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Original article

Variation in chemical components and biological activity of *Pterocarya fraxinifolia* Lam. stems at different developmental stages

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Abstract

Background and objectives: *Pterocarya fraxinifolia* Lam. is a deciduous, fast-growing tree from walnut family. The stem barks and fruits of the plant have been used as diaphoretic in traditional medicine. Variation in the quantity and quality of the essential oil and extract of stems of the plant at different developmental stages was evaluated in addition to assessing the antimicrobial, cytotoxic and radical scavenging activities in the present study. **Methods:** Different developmental stages of the plant's stem (i.e. vegetative, flowering, immature fruit and mature fruit) were subjected to hydro-distillation for obtaining the essential oil. The methanol extract of the samples was obtained by Soxhlet apparatus. Chemical composition of the oils was analyzed by gas chromatography/mass spectroscopy (GC/MS). Antimicrobial activity of the oils and extracts were determined against three Gram-positive and five Gram-negative bacteria and two fungi by disc diffusion method. Antioxidant activity of the samples was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene assays. Total phenolics content of extracts was determined using Folin-Ciocalteau reagent and cytotoxic effect was determined by brine shrimp lethality bioassay. **Results:** Hexadecanoic acid was one of the major components in all essential oil samples. All samples showed good antimicrobial activity against tested strains. Antioxidant activity of the extracts was comparable to the synthetic standard (butylated hydroxytoluene). The highest total phenolic content and cytotoxic effect were detected for the mature fruit stage of the plant extract and essential oil, respectively. **Conclusion:** Showing considerable antioxidant and cytotoxic effects, suggested the plant as a good candidate for further investigations.

Keywords: antimicrobial activity, antioxidant activity, cytotoxic effect, essential oil, *Pterocarya fraxinifolia*

Introduction

Pterocarya fraxinifolia Lam. is a deciduous, fast-growing tree from walnut family (Juglandaceae). It is native to the Caucasus from northern forests of Iran to the Ukraine and distributed throughout a wide area in western black sea region [1].

Literature review has revealed that the leaves

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have antibacterial, antifungal, larvicidal and antioxidant activities [2] and have been used as a natural dye and anaesthetic agent by Caucasians for fishing [1]. It is found that juglone is one of the major compounds of this plant, with potent antimicrobial effect [3,4]. The stem barks and fruits have been used as a diaphoretic agent in traditional medicine [5]. There are some reports about the *in vitro* evaluation of antioxidant activity of different parts of the tree [6,7], secondary metabolites, biological activity, toxicity and mechanical properties of the wood of *P. fraxinifolia* [1,8-10].

Chemical composition of the essential oils and extracts of medicinal plants can be varied depending on origin, environmental conditions, and developmental stage of collecting plant materials [11]. Antioxidant and other biological activities of the essential oils also vary, based on the variations in chemical composition [12-18].

Despite having valuable phytochemicals, there is no information about the chemical composition of *P. fraxinifolia* from Gilan province of Iran and its variations during the phonological cycle in the literature. The aim of the study was to evaluate the chemical composition, radical scavenging and antimicrobial activity and cytotoxic effect of *P. fraxinifolia* stems in different developmental stages.

Experimental

Plant material

Samples of *P. fraxinifolia* stem were collected at different developmental stages (vegetative, flowering, immature fruit and mature fruit) from its habitat in Fuman, Gilan province, Iran (South-West of Caspian Sea, latitude 37˚ N and longitude 49˚ E), at an altitude of 100 m, in May, July, September and November 2011. They were identified and deposited at the Herbarium of Research Institute of Forests and Rangelands, Kashan, Iran (Voucher No. KBGH 8114). They were then dried at room temperature and ground in a blender.

Chemicals

Anhydrous sodium sulfate, methanol, dimethyl sulfoxide (DMSO), Mueller-Hinton agar (MHA) medium, potato dextrose agar (PDA) medium, Sabouraud 4% dextrose agar (SDA) medium, brain heart infusion (BHI) broth medium, butylated hydroxytoluene (BHT), ethanol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, linoleic acid, Tween 40, Folin-Ciocalteau reagent, sodium carbonate, gallic acid, Magnesium chloride hexahydrate ($MgCl_2.6H_2O$), Calcium chloride dihydrate ($CaCl_2.2H_2O$), Potassium chloride (KCl) and sodium chloride (NaCl) were purchased from Merck (India).

