AILANTHUS ALTISSIMA (MILLER) SWINGLE: A SOURCE OF BIOACTIVE COMPOUNDS WITH ANTIOXIDANT ACTIVITY

Ângelo Luís,[a] Nuno Gil,[b] Maria Emília Amaral,[b] Fernanda Domingues,[a] and Ana Paula Duarte [a,]*

Ailanthus altissima (Miller) Swingle is a tree used in Chinese traditional medicine as a bitter aromatic drug and in the treatment of colds and gastric diseases. Previous phytochemical studies have demonstrated the presence of quassinoids in the plant, as well as indole alkaloids. The purpose of this work was to determine the phenolic, flavonoid, and total alkaloid contents of the ethanolic, methanolic, acetone, and hydroalcoholic crude extracts of *A. altissima* and then try to correlate them with antioxidant activity of corresponding extracts. Moreover, the phenolic compounds present in the extracts were analyzed by RP-HPLC. Extracts from leaves have greater phenolic content than the other parts of this tree. Concerning the extraction process, it is possible to conclude that the mixture of water and ethanol is the best solvent to extract substances with antioxidant activity. Analysis by RP-HPLC showed that ferulic acid was the most dominant hydroxycinnamic acid, with an occurrence percentage of 25.59%. These results presented a positive linear correlation between antioxidant activity index and total phenolic content of all the extracts.

Keywords: Ailanthus altissima; Phenolic compounds; Flavonoids; Antioxidant activity; RP-HPLC

Contact information: a: CICS-UBI - Health Sciences Research Centre, University of Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal; b: Research Unit of Textile and Paper Materials, University of Beira Interior, Rua Marquês d'Ávila e Bolama, 6201-001 Covilhã, Portugal;
**Corresponding author: apcd@ubi.pt*

INTRODUCTION

Invasive exotic species have significant negative impacts on socio-ecological systems. Some invasive species cause acute socio-ecological problems; other impacts are subtler, and are the cause of longer-term change, such as changes to the composition and function of ecosystems and the loss of biodiversity. *Ailanthus altissima* (Miller) Swingle is an example of a vegetal species that is invasive and exotic in various countries (Bardsley and Edwards-Jones 2007), including in Portugal. *A. altissima*, the tree-of-heaven, is native in China and was introduced in Europe around the end of 18th century. Although they are scentless on the tree, the leaves and flowers have an unpleasant odor when crushed. *A. altissima* is used in Chinese traditional medicine as a bitter aromatic drug in the treatment of colds and gastric diseases. Previous phytochemical studies have demonstrated the presence of quassinoids in the plant, as well as indole alkaloids. Lipids and fatty acids, phenolic derivatives, and volatile compounds from leaves have also been characterized. Extracts with organic solvents of tree-of-heaven and some isolated compounds have demonstrated medicinal properties (Feo et al. 2003).

The ways in which plants interact with other organisms in an environment are complex. The production and/or the accumulation of secondary metabolites could have different functions: self-defense, sexual attraction, symbiosis, and development (Feo et al. 2003). There is an increasing interest in the measurement and use of plant antioxidants for scientific research, as well as industrial purposes (dietary, pharmaceutical, and cosmetic). This is mainly due to their strong biological activity, exceeding those of many synthetic antioxidants that have possible activity as promoters of carcinogenesis. Therefore, the need exists for safe, economic, powerful, and natural antioxidants to replace these synthetic ones. In addition, antioxidants are an important group of medicinal preventive compounds, as well as being food additives inhibiting detrimental changes of easily oxidizable nutrients (Ksouri et al. 2009). Amongst the antioxidants, several groups of polyphenols are currently used in the industry as nutraceuticals and/or functional foods. Some antioxidant compounds are extracted from easily available sources, such as agricultural and horticultural crops, or medicinal plants.

Phenolic compounds play an important role for normal growth in plant development, as well as defense against infection and injury. The presence of phenolics in injured plants may have an important effect on oxidative stability and microbial safety (Ksouri et al. 2009). On the other hand, the number of contributions on isolation and activity-testing of plant antioxidants has significantly increased in recent years. Several studies have described the antioxidant properties of medicinal plants, foods, and beverages that are rich in phenolic compounds because of their high redox potentials. Such compounds act as reducing agents, hydrogen donors, singlet oxygen quenchers, free radical scavengers, and as chelating agents of pro-oxidant metals, such as transition metals. It was recently showed that phenolic antioxidants in herbs have capacities of quenching lipid per-oxidant, preventing DNA oxidative damage, and scavenging reactive oxygen species (Ksouri et al. 2009).

Although the potential benefits of antioxidants such as phenolic compounds, against reactive oxygen species (ROS), it is well known that ROS are not only harmful agents that cause oxidative damage in pathologies, they also have important roles as regulatory agents in a range of biological phenomena (Murphy et al. 2011).

The purpose of this work was to determine the phenolic, flavonoid, and total alkaloid contents of the ethanolic, methanolic, acetone, and hydroalcoholic crude extracts of *A. altissima* and then try to correlate them with antioxidant activity of the same extracts. Then, the phenolic compounds present in the extracts were analyzed by reversed-phase-HPLC (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 for alkaloids estimation. The methods of DPPH (2,2-diphenyl-1-picrylhydrazyl) and β-carotene bleaching test were used to assess the antioxidant activity of extracts.

