Pharmacognosy Communications
Volume 3 | Issue 2 | Apr–Jun 2013
www.phcogcommn.org

Research Article

Antioxidant proprieties of *Pistacia lentiscus* L. leaves extracts

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ABSTRACT: **Introduction:** *Pistacia lentiscus* L. (Anacardiaceae) is used traditionally in Algeria for gastro-intestinal disorders. In the present study, different antioxidant tests were employed to evaluate the antioxidant activities of crude chloroform, ethyl acetate and aqueous leaf extracts of this plant. **Methods:** The antioxidant capacity of extracts were determined by the determining the scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH), free hydroxyl radicals (HO.), β-carotene/linoleic acid model system, lipid peroxidation and reducing power. Total phenolic content in these extracts was determined using Folin–Ciocalteu's reagent. **Results:** Total phenolics varied between 207 ± 0.021 and 390 ± 0.005 mg/g dry weight, expressed as gallic acid equivalents for chloroform extract (CHE) and crude methanolic extract (CE) respectively. Total flavonoid contents were determined using 2% aluminum chloride and they varied between 13 ± 0.003 and 82.37 ± 0.0043 mg quercetin equivalent/g dry weight for aqueous extract (AQE) and ethyl acetate extract (EAE) respectively. The ethyl acetate extract showed the highest antioxidant activity using DPPH, hydroxyl radical scavenging and reducing power with an IC50 value of 0.0068 ± 0.0013 mg/ml, 0.16 ± 0.0082 mg/ml and 1.55 ± 0.025 mg/ml respectively. Using the β-carotene/linoleic acid bleaching assay, crude methanolic extract had the highest antioxidant activity with 94% inhibition. BHT was used as positive control in this test. In addition ethyl acetate extract (EAE) and crude methanolic extract (CE) exhibited good anti- lipid peroxidation, with 42.7% and 41%, inhibiting activity respectively in the linoleic acid emulsion system. **Conclusion:** *Pistacia lentiscus* L. extracts contain high amounts of phenolics and exhibited high antioxidant activity which is related to these compounds.

KEYWORDS: *Pistacia lentiscus* L., polyphenols, antioxidant activity, lipid peroxidation, scavenging activity

INTRODUCTION

Free radicals and other reactive species present in the body can be generated both endogenously and exogenously. Oxidative damage caused by free radicals to living cells mediate the pathogenesis of many chronic diseases such as atherosclerosis, Parkinson's disease, Alzheimer's disease, stroke, arthritis, chronic inflammatory diseases, cancers and other degenerative diseases.[1]

The human organism has developed defense systems to deal with this oxidative stress. These include enzymatic systems, especially superoxide dismutase, catalase, glutathione peroxidase and thioredoxin systems, which are highly efficient in ROS detoxification. The nonenzymatic antioxidants include glutathione, bilirubin, estrogenic sex hormones, uric acid, coenzyme Q, melanin, melatonin, α-tocopherol and lipoic acid.[1] Moreover, many studies have now confirmed that exogenous antioxidants, are essential for counteracting oxidative stress. These antioxidants come from plants in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids.[2]

Polyphenols constitute a large group of naturally occurring substances in the plant kingdom. These substances have considerable interest in the field of food chemistry, pharmacy and medicine due to their wide range of biological effects.[3] The antioxidant activity of phenolics is

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DOI: 10.5530/pc.2013.2.7

mainly due to their redox properties. They act as reducing agents (free radical terminators), hydrogen donors, singlet oxygen quenchers and metal chelators.[3]

Pistacia lentiscus L is an evergreen member of the Anacardiaceae family, largely distributed in the Mediterranean area, which are characterized by nutrient and water scarcity and long term exposures to extensive solar radiation and high temperatures.[4] Its mastic has been utilized in folk medicine since the time of the ancient Greeks.[5] The aerial part of this plant has traditionally been used as a stimulant, for its diuretic properties, and to treat hypertension, coughs, sore throats, eczema, stomach aches, kidney stones and jaundice.[5,6]

In this study, we have attempted to evaluate the antioxidant activity of different extracts from the leaves of *P. lentiscus*. The radical scavenging capacity, the anti-lipid peroxidation and reducing power of crude chloroform, ethyl acetate and aqueous fractions were evaluated.

