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Essential oil Composition and Antioxidant Activity of *Pterocarya fraxinifolia*

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Abstract: Current research into free radicals has confirmed that plants rich in antioxidants play an essential role in the prevention of many diseases. The potential antioxidant activities of *Pterocarya fraxinifolia* bark and leaves investigated employing six in vitro assay systems. IC_{50} for DPPH radical-scavenging activities were 3.89±0.09 for leaves and 41.57±1.30 μg mL^{-1} for bark, respectively. The leaf extract exhibited a good reducing power at 2.5 and 80 μg mL^{-1} that was comparable with Vit C ($p>0.05$). The extracts also showed weak nitric oxide-scavenging activity and Fe^{2+} chelating ability. The peroxidation inhibition of extracts exhibited values from 92 to 93% at 72nd h, almost at the same pattern of Vitamin C activity ($p>0.05$). Based on higher total phenol and flavonoid contents in leaves, higher antioxidant activities were observed in leaf extract. In addition, chemical composition of leaf essential oil was determined. The major compound was bisabolol oxide A (23.6%). Sesquiterpenes and monoterpenes are the major compounds in leaves essential oil. Presence of these compounds may be a reason for the good antioxidant activity of leaf extract.

Key words: Antioxidant activity, essential oil composition, Folin-Ciocalteau method, *Pterocarya fraxinifolia*, radical scavenging activity

INTRODUCTION

The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (Nabavi *et al*., 2009b). The harmful action of the free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activity (Zheng and Wang, 2001). The search for newer natural antioxidants, especially of plant origin, has ever since increased. *Pterocarya fraxinifolia* (Juglandaceae) is widely distributed in Northern area of Iran (Botanical Dermatology Database, 1999). Native people use the leaf of this tree as an anesthetic agent for catching fish (Azadbakht *et al*., 2005), dyeing and as an antifungal agents (Hadjmohammadi and Kamel, 2006). Liss information is available about its chemical composition and biological activity. Good antibacterial and antioxidant activities were reported by bioautographic (TLC) method (Azadbakht *et al*., 2005). In addition, good antioxidant activity in thiobarbituric acid model has been reported from *P. fraxinifolia* (Souri *et al*., 2004). Coumaroylspermidine have been isolated from *P. fraxinifolia* (Meurer *et al*., 1988). Recently, a new distribution of this species has been explained (Saribaz *et al*., 2007; Avsar and Ok, 2004). Reaction of walnut to Phytophthora species has been investigated recently (Banihashemi and Ghaderi, 2006). Juglone, a naphthoquinone compound, have extracted from leaf and hulls of *P. fraxinifolia* recently (Hadjmohammadi and Kamel, 2006). In this study, we examined the antioxidant activity of *P. fraxinifolia* bark and fresh leaf, employing various in vitro assay systems, such as DPPH and nitric oxide radical scavenging, reducing power, linoleic acid and iron ion chelating power, in order to evaluate its antioxidant activity in other tests and to understand the mechanism of action of its activity and usefulness of this plant in our living. In addition, chemical composition of leaf essential oil was determined.

MATERIALS AND METHODS

This study was performed during the summer and autumn 2008 in Pharmaceutical Sciences Research Center, School of pharmacy, Mazandaran University of Medical Sciences, Sari, Iran.

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Plant material and preparation of freeze-dried extract: Bark and fresh leaf of *P. fraxinifolia* were collected from Sari forest, Iran, in summer 2008. After identification of the plant by Dr. Bahman Eslami a voucher (No. 870 and 871) has been deposited in the Sari School of Pharmacy herbarium. Leaf and bark were dried at room temperature and coarsely ground before extraction. A known amount of each part was extracted at room temperature by percolation method using methanol. The resulting extract was concentrated over a rotary vacuum until a crude solid extract was obtained. The bark extract, 5% and leaf extract, 23% were obtained, respectively.

Isolation of the essential oil: The air dried and ground leaf of plant collected was submitted for 3 h to water-distillation using a British-type Clevenger apparatus. The obtained essential oil (0.5%) was dried over anhydrous sodium sulphate and after filtration, stored at 4°C until tested and analyzed.

