higher amount of glucose in its chemical composition.

Observing the Table 2, it is possible to conclude that hydroalcoholic extracts had higher extraction yields. Generally *Acacia melanoxylon* gives rise to higher extraction yields.

Table 1: Table shows the chemical composition of *Olea europaea*, *Acacia melanoxylon* and *Acacia dealbata.*

Tree	***Olea europaea***	***Acacia melanoxylon***	***Acacia dealbata***
Composition	**% Dry matter ***		
Extractives (ethanol)	13.30 ± 0.78	14.05 ± 0.08	6.75 ± 0.03
Glucose[a]	34.24 ± 1.78	31.79 ± 0.75	40.24 ± 0.88
Xylose[a]	12.23 ± 1.06	10.02 ± 0.17	18.03 ± 0.11
Arabinose[a]	2.44 ± 0.65	2.24 ± 0.20	Not detected
Acid insoluble lignin	20.36 ± 0.53	28.15 ± 0.91	22.46 ± 0.05
Acid soluble lignin	7.22 ± 0.78	4.18 ± 0.04	4.78 ± 0.12
Ash	4.37 ± 0.00	3.40 ± 0.00	1.50 ± 0.00

* Results in terms of mean ± standard deviation.

[a] Sugar monomers are presented as anhydromonomers.

Total phenolics

Total phenolics were most conveniently assessed by spectrophotometric measurement on a simple extract of the plant material. Colorimetric procedures rely on the reaction of the phenolic with one of a number of reagents of varying selectivity. Folin-Ciocalteu reagent, which has been used before and after precipitation of phenolic compounds in acidic methanol, is the classic reagent [19].

Table 2: Table shows the extraction yields of the different types of extractions.

Extracts	**Tree**	**Extraction Yield (%) ***
Ethanolic	*Olea europaea*	13.30 ± 0.78
	Acacia melanoxylon	14.05 ± 0.08
	Acacia dealbata	6.75 ± 0.03
Hidroalcoholic	*Olea europaea*	14.69 ± 1.80
	Acacia melanoxylon	15.41 ± 0.66
	Acacia dealbata	9.51 ± 0.68

Methanolic	*Olea europaea*	16.31 ± 1.74
	Acacia melanoxylon	11.88 ± 1.36
	Acacia dealbata	7.68 ± 0.39
Acetone	*Olea europaea*	6.33 ± 0.29
	Acacia melanoxylon	5.68 ± 0.15
	Acacia dealbata	3.26 ± 0.33

* Results in terms of mean ± standard deviation.

As can be seen by the Table 3, the extract that displayed the highest concentration of total phenols is the hydroalcoholic extract of *Acacia dealbata*. However, this method can also quantify some derivatives of sugars that can be extracted by this mixture of solvents. The species that has the highest amount of phenols is *Acacia dealbata*. Comparing all the extracts of same species with different solvent, it can be observed that solvent does not appear to influence the extraction of phenols. In general, *Olea europaea* is the tree that has the poorest content in total phenolics. The results obtained for total phenolics for the species now studied are very similar to the ones obtained by other [6,20].

Flavonoids

Flavonoid is a general name of a class of more than 6500 molecules based upon a 15-carbon skeleton. The core structure is a 2-pheylbenzopyranone, in which the three carbon bridge between the phenyl groups is commonly cyclised with oxygen [19]. In general, the search for a function for these compounds in plants has focused on the interaction that may take place between the plant and other living organisms and, in particular, on the effects of flavonoids on microorganisms which may infect plants and on animals which graze on plants [19]. The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. Flavonoids can interfere with at least three different free-radical producing systems. Due to their lower redox potentials, they are able to reduce highly oxidizing free radicals and so prevent, for example, lipid peroxidation, one of the most important actions of free radicals that leads to cellular membrane damage and, ultimately, to cell death [19].

In this way it is reasonable to determine these compounds in the extracts. The analysis of Table 3 allows concluding that *Acacia melanoxylon* is the specie with more flavonoids, and of all the extracts, the acetone extract that had the highest concentration in these compounds. Apparently the solvent used in extraction does not influence the extraction of flavonoids. The results obtained in this study are consistent to the one obtained in a previous report [21].

