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Evaluation of Biological Activity and Analysis of Volatile Fraction from *Pterocarya fraxinifolia* in Vegetative Stage from Iran

M. Akhbari*, S. Tavakoli, Z. Ghanbari, M. Dadgarnia and A. Mazoochi

Essentialoils Research Institute, University of Kashan, Kashan, Islamic Republic of Iran

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Abstract

This study reports chemical composition of the essential oils and *in vitro* antioxidant, antimicrobial and cytotoxic activity of the volatile fraction, extracted using simulations steam distillation-solvent extraction (SDE) method, and methanolic extract from the stems and young leaves of *Pterocarya fraxinifolia* from Gilan, west-north of Iran for the first time. The extraction yield of volatile fraction from stems was about two times more than that of leaves. GC and GC/MS analysis of the stem oil, exhibited 44 components; the most abundant constituents was hexadecanoic acid. On the other hand, 23 components were identified in the oil from leaves, with 3¸7-guaiadiene as the major components. The oils and extracts from both examined plant samples showed excellent antioxidant activities in 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (IC_{50} values <45 µg/mL). In ß-carotene bleaching assay, only the extracts showed high activities with inhibition percentages more than 80%. Total phenolic contents, based on gallic acid equivalent, for the leaves and stems extracts were also very high. Screening of cytotoxic activity of the extracts via brine shrimp lethality assay exhibited higher activity for stem. About antimicrobial properties, each sample showed high activity against at least 1 tested microorganism. In comparison, stem extract and oil exhibited higher biological activities than that of leaves.

Keywords: *Pterocarya fraxinifolia*; Essential oil analysis; Antioxidant activity; Antimicrobial activity; Brine shrimp bioassay.

Introduction

Recently, there is growing interest in naturally-occurring antioxidants that can be used to protect human beings from oxidative stress damage. Herbs and species are amongst the most important targets to search for natural antioxidants for their safety considering. Similarly, many scientific researches focused on finding new natural antimicrobial agents to achieve novel antibiotics with no antimicrobial resistance. Antimicrobial and antioxidant properties are caused of many active phytochemicals. Many reports demonstrate that polyphenolic compounds, which are the secondary metabolites of plants, have high antioxidant and antimicrobial potential. *Pterocarya fraxinifolia* belongs to the family juglandaceae is a huge tree, cloven bark,

* Corresponding author: Tel/Fax: +9855643292; Email: m_akhbari@kashanu.ac.ir

dark grey colour with height 35 m [1]. It is a beautiful tree and also an attractive garden and street plant because of its wide crown, pendulous fruiting spikes and large pinnate leaves. The plant is a fast-growing tree species, naturally distributed throughout western region of Black Sea in Turkey and is native to the Caucasus from northern Iran to the Ukraine [2,3]. In Iran, This species grows wildly in Golestan, Gillan and Mazandaran provinces; also in recent years were reported in Ilam and Lorestan provinces [1]. It has been shown that the leaves of this plant have antimicrobial activity against *Bacillus subtilis*, *Candida albicans* and *Cladosporium cucumericum*, as well as larvicidal activity against *Aedes aegypti* [4]. Native people also have used its young leaves as anesthetic agent for catching fish [5], dyeing and antifungal agent for a long time [2-4]. Sudorific property is the only remedy effect that has been reported for this plant [2,3].

As far as existing active components were concerned, 5-hydroxy-1,4-naphthoquinone components or juglone that shows high antimicrobial activity against some bacteria and fungi strains, were found in the leaves of this tree [5]. Factually it is well documented that many of 1,4–naphthoquinone derivatives show antimicrobial activity, especially if a hydroxyl group is present at the C-5 position [2,3]. Isolation of coumaroylspermidine from *P. fraxinifolia* from Mazandaran province of Iran have also been reported [2].

In addition, good antioxidant activity in thiobarbituric acid model and neurotoxicity potential based on cholinesterase activity and histopathology has been reported from *P. fraxinifolia* [2].