EXPERIMENTAL

Plant Material

Aerial parts (stems, stalks, and leaves) of *A. altissima* were collected in *Serra da Estrela* (GPS coordinates: N 40º 16,719'; W 07º 30,744'; altitude: 649 m). Plant

materials were dried at 35 ºC in a ventilated oven during 48 hours and reduced to coarse powder (< 2 mm) using a laboratory cutting mill. Harvesting and transport of this plant were authorized by *Instituto da Conservação da Natureza e da Biodiversidade*. The vegetal specie was identified (*Ailanthus altissima* (Miller) Swingle) by a botanist, and a voucher specimen has been deposited in the Herbarium (LISI 19/2011) of the *Instituto Superior de Agronomia* (*Jardim Botânico d'Ajuda*, *Lisboa*).

Extraction Process

Ethanolic, methanolic, and acetone extracts were carried out with a Soxhlet apparatus, 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 with 1000 mL of solvent. The extract solutions were filtered under vacuum using a crucible of porosity #2 and then evaporated 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 phenolics were determined by Folin-Ciocalteu's colorimetric method. The methanolic 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 0.2 N Folin-Ciocalteu's reagent (diluted with distilled water) was added. The mixtures were allowed to stand for 5 minutes, and then 2 mL of aqueous Na_2CO_3 (75 g/L) was added. After incubation of these reaction mixtures (90 minutes / 30 ºC), 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 phenolic values were expressed as gallic acid equivalents (mg GAE / g of dry mass), which is a common reference compound for phenolic compounds (Tawaha et al. 2007; Pourmorad et al. 2006). The tests were conducted in triplicate.

Flavonoids Determination

Aluminum chloride colorimetric method was used for flavonoids determination according to Pourmorad et al. (2006). 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 minutes; 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 ($y=0.0074x$; $R^2=0.9980$). Total flavonoid values were expressed as quercetin equivalents (mg QE / g of dry mass), which is a common reference compound for flavonoids (Pourmorad et al. 2006). These determinations were made in duplicate.

Alkaloids Estimation

The alkaloids estimation was performed by the spectrophotometric method of Dragendorff's reagent, as described by Sreevidya and Mehotra (2003). Briefly, 10 mL of each crude extract was centrifuged for 10 minutes (3000 rpm) to remove residual suspended particles, and then 5 mL of the supernatant was mixed with 1 mL of 0.1 N HCl. Then, 2.5 mL of Dragendorff's reagent was added to the previous mixture for precipitation, and the precipitate was centrifuged for 5 minutes (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 minutes, 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 0.1N HCl ($y=0.0013x-0.2750$; $R^2=0.9957$). Alkaloid contents were expressed as pilocarpine nitrate equivalents (mg PNE / g of dry mass) (Sreevidya and Mehotra 2003). 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 described by Scherer and Godoy (2009). Briefly, 0.1 mL aliquots of methanolic solutions of the extracts or standards at different concentrations were added to 3.9 mL of a DPPH methanolic 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,

$$I\ \% = [(Abs_0 - Abs_1) / Abs_0] \times 100 \quad (1)$$

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 (Scherer and Godoy 2009):

$$AAI = (\text{final concentration of DPPH in the control sample} - \mu g.mL^{-1})/(IC_{50} - \mu g.mL^{-1}) \quad (2)$$

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 the sample used. In this work, it was observed that shrub extracts showed poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when AAI was between 0.5 and 1.0, strong antioxidant activity when AAI between 1.0 and 2.0, and very strong antioxidant activity when AAI > 2.0, according to Scherer and Godoy (2009). Assays were carried out in duplicate, and all the samples and standard solutions, as well as the DPPH solutions, were prepared daily (Scherer and Godoy 2009).

β-carotene bleaching test

The β-carotene bleaching test was also used to evaluate the antioxidant activity of the extracts. Twenty µL of β-carotene solution (20 mg/mL in chloroform) 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 ºC for 5 minutes 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. 5 mL of the emulsion were transferred into test tubes containing 300 µL of extracts in methanol at different concentrations. 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 the samples, was used as reference. The tubes were then gently shaken and placed at 50 ºC in a water bath for 2 hours. The absorbances of the standard and control extracts 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 h) and at final time (t=2 h). All samples were assayed in duplicate. The antioxidant activity was measured in terms of percentage of inhibition of β-carotene's oxidation by,

$$\% \text{ Inhibition} = (Abs_{t=2sample} - Abs_{t=2control})/(Abs_{t=0} - Abs_{t=2control}) \quad (3)$$

where $Abs_{t=2}$ was the absorbance of the sample or control at the final time of incubation and $Abs_{t=0}$ was the absorbance in the control at the initial time of incubation (Luís et al. 2009).

RP-HPLC Analysis of Phenolics

A simple and quick 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. The chromatographic system 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 a 150×4.60 mm, Phenomenex Kinetex Luna 2.6 µm PFP 100A reversed phase column equipped with

Phenomenex KrudKatcher Ultra HPLC In-Line Filter with 0.5 μm porosity. Standard solutions were filtered through a 0.22 μm pore size membrane filter before injection, and crude methanolic extracts were centrifuged and then 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, the column temperature 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), and results were recorded for all peaks. Duplicate analyses were performed for each sample. The identification of phenolic compounds was 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. Standard compounds used were: gallic acid, vanillic acid, caffeic 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) (Kelebek et al. 2009; Fernández-Arroyo et al. 2010; Tarnawski et al. 2006).

RESULTS AND DISCUSSION

From the results presented in Table 1, generally leaves gave rise to a higher extraction yield than the other parts of the plant. The extraction yields obtained in the present study were very similar to those obtained for some medicinal plants reported in literature (Silva et al. 2007).

Phenolic compounds are one of the most important groups of compounds that occur in plants. These compounds are reported to exhibit anticarcinogenic, anti-inflam-atory, antiatherogenic, antithrombotic, immune-modulating, and analgesic activities, among others (Kassim et al. 2010). In this study, these compounds were determined with the colorimetric method of Folin-Ciocalteu's reagent, which is a very well established and widely used method for total phenolics quantitation. Nevertheless, this method has some limitations; namely, the Folin-Ciocalteu's reagent can react with other non-phenolic reducing compounds and can lead to the overvaluation of the phenolic compounds. These values only intend to provide a relative result for comparison between the different extracts. As can be seen in Table 2, generally, extracts from leaves yielded higher amounts of phenolic compounds than the extracts from stems and stalks.