Isolation of the volatile oil

The dried powders of plant stems were subjected to hydro-distillation for 4 h using a Clevenger type apparatus. The oils were dried over anhydrous sodium sulfate and after filtration stored at 4 °C in the sealed vials until analyzed.

Extraction

Dried stem powders (25 g) was extracted by soxhlet apparatus using methanol. The resulting solution was filtered, and the solvent was evaporated in a rotary evaporator (Buchi/Rotavapor R-200, Switzerland). The brown sticky extracts were dried in a 1.3 kPa vacuum oven for 48 h.

GC and GC/MS analysis of the essential oil

Analytical gas chromatography (GC) of volatile oils were 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 programed at 50 °C for 10 min and subsequently heated to 240 °C at a rate of 4 °C/min and then kept constant at 240 °C for 15 min. Gas chromatography/mass spectroscopy 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; 1 mL/min. The column was held at 50 °C for 10 min and programmed up to 240 °C at rate of 4 °C/min, then kept constant at 240 °C for 15 min. The MS was operated at 70

eV ionization energy. Retention indices were calculated by using retention time of *n*-alkanes that were injected after the oil at the same chromatographic conditions. Quantitative data were obtained from the electronic integration of the FID peak areas. Acquisition mass range was 40-400 *m/z*. The components of the oils were identified by comparison of their mass spectra and kovats indexes with Wiley library and data published in the references [19-21].

Antimicrobial activity

Antimicrobial activity of the essential oils and extracts were determined against three Gram-positive bacteria (*Staphylococcus epidermidis* ATCC 12228, *Staphylococcus aureus* ATCC 29737, *Bacillus subtilis* ATCC 6633) and five Gram-negative bacteria (*Salmonella paratyphi*-A serotype ATCC 5702, *Shigella dysenteriae* PTCC 1188, *Proteus vulgaris* PTCC 1182, *Escherichia coli* ATCC 10536 and *Klebsiella pneumonia* ATCC 10031), one yeast (*Candida albicans* ATCC 10231) and one fungi (*Aspergillus niger* ATCC 16404). The antimicrobial activities of the samples were determined by disc diffusion method by determining of diameter of inhibition zones [22]. Bacterial strains and yeast sensitive to the plant samples in disc diffusion assay were studied for minimal inhibition concentration (MIC) values using micro-well dilution assay method. Gentamicin and rifampin were used as the standard positive drugs for bacteria and nystatin for yeast in conditions identical to tests materials.

The dried plant extracts were dissolved in 2% dimethyl sulfoxide (DMSO) in distilled water to a final concentration of 30 mg/mL and filtered by 0.45 µm polypropylene filters (OlimPeak™, Spain) for sterilization. Antimicrobial tests were carried out using 100 µL of suspension containing 10^8 CFU/mL of bacteria on muller-hinton agar media, 10^6 CFU/mL of yeast on potato dextrose agar media and 10^4 spore/mL of fungus spread on the sabouraud dextrose agar media, respectively. The discs (6 mm in diameter) impregnated with 10 µL of the essential oil or 300 µg/disc of the extract solution and DMSO (as the negative control) were placed on the inoculated agar. After 24 h of incubation at 37 °C for bacterial strains and 48 h at 30 °C for the yeast and 72 h at 30 °C for fungi [23], the diameters of inhibition zones were evaluated as a measure of antimicrobial activity. Each assay was repeated twice.

For assessing the minimum inhibitory concentration values (MIC) the inocula of the microbial strains were prepared from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The extract dissolved in 10% DMSO solution were first diluted to the highest concentration (2 mg/mL) to be tested, and then serial twofold dilutions were made in a concentration range from 0.078 up to 2 mg/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 culture media and 5 µL of the inoculum into each well. A 100 µL aliquot from the stock solutions of the plant extracts initially prepared at the concentration of 2 mg/mL was added into the first wells. Then, 100 µL volumes from their serial dilutions were transferred into six consecutive wells. The last well containing 195 µL of the culture media without the test materials and 5 µL of the inoculum on each strip was used as the negative control. The plates were covered with sterile plate sealers. Contents of each well were mixed on plate shaker at 300 rpm for 20 s and then incubated at appropriate temperatures 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 appropriate medium. The MIC value was defined as the lowest concentration of the plant samples required for inhibiting the growth of microorganisms [24].