Chemicals: Ferrozine, Linoleic acid, trichloroacetic acid (TCA), 1,1-diphenyl-2-picryl hydrazyl (DPPH), potassium ferricyanide were purchased from Sigma Chemicals Co. (USA). Gallic acid, quercetin, Butylated hydroxyanisole (BHA), ascorbic acid, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Gas Chromatography-Mass Spectrometry (GC-MS): GC-MS was carried out using a Hewlett-Packard 5975B series instrument and a Agilent 19091J-433 HP-5 capillary column (30 m, 250 μm i.d., film thickness 0.25 μm) which was programmed to 50°C for 10 min, then rising at 4°C min^{-1} to 240°C; carrier gas, helium at a flow rate of 1 mL min^{-1}. split ratio, 1:10; ionization energy, 70 eV; scan time, 1 sec; acquisition mass range, m/z 40-400. The compounds were identified according to their retention indexes and by comparison of their mass spectra with those of a computer library or with authentic compounds (Dehpour *et al*., 2009).

Determination of total phenolic compounds and flavonoid content: Total phenolic compound contents were determined by the Folin-Ciocalteau method (Ebrahimzadeh *et al*., 2008b; Nabavi *et al*., 2008a; Ebrahimzadeh *et al*., 2009b). The extract samples (0.5 mL) were mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 mL of 75 g L^{-1} sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Results were expressed as gallic acid equivalents. Total flavonoids were estimated using $AlCl_3$ method (Ebrahimzadeh *et al*., 2008b; Nabavi *et al*., 2008a). Briefly, 0.5 mL solution of each plant extracts in methanol were 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 and left at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid contents were calculated as quercetin from a calibration curve.

DPPH radical-scavenging activity: The stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the extracts (Ebrahimzadeh *et al*., 2008a; Nabavi *et al*., 2008b; Ghasemi *et al*., 2009). Different concentrations of each extracts were added, at an equal volume, to methanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Reducing power determination: The reducing power of *P. fraxinifolia* was determined according to our recently published paper (Nabavi *et al*., 2009b). About 2.5 mL of each extracts (25-800 μg mL^{-1}) in water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Assay of nitric oxide-scavenging activity: The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of each extracts dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract but with an equivalent amount of water, served as control. After the incubation period, 0.5 mL of Griess reagent was added. The absorbance of the

chromophore formed was read at 546 nm. Quercetin was used as positive control (Ebrahimzadeh *et al*., 2009c).

Metal chelating activity: The chelating of ferrous ions by *P. fraxinifolia* was estimated by our recently published paper (Ebrahimzadeh *et al*., 2008c, 2009a). Briefly, the extract (0.2-3.2 mg mL^{-1}) was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine- Fe^{2+} complex formation was calculated as:

$$\frac{A_0-A_s}{A_s}\times 100$$

where A_0 was the absorbance of the control and A_s was the absorbance of the extract/ standard. Na_2EDTA was used as positive control.

Determination of Antioxidant Activity by the FTC Method: The inhibitory capacity of *P. fraxinifolia* extracts against oxidation of linoleic acid by FTC method was tested. This method was adopted from Osawa and Namiki (1981) (Ebrahimzadeh *et al*., 2009b; Nabavi *et al*., 2009a). Twenty Milliliter per liter of samples dissolved in 4 mL of 95% (w/v) ethanol were mixed with linoleic acid (2.51%, v/v) in 99.5% (w/v) ethanol (4.1 mL), 0.05 M phosphate buffer pH 7.0 (8 mL) and distilled water (3.9 mL) and kept in screwcap containers at 40°C in the dark. To 0.1 mL of this solution was then added 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured and it was measured again every 24 h until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as:

$$\text{Inhibition (\%)} = 100 - \frac{\text{Absorbance increase of the sample}}{\text{Absorbance increase of the control}}\times 100$$

All tests were run in duplicate and analyses of all samples were run in triplicate and averaged. Vit C and BHA used as positive control.