MATERIALS AND METHODS

Chemicals

Linoleic acid, ammonium thiocyanate, β-carotene, butylated hydroxytoluene (BHA), were purchased from Fluka Chemical Co. (Buchs, Switzerland). 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), ethylenediamine tetraacetic acid (EDTA) and gallic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium ferricyanide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), ferrous and ferric chloride were obtained from Merck. All other reagents were of analytical grade

Plant material

The leaves of *P. lentiscus* L were collected from Sidi Ibrahim forest (Bordj Bou Arerridj) in eastern Algeria. The plant was identified by Pr. Hocine Louar from the laboratory of Botany, Faculty of Natural and Life Sciences, University Ferhat Abbas, Setif, Algeria. A voucher specimen was kept at the laboratory.

Preparation of P. lentiscus leaves extract

The extraction of phenolic compounds was carried out according to Markham.[7] Leaves of *P. lentiscus* (100 g) were dried in shadow, powdered and mixed with one liter of methanol-water solution (85:15 v/v) and kept at room temperature for 3 days. The resulting solution was then filtered and the solvent was evaporated under reduced pressure to get crude methanolic extract (CE). The aqueous solution was washed with hexane several times until a clear upper layer of hexane was obtained. The lower layer was then extracted with chloroform and ethyl acetate to obtain chloroform (CHE), ethyl acetate (EAE) and aqueous extracts (AQE). Each fraction was dehydrated and stored at –20°C.

Determination of total phenolic content

Total soluble phenolics in the methanol extract, as well as the chloroform, ethyl acetate and water extract fractions of *P. lentiscus* leaves were determined using Folin–Ciocalteu reagent, according to the Li *et al*[8] with slight modifications. Briefly, 0.1 ml of sample or standard was mixed with 0.5 ml of Folin-Ciocalteau reagent (diluted 10 times). After 4 min, 0.4 ml of 7.5% sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 1 h and 30 min in dark at room temperature. The absorbance of all samples was measured at 760 nm and the results are expressed in mg gallic acid per g (GEA) dry weight of plant material.

Determination of flavonoid content

Total flavonoid contents of each fraction was determined using the aluminum chloride colorimetric method.[8] This assay is based on the formation of a flavonoid–aluminum complex, having the absorbance maximum at 430 nm. Quercetin was used as a standard. 1 ml of diluted sample was mixed with 1 ml of 2% aluminum chloride methanolic solution. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 430 nm and flavonoids content was expressed in mg per g of quercetin equivalent (QE).

DPPH radical scavenging assay

Free radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured using the method described by Burits and Bucar.[9] 50 µl of different dilutions of the extracts were added to 1250 µl of 4% solution DPPH dissolved in methanol. After 30 min at room temperature, the absorbance was measured at 517 nm. BHT and quercetin were used as standards. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = $(A_{control} - A_{sample} / A_{control}) \times 100$ where Abs control is the absorbance without extract and Abs sample is the absorbance in the presence of sample. IC_{50} values (the concentration required to scavenge 50% DPPH free radicals) were calculated.

Hydroxyl radical-scavenging by phenanthroline-Fe (II) oxidation assay

The Fenton reaction is a key reaction in organisms which produces hydroxyl radicals. Hydroxyl radicals can attack

aromatic compound (eg salicylic acid) hydroxylation to form hydroxylation products eg (2, 3-dihydroxyl benzoic acid), which can be detected by colorimetric methods. The adding of antioxidants can reduce hydroxyl radicals and hydroxylation so that the efficiency of scavenging hydroxyl radical can be determined. The ability of the hydroxyl radical-scavenging was carried out as described previously.[10] Briefly, 600 µL of (5 mM) phenanthroline, 600 µL (5 mM) FeSO4, 600 µl of EDTA (15 Mm), 400 µl phosphate buffer (0.2 M, pH = 7.4) and 800 µl (0.01%) H_2O_2 were added into 600 µl of extract. After 1 h of incubation at 37°C, the absorbance at 536 nm was recorded.