Alkaloids

The determination of alkaloids is important, since these compounds are known to have many pharmacological properties. Concerning its determination, it is possible to conclude that the solvent more appropriate to their extraction is acetone. According to Table 3, the extract that had the highest concentration in these compounds is the acetone extract of *Acacia dealbata*. *Olea europaea* is the species that presents fewer alkaloids content.

Table 3: Table shows the total phenolic compounds, flavonoids, alkaloids and antioxidant properties of extracts of *Olea europaea*, *Acacia melanoxylon* and *Acacia dealbata*

Extracts	Tree	Total phenolic content (mg GAE / g of dry mass) *	Flavonoids (mg QE / g of dry mass) *	Alkaloids (mg PNE / g of dry mass) *	IC_{50} (mg/L) *	AAI *	Antioxidant Activity
Ethanolic	*Olea europaea*	115.89 ± 0.11	31.60 ± 0.51	2.96 ± 0.27	44.08 ± 0.47	1.08 ± 0.01	Strong
	Acacia melanoxylon	127.33 ± 0.41	45.82 ± 0.86	10.12 ± 0.94	29.72 ± 1.58	1.66 ± 0.11	Strong
	Acacia dealbata	243.80 ± 2.50	18.37 ± 0.23	5.82 ± 0.20	13.82 ± 0.32	3.43 ± 0.10	Very strong
Hidroalcoholic	*Olea europaea*	112.01 ± 2.46	18.75 ± 0.11	4.82 ± 0.09	49.97 ± 1.58	0.95 ± 0.03	Moderate
	Acacia melanoxylon	138.76 ± 1.23	22.21 ± 1.09	4.15 ± 0.16	22.49 ± 0.64	2.09 ± 0.06	Very strong
	Acacia dealbata	290.65 ± 5.87	13.46 ± 0.19	5.81 ± 0.34	11.85 ± 0.20	3.98 ± 0.09	Very strong
Methanolic	*Olea europaea*	105.30 ± 1.19	27.40 ± 0.29	2.76 ± 0.36	48.77 ± 2.35	0.98 ± 0.04	Moderate
	Acacia melanoxylon	112.03 ± 2.32	43.47 ± 0.87	18.18 ± 0.42	29.32 ± 0.79	1.59 ± 0.05	Strong
	Acacia dealbata	241.81 ± 2.28	16.89 ± 0.03	14.88 ± 0.56	13.49 ± 0.20	3.45 ± 0.06	Very strong
Acetone	*Olea europaea*	116.55 ± 7.11	48.70 ± 2.25	18.87 ± 0.55	54.23 ± 2.36	0.91 ± 0.03	Moderate
	Acacia melanoxylon	100.10 ± 4.74	99.24 ± 2.57	13.70 ± 0.77	45.97 ± 1.06	1.05 ± 0.02	Very strong
	Acacia dealbata	203.10 ± 3.84	21.70 ± 3.12	19.46 ± 0.92	15.49 ± 0.99	3.11 ± 0.20	Very strong
Trolox	-	-	-	-	8.38 ± 0.13	6.62 ± 0.10	Very strong
Gallic acid	-	-	-	-	2.23 ± 0.02	22.77 ± 0.25	Very strong
Rutin	-	-	-	-	10.66 ± 0.39	4.90 ± 0.21	Very strong
Quercetin	-	-	-	-	4.32 ± 0.39	12.17 ± 1.71	Very strong

***** Results in terms of mean ± standard deviation

Antioxidant activity

DPPH scavenging assay

DPPH is a stable free radical that has a maximal absorption at 517 nm. When purple-colored DPPH radical reacts with a hydrogen donor, such as an antioxidant, the solution discolors, because of DPPH reduction to the corresponding hydrazine [22,23]. As can be seen

in Table 3, the extract that displayed the highest concentration of total phenols is the hydroalcoholic extract of *Acacia dealbata*. Simultaneously this extract had the greatest antioxidant activity, i.e., this extract is the one that requires the lowest concentration to promote 50% of inhibition (IC_{50}) and higher value of AAI, which classifies the activity of this extract as being a very strong antioxidant. All the extracts of *Acacia dealbata* present very strong antioxidant activity. *Olea europaea* is the specie with extracts displaying weaker antioxidant activity.