Statistical analysis: Experimental results are expressed as Means±SD. All measurements were replicated three times. The data were analyzed by an analysis of variance ($p<0.05$) and the means separated by Duncan's multiple range test (by InStat3 software). The EC_{50} values were calculated from linear regression analysis.

RESULTS

Chemical composition of the essential oil: Thirty three compounds were identified, representing 94.24% of the total oil. Oil yield of plant was determined as 0.54% v/w. as a result of GC-MS analysis the major compound was bisabolol oxide A (23.6%). Additionally, other major compounds were hexadecanoic acid dihydroxypropyl ester (14.4%) and bisabolone oxide (6.62%). Sesquiterpenes and monoterpenes are the major compound in leaves essential oil Table 1.

Total phenol and flavonoid contents: Total phenol compounds, was determined by folin Ciocalteu method and reported as gallic acid equivalents, by reference to standard curve ($y = 0.0063x$, $R^2 = 0.987$). The total phenolic contents of leaf and bark were 429.51±13.60 and 88.53±2.20 mg gallic acid equivalent/g of extract powder, respectively. The total flavonoid contents of leaf and bark

Table 1: Chemical composition of the essential oil of *Pterocarya fraxinifolia* leaf

Components	K.I	Composition (%)
4-Decen-6-yne	994	0.07
R-Limonen	1094	1.07
Hi-oleic safflower oil	1146	1.41
1-Menthen-8-yl acetate	1180	0.10
Cyclocitral	1208	0.37
2-Pinene	1231	0.43
Octadecatrienoate metyl ester	1303	0.09
Naphthalene 1,4-dihydro-2,5,8- trimethyl	1337	0.25
Heptadecadiene-4,6-diyn-8-ol	1377	0.66
trans-Caryophyllene	1403	4.81
Oxacyclotetradeca-4,11-diyne	1417	0.42
α-Humulene	1428	0.05
Undecadien-2-one, 6,10-dimethyl	1446	3.80
Pteridinetriamine, 6-methyl	1451	2.18
Cubebene	1468	4.98
Ionone	1472	1.28
Cholic acid	1480	0.33
Methyl-cis-decalin-1,8-dione	1509	1.13
Dehydroaromadendrene	1562	4.01
Nerolidol-epoxy acetate	1641	5.65
Bisabolone oxide	1667	6.62
Cyclohexane, (1-hexadecylheptadecyl)	1699	0.66
Naphthalene, 1-methyl-7-(1-methylethyl)	1706	1.35
Bisabolol oxide A	1736	23.60
Acetoxy-16-ketocinobufagin	1826	0.44
Dideutero octadecanal	1832	2.44
Dioxocane, 2-pentadecyl	1872	0.89
Dimethyl (tetramethyl heneicosa pentaenyl) oxirane	1901	2.37
Hexadecanoic acid dihydroxypropyl ester	1987	14.40
Flavone-4'-OH, 5-OH, 7-di-O-glucoside	2097	4.19
Ethanol, 2-(9,12-octadecadienyloxy)	2112	0.60
Phenyl 1,3-dioxolane-4-methyl octadec-9,12,15-trienoate	2138 2.18	
Nanacosane	2487	1.41
Total		94.24

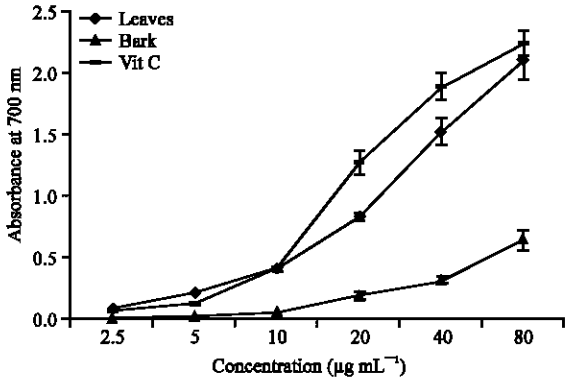

Fig. 1: Reducing power of methanol extract of *P. fraxinifolia*. Vitamin C used as control