Recently, we and other members of our research group have published two reports about chemical composition and biological activity of *P. fraxinifolia*. One of them is about leaves, barks and fruits [7] and the other one is about identification of some phytochemicals isolated from methanol extract [8]. Exept of these reports, literature survey indicates that, there is only one study on the antioxidant activity and essential oil composition of the *P. fraxinifolia* which is related to leaves of the plant from Mazandaran province of Iran, with bisabolol oxide as the major component [2-5].

To the best of our knowledge, there is no information, concerning the essential oil composition and biological activity of the leaves and stems of *P. fraxinifolia* compare to each other,. Because local people used to use young leaves of this plant for its traditional applications as mentioned above, in this study, the leaves and stems of *P. fraxinifolia* was collected in vegetative stage and volatile Secondary Metabolites (VSM) composition as well as some *in-vitro* biological activities of methanolic extracts were investigated.

Materials and Methods

Plant materials

Young leaves and stems of *P. fraxinifolia* were collected in May 2011 from Hashtpar, Gillan province, west north of Iran. A voucher specimen was deposited in the herbarium of Research Institute of Forests and Rangelands, Kashan, Iran (Voucher No. KBGH 8114).

Volatile fraction separation

The plant materials were dried in shadow at room temperature and then ground with a rolling mill (Retschmuhle, GmbH, 5657 HAAN, Germany). The volatile fraction of each crushed leaves and stems (100g) were individually extracted via simultaneous distillation- extraction (SDE) [6] method for 1.5 h using *n*-pentane as solvent. In this method the yield of extraction is elevated via trapping the volatile compounds in *n*-pentane. The *n*-pentane solution was then dehydrated by anhydrous sodium sulphate; and was evaporated at room temperature under vacuum to yield 0.43% and 0.6% of the stem and leaves yellow oils. The oils were recovered and stored at low temperature (4^0C) under nitrogen atmosphere in amber vials and were used for analyses within a few days.

GC-FID analysis

Oils obtained from the plant were analyzed using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 µm filmthickness; Restek, Bellefonte, PA) equipped with a FID detector. Oven temperature was kept at 60^0C for 3 min initially, and then raised with a rate of 3^0C/min to 250^0C. Injector and detector temperatures were set at 220 and 290^0C, respectively. Helium (1 mL/min) was used as carrier gas and diluted samples (1/1000 in *n*-pentane, v/v) of 1.0 µL were injected manually in the split less mode. Peaks area percents were used for obtaining quantitative data.

GC-Mass analysis

GC/MS analysis of the oils were carried out on an Agilent HP- 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) with a HP-5MS 5% phenylmethylsiloxane capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Restek, Bellefonte, PA) equipped with an Agilent HP-5973 mass selective detector in the electron impact mode (ionization energy: 70 eV) operating under the same conditions as described

above. Retention indices (RI) were calculated for all components using a homologous series of n-alkanes injected in conditions equal to the samples ones.

Identification of components of the volatile fraction was based on retention indices relative to n-alkanes and computer matching with the Wiley275.L and Wiley7n.L libraries as well as comparisons of the fragmentation pattern of the mass spectra with the data published in the literature [9].

Preparation of extracts

The portions (50 g) of each ground leaves and stems were individually transferred to cellulose thimbles and subjected to soxhlet extraction with 500 mL methanol for 8 h at the boiling temperature of the solvent The extracts were concentrated using a rotary evaporator (Buchi Rotavapor R-200, Flawil, Switzerland) at a maximum temperature of 45^0C. All volatile components of the extract, and traces of the solvent (methanol) used in the extraction process, were completely removed using a vacuum oven (Memmert, VO400, Germany), working at temperature and pressure of 50 ^{0}C and 7.5 torr, respectively. The extracts of the leaves and stems, yielded 34% and 14% (w/w) on the basis of the weight of dried material, were stored in amber vials in the refrigerator and used for analyses within a few days.