Table 1. Extraction Yields of the Different Types of Extraction for the Several Parts of the Plant (%)

Solvent	Plant part	Extraction yield (%) *
Ethanol	Stems	6.28 ± 0.45
	Stalks	6.32 ± 0.46
	Leaves	24.70 ± 0.75
Acetone	Stems	3.59 ± 0.39
	Stalks	2.67 ± 0.33
	Leaves	24.25 ± 0.60
Hydroalcoholic (50/50 v:v)	Stems	8.42 ± 0.20
	Stalks	13.38 ± 0.16
	Leaves	29.75 ± 2.60
Methanol	Stems	8.69 ± 1.13
	Stalks	12.47 ± 0.10
	Leaves	24.96 ± 5.13
* Results in terms of mean ± standard deviation		

Referring to Table 2, hydroalcoholic extracts presented the biggest concentration of total phenolics, which indicates that this mixture of solvents is the best to use in the extraction of these compounds from plants. However, this method can also quantify some derivatives of sugars that can be extracted by this mixture of solvents. Acetone is the solvent used that extracted the least concentration of phenolic compounds. The acetone extracts of stalks of *A. altissima* were the ones with the lowest quantity of phenolics, in contrast to the hydroalcoholic extract of leaves, which was the richest in phenolics.

Flavonoids, which are partly responsible for the pigmentation of flowers, fruits, and leaves, are polyphenolic compounds that occur ubiquitously in edible plants and structurally have variations in the C ring that characterizes the different types, namely flavonols, flavones, isoflavones, flavonones, flavanol, and anthocyanins (Aruoma 2003; Surveswaran et al. 2007). Flavonoids could act as antioxidants because they can readily donate an electron or a hydrogen atom to a peroxyl or alkoxy radical to terminate a lipid peroxidation chain reaction or to regenerate a phenolic/flavonoid compound; or they can effectively chelate a pro-oxidant transition metal (Aruoma 2003). The process used in quantitative methods for the flavonoids determination is performed by precipitating them with aluminum chloride in an alkalinized medium (Alim et al. 2009). Table 2 shows the results of the determination of flavonoids in all the extracts of *A. altissima*. It is possible to conclude that the methanolic extracts of stalks are the ones that present the lower concentration in flavonoids, or in other way, that the acetone extract of leaves is the richest extract in flavonoids. These results are very similar to those obtained previously by Andrade et al. (2009).

In the most cases 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 (Sreevidya and Mehotra 2003). Alkaloids are low molecular weight nitrogen-containing substances with characteristic toxicity and pharmacological activity (De Luca and Pierre 2000). 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 (Sreevidya and Mehotra 2003). All the extracts contain large amounts of alkaloids (Table 2), which is in agreement with the literature. Other studies have reported the presence of alkaloids in *A. altissima* (Feo et al. 2003). In particular, the acetone extract of stalks is the one that exhibited the highest amount of these compounds.

Table 2. Contents of Phenolics, Flavonoids, and Alkaloids of the Extracts

Solvent	Plant part	Total phenolic compounds (mg GAE / g dry matter) *	Flavonoids (mg QE / g dry matter) *	Alkaloids (mg PNE / g dry matter) *
Ethanol	Stems	44.95 ± 0.97	8.04 ± 0.05	17.91 ± 1.49
	Stalks	52.48 ± 1.69	17.30 ± 0.63	9.37 ± 1.25
	Leaves	136.55 ± 1.31	87.09 ± 0.94	14.42 ± 1.88
Acetone	Stems	48.60 ± 1.59	20.62 ± 0.22	15.37 ± 0.91
	Stalks	25.96 ± 1.35	31.51 ± 1.57	28.84 ± 0.91
	Leaves	106.96 ± 0.35	122.53 ± 0.68	6.53 ± 1.38
Hydroalcoholic (50/50 v:v)	Stems	65.61 ± 1.54	7.47 ± 0.36	15.44 ± 1.08
	Stalks	149.52 ± 2.04	22.82 ± 0.17	27.58 ± 0.20
	Leaves	158.72 ± 0.38	29.59 ± 0.25	19.19 ± 0.19
Methanol	Stems	53.49 ± 2.55	7.73 ± 1.29	14.08 ± 1.18
	Stalks	15.88 ± 0.49	3.69 ± 0.05	18.38 ± 0.57
	Leaves	151.80 ± 1.02	68.39 ± 0.13	16.68 ± 0.45

* Results in terms of mean ± standard deviation

One problem in the determination of the antioxidant activity is that this activity is variable depending on the method used. It is known that an antioxidant mechanism in various biological matrices is very complex and many other factors may intervene in this mechanism (Çam et al. 2009). Given this complexity, the use of only one method to determine the antioxidant activity of the extracts is not sufficient to reach a conclusion. In this sense, we have applied two different methods and diverse antioxidant properties can then be determined.

It is well known that the antioxidants react with the stable free radical DPPH (deep violet color) and convert it to 2,2-diphenyl-1-picrylhydrazine (DPPH-H) with discoloration. The antioxidant effect of plant extracts on DPPH radical scavenging may

be due to their hydrogen donating ability, as well as the reduction of the stable violet DPPH radical to yellow DPPH-H (Ahmadi et al. 2010).