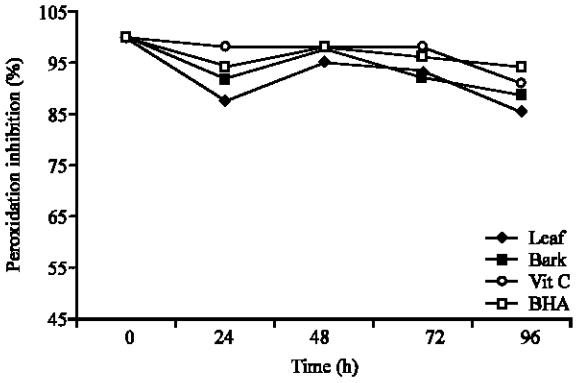

Fig. 2: Antioxidant activity of *P. fraxinifolia* in FTC method at different incubation times. Leaf and bark methanol extract in 0.4 mg mL^{-1}, Vitamin C and BHA in 0.1 mg mL^{-1}

were 24.32±0.98, 11.82±0.27 mg quercetin equivalent/g of extract powder, respectively, by reference to standard curve ($y = 0.0067x + 0.0132$, $R^2 = 0.999$).

DPPH radical-scavenging activity: It was found that the radical- scavenging activities of extracts increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was in the order: leaf (3.89±0.09) > bark (41.57±1.30) µg mL^{-1}, respectively. Based on the IC_{50} results, according to higher total phenol and flavonoid contents, it was shown that leaf extract had more than 10 times higher DPPH-scavenging activity than bark extract. The IC_{50} values for Ascorbic acid, quercetin and BHA were 1.26±0.11, 1.32±0.07 and 13.49±1.04 µg mL^{-1}, respectively.

Reducing power of *P. fraxinifolia* extracts: It was found that the reducing powers of extracts also increased with the increase of their concentrations. There were significant differences ($p<0.001$) among the different extracts in reducing power. The leaf extract had shown better reducing power than bark extract. The leaf extract exhibited a good reducing power at 2.5 and 80 µg mL^{-1} that was comparable with Vitamin C ($p> 0.05$). The dose-response curves for reducing powers of the extracts have been showed in Fig. 1.

Assay of nitric oxide-scavenging activity: The extracts showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg mL^{-1}. The % inhibition was increased with increasing concentration of the extract. The leaf extract had shown better reducing power than bark extract (IC_{50} were 65±3 for leaf and 985±35 µg mL^{-1} for bark) vs. 17.01±0.03 µg mL^{-1} for quercetin that used as control.

Fe^{2+} chelating activity of *P. fraxinifolia* extracts: The chelating of ferrous ions by the extract was estimated by the method of Dinis (Ebrahimzadeh *et al*., 2008c). The absorbance of Fe^{2+} -ferrozine complex was decreased dose-dependently, i.e. the activity was increased on increasing concentration from 0.2-3.2 mg mL^{-1}. *Pterocarya fraxinifolia* extracts showed weak Fe^{2+} chelating ability. IC_{50} were 1.81±0.14 for leaf and 1.33±0.09 for bark mg mL^{-1}. EDTA showed very strong activity (IC_{50} = 18 µg mL^{-1}).

FTC method: The peroxidation inhibition (antioxidant activity) of extracts exhibited values from 95.1 to 97.7% (at 48th h) and from 92 to 93% (at 72nd h). All tested extracts exhibited high antioxidant activity. There were no significant differences ($p>0.05$) among extracts in antioxidative activity. All of extracts manifested almost the same pattern of activity as Vitamin C at different incubation times ($p>0.05$) (Fig. 2).