Antioxidant activity

Antioxidant Activity in the ß-Carotene-Bleaching assay

In this assay, antioxidant activity was determined by measuring the inhibition of volatile organic compounds and conjugated diene hydroperoxides arising from linoleic acid oxidation. The method described by Akhbari & Batooli, et al [10] was used with slight modifications. A stock solution of *β*-carotene and linoleic acid was prepared with 0.5 mg of *β*-carotene in 1 mL chloroform, 25 µL of linoleic acid and 200 mg tween 40. The chloroform was evaporated under vacuum and 100 mL of oxygen-saturated distilled water was then added to the residue. The samples (2 g/L) were dissolved in DMSO and 350 µL of each sample solution was added to 2.5 mL of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50 °C for 2 h, together with two blanks, one contained the antioxidant BHT as a positive control and the other contained the same volume of DMSO instead of the extracts. The test tube with BHT maintained its yellow color during the incubation period. The absorbance was measured at 470 nm on an ultraviolet spectrophotometer (Cintra 6, GBC, Australia). Antioxidant activities (inhibition percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene}} / A^0_{\beta\text{-carotene}}) \times 100$$

where $A_{\beta\text{-carotene}}$ is the absorbance of *β*-carotene after 2 h assay remaining in the samples and $A^0_{\beta\text{-carotene}}$ is the absorbance of *β*–carotene at the beginning of the experiments. All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical-Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay usually involves hydrogen atom transfer reaction but, based on kinetic data, an electron transfer mechanism has also been suggested for this assay [11]. Radical-scavenging activity of the plant volatile fraction and extracts was determined using a published DPPH radical scavenging activity assay method [12,13] with minor modifications. Briefly, stock solutions (10 mg/mL each) of the volatile fraction, extracts and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions are made to obtain concentrations ranging from 1 to 5×10^{-10} mg/mL. Diluted solutions (1 mL each) were mixed with 1 mL of a freshly prepared 80 µg/mL DPPH methanol solution and allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Ultraviolet (UV) absorbance of these solutions were recorded on a spectrophotometer (Cintra 6, GBC, Australia) at 517 nm using a blank containing the same concentration of oil or extracts or BHT without DPPH. Inhibition of free radical DPPH in percent (I%) was calculated as follow:

$$I\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

Assay for Total Phenolic Content

In this part, total phenolic contents of the methanolic extracts of *P. fraxinifolia* were determined using published procedure involving Folin–Ciocalteu phenol reagent and gallic acid standard [14]. Solutions of the extracts (0.1 mL of each) containing 1000 µg of the extracts were taken individually in volumetric flasks, 46 mL of distilled water and 1 mL of Folin–Ciocalteu phenol reagent were added to them and the flasks were thoroughly shaken. After 3 min, 3 mL of 2% (w/v) Na_2CO_3 solution was added and the mixtures were

allowed to stand for 2 h with intermittent shaking. Absorbance were measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (0–1000 µg/mL) and a standard curve was obtained with the following equation:

$$\text{Absorbance} = 0.0012 \times \text{gallic acid (µg)} + 0.0033$$

Total phenols of each extract, as gallic acid equivalents, were determined using its absorbance measured at 760 nm as input to the obtained standard curve and its equation. All tests were carried out in triplicate and phenolic contents as gallic acid equivalents were reported as means ± SD of triplicate determinations.

Antimicrobial activity

Microbial strains: The oils and extracts of *P. fraxinifolia* were individually tested against a set of 11 microorganisms. Following microbial strains were provided by Iranian Research Organization for Science and Technology (IROST) and used in this research:, *Escherichia coli (ATCC 10536), Bacillus subtilis (ATCC 6633), Staphylococcus aureus (ATCC 29737), Klebsiella pneumoniae (ATCC 10031), Staphylococcus epidermidis (ATCC 12228), Shigella dysenteriae (PTCC 1188), Proteus vulgaris (PTCC 1182), Salmonella paratyphi-A serotype (ATCC 5702), Candida albicans (ATCC 10231), Aspergillus niger (ATCC 16404) and Aspergillus brasiliensis (PTCC 5011).* Bacterial strains were cultured overnight at 37 ^{o}C in nutrient agar (NA) and fungi were cultured overnight at 30 ^{o}C in sabouraud dextrose agar (SDA).