β-carotene bleaching test

In the β-carotene bleaching test, β-carotene undergoes rapid discoloration in the absence of an antioxidant. This fact occurs because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As a result, β-carotene is oxidized and broken down in part; subsequently, the system loses its cromophore and characteristic orange colour, which is monitored spectrophotometrically [21].

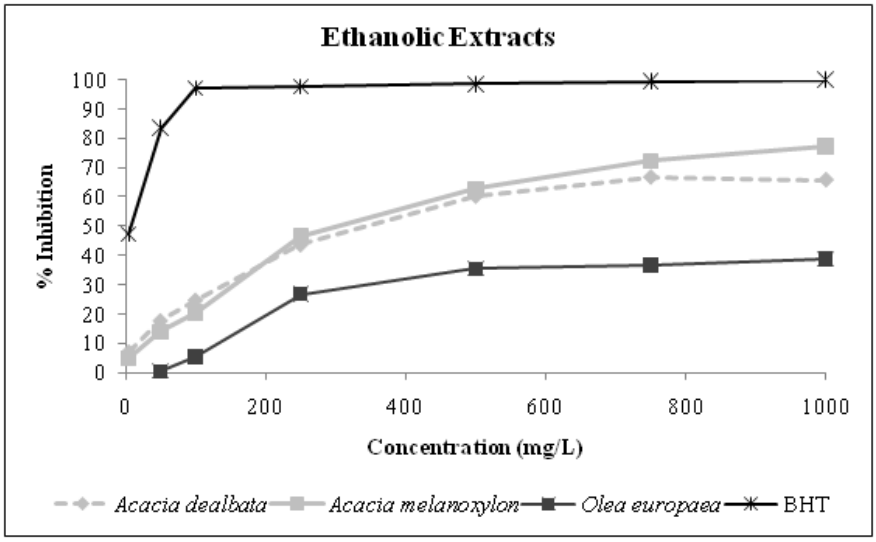

Fig. 1: It shows the antioxidant activity measured by β-carotene bleaching test of ethanol extracts

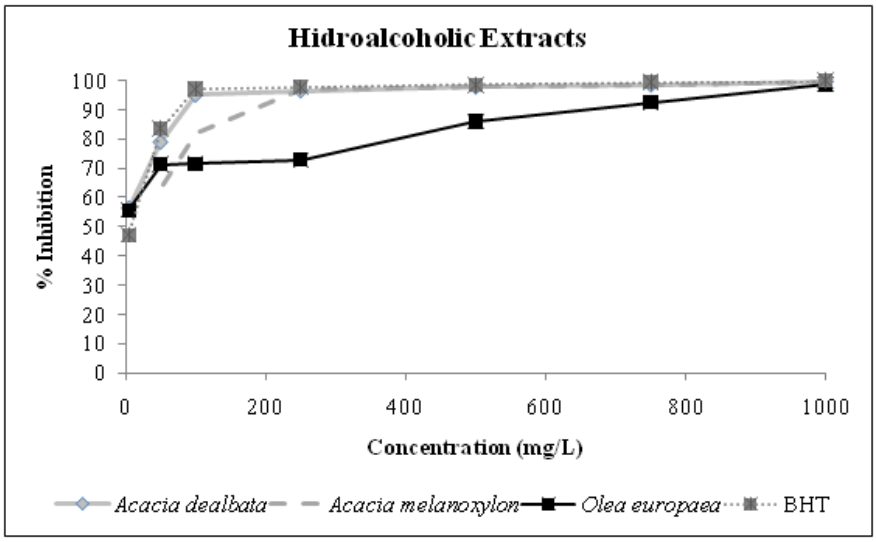

Fig. 2: It shows the antioxidant activity measured by β-carotene bleaching test of hydroalcoholic extracts