Reducing power

Fe^{+3} reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties.[11] The reducing power of the extracted samples (dissolved in different solvents) was determined according to the method of Chung *et al*.[12] A 0.1 ml aliquot of different concentrations of each fraction, were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, to reduce ferricyanide into ferrocyanide. After that, 0.25 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction and centrifuged at 3000 rpm for 10 min. The supernatant (0. 25 ml) was added to distilled water (0.25 ml) and 0.1% ferric chloride (0.5 ml). Absorbance was measured by a spectrophotometer to determine the amount of ferric ferrocyanide (Prussian blue) formed at 700 nm against a blank.

β-carotene/linoleic acid assay

The ability of plant extracts to prevent bleaching of *β*-carotene was assessed as previously described.[13] A stock solution of β-carotene/linoleic acid was prepared by dissolving 0.5 mg of β-carotene in 1 ml chloroform, 25 µl of linoleic acid and 200 mg of Tween 40. The chloroform was completely evaporated under vacuum in a rotatory evaporator at 40°C, then 100 ml of distilled water were added and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. Aliquots (2.5 ml) of the β-carotene/linoleic acid emulsion were transferred to test tubes containing different concentrations. The absorbance of each sample was measured at 470 nm after 0, 1, 2, 4, 6, 24 and 48 hours of incubation. BHT was used as a positive standard. The control tube contained no sample. The antioxidant activity was calculated as following equation: $Abs_{sample}/Abs_{BHT} \times 100$.

Ferric thiocyanate (FTC) method

The FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation[14] with slight modifications. Linoleic acid emulsion (0.02 M) was prepared with linoleic acid (155 µL) and Tween 20 (155 µL) in phosphate buffer (50 ml, 0.02 M, pH 7.4). A reaction solution, containing extracts with different concentrations (0.5 ml), linoleic acid emulsion (2.5 ml), and phosphate buffer (2 ml, 0.02 M, pH 7.0) was placed in a glass vial with a screw cap and mixed with a vortex mixer. The reaction mixture was incubated at 40°C in the dark. To 0.1 ml of reaction mixture, 4.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate were added. Exactly, 3 min after the addition of 0.1 ml of 0.02 M $FeCl_2$ in 3.5% HCl, the peroxide value was determined by recording the absorbance at 500 nm every 24 hours until the absorbance of the control reached a maximum. The positive and negative controls were subjected to the same procedures as the sample, except for the negative control, in which only the solvent was added, and for the positive control in which the sample was replaced with BHT and Vitamin C. The inhibition percentage of linoleic acid peroxidation was calculated as: Inhibition% = (1 – Absorbance of sample at 500 nm/Absorbance of control at 500 nm) × 100.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

Total phenolic and flavonoid contents of the extract and different fractions of *P. lentiscus* were estimated using the Folin–Ciocalteu colorimetric assay and $AlCl_3$ methods respectively. Total phenolic compounds form a blue color complex with phosphomolybdic–phosphotungstic acid reagent with maximum absorbance at 765 nm. The total phenolic content of each extract was expressed as mg gallic acid equivalent per gram dried extract. The results showed that *P. lentiscus* fractions contained phenolic compounds with levels in the following order: CE > AQE > EAE > CHE (Table 1). In the other colorimetric method, aluminum chloride forms acid stable complex with the keto and/or the hydroxyl groups in the A or C ring of flavonoids. In addition, it forms acid labile complexes with orthodihydroxyl groups of the A or B ring of flavonoids. The $AlCl_3$ complexes of flavonoid compounds show strong absorbance at 430 nm and flavonoids with more functional groups absorb stronger at 430 nm.[14] Total flavonoid contents of different *P. lentiscus* fractions were reported as mg quercetin equivalent per gram dried extract (Table 1). Flavonoid contents were in the order: EAE > CE > CHE > AQE. Based on these data, it can be concluded that the CE extract of *P. lentiscus* contains the highest amount