DISCUSSION

The Folin-Ciocalteu reagent is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic and polyphenolic antioxidants (Singleton *et al*., 1999). It works by measuring the amount of the substance being tested needed to inhibit the oxidation of the reagent (Vinson *et al*., 2005). Total phenol compounds, as determined by Folin Ciocalteu method and reported as Gallic acid equivalents, by reference to standard curve. The total flavonoid contents determined as mg quercetin equivalent/g of extract powder by reference to standard curve. Leaf extract had significant higher total phenol and flavonoid contents than did bark extract ($p<0.001$). Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived

from plant sources and they have been shown to possess significant antioxidant activities (Van Acker *et al*., 1996). Studies have shown that increasing levels of flavonoids in the diet could decrease certain human diseases (Hertog *et al*., 1993).

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples (Lee *et al*., 2003). DPPH is a stable nitrogen-centered free radical the color of which changes from violet to yellow upon reduction by either the process of hydrogen- or electron- donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers (Brand-Williams *et al*., 1995). It was found that the radical- scavenging activities of extracts increased with increasing concentration. According to higher total phenol and flavonoid contents of leaf, this part showed more than 10 times higher DPPH-scavenging activity than bark extract. Such a good radical-scavenging activities has been reported by bioautographic (TLC) method from its aerial parts (Azadbakht *et al*., 2005). In the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability (Ebrahimzadeh *et al*., 2008b). It was found that the reducing powers of extracts also increased with the increase of their concentrations. There were significant differences ($p<0.001$) among the two extracts in reducing power. The leaf extract showed better reducing power than bark extract. The leaf extract exhibited a good reducing power at 2.5 and 80 μg mL^{-1} that was comparable with Vit C ($p>0.05$). Because the reductive abilities of the leaf extract of *P. fraxinifolia* were significantly comparable to Vit C, it was evident that *P. fraxinifolia* did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

The extracts showed weak nitric oxide-scavenging activity between 0.1 and 1.6 mg mL^{-1} but leaf extract was better than bark extract. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada *et al*., 1991). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. Iron chelators mobilize tissue iron by forming soluble, stable complexes that are then excreted in the feces and/or urine. Chelation therapy reduces iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as Thalassemia major (Hebbel *et al*., 1990). In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in Alzheimer's disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD (Reznichenko *et al*., 2006). Foods are often contaminated with transition metal ions which may be introduced by processing methods. The transition metal, e.g. iron, is capable of generating free radicals from peroxides by Fenton reactions and may be implicated in human cardiovascular disease (Halliwell and Gutteridge, 1990). Because Fe^{2+} also has been shown to cause the production of oxyradicals and lipid peroxidation, minimizing Fe^{2+} concentration in Fenton reactions affords protection against oxidative damage. The chelating of ferrous ions by the extract was estimated by the method of Dinis (Ebrahimzadeh *et al*., 2008c). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. In this assay, both extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+} -ferrozine complex was decreased dose-dependently. Metal chelating capacity was significant since the extract reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh *et al*., 1999). It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gordon, 1990). *Pterocarya fraxinifolia* extracts showed weak Fe^{2+} chelating ability.

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (Yu, 2001). The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (Ordonez *et al*., 2006). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Figure 2 shows the time-course plots for the antioxidative activity of the different extracts of *P. fraxinifolia* using the FTC method. All tested extracts exhibited high antioxidant activity. There were no

significant differences ($p>0.05$) among extracts in antioxidative activity. All of extracts manifested almost the same pattern of activity as Vit C at different incubation times ($p>0.05$). Such a strong peroxidation inhibition activity has been reported recently by Souri *et al.* (2004) by diethyl thiobarbituric acid test (IC_{50} = 2.60±0.11 μg).

The results of GC-MS analysis improved the sesquiterpenes and monoterpenes are the major compound in leaves essential oil. Presence of bisabolol oxide A and bisabolol oxide (about 1/3 of total essential oil) may be a reason for good antioxidant activity of leaf extract.

CONCLUSION

The leaf and bark extracts of *P. fraxinifolia* exhibited good but different levels of antioxidant activity in all the models studied. The extracts had good reducing power, anti-lipid peroxidation activity and DPPH radical-scavenging activity. These good activities may introduce its leaf as a useful medicinal plant. Further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is needed.

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