Free-radical scavenging properties of extracts of *A. altissima* are presented in Table 3. Hydroalcoholic extracts are the ones that exhibited the greatest antioxidant properties measured by the DPPH scavenging method. These results indicate that the mixture of water and ethanol is the solvent more appropriate to extract substances with antioxidant activity. This is in agreement with the results obtained for the phenolics determination, which suggests that the total phenolic compounds were responsible for the antioxidant activity of the extracts. The hydroalcoholic extract of leaves is the extract with the greatest value of AAI, which classifies the antioxidant activity of this extract as "very strong." The methanolic extract of stalks showed a poor antioxidant activity. Generally, all the extracts of *A. altissima* presented great antioxidant activity when compared with the standards. These results are in agreement with the study of Rahman et al. (2009), who also reported a high antioxidant activity and high levels of phenolic compounds in extracts of leaves from *A. altissima*. However, in this work a more detailed characterization of the bioactive compounds and antioxidant activity of different extracts, from the aerial parts of *A. Altissima*, was done.

Table 3. Antioxidant Properties of *A. altissima* Extracts and Standards

Solvent	Plant part	IC_{50} (mg/L) *	AAI *	Antioxidant Activity
Ethanol	Stems	65.69 ± 2.58	0,88 ± 0,03	Moderate
	Stalks	77.49 ± 3.34	0,78 ± 0,14	Moderate
	Leaves	28.53 ± 0.34	2,01 ± 0,03	Very Strong
Acetone	Stems	58.00 ± 3.53	0,99 ± 0,06	Moderate
	Stalks	96.24 ± 3.52	0,59 ± 0,02	Moderate
	Leaves	36.29 ± 1.18	1,58 ± 0,05	Strong
Hydroalcoholic (50/50 v:v)	Stems	65.45 ± 2.12	0,87 ± 0,03	Moderate
	Stalks	26.23 ± 0.63	2,15 ± 0,06	Very Strong
	Leaves	22.98 ± 0.42	2,44 ± 0,06	Very Strong
Methanol	Stems	80.11 ± 3.29	0,67 ± 0,03	Moderate
	Stalks	162.46 ± 2.10	0,33 ± 0,01	Poor
	Leaves	20.23 ± 0.31	2,64 ± 0,05	Very Strong
Rutin		10.66 ± 0.39	4.90 ± 0.21	Very Strong
Quercetin		4.32 ± 0.39	12.17 ± 1.71	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

* Results in terms of mean ± standard deviation

In the β-carotene bleaching test, oxygen is needed for linoleic acid oxidation and to convert it to lipid hydroperoxide. This compound is very active and attacks the highly unsaturated β-carotene molecules, giving rise to the bleaching of its characteristic yellow color in solution. Antioxidants perform their inhibition by various mechanisms such as peroxide decomposition or chain stopping and neutralization of linoleate-free radical and other free radicals formed in the system (Ahmadi et al. 2010; Rajaei et al. 2010). All the extracts presented antioxidant properties when compared with the synthetic antioxidant BHT, as measured by this method. Based on Figures 1 to 4, it is possible to conclude that extracts from leaves of *A. altissima* are potent antioxidants, allowing high percentages of inhibition of oxidation of β-carotene molecules. These results indicate that this plant is a potential source of compounds with the ability to inhibit lipid peroxidation. Figure 1 presents the behavior of ethanolic extracts, and as mentioned above, extracts of leaves showed the greatest antioxidant activity measured by this method.

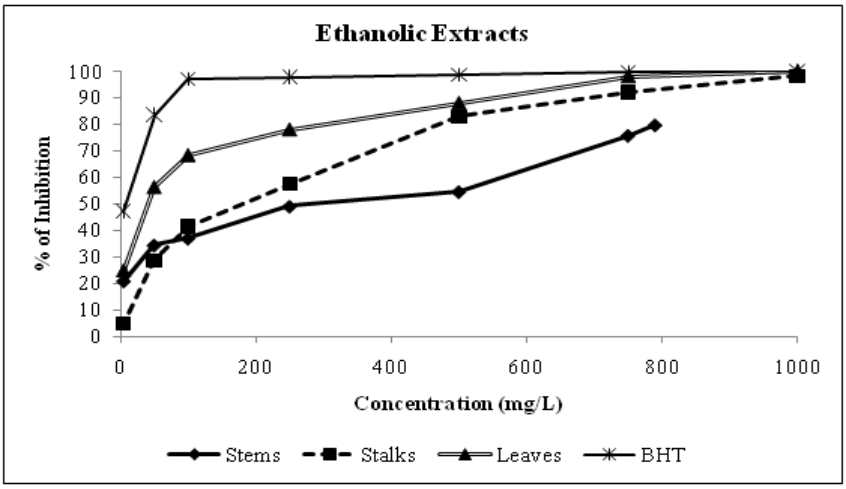

Fig. 1. Antioxidant activity of ethanolic extracts measured by β-carotene bleaching test

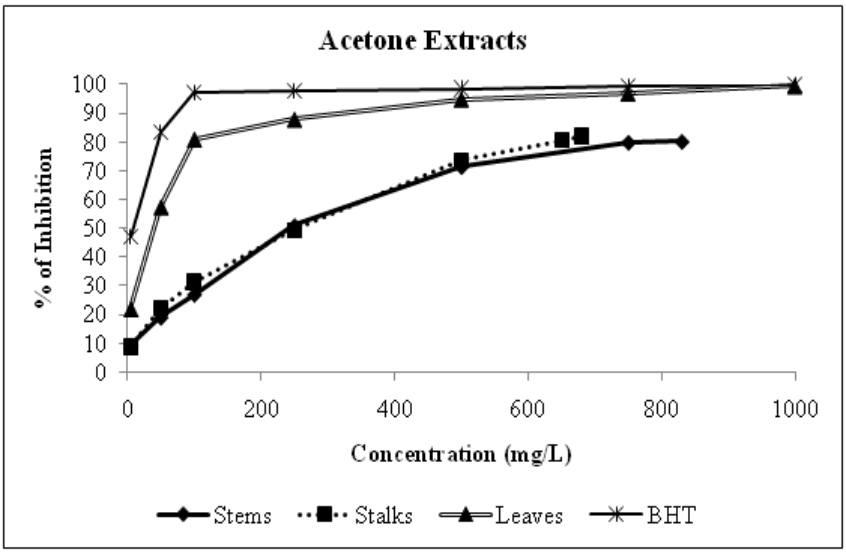

Fig. 2. Antioxidant activity of acetone extracts measured by β-carotene bleaching test