Table 1: Total phenolic and flavonoid contents of *Pistacia lentiscus* L. leaves extracts

Extracts	Total phenolic content (mg GA.Eq/g extract)	Total flavonoids (mg Q.Eq/g extract)
CE	390 ± 0.05	25.53 ± 0.029
CHE	207 ± 0.021	25.23 ± 0.015
EAE	262 ± 0.085	82.37 ± 0.043
AQE	301.5 ± 0.015	13.38 ± 0.03

CE; crud extract, CHE; chloroform extract, EAE; ethyl acetate extract, AQE; aqueous extract.
Results are expressed as means ± SD (n = 3).

of phenolics (390 ± 0.05 mg GA equivalent/g dry crud extract) because most of these compounds are soluble in this hydro-methanolic solution including hydrophilic and hydrophobic molecules (Phenolic acids, flavonoids, high molecular weight phenolics). However, the EAE contains the highest content of flavonoids (82.37 ± 0.043 mg Quercetin/g of dry EAExtract) because this fraction contains most flavonoids according to their polarity and these molecules are more concentrated in this fraction.

DPPH radical scavenging activity

The DPPH is a stable free radical, which has been widely used as a tool for estimating free radical scavenging activities of antioxidants.[15] When the antioxidants react with DPPH, the purple color of the stable free radical changes to a yellow color. The quantity of DPPH reduced was quantified by measuring a decrease in absorbance at 517 nm. The capacity of *P. lentiscus* leaves extracts to scavenge DPPH was measured and the results are shown in Figure 1. All the extracts exhibited an antiradical effect but differed in their inhibiting activities.

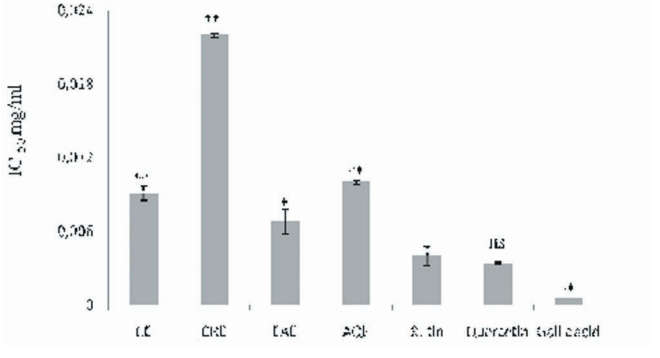

Figure 1. IC_{50} values of plant extracts for free radical scavenging activity by DPPH method. Lower IC_{50} value indicates higher antioxidant activity. CE; crud methanolic extract, CHE; chloroform extract, EAE; ethyl acetate extract, AQE; aqueous extract. Values are means ± SD (n = 3). ns: no significant difference, **($p < 0.001$), * ($p < 0.01$) compared to Rutin as standard.

The highest scavenging activity was observed for the ethyl acetate fraction, with IC_{50} = 0.0068 ± 0.001 mg/ml, whilst the lowest was observed for the chloroform fraction, with IC_{50}= 0.022 ± 0.00017 mg/ml. All extracts were significantly ($p < 0.05$) lower than that of antioxidant references (gallic acid; 0.00058 ± 0.000001 mg/ml, quercetin; 0.0034 ± 0.00005 mg/ml, rutin; 0.004 ± 0.0008 mg/ml). The IC_{50} (DPPH•) values of the extracts increased in the following order: EAE < CE < AQE < CHE. This may be related to the difference in the amount of flavonoid and phenolic compounds in these extracts. Previous chemical studies on *P. lentiscus* have shown the presence of polyphenols, flavonoids and essential oils.[16] The methanol extracts of Turkish *Pistacia terebinthus* exhibited a DPPH scavenging activity of more than 90% from total inhibition at 100 µg/ml compared to BHT.[17] The high scavenging activity of the leaves extracts/fractions may be due to phenolic compounds which possess ideal structural chemistry for free radical scavenging activity. Antioxidant properties of these molecules arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol derived radical to stabilize the unpaired electron.[18]