Evaluation of antioxidant activity by DPPH assay

DPPH assay [25] was used for determination of

free radical scavenging activity of the essential oils and total extracts. An aliquot of the sample or BHT standard antioxidant, as positive control, (100 mg) was mixed with 1.5 mL of ethanol and then added to 1 mL of 0.004% DPPH in ethanol. The mixture was allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Ultraviolet (UV) absorbencies of these solutions were recorded using a spectrometer (Cintra 6, GBC, Australia) at 517 nm. The control contained the same concentration of oil, extracts or BHT (positive control) without DPPH and the blank contained the solvent and DPPH. Inhibitions of DPPH radical (I %) were calculated as follow:

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

A_{sample} was the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated by plotting the inhibition percentages against concentrations of the sample. All tests were carried out in triplicate and IC_{50} values were reported as means ± Standard deviation (SD) of triplicates.

Evaluation of antioxidant activity by β-carotene assay

The inhibition of β-carotene oxidation was determined using the organic compounds of the extract and volatile oil in presence of linoleic acid peroxyl radicals [26]. A reagent solution was prepared by adding β-carotene (0.7 mg), linoleic acid (37.5 mL) and 300 mg Tween 40 (as co-solvent) to 150 mL of oxygen-saturated distilled water. Then the resulting mixture was stirred well until a homogenous clear solution was obtained (solution R). Three types of samples were prepared in this investigation; the first (type a) contained 2.5 mL solution R to which 1 mL of the samples dissolved in ethanol with a concentration of 2 mg/mL was added; the second sample (type b) was used as a blank and contained only 2.5 mL of solution R and the third sample (type c) used for positive control contained 1 mL of BHT solution in ethanol (concentration of 2 mg/mL). All samples (type a-c) were added up in a 5 mL volumetric balloon using ethanol. They were then maintained in hot water (50 °C) for 2 h except type b samples, for which, the absorbance reading was taken immediately after it was totaled to 5 mL. The absorbance was measured at 470 nm. Antioxidant capacities (Inhibition percentages, I%) of the tested solutions were calculated using the following equation:

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

where the numerator indicates the absorbance made from the types a or c samples and the denominator indicates the absorbance made from type b samples (t= 0 min). Inhibition percentage of the "type a"' sample was compared to that of the positive control from type c sample. It should also be mentioned that in the experiments with BHT, the yellow color was maintained during the incubation period. The tests were carried out in triplicate and inhibition percentages were reported as means ±SD.

Total phenolics content

Total phenolics content of *P. fraxinifolia* extracts was determined by Folin-Ciocalteau reagent [25]. Each extract (0.5 mL of sample) was mixed with 2.5 mL of 0.2 N Folin-Ciocalteau reagent for 5 min. Then 2 mL of 75 g/L sodium carbonate was added to the mixture. The absorbencies were measured at 760 nm after 2 h incubation in room temperature. The standard curve was prepared using different concentrations of gallic acid (50-250 µg/mL of gallic acid in 50% methanol). Total phenolics content of the extracts was determined as gallic acid equivalent (GAE). The tests were carried out in triplicate and the results were reported as means±SD.

Assessment of cytotoxic effect using brine shrimp lethality test (BST)

A brine shrimp lethality bioassay was carried out for evaluation of the cytotoxic effect of the extracts and the oil [27]. Brine shrimps (*Artemia*

salina Leach) were hatched in sterile artificial seawater, prepared by mixing water (2 L), NaCl (46 g), $MgCl_2.6H_2O$ (22 g), Na_2SO_4 (8 g), $CaCl_2.2H_2O$ (2.6 g), and KCl (1.4 g) with, pH of 9.0 adjusted by Na_2CO_3, under constant aeration for 48 h. After hatching, ten active nauplii (larvae) were collected by pipette and placed in vials containing 5 mL of prepared artificial seawater solution. The extracts were dissolved in DMSO and diluted with artificial seawater to obtain solutions with concentrations of 10, 100, 300, 500, 700 and 1000 μg/mL. The experiments were conducted by adding sample solutions to 5 mL of brine solution in a set of three tubes per each concentration. The vials were maintained at room temperature for 24 h under light, and then the surviving larvae were counted. The percentage lethality was determined by comparing the mean surviving nauplii of the test and control tubes. The concentration values related to 50% lethality (LC_{50}) were obtained from the best-fit line plotting concentration versus lethality percentage.