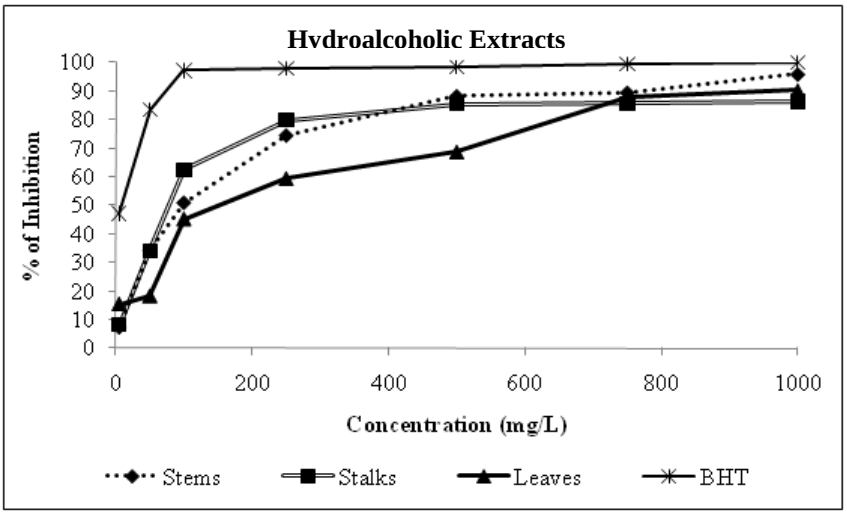

Fig. 3. Antioxidant activity of hydroalcoholic extracts measured by β-carotene bleaching test

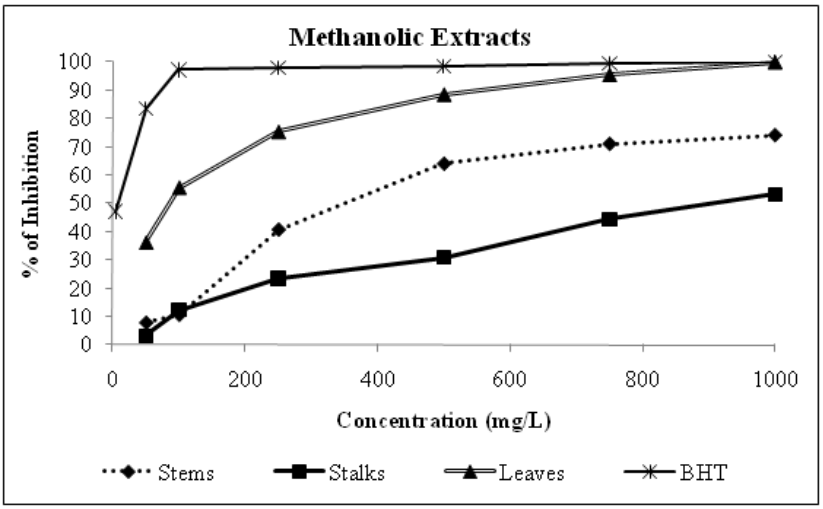

Fig. 4. Antioxidant activity of methanolic extracts measured by β-carotene bleaching test

In the case of acetone (Fig. 2) and methanolic (Fig. 4) extracts, the results were similar to those obtained for ethanolic extracts. In the case of hydroalcoholic extracts (Fig. 3), the extract of stems possessed the greatest antioxidant activity, but it was very identical to the antioxidant activity of extracts of stalks and leaves.

A. altissima is a potential source of natural occurring antioxidant compounds that can be used for medicinal or industrial purposes. However, the excess consumption of antioxidants can cause damage. Several studies summarized by Ristow and Schemeisser (2011) evidence that several longevity-promoting interventions may converge by causing an activation of mitochondrial oxygen consumption to promote increased formation of ROS. These serve as molecular signals to exert downstream effects to ultimately induce endogenous defense mechanisms culminating in increased stress resistance and longevity,

an adaptive response more specifically named mitochondrial hormesis or mitohormesis (Ristow and Schemeisser 2011).

According to Fig. 5, it is possible to observe a positive linear correlation between antioxidant activity index and total phenolic content of all the extracts of *A. altissima* (R^2=0.9593). These results indicate that the phenolic compounds could be the main contributor of the antioxidant activities of this tree. This result was in agreement with previous studies (Fu et al. 2010). These authors showed results with a positive linear correlation between the antioxidant capacities and total phenolic content (R^2>0.86). The same analysis was done for the flavonoids (Fig. 5), and it was verified that there was no correlation (R^2=0.2901) between these compounds and the antioxidant activity of the extracts. It is known that only flavonoids of a certain structure and particularly hydroxyl position in the molecule determine antioxidant properties; in general these properties depend on the ability to donate a hydrogen or electron to a free radical (Miliauskas et al. 2004).

