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Identification of Volatile Components and Biological Properties of the extracts of aerial parts of *Pterocarya fraxinifolia* L.

Zahra Aghajani*, Ali-Asghar Engashte-Vahed* and Maryam Akhbari**
**Department of Chemistry, Qom Branch, Islamic Azad University, Qom, IRAN.*
***Essential oil research institute, University of Kashan, Kashan, IRAN.*

(Corresponding author: Zahra Aghajani)
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ABSTRACT: The volatile components of hexane extract of the leaf and stem of the *Pterocarya fraxinifolia* L., a native plant of the northern Iran Caspian coastal, obtained by cold percolation method and analyzed using GC/FID and GC/MS and their antioxidant and anti-bacterial activity were assessed. Palmitic acid (53.66 %) and 9, 17-Octadecadienal (22.36%) were the main components of the stem hexane extract and Aromadendrene and 9, 17-Octadecadienal in the leaves hexane extract of the plants with 27.86 % and 18.49 % were the major components respectively. The number of identified compounds in the volatile components of hexane leaves extract was 11 while the number in hexane stem extract was 9. In this research antioxidant activity of hexane and acetone extracts of leaves and stems of the *P. fraxinifolia* were evaluated via DPPH radical scavenging and beta-carotene bleaching assays and also the antibacterial properties of these extracts against five strains of bacteria were assessed.

Keywords: *Pterocarya fraxinifolia*, volatile components, antibacterial activity, antioxidant activity

INTRODUCTION

Natural plant products have been used throughout human history for various purposes. Having coevolved with life, these natural products are billions of years old. Tens of thousands of them are produced as secondary metabolites by the higher plants as a natural defense against disease and infection.

Pterocarya fraxinifolia (*P. fraxinifolia*) belongs to the juglandaceae family; a large tree with cloven bark, dark grey color, and as tall as 35 meters. The plant is a fast-growing tree species naturally distributed throughout Western Black Sea Region of Turkey and is native to the Caucasus from northern Iran to the southern Ukraine (Nabavi *et al.,* 2008). It is an indigenous plant found in northern Iran. Local people use leaves of this tree as an anesthetic agent for fishing, hair dye, and treatment of tinea as well as other parasites (Nabavi *et al.,* 2008; Hadjmohammadi and Kamel 2006).

Despite being widely spread, there is limited information on the features of this plant in the literature, especially the chemical and biochemical properties of *P. fraxinifolia*. To the best of our knowledge, there is only one report on the volatile components of the leaves and stem and their effects by Ebrahimzadeh *et al.,*(2009) for *P. fraxinifolia* in Mazandaran province; (Ebrahimzadeh *et al.,*2009). The high antioxidant activity for the leaves of this plant was among the other reports by the same group and others in the same province (i.e. Mazandaran) (Ebrahimzadeh *et al.,* 2009; Ebrahimzadeh *et al.,* 2010; Souri *et al.,* 2009). In the other report Sadighara, *et al.,* (2009) have studied toxicological effect of *P. fraxinifolia* on the chicken embryo (Sadighara *et al.,* 2009).

In this research the composition of the hexane extract of leaves and stem of *P. fraxinifolia* from Gilan province, north of Iran, were assessed and antioxidant activity of hexane and acetone extracts of leaves and stems of this plant were evaluated via DPPH radical scavenging and beta-carotene bleaching assays in a competitive reaction in the proximity of Hydroperoxide linoleic acid. Also the antibacterial properties of these extracts against five strains of bacteria by disc diffusion method were assessed.

MATERIAL AND METHODS

A. Materials

Leaves and stems from *P. fraxinifolia* were collected from Gilan province, north of Iran, coastal province year 2013.Samples were deposited in the herbarium of Research Institute of forests and Rangelands, Kashan, Iran (Voucher No. KBGH 8114). Samples were dried and subsequently ground in a blender to obtain fine powder. All reagents and chemicals used in this study were from Merck or Sigma Companies.

B. Method of extraction

Plant extracts were prepared by cold percolation method. The plant materials were dried under shade and ground into fine powder using electric blender. Ten gram of dried powder was taken in 300 ml of solvent in a conical flask, for 48 hours with intermittent shaking. After that the extract was filtered through Whatman No. 1 filter paper and then the solvent removed by using a rotary evaporator and then dried until a constant dry weight of each extract was obtained. The residues were stored at 4°C for further use (Kalia *et al.*, 2002).

(i) Gas Chromatography/Mass spectroscopy (GC/MS): Analytical gas chromatography of the volatile components was carried out using a Hewlett-Packard 5975B series gas chromatograph with Agilent HP-5 capillary column (30 m × 0.25 mm, f.t 0.25 µm); carrier gas, He; split ratio, 1:10, and using a flame ionization detector. The column temperature was adjusted at 50°C for 10 min and programmed to rise up to 240°C at a rate of 4°C/min and then kept constant at 240 °C for 15 min. GC/MS was performed on a HP 5975B with a Hewlett-Packard 5973 quadruple detector, on capillary column HP-5 (30 m × 0.25 mm; f.t 0.25 µm); carrier gas; He, flow rate; 1ml/min. The column was held at 50°C for 10 min and programmed up to 240°C at rate of 4°C/min, and then kept constant at 240° C for 15 min.