Results and Discussion

The yield of oil and extracts has been summarized in table 1. They were relatively similar in different growth stages.

A comparative study of the essential oils composition during the mentioned stages showed quantitative and qualitative difference (table 2). Hexadecanoic acid (6.04%-32.60%) was the most dominant compound at the flowering and vegetative stages, while biotol and caryophyllene oxide were the major components of immature and mature fruit stage, respectively. Methyl linoleate (6.30%) was only available at the immature fruit while ethyl linoleate was found at the vegetative stage. On the other hand, linoleic acid was present in all developmental stages in different amounts (1.18-12.36%). Juglone, which is one of the known compounds for antimicrobial effect, isolated from the methanol extract of this species [2], was only present at the immature and mature fruit stages oils. Another antimicrobial agent; eugenol is present in all stages, but in much higher quantities in mature fruit stage (5.55%).

Oxyginated sesquiterpenes were the dominant classes of compounds, present in oil of all stages.

The results of antimicrobial assessment of the essential oil and the extract of *P. fraxinifolia* stems at different developmental stages have been summarised in table 3.

There is no report about the antimicrobial activity of the essential oil of *P. fraxinifolia* stem in the literature. Hexadecanoic acid (palmitic acid) which is known for its antimicrobial and antifungal activity [28] was present in all samples in relatively high amounts. Menthol, which is also a known antimicrobial compound [29], was only present at the immature fruit stage (3.01%) and methyl salicylate, the other known antimicrobial agent was present in the oil at the vegetative and the mature fruit stages (1.40% and 3.39%, respectively).

On the other hand, caryophyllene oxide and eugenol which both are well known antimicrobial agents [30], were present in significant amounts in the oil, only at the mature fruit stage (16.20 and 5.55%, respectively). Essential oils, particularly from flowering, the immature and mature fruit stage, showed the best activity against *Staphylococcus* species (*S. aureus* and *S. epidermidis*). The immature fruit and mature fruit stages were the only ones owing juglone; on the other hand, essential oil of the vegetative stage was the weakest between four samples which may be due to lack of some known antimicrobial components (e.g. caryophyllene oxide, menthol and juglone); while having hexadecanoic acid in relatively high quantity (22.09%). There were some difference between antimicrobial effect of essential oil and extract of *P. fraxinifolia* stem microorganisms. *P. vulgaris* and *S. dysenteriae* showed some susceptibility to the extract, while resistant to the oil in tested concentrations. Meanwhile, both were ineffective against *K. pneumonia*, *C. albicans* and *S. paratyphi*-A serotype (table 3). This may be in some extent due to the presence of caryophyllene oxide (mature fruit), eugenol (mature fruit) and trans-farnesol [31] (flowering state).

Table1. The yield of oil and extracts of *P. fraxinifolia* stem, in different growth stages of the plant

	immature fruit		**flowering**		**Mature fruit**		**vegetative**	
	oil	extract	oil	extract	oil	extract	oil	extract
The yield	0.26%[a]	27%[b]	0.26%	26%	0.34%	24%	0.31%	20%

[a] v/w for the oils
[b] w/w for the extracts

Table 2. Essential oil composition of *Pterocarya fraxinifolia* Lam. stems at different developmental stages