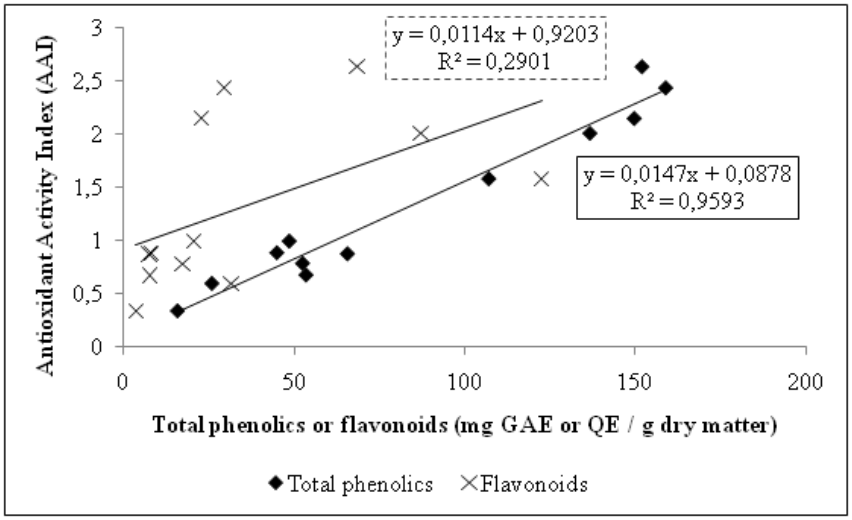

Fig. 5. Correlation between antioxidant activity, total phenolics and flavonoids of extracts

The RP-HPLC analysis was employed to identify major phenolic compounds in the extracts of *A. altissima*. These data will be helpful for comparison between the antioxidant activities and phenolic compounds of the different studied extracts and will also be useful for understanding their chemical constituents and functionality. RP-HPLC analysis is the most used method for identification of plant phenolics. Because of the diversity and complexity of natural phenolic compounds in hundreds of medicinal plant extracts, it is rather difficult to characterize every compound and elucidate its structure. It is not difficult, however, to identify major categories of phenolic compounds and representative phenolics. Namely, quercetin is an example of one flavonoid aglycone, and the *p*-coumaric and ferulic acids are examples of hydroxycinnamic acids (Cartea et al. 2011). In the present study, we conducted identification of representative natural phenolic compounds from selected extracts. In the Fig. 6 an example can be seen of an HPLC chromatogram of the standard phenolic compounds used in this study. The phenolic compounds were detected at 280 nm (although 320 and 350 nm were more adequate

wavelengths to detect hydroxycinnamic acids and flavonoids derivatives, respectively). A total of nine phenolic compounds were identified and quantified in the extracts of the plant (Table 4), including hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids. The total amount of phenolic compounds in all the extracts was 268.15 mg / g of dry matter. Gallic acid is one of the most important hydroxybenzoic acids, which is a naturally abundant plant phenolic compound, and it is present in food of plant origin; since it was found to exhibit antioxidant properties, it has attracted considerable interest. Surprisingly, in the extracts now analyzed, gallic acid was the phenolic compound that was less abundant, having an occurrence percentage of 1.45%. The hydroxycinnamic acids identified (caffeic acid, chlorogenic acid, *p*-coumaric acid, ferulic acid) were abundantly present. Ferulic acid was the most dominant hydroxycinnamic acid, presenting an occurrence percentage of 25.59%. These results are in agreement with other previously published data (Kelebek et al. 2009). Acetone was the solvent that extracted the lowest quantity of phenolics, at about 40.67 mg / g of dry matter. Methanol extracted the biggest concentration of phenolic compounds, at about 81.63 mg / g of dry matter. Generally, extracts from *A. altissima* leaves yielded more phenolic compounds, when compared with the extracts of stems and stalks.

Table 4. Phenolic Compounds in *A. altissima* Extracts Analyzed by HPLC

Standard Phenolic Compounds (mg / g dry matter) *										
Solvent	**Plant part**	**Gallic Acid**	**Vanillic Acid**	**Caffeic Acid**	**Chlorogenic Acid**	**Syringic Acid**	***p*-Coumaric Acid**	**Ferulic Acid**	**Ellagic Acid**	**Quercetin**
Ethanol	Stems	1.65 ± 0.16	2.59 ± 0.39	1.37 ± 0.14	1.81 ± 0.08	1.80 ± 0.24	1.18 ± 0.22	5.80 ± 0.28	4.08 ± 0.68	N/D
	Stalks	0.69 ± 0.06	0.96 ± 0.08	N/D	0.90 ± 0.03	N/D	0.82 ± 0.12	4.38 ± 0.09	0.74 ± 0.00	N/D
	Leaves	N/D	4.08 ± 0.24	2.82 ± 0.39	11.30 ± 0.17	2.53 ± 0.56	6.98 ± 0.83	14.21 ± 0.65	3.99 ± 0.12	N/D
Acetone	Stems	N/D	N/D	N/D	N/D	N/D	4.65 ± 0.69	3.93 ± 0.38	5.17 ± 0.21	N/D
	Stalks	N/D	N/D	N/D	N/D	N/D	N/D	2.43 ± 0.52	10.46 ± 0.61	N/D
	Leaves	N/D	N/D	N/D	N/D	N/D	N/D	3.99 ± 0.33	5.54 ± 0.28	4.50 ± 0.41
Hydroalcoholic	Stems	N/D	1.48 ± 0.09	0.59 ± 0.07	1.35 ± 0.20	3.17 ± 0.26	1.66 ± 0.09	4.80 ± 1.11	N/D	N/D
	Stalks	N/D	3.54 ± 0.65	2.84 ± 0.68	8.72 ± 0.82	1.43 ± 0.17	1.94 ± 0.00	3.09 ± 0.14	3.19 ± 0.38	1.03 ± 0.06
	Leaves	1.56 ± 0.16	1.49 ± 0.11	0.98 ± 0.07	4.41 ± 0.59	6.64 ± 0.62	2.48 ± 0.04	10.26 ± 0.77	4.52 ± 0.89	N/D
Methanol	Stems	N/D	0.57 ± 0.05	N/D	1.10 ± 0.12	0.87 ± 0.01	0.54 ± 0.03	1.23 ± 0.10	1.78 ± 0.13	N/D
	Stalks	N/D	1.48 ± 0.16	N/D	N/D	1.18 ± 0.08	0.72 ± 0.14	1.91 ± 0.14	0.85 ± 0.17	N/D
	Leaves	N/D	2.71 ± 0.25	0.90 ± 0.03	17.59 ± 0.49	2.02 ± 0.24	12.81 ± 0.71	12.58 ± 0.71	19.02 ± 0.77	1.77 ± 0.04