Disk diffusion assay

Determination of antimicrobial activities of the oils and extracts of *P. fraxinifolia* were accomplished by agar disc diffusion method [10]. The dried plant extracts were dissolved in DMSO to a final concentration of 30 mg/mL and filtered by 0.45 µm Millipore filters for sterilization. Antimicrobial tests were carried out using employing 100 µL of suspension containing 10^8 CFU/mL of bacteria, 10^6 CFU/mL of yeast and 10^4 spore/mL of fungi spread on the nutrient agar (NA), sabouraud dextrose (SD) agar and potato dextrose (PD) agar mediums, respectively. The discs (6 mm in diameter) impregnated with 10 µL of the essential oils or the extracts solution (300 µg/disc) and DMSO (as negative control) were placed on the inoculated agar. The inoculated plates were incubated for 24 h at 37^{o}C for bacterial strains and 48 h and 72 h at 30 ^{o}C for yeast and mold isolates, respectively. Gentamicin (10 µg/disc) and rifampin (5 µg/disc) were used for bacteria and nystatin (100 I.U.) was used for fungi as positive controls. The diameters of inhibition zones were used as a measure of antimicrobial activity and each assay was repeated twice.

Micro-well dilution assay

Bacterial strains and yeast sensitive to the plant extracts and volatile fraction in disc diffusion assay were studied for their minimal inhibition concentration (MIC) values using micro-well dilution assay method [10]. The inocula of the microbial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The extracts and oils of *P. fraxinifolia* dissolved in 10% DMSO were first diluted to the highest concentration (500 µg/mL) to be tested, and then serial twofold dilutions were made in a concentration range of 7.8–500 µg/mL in 10 mL sterile test tubes containing brain heart infusion (BHI) broth for bacterial strains and sabouraud dextrose (SD) broth for the yeast. The 96-well plates were prepared by dispensing 95 µL of the cultures media and 5 µL of the inoculum into each well. A 100 µL aliquot from the stock solutions of the plant products initially prepared at the concentration of 500 µg/mL was added into the first well. Then, 100 µL from their serial dilutions were transferred into six consecutive wells. The last well containing 195 µL of the cultures media without the test materials and 5 µL of the inoculum on each strip was used as negative control. The final volume in each well was 200 µL. Gentamicin and rifampin for bacteria and nystatin for yeast were used as standard drugs for positive control in the conditions identical to that of the tests materials. The plates were covered with sterile plate sealers. Contents of each well were mixed on the plate shaker at 300 rpm for 20 s and then incubated at appropriate temperature for 24 h. Microbial growth was determined by the presence of a white pellet on the well bottom and confirmed by plating 5 µL samples from clear wells on NA medium. The MIC value was defined as the lowest concentration of the plant extract required for inhibiting the growth of each microorganism. All tests were repeated three times.

Cytotoxic activity

Brine shrimp assay

A brine shrimp lethality bioassay was carried out to investigate the cytotoxicity of the extracts. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a glass rectangular vessel (5 L), filled with sterile artificial seawater, prepared using water (2 L), NaCl (46 g), $MgCl_2 \cdot 6H_2O$ (22 g), Na_2SO_4(8 g), $CaCl_2 \cdot 2H_2O$ (2.6 g), and KCl (1.4 g), with a pH of 9.0 adjusted with Na_2CO_3, under constant aeration for 48 h. After hatching, active nauplii free from egg shells were

collected from the brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in vials containing 5 mL of brine solution. In each experiment the extract solutions were prepared using the brine solution and DMSO. From them were obtained six other solutions at different concentrations, which were then added to the 5 mL brine solution. The vials were maintained at room temperature for 24 h under light, and the surviving larvae were counted. Experiments were conducted along with control and different concentrations (10, 100, 300, 500, 700 and 1000 μg mL^{-1}) in a set of three tubes per dose. The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. Lethal concentration (LC_{50}) values were obtained from the best-fit line plotting concentration versus percentage lethality [15]. All tests were carried out in triplicate and LC_{50} were reported as means±SD of triplicate determinations.