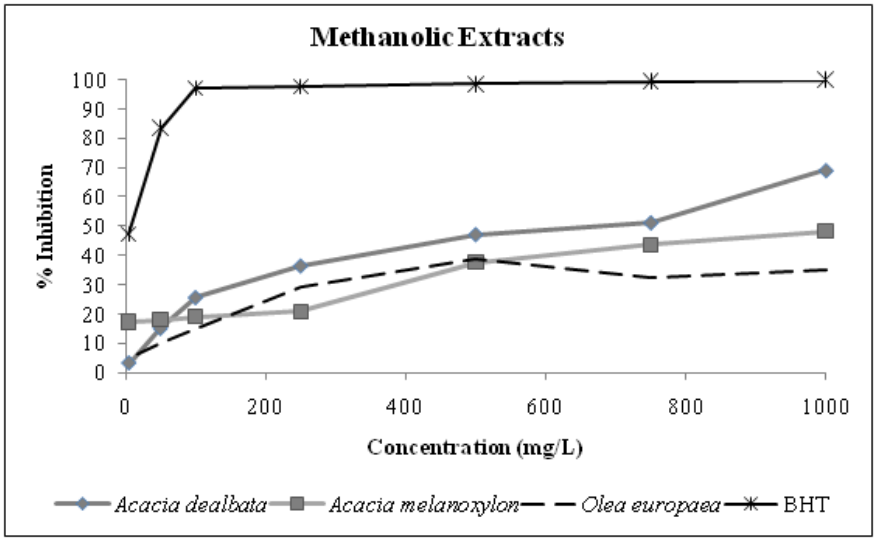

Fig. 3: It shows the antioxidant activity measured by β-carotene bleaching test of methanol extracts

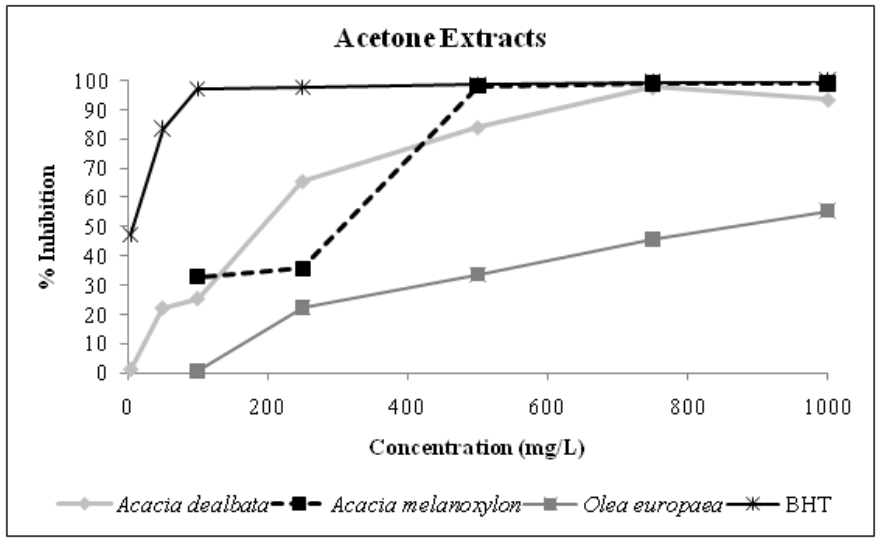

Fig. 4: It shows the antioxidant activity measured by β-carotene bleaching test of acetone extracts

Regarding the Figures 1 to 4, it is observed that *Olea europaea* is the species with lower antioxidant activity, as measured by this method, contrariwise *Acacia dealbata* showed great potential to inhibit the oxidation of linoleic acid. Comparing all solvent studied, it appears that the mixture of water/ethanol is the most suitable for the extraction of compounds with the ability to inhibit lipid peroxidation.