* Results in terms of mean ± standard deviation

N/D – Not detected

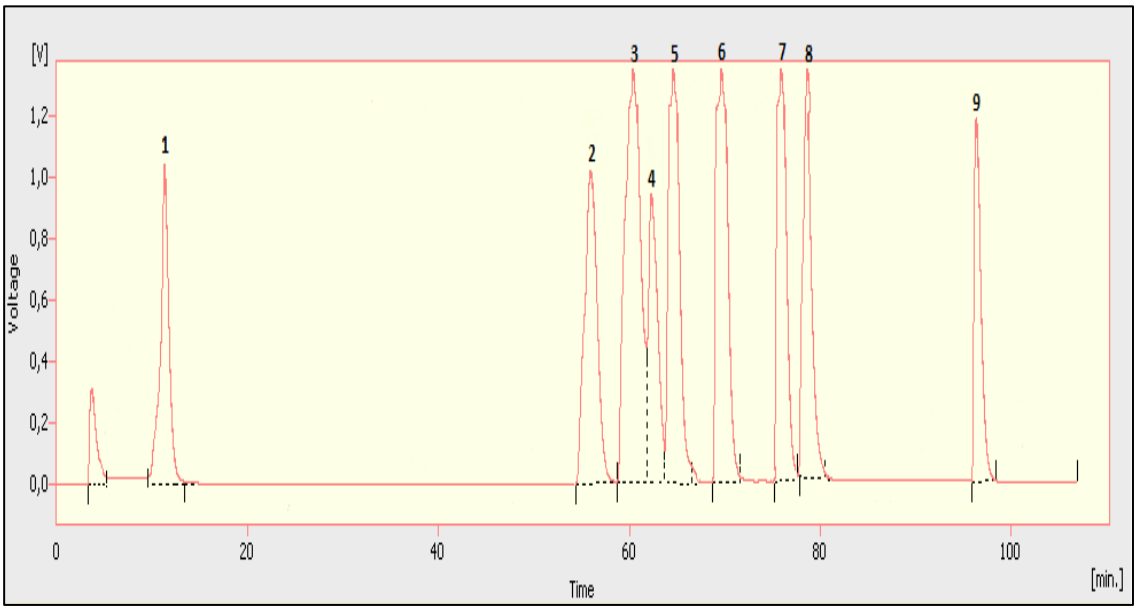

Fig. 6. Example of HPLC chromatogram of one mixture of the standard compounds used (1 mg/mL). *Peaks: 1)* gallic acid; *2)* vanillic acid; *3)* caffeic acid; *4)* chlorogenic acid; *5)* syringic acid; *6) p*-coumaric acid; *7)* ferulic acid; *8)* ellagic acid; *9)* quercetin.

CONCLUSIONS

1. *A. altissima* is a potential source of bioactive compounds with antioxidant activity, namely phenolic compounds and flavonoids. This species is also rich in alkaloids.
2. Extracts from leaves have greater amounts in phenolics than the other parts of this tree.
3. The mixture of water and ethanol is the solvent most suitable to extract substances with antioxidant activity.
4. There was a positive linear correlation between antioxidant activity index and total phenolic content of all the extracts of *A. altissima*.
5. Analysis by RP-HPLC shows that ferulic acid was the most dominant hydroxyl-cinnamic acid, presenting an occurrence percentage of 25.59%.

ACKNOWLEDGMENTS

The authors acknowledge Fundação Para a Ciência e a Tecnologia for the PhD. fellowship to Ângelo Luís (Reference: SFRH / BD / 65238 / 2009). The authors wish to thank to Prof. Dr. Dalila Espírito Santo and Dr. Paula Paes, from the Herbarium of the Instituto Superior de Agronomia (Jardim Botânico d'Ajuda, Lisboa), for the identification and deposit of the vouchers specimens of the plant species used in this work.

REFERENCES CITED

Ahmadi, F., Sadeghi, S., Modarresi, M., Abiri, R., and Mikaeli, A. (2010). "Chemical composition, in vitro anti-microbial, antifungal and antioxidant activities of the essential oil and methanolic extract of *Hymenocrater longiflorus* Beth., of Iran," *Food Chem. Toxicol.* 48, 1137-1144.

Ali, S., Kasoju, N., Luthra, A., Singh, A., Sharanabasava, H., Sahu, A., and Bora, U. (2008). "Indian medicinal herbs as sources of antioxidants," *Food Res. Int.* 41, 1-15.

Alim, A., Goze, I., Goze, H., and Tepe, B. (2009). "In vitro antimicrobial and antiviral activities of the essential oil and various extracts of *Salvia cedronella* Boiss," *J. Med. Plants Res.* 3, 413-419.

Andrade, D., Gil, C., Breitenfeld, L., Domingues, F., and Duarte, A. (2009). "Bioactive extracts from *Cistus ladanifer* and *Arbutus unedo* L.," *Ind. Crops Prod.* 30, 165-167.