Hydroxyl radical scavenging activity

The hydroxyl radical is the most reactive of the free radicals and induces severe damage to adjacent biomolecules[19] Phenanthroline-Fe (II) is an oxidation-reduction indicator in common use, where the change of color can be used to indicate the alteration of the state of oxidation–reduction reaction. In the phenanthroline- Fe (II) assay, hydroxyl radicals (OH°) produced in the H_2O_2/Fe^{2+} system can oxidise phenanthrolene-Fe^{2+} into phenanthrolene-Fe^{3+}, and the absorption was measured at 536 nm. Figure 2 shows that all extracts of *P. lentiscus* and vitamin C exhibited scavenging activities against hydroxyl radicals. The radical scavenging activity for *P. lentiscus* leaves plant extracts decreased in the following order EAE> CE> AQE> CHE.

It was found that IC_{50} values of CE (0. 31 ± 0, 0002 mg/ml), CHE (0.52 ± 0.003 mg/ml), and AQE (0.34 ± 0.003 mg/ml) in scavenging abilities on hydroxyl radical, were significantly different ($p < 0.05$) from the IC_{50} values obtained for vitamin C (0.15 ± 0.005 mg/ml). No significant differences ($p > 0.05$) in antioxidant activities were found among vitamin C and EAE. We observed that EAE (0.16 ± 0.008 mg/ml) possess high scavenging against DPPH and OH° radicals. This may be related to the high content of flavonoid in this fraction. Other studies previously reported that aqueous extract of *Pistacia lentiscus* has a high antioxidant potential established in vitro.[20,21] The leaves of *Pistacia lentiscus* contain large amounts of

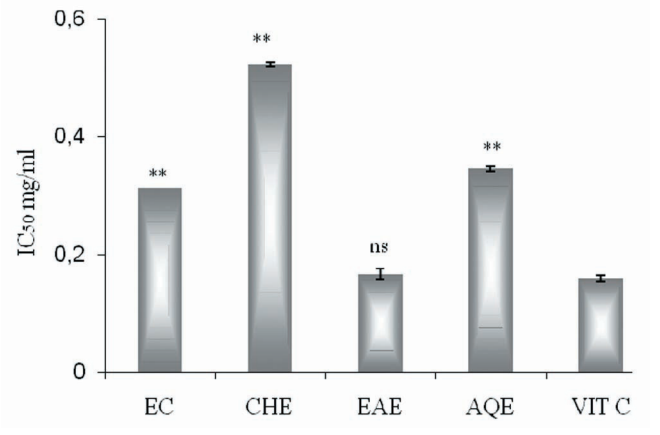

Figure 2. IC_{50} values of plant extracts for hydroxyl radical scavenging activity by phenantrine method. Lower IC_{50} value indicates higher antioxidant activity. CE; crud methanolic extract, CHE; chloroform extract, EAE; ethyl acetate extract, AQE; aqueous extract, VIT C; vitamin C. Values are means ± SD (n = 3). ns: no significant difference, **($p < 0,001$) compared to vitamin C as standard.