The MS operated at 70eV ionization energy. Retention indices were calculated using retention time of n-alkanes that were injected after volatile components at the same chromatographic conditions. Quantitative data were obtained from the electronic integration of the FID peak areas. The components of hexane extracts were identified by comparing their mass spectra and kovats indexes indicate with Wiley library and those published books, data bases available and websites (Adams, 2001).

Antimicrobial activity: Antibacterial activity of the samples was investigated against Gram-positive bacteria *Staphylococcus aureus* (ATTC 29737), *Bacillus subtilis* (PTCC 1023) and *Staphylococcus epidermidis* (ATCC 12228) as well as Gram-negative bacteria *Salmonella entericaserovar Paratyphi* A (PTCC 1230) and *Escherichia coli* (ATCC 10536). Activities were assessed by measuring the growth inhibition zone diameter applying agar disc diffusion method (NCCLS 1997). Following to dissolution of the dried plant extracts in DMSO to a final concentration of 30 mg/ml, filtration was done by passing the extracts through 0.45 µm Millipore filter for sterilization. Antimicrobial activity was determined using 100 µl of suspension containing 10^8 CFU/ml of bacteria spread onto the nutrient agar and potato dextrose agar media, respectively. 10 µl of the each extract containing 300 µg/disc of the sample were applied to the discs (6mm in diameter) placed on agar plates. DMSO was used as negative control in the assessment of the antimicrobial activity. Following to 24 h at 37°C for bacterial strains (Huang *et al.*, 2011) the inhibition zone around each filter disc was measured. Each test was done twice.

Antioxidant activity:

(i) DPPH radical assay: Radical-scavenging activities of the plant extract were determined using a published DPPH radical scavenging activity assaying method with minor modifications (Foti *et al.*, 2004, Huang *et al.*,2005, Garcia *et al.*, 2012). Briefly, stock solutions (10 mg/ml) each of the extract and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions were made to obtain concentrations ranging from 0.8 to 5×10^{-4} mg/ml. Diluted solutions (1ml) each were mixed with 1 ml of a freshly prepared 1mg/ml DPPH radical methanol solution and allowed to stand for 30 min in the dark at room temperature for reactions to take place. Absorbance values of these solutions were recorded on an ultraviolet and visible (UV-Vis) spectrometer at 517 nm using a blank containing the same concentration of the extract or BHT without DPPH radicals. Inhibition of DPPH radical in percent (I %) was calculated as follows:

$$I\% = [(A \text{ blank} \_ A \text{ sample}) / A \text{ blank}] \times 100$$

Where A blank is the absorbance value of the control reaction (containing all reagents except the test compound) and A sample is the absorbance values of the test compounds. The sample's concentration is expressed in terms of IC_{50} which was calculated by drawing the chart of inhibitory percentages against concentrations of the sample. All the tests were carried out in triplicate and IC_{50} values were reported as means ± SD.

(ii) β-carotene /linoleic acid bleaching assay: In the β-carotene /linoleic acid test, the antioxidant competes with β-carotene for transferring hydrogen atoms to the proxy radicals (R1R2HCOO.) formed from the oxidation of linoleic acid in the presence of molecular oxygen (O_2) and converts them to hydroperoxides (R1R2HCOOH) leaving the β-carotene molecules intact (Huang *et al.*, 2005). Assaying the remained β-carotene gives an estimation of antioxidative potential of the sample. A mixture of β -carotene and linoleic acid was prepared by adding 0.5 mg of β-carotene to 1 ml of chloroform (HPLC grade), 50 mg of linoleic acid and 200 mg of Tween 40. The chloroform was then completely evaporated under vacuum and 100 ml of oxygenated distilled water were subsequently added to the residue and mixed gently to form a clear yellowish emulsion. The extract and BHT (positive control) were individually dissolved in methanol (2 g/l) and 350 µl of each of them were added to 2.5 ml of the above mentioned emulsion in test tubes and mixed thoroughly.

The test tubes were incubated in a water bath at 50 °C for 2 h together with a negative control (blank) that contained the same volume of methanol instead of the extracts. The absorbance values were measured at 470 nm on an ultraviolet and visible (UV-Vis) spectrometer. Antioxidant activities (inhibition percentages, I %) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{ -carotene after 2 h assay}} / A_{\text{initial }\beta\text{ -carotene}}) \times 100$$

Where $A_{\beta\text{ -carotene}}$ after 2 h assay is the absorbance values of β -carotene after 2 h assay remaining in the samples and A initial β-carotene is the absorbance value of β-carotene at the beginning of the experiments. All the tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

RESULTS AND DISCUSSION

The number of identified volatile compounds in the hexane extract of leaves was 11 while the number in hexane stem extract was 9 which were separated and identified by GC/FID and GC/Mass analysis. Total identified volatile constituents of the hexane extracts were 84.28% and 92.29% for the leaves and stem respectively (Table 1).