Statistical Analysis

As mentioned above, all the results values are presented as means ± SD. Differences between group means were estimated using a one-way analysis of variance followed by Duncan's multiple range tests. Results were considered statistically significant when $p<0.05$.

Results and Discussion

Chemical composition of the volatile fraction

SDE extraction of volatile fraction from leaves and stems of *P. fraxinifolia*, yielded 0.6% and 0.43 v/w yellow colored oils on the basis of the weight of dried, for leaves and stems, respectively. Table 1 shows the percentage of volatile fraction compositions. 23

Table 1. Chemical Composition of *P. fraxinifolia* Essential Oils

No.	Components [a]	Stem (%)	Leaf (%)	KI[b]	KI[c]
1	n-Hexanol	0.20	-----	862	870
2	α-Pinene	-----	0.84	934	939
3	β-Pinene	-----	1.02	980	979
4	p-cymene	-----	0.08	1025	1024
5	2-Ethyl-1-hexanol	0.94	-----	1027	1028
6	δ-Limonene	-----	0.53	1030	1029
7	1-Octanol	0.36	-----	1071	1068
8	Borneol	0.87	-----	1168	1169
9	Terpinen-4-ol	-----	0.11	1179	1177
10	methyl salicylate	1.35	-----	1197	1191
11	(-)-Myrtenol	-----	0.09	1198	1195
12	β-Citronellol	1.04	-----	1230	1225
13	D-Carvone	0.88	-----	1247	1255
14	Thymol	0.98	-----	1294	1290
15	Carvacrol	0.98	-----	1305	1299
16	Vinylguajacol	0.60	-----	1316	1320
17	2,4 Decadienal	1.19	-----	1320	1325
18	1,5,5-Trimethyl-6-methylene-cyclohexene	-----	0.31	1340	1338
19	Eugenol	0.68	-----	1360	1359
20	γ-n-Amylbutyrolactone	1.14	-----	1368	1371
21	Methyleugenol	0.87	-----	1408	1403
22	Trans-Caryophyllene	0.43	2.13	1423	1427
23	2-Norpinene	2.62	-----	1440	1430
24	3,7-Guaiadiene	3.51	27.32	1449	1440
25	α-Humulene	1.34	-----	1459	1454
26	Alloaromadendrene	5.25	-----	1466	1462
27	β-selinene	1.37		1491	1490
28	Viridiflorene	-----	21.58	1496	1491
29	α-Curcumene	1.72	8.44	1500	1492
30	Selinene<-δ>	-----	4.36	1506	1502
31	Zingiberene	1.83	5.37	1508	1509
32	β-Bisabolene	1.33	3.51	1512	1512
33	δ-cadinene	1.68	-----	1528	1523
34	β-Sesquiphellandrene	-----	2.27	1543	1534
35	Germacrene B	-----	0.58	1572	1561
36	Palustrol	0.77	0.64	1582	1581

Table 1. Cntd

37	Spathulenol	-----	4.41	1589	1583
38	Veridiflorol	3.47	7.22	1602	1598
39	Ledol	1.22	2.03	1611	1605
40	Humulene epoxide II	2.65	-----	1619	1608
41	Helifolen-12-al D	7.37	-----	1625	1620
42	Dillapiole	0.90	-----	1631	1620
43	Tau-Murolol	0.53	-----	1636	1642
44	Agarospirol	0.60	-----	1651	1648
45	isospathulenol	-----	0.69	1653	1649
46	β-Eudesmol	0.70	-----	1658	1650
47	α-Cadinol	0.58	0.39	1662	1654
48	Germacra-4(15),5,10,(14)-trien-1-α-ol	7.98	-----	1701	1686
49	trans-Farnesol	1.23	-----	1726	1743
50	Tetradecanoic acid	1.95	-----	1772	1763
51	Hexahydrofarnesylacetone	0.58	-----	1848	-----
52	nonadecane	0.7	-----	1901	1900
53	Hexadecanoic acid	21.74	-----	1997	1991
54	Heneicosane	0.64	-----	2099	2100
55	Phytol	1.00	0.32	2113	2110
56	Linoleic acid ethyl ester	5.11	-----	2163	2162
57	Linolic acid	1.57	-----	2171	2180
	Total	94.45%	94.24%		