Table 4: Table shows the content of phenolics in extracts determinate by HPLC analysis

		Standard Phenolic Compounds (mg / g dry matter)*								
Extracts	**Tree**	**Gallic Acid**	**Vanillic Acid**	**Cafeic Acid**	**Chlorogenic Acid**	**Syringic Acid**	**p-coumaric Acid**	**Ferulic Acid**	**Ellagic Acid**	**Quercetin**
Ethanolic	*Acacia dealbata*	N/D	N/D	N/D	2.65 ± 0.01	1.02 ± 0.06	3.14 ± 0.32	3.77 ± 0.16	8.16 ± 0.76	1.65 ± 0.56
	Acacia melanoxylon	N/D	N/D	N/D	3.32 ± 0.16	N/D	N/D	2.44 ± 0.15	4.37 ± 0.41	3.07 ± 0.80
	Olea europaea	0.72 ± 0.08	0.63 ± 0.10	0.88 ± 0.13	N/D	1.00 ± 0.14	1.14 ± 0.08	4.25 ± 0.85	17.84 ± 0.41	6.69 ± 0.04
Acetone	*Acacia dealbata*	N/D	N/D	N/D	4.20 ± 0.67	4.00 ± 0.81	5.48 ± 0.94	12.44 ± 1.08	6.98 ± 0.53	N/D
	Acacia melanoxylon	N/D	N/D	1.77 ± 0.22	7.10 ± 0.53	N/D	N/D	5.22 ± 0.43	3.81 ± 0.35	4.58 ± 0.18
	Olea europaea	N/D	N/D	0.86 ± 0.01	N/D	1.54 ± 0.47	1.02 ± 0.03	26.97 ± 2.23	20.37 ± 1.96	4.36 ± 0.37
Hidroalcoholic	*Acacia dealbata*	N/D	N/D	0.99 ± 0.05	3.28 ± 0.05	1.52 ± 0.07	1.52 ± 0.14	6.15 ± 0.08	6.43 ± 0.53	1.14 ± 0.23
	Acacia melanoxylon	N/D	1.20 ± 0.33	0.98 ± 0.17	4.10 ± 0.57	2.90 ± 0.44	3.19 ± 0.77	7.61 ± 0.11	3.48 ± 0.44	0.87 ± 0.26
	Olea europaea	N/D	1.30 ± 0.38	0.52 ± 0.05	1.22 ± 0.21	0.60 ± 0.08	N/D	0.96 ± 0.11	5.47 ± 0.52	1.45 ± 0.26
Methanol	*Acacia dealbata*	N/D	N/D	N/D	1.15 ± 0.02	0.65 ± 0.04	2.00 ± 0.11	3.82 ± 0.09	2.08 ± 0.05	3.44 ± 0.21
	Acacia melanoxylon	N/D	N/D	0.91 ± 0.07	N/D	N/D	1.30 ± 0.05	2.33 ± 0.03	5.78 ± 0.23	0.77 ± 0.15
	Olea europaea	N/D	1.76 ± 0.14	N/D	1.41 ± 0.15	N/D	N/D	18.01 ± 0.31	8.70 ± 0.27	9.67 ± 0.67

* Results in terms of mean ± standard deviation

N/D – Not detected

RP-HPLC analysis

The HPLC technique has been gaining importance in the development of new methods to identify the main compounds present in plant extract that are responsible for biological activity. *Table 4* shows de results of the phenolic content in all the extracts analyzed in this study. A total of nine phenolic compounds were identified and quantified in the tree extracts, including hydroxybenzoic acids, hydroxycinnamic acids and flavonoids.

The total amount of phenolic compounds in all the extracts was 298.10 mg/g of dry matter. Gallic acid is the phenolic compound that is less abundant, having an occurrence percentage of 0.24%. Otherwise, the hydroxycinnamic acids identified (cafeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid) are abundantly present. Ferulic acid was the most dominant hydroxycinnamic acid, having an occurrence percentage of 31.52%. Acetone is the solvent that extracts the higher amount of phenols, which corresponds to a total of phenols extracted of 110.70 mg/g of dry matter. The ethanol extract of *Olea europaea* is the richest in ellagic acid, in terms of percentages of occurrence (53.82%). Literature specifies many methods, used with good results, for chromatographic analysis of phenolic compounds [24]; most of them involve gradient separations, like the method used in this study. It can be noticed that satisfactory separation with good resolution can be achieved with the method now used.

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