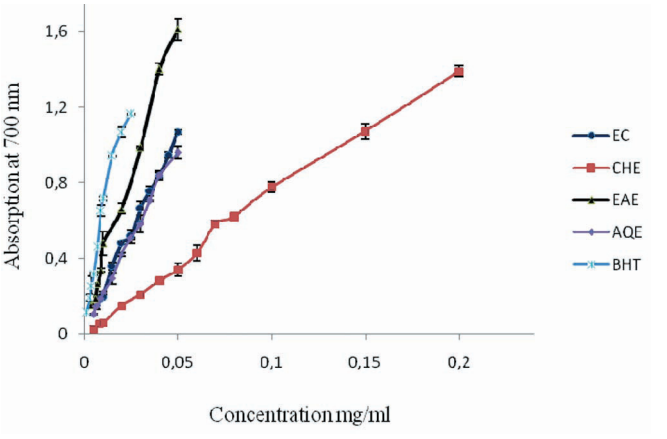

Figure 3. Antioxidant activity of P. lentiscus extracts expressed as reducing power. CE; crud methanolic extract, CHE; chloroform extract, EAE; ethyl acetate extract, AQE; aqueous extract, BHT: Positive standard. Values are means ± SD (n = 3).

gallic acid and galloyl derivatives.[22] These compounds are also efficient antioxidants, capable of scavenging hydroxyl radicals and effectively reducing the extent of oxidation of low density lipoproteins.[20] Therefore, it is not unreasonable to suppose that the widespread use of *Pistacia lentiscus* in traditional Arab medicine can be attributed to the content of these antioxidant phytochemicals.

Reducing power

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties.[23] In this assay, all extracts showed the ability to reduce Fe^{3+} to Fe^{2+}. The increase in the absorbance at 700 nm of the reaction mixture caused by the extracts of *Pistacia* is indicative of their increased reducing power (Figure 3). EAE showed significantly higher reducing potential than other extracts at 0.05 mg/ml with a value of absorption of 1.612 ± 0.057. All the extracts showed a degree of electron donation capacity in a concentration-dependent manner, but the capacities were inferior to that of vitamin C. The reducing power of the extract/fractions may be due to the presence of phenolic compounds in these fractions. Similar relations between Fe^{3+} reducing activity and total phenol content have been reported in the literature,[24] however the correlation may not always be linear as compared to other methods.[25]

β-carotene/linoleic acid assay

In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation. The presence of antioxidants in the extract will minimize the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system will be neutralized by the antioxidants from the extracts. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts. There was a correlation between degradation rate and the bleaching of β-carotene; where the extract with the lowest β-carotene degradation rate exhibited the highest antioxidant activity. Figure 4 shows the inhibition of β-carotene oxidation in the presence of 2 mg/ml of the extracts or reference antioxidant (BHT) after 24 hours of incubation. Most effective were the initial methanolic extract (94.73 ± 1.16%), aqueous fraction (91.12 ± 3.64) and ethyl acetate fraction (84.84 ± 1.98%),

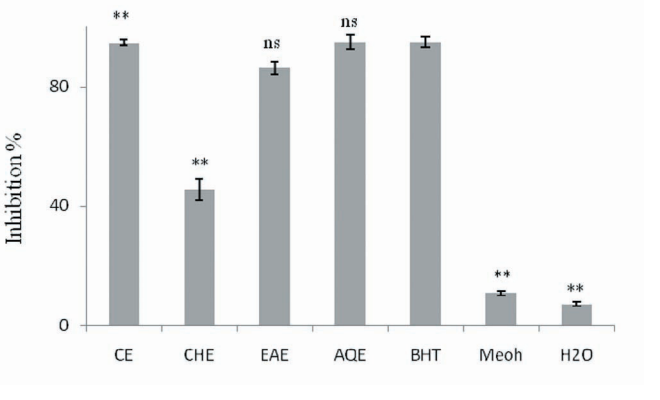

Figure 4. Antioxidant activities of *P. lentiscus* extracts (2 mg/ml at 24 hours of incubation) measured by β-carotene bleaching method. CE; crud methanolic extract, CHE; chloroform extract, EAE; ethyl acetate extract, AQE; aqueous extract. BHT was used as reference antioxidant. Values are means ± SD (n = 3). ns: no significant difference, **($p < 0,001$) compared to BHT as standard.