[a] Compounds listed in order of elution from HP-5MS column.
[b] Relative retention indices to C_8-C_{24}*n*-alkanes on HP-5MS column.
[c] Literature retention indices.

components (94.24%) were identified in the oil from the leaves, with 3,7-guaiadiene (27.32%), viridiflorene (21.58%), α-curcumene (8.44%) and veridiflorol (7.22%) as major components. On the other hand, in the stems oil, 44 components were identified, which made up 94.45% of the total oil. The most abundant constituents were hexadecanoic acid (21.74%), germacra-4(15),5,10,(14)-trien-1-α-ol (7.98%), helifolen-12-al D (7.37%) and linoleic acid ethyl ester (5.11%). There is only one report on the chemical composition of the essential oils of *P. fraxinifolia* in the literature, in which bisabolol oxide A (23.6%), hexadecanoic acid dihydroxypropyl ester (14.4%) and bisabolone oxide (6.62%) were found in the leaves essential oil of the plant as major components [3].

Such a high difference between the oil compositions of the same species from different regions is probably depended on climate and environmental factors.

Antioxidant activity

According to the results from DPPH free radical scavenging assay (Table 2), steam oil and extract showed very high activities which were comparable with BHT standard antioxidant. Such a high antioxidant activity would not be expected from plant volatile components, generally.

In the case of ß-Carotene-Bleaching assay, methanolic extracts of the leaves and stems were showed an inhibition percentage (80.99% and 86.74%) comparable to that of synthetic standard BHT. Peroxy radicals usually initiate lipid peroxidation by abstraction of an allylic or benzylic hydrogen atom from the molecule under oxidation; thus, considerable antioxidant activity in the *β*-carotene bleaching assay exhibits the possible presence of allylic and/or benzylic hydrogen containing secondary metabolites in the above mentioned extract. However, leaves and stems oils exhibited low inhibition percentage (18.66% and 14.18%).

Due to the major contribution of phenolic compounds in antioxidant activity, total phenolic content of the methanolic extracts were determined as gallic acid equivalent. Relatively high antioxidant activity of the plant extracts may be a consequence of its high phenolic compounds content which was reflected in its Folin-Ciocalteu test (137.96 ± 0.8 and 255.30 ± 1.2 µg mg^{-1}, as gallic acid equivalent, for leaves and stems respectively).

Based on our research, there are 3 reports on the antioxidant activity and total phenolic contents of the methanol extract of *P. fraxinifolia* from Iran which is from Mazandaran province (north of Iran), in the literature [2-5]. In one of these reports, IC_{50} for DPPH radical scavenging activity shows that leaves extract had more than 10 times higher DPPH radical scavenging activity than bark, which is in contrast of our results [2].

In the other report, IC_{50} for DPPH radical scavenging activity was in the order: leaves (15.59 ±

Table 2. Effects of *P. fraxinifolia* Oils and Extracts and Positive Control on the *in Vitro* Free Radical (DPPH) Scavenging, β-Carotene/Linoleic Acid and Brine Shrimp Lethality Assays.

Sample	DPPH IC_{50} (µg/mL)	β-carotene/linoleic acid Inhibition (%)	Total phenolics (µg/mg)	Brine shrimp bioassay LC_{50}(µg/mL)
Leaf Oil	43.45± 0.12	18.66 ± 0.70	NA	ND [b])
Stem Oil	19.25 ± 0.10	14.18 ± 0.40	NA	ND
Leaf Extract	39.80 ± 0.09	80.99 ± 0.60	137.96± 0.80	930 ± 9
Stem Extract	15.84 ± 0.08	86.74 ± 0.80	255.30 ± 1.20	561 ± 6
BHT	19.72 ± 0.80	89.32 ± 0.87	NA	NA
Negative control	NA [a])	0.00	NA	NA