After studying the hexane extract volatile compounds from the leaves and stem, a great difference was observed in the obtained compounds. For instance the primary of volatile components in hexane extract of the leaves were Aromadendrene (27.86%) and 9, 17-Octadecadienal (18.49%), while the primary compounds in the stem were Palmitic acid (53.66%) and 9, 17- Octadecadienal (22.36%) (Table 1). As shown in Table 3, volatile components of hexane leaf extract has a smaller average molecular weight and low average boiling point than the stem volatile components, this makes the high solubility of these compounds in comparison with components of hexane stem extract. According to Table 2, it can be concluded that the terpenic compounds in the leaves extract of this plant is higher than other combinations (53.39%), however there is a very high percentage of non terpenic volatile compounds in hexane extract of the stem (92.80%). These results can be due to physiological differences in leaf structure as compared to the stem. In general, since the secondary metabolism and photosynthesis is done more in leaves to stem organs, compounds with low boiling point and less molecular weight, seems normal.

Table1: Volatile components of hexane extract of stem and leaves of *P. fraxinifolia*

NO	Components	Hexane extract stem	Hexane extract leaf	RI [a]
1	Isopropyl acetone	0.21	-	740
2	Octane	1.24	1.27	840
3	o-Xylene	-	8.22	957
4	trans - limonene oxide	-	3.32	1139
5	Alloaromadendrene	-	2.89	1461
6	Aromadendrene	-	27.86	1480
7	1-Tridecanol	0.85	-	1498
8	α-Curcumene	-	5.74	1500
9	α-Caryophyllene	-	3.68	1506
10	α-Zingiberene	-	8.22	1512
11	Palmitic acid	53.66	-	1994
12	Phytol	-	1.68	2140
13	9,17- Octadecadienal	22.36	18.49	2178
14	Octadecanoic acid	5.19	-	2184
15	1-Octadecanol	1.35	-	2302
16	Hexanedioic acid, dioctyl ester	0.28	2.91	2309
17	Hexanedioic acid, bis(2-ethylhexyl) ester	7.15	-	2400

RI[a] : Retention index on a HP-5 MS column

Table 2: The category of volatile components of hexane extracts of *P. fraxinifolia*

Category	Hexane leaf extract	Hexane stem extract
Non terpenoid oxygenated	21.80	91.56
Non terpenoid hydrocarbons	9.49	1.24
Mono terpenoid hydrocarbons	5.74	-
Mono terpenoid oxygenated	3.32	-
Sesquiterpenoid hydrocarbons	42.65	-
Oxygenated diterpenoid	1.68	-

Table 3: Comparison of volatile components of hexane extracts of *P. fraxinifolia*by three measured parameters.

Parameter measured	Hexane stem extract	Hexane leaf extract
Average molecular weight	247.97	211.26
Average Solubility in water (In 25 ^{o}C , ppm)	2.04	28.75
Average boiling point (In 760 mm Hg)	276.87	212.86

Table 4:Antioxidant activities of positive control (BHT) and extracts of *P. fraxinifolia.*

Sample	DPPH IC_{50} (µg/ml)	β-carotene /linoleic acid Inhibition (%)
Acetone leaf extract	707± 0.75	50.45±0.29
Acetone stem extract	841.5±0.68	82.26±0.64
Hexane leaf extract	900≤	33.47±0.57
Hexane stem extract	900 ≤	44.71±0.58
BHT	19.82 ± 0.52	88.34 ± 0.71
Negative control	NA	5.5 ± 0.52

Table5: Antibacterial activity of acetone and hexane extracts of *P. fraxinifolia.*

Bacterial species	**Source**	**acetone stem extract**	**acetone leaf extract**	**Hexane leaf extract**	**Hexane stem extract**
Gram positive bacteria					
Staphylococcus aureus	(ATTC 29737)	12	14	-	10
Bacillus subtilis	(PTCC 1023)	-	10	-	-
Staphylococcus epidermidis	(ATCC 12228)	12	14	-	-
Gram-negative bacteria					
Salmonella enterica serovar Paratyphi A	(PTCC 1230)	11	11	14	-
Escherichia coli	(ATCC 10536)	11	12	-	-

The bleaching test of acetone stem extract of this plant showed more antioxidant activity compared to leaves acetone extract, so that its inhibition power is similar to that of the BHT. Overall, extracts of this plant showed no significant antioxidant effect.

According to the results reported in Table 4 and Table 5, the hexane extracts of this plant has lower antioxidant and antibacterial effect in comparison with acetone extract. This may reflect the effect of solvent polarity on the extracted components (dipole moment of acetone is 2.91 D and for hexane is 0.08).

Although hexane extract of the *P. fraxinifolia* contains compounds like Aromadendrene α-Zingiberene and α-Curcumene with antifungal, antibacterial and antiviral properties hexane extract of this plant does not show good antibacterial effect. This may be related to the complex composition of the extract (synergic effect).

Based on these results, it can be concluded that the plant acetone extract have potential as antimicrobial compounds against microorganisms and can be used in the treatment of infectious diseases caused by resistant microorganisms.

Another interesting points is that the volatile components in hexane extract of the stem of this plant containing more than 50% Palmitic acid. Therefore, hexane extract of this plant is applicable in food and pharmaceutical industries.

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