whereas the effect of the chloroform fraction (42.83 ± 3.69%) was not as strong. CE, EAE and AQE were less effective in comparison with BHT (94.9 ± 2.11%) at the same concentration, but were not significantly different ($p > 0.05$). The results indicated that the extracts had acted as an effective antioxidant in a β-carotene linoleic acid model system. In this study, four extraction mediums were used for preparing *Pistacia* extracts. A previous study had reported that antioxidant activity and the yield of phenolic content were influenced by different extracting solvents.[26]

The antioxidative effectiveness in natural sources has been reported to be mostly due to phenolic compounds. Some authors have found a correlation between the phenolic content and the antioxidant activity,[27] whilst others have found no such relationship.[26]

Ferric thiocyanate (FTC) method

The inhibition of self-oxygenation of unsaturated fatty acids is one of action mechanism in antioxidant activity. The inhibitory effect of different plant extracts and references antioxidant (BHT and vitamin C) were determined spectrophotometrically by thiocyanate method (Figure 5). All leaf extracts/fractions exhibited varying degrees of antioxidant capacity when compared BHT. EAE and CE exhibited good peroxidation inhibiting activity, with 42.7% and 41% respectively against the linoleic acid emulsion system respectively.

In the leaf extract/fractions of *P. lentiscus*, the antioxidant activity decreases in the following order AQE > CHE > CE > EAE after three days of incubation. The difference in antioxidant activities of the leaves extracts of

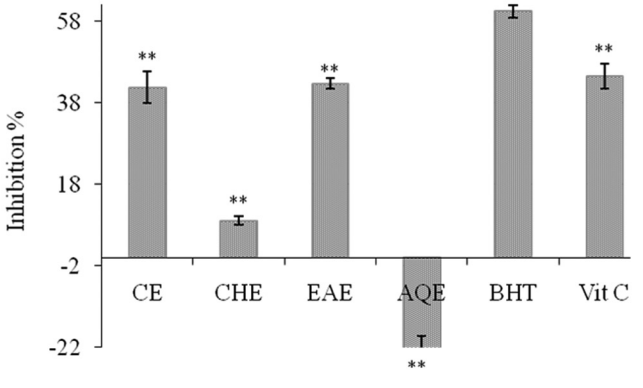

Figure 5. Antioxidant activities of *P. lentiscus* extracts (2 mg/ml at 72 hours of incubation) measured by Ferric thiocyanate (FTC) method. CE; crude extract, CHE; chloroform extract, EAE; ethyl acetate extract, AQE; aqueous extract. BHT and Vitamin C were used as reference antioxidants. Values are means ± SD (n = 3). ns: no significant difference, **(p< 0,001) compared to BHT as standard.

P. lentiscus might be attributed to a difference in total phenolic content. Several studies showed that two compounds (gallic acid and 1,2,3,4,6-pentagalloylglucose) exert strong inhibitory action against lipid peroxidation and no $O_2^{\bullet}$ scavenging properties.[28] These results were similar for some polyphenols, tested by Hanasaki *et al*.[29]

CONCLUSION

This study shows that extracts of *P. lentiscus* leaves contain high levels of total phenolic compounds and were capable of scavenging free radicals to terminate the radical chain reaction, acting as reducing agents and inhibiting lipid peroxidation. To explore the suitability of extracting with solvents with different polarity, we have compared total phenolic content and antioxidant properties of an initial methanolic extract with chloroform, ethyl acetate and aqueous fractions from the initial extract. Ethyl acetate extract showed the highest antioxidant activity using DPPH scavenging, hydroxyl radical scavenging, reducing power and lipid peroxidation inhibition. The ethyl acetate fraction seems to be the most promising source for the isolation of natural antioxidant compounds from the leaves of *P. lentiscus*.

ACKNOWLEDGEMENTS

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MERS) and by the Algerian Agency for the Development of Research in Health (ANDRS). We express our gratitude to these organizations.

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