Values V: Values are mean ± SD (n = 3), [a]) NA (Not applicable) [b]) ND (Not determined)

Table 3. Antimicrobial Activities of the Oils and Extracts from *Pterocarya fraxinifolia* against the Bacteria Strains Tested

	Essential oils				Extracts				Antibiotics					
	Leaf		Stem		Leaf		Stem		Rifampin		Gentamicin		Nystatin	
Test microorganisms	DD [a)]	MIC[b)]	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC	DD	MIC
A.brasiliensis	- [c])	-	-	-	10	>500	-	-	-	-	23	500	NA[d])	NA
B.subtilis	9	-	-	-	-	-	-	-	13	125	21	500	NA	NA
E.coli	-	-	11	500	-	-	13	500	11	500	20	500	NA	NA
S.aureus	-	-	-	-	-	-	13	250	10	500	21	500	NA	NA
k.pneumoniae	-	-	-	-	-	-	-	-	7	500	22	250	NA	NA
S.epidermidis	22	250	12	500	-	-	-	-	40	500	35	500	NA	NA
S.dysenteriae	-	-	-	-	10	500	15	250	8	500	18	500	NA	NA
P.vulgaris	-	-	-	-	-	-	11	>500	10	500	23	500	NA	NA
S.paratyphi-Aserotyp	-	-	-	-	-	-	-	-	-	500	21	500	NA	NA
C.albicans	-	-	-	-	-	-	-	-	NA	NA	NA	NA	33	125

Values are mean ± SD (n = 3)
[a]) Inhibition zone in diameter (mm) around the impregnated discs.
[b]) Minimal Inhibition concentrations (as µg/mL).
[c]) A dash (-) indicate no antimicrobial activity.
[d]) NA (not applicable).

0.11) and bark (166.24 ±2.30) µg mL^{-1}. Also, the total phenolic contents of leaves and bark were 85.93± 2.20 and 17.88± 1.32 mg gallic acid equivalent/g of the extract powder [1-4].

Comparing the reported results with those of the present study, it could be seen that *P. fraxinifolia* from Gillan, is more active than that from Mazandaran, and interestingly, total bioactivity of stems is higher than leaves for this chemotype of *P. fraxinifolia*; however, some observed properties may be related to the season of sampling (vegetative stage).

Antimicrobial activity

The antimicrobial activity of *P. fraxinifolia* volatile fraction and methanolic extracts against a panel of 11 microorganisms was examined and their potencies were assessed both qualitatively and quantitatively by the presence or absence of inhibition zones, zone diameters and MIC values (Table 3). The volatile fraction, showed antimicrobial activities against three of the tested microorganisms. Maximum inhibition zones and minimum MIC values were 22 mm and 250 µg/mL, respectively, which showed high sensitivity for *S. epidermidis* microbial strain to the leaves volatile fraction. Although methanolic extract of the plant leaves showed also antimicrobial activities against *A. brasiliensis* and *S. dysenteriae* in both disc diffusion and micro-well dilution tests, stems extract showed stronger and broader spectrum of antimicrobial activity.

To the best of our knowledge, the antimicrobial activity of this plant has not been reported before in the literature and this report is the first one.

Cytotoxic activity

Brine shrimp lethality bioassay was performed to determine cytotoxic activity of the methanolic extracts.

The 50% lethal concentrations (LC_{50}) for leaves and stems (see Table 2) indicates existence of cytotoxicity, although this activity was not very high.

As a conclusion of this study, it could be state that *P. fraxinifolia* from Gillan (northwest of Iran) contains specific secondary metabolites and shows different types of biological activities both for the volatile fraction and polar extracts. It is also interesting that the stems of this plant showed comparable and even higher activity than the leaves; since the tree is from walnut family, with valuable wood, having good biological properties of this part of the mentioned plant increase notability of this research work. According to significant antioxidant activity, on the one hand, large body and existence of huge mass of leaves of this plant, on the other, doing more advanced researches specially evaluation of *in-vivo* protective effects for this plant should be very useful to find new antioxidant agents.

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