Aruoma, O. (2003). "Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods," *Mutation Res.* 523-524, 9-20.

Bardsley, D., and Edwards-Jones, G. (2007). "Invasive species policy and climate change: social perceptions of environmental change in the Mediterranean," *Environ. Sci. Policy.* 10, 230-242.

Çam, M., Hışıl, Y., and Durmaz, G., (2009). "Classification of eight pomegranate juices based on antioxidant capacity measured by four methods," *Food Chem.* 112, 721-726.

Cartea, M., Francisco, M., Soengas, P., and Velasco, P. (2011). "Phenolic compounds in brassica vegetables," *Molecules.* DOI:10.3390/molecules16010251.

De Luca, V., and Pierre, B. (2000). "The cell and developmental biology of alkaloid biosynthesis," *Trend Plants Sci.* 5, 168-173.

Feo, V., Martino, L., Quaranta, E., and Pizza, C. (2003). Isolation of phytotoxic compounds from tree-of-heaven (*Ailanthus altissima* Swingle)," *J. Agric. Food Chem.* 51, 1177-1180.

Fernández-Arroyo, S., Barrajón-Catalán, E., Micol, V., Segura-Carretero, A., and Fernández-Gutiérrez, A. (2010). "High-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight and ion-trap tandem mass spectrometry to identify phenolic compounds from a *Cistus ladanifer* aqueous extract," *Phytochem. Anal.* 21, 307-313.

Fu, L., Xu, B., Xu, X., Qin, X., Gan, R., and Li, H. (2010). "Antioxidant capacities and total phenolic contents of 56 wild fruits from South China," *Molecules*. DOI:10.3390/molecules15128602.

Kassim, M., Achoui, M., Mustafa, M., Mohd, M., and Yousoff, K. (2010). "Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity," *Nutrition Res.* 30, 650-659.

Kelebek, H., Selli, S., Canbas, A., and Cabaroglu, T. (2009). "HPLC determination of organic acids, sugars, phenolic compositions and antioxidant capacity of orange juice and orange wine made from a Turkish cv. Kozan," *Microchem. J.* 91, 187-192.

Ksouri, R., Falleh, H., Megdiche, W., Trabelsi, N., Mhamdi, B., Chaieb, K., Bakrouf, A., Magné, C., and Abdelly, C. (2009). "Antioxidant and antimicrobial activities of the

edible medicinal halophyte *Tamarix gallica* L. and related polyphenolic constituents," *Food Chem. Toxicol.* 47, 2083-2091.

Luís, A., Domingues, F., Gil, C., and Duarte, A. P. (2009). "Antioxidant activity of extracts of Portuguese shrubs: *Pterospartum tridentatum*, *Cytisus scoparius* and *Erica* spp.," *J. Med. Plant Res.* 3, 886-893.

Miliauskas, G., Venskutonis, P., and van Beek, T. (2004). "Screening of radical scavenging activity of some medicinal and aromatic plant extracts," *Food Chem.* 85, 231-237.

Murphy, M., Holmgren, A., Larsson, N., Halliwell, B., Chang, C., Kalyanaraman, B., Rhee, S., Thornalley, P., Partridge, L., Gems, D., Nyström, T., Belousov, V., Schumacker, P. and Winterbourn, C. (2011). "Unravelling the Biological Roles of Reactive Oxygen Species," *Cell Metabol.* 13, 361-366.

Pourmorad, F., Hosseinimehr, S., and Shahabimajd, N. (2006). "Antioxidant activity, phenol and flavonoids contents of some selected Iranian medicinal plants," *African J. Biotechnol.* 5, 1142-1145.

Rahman, A., Kim, E. and Kank, S. (2009). "Antibacterial and antioxidant properties of *Ailanthus altissima* Swingle leave extract to reduce foodborne pathogens and spoiling bacteria," *J. Food Safety* 29, 499-510.

Rajaei, A., Barzegar, M., Mobarez, A., Sahari, M., and Esfahani, Z. (2010). "Antioxidant, anti-microbial and antimutagenic activities of pistáchio (*Pistachia vera*) gree hull extract," *Food Chem. Toxicol.* 48, 107-112.

Ristow, M. and Schmeisser, S. (2011). "Extending life span by increasing oxidative stress," *Free Rad. Biol. Med.* 51, 327-336.

Scherer, R., and Godoy, H. (2009). "Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method," *Food Chem.* 112, 654-658.

Silva, E., Souza, J., Rogez, H., Rees, J., and Larondelle, Y. (2007). "Antioxidant activities and polyphenolic contents of fifteen selected plant species from the Amazonian regions," *Food Chem.* 101, 1012-1018.

Sreevidya, N., and Mehotra, S. (2003). "Spectrophotometric method for estimation of alkaloids precipitable with Dragendorff's reagent in plant materials," *J AOAC Int.* 86, 1124-1127.

Surveswaran, S., Cai, Y., Corke, H., and Sun, M. (2007). "Systematic evaluation of natural phenolic antioxidant from 133 Indian medicinal plants," *Food Chem.* 102, 938-953.

Tarnawski, M., Depta, K., Grejcium, D., and Szelepin, B. (2006). "HPLC determination of phenolic acids and antioxidant activity in concentrated peat extract - A natural immunomodulator," *J. Pharm. Biomed. Anal.* 41, 182-188.

Tawaha, K., Alali, F., Gharaibeh, M., Mohammad, M., and El-Elimat, T. (2007). "Antioxidant activity and total phenolic content of selected Jordanian species," *Food Chem.* 104, 1372-1378.

Article submitted: January 12, 2012; Peer review completed: March 16, 2012; Revised version received and accepted: March 18, 2012; Published: March 22, 2012.