No.	**Component**	**Percentage in different stages**					
		Vegetative	**Flowering**	**Immature Fruit**	**Mature Fruit**	**RI_s[a]**	**RI_r[b]**
1	n-Hexanol	00.35	00.48	00.21	00.76	862	870
2	*α*-Pinene	-	-	-	00.23	936	939
4	Benzaldehyde	-	00.39	-	-	960	960
3	*β*-Pinene	-	-	-	00.51	980	979
5	2-Pentylfuran	-	00.89	-	00.36	992	993
6	2,4-Heptadienal	-	01.17	00.14	-	1011	1007
7	2-Ethyl-1-hexanol	00.84	-	-	-	1027	1028
8	1,8-Cineole	-	00.23	-	-	1033	1031
9	2-Pyrrolidinone	00.09	00.74	01.20	-	1043	1045
10	1-Octanol	00.32	00.59	00.26	00.71	1070	1068
11	Nonanal	-	00.71	-	00.83	1106	1100
12	Benzeneethanol	-	00.75	-	-	1116	1107
13	1-Menthone	-	-	01.51	-	1157	1152
14	2-Nonenal	-	00.53	-	-	1161	1161
15	Borneol	00.55	-	-	-	1168	1169
16	Menthol	-	-	03.01	-	1178	1171
17	methyl salicylate	01.40	-	-	03.39	1197	1191
18	Decanal	-	00.64	-	00.77	1207	1201
19	*β*-Citronellol	01.10	-	-	-	1230	1225
20	Hexyl(2*E*)-butanoate	-	02.51	-	-	1233	1242
21	D-Carvone	00.83	-	-	-	1247	1255
22	Ascaridole	-	-	-	00.96	1265	1273
23	Thymol	01.08	-	-	-	1294	1290
24	Menthyl acetate	-	-	00.44	-	1296	1295
25	Carvacrol	00.93	-	-	-	1305	1299
26	Vinylguajacol	-	-	00.28	01.20	1316	1320
27	2,4-Decadienal	01.19	03.01	-	-	1320	1325
28	Eugenol	00.68	01.54	00.28	05.55	1360	1359
29	*γ*-n-Amylbutyrolactone	01.14	03.71	-	-	1368	1371
30	Methyleugenol	00.82	-	-	-	1408	1403
31	Trans-Caryophyllene	00.48	-	00.29	00.38	1423	1427
32	2-Norpinene	02.57	-	-	02.73	1440	1430
33	3,7-Guaiadiene	03.00	-	00.59	03.75	1447	1440
34	5,9-Undecadien	02.23	00.83	-	-	1456	1451
35	*α*-Humulene	01.41	-	00.25	-	1458	1454
36	(*E*)-*β*-Famesene	-	-	-	01.27	1461	1458
37	Alloaromadendrene	05.28	00.67	00.45	06.71	1466	1462
38	*α*-Curcumene	01.66	01.02	01.00	-	1486	1480
39	*β*-Ionone	00.91	01.48	-	-	1491	1488
40	*β*-selinene	01.24	-	00.60	01.00	1490	1490
41	Bicyclogermacrene	-	-	-	03.08	1503	1503
42	Zingiberene	01.92	-	01.33	-	1499	1509
43	Juglone	-	-	02.10	03.60	1510	o
44	*β*-Bisabolene	01.34	-	-	01.01	1512	1512
45	*β*-Sesquiphellandrene	-	-	00.61	00.58	1527	1522
46	*δ*-Cadinene	01.36	-	-	-	1528	1523

Table 2. Continued.

No.	Component	Percentage in different stages					
		Vegetative	**Flowering**	**Immature Fruit**	**Mature Fruit**	**RI_s[a]**	**RI_r[b]**
47	Nerolidol	-	01.18	01.03	01.37	1569	1564
48	Palustrol	00.45	-	-	-	1572	1581
49	Caryophyllene oxide	-	01.26	-	16.20	1590	1583
50	Veridiflorol	03.19	00.97	-	02.96	1602	1598
51	Ledol	01.01	-	-	01.38	1611	1605
52	Humulene epoxide II	02.76	02.17	02.30	01.37	1616	1608
53	Biotol	-	-	36.80	-	1618	1613
54	Helifolen-12-al D	07.45	02.00	-	-	1625	1620
55	Dillapiole	01.12	-	-	-	1631	1620
56	Cubenol	00.11	01.74	01.06	-	1638	1628
57	Tau-Murolol	00.33	-	-	-	1649	1642
58	Agarospirol	00.85	06.38	-	-	1651	1648
59	*β*-Eudesmol	00.65	03.12	01.40	-	1658	1650
60	*α*-Cadinol	00.28	-	-	-	1662	1654
61	Germacra-4(15),5,10, (14)-trien-1-α-ol	07.83	05.98	02.12	08.47	1698	1686
62	Heptadecane	-	-	00.24	-	1702	1700
63	trans-Farnesol	01.34	04.18	-	-	1728	1743
64	Tetradecanoic acid	02.00	01.95	00.82	-	1772	1763
65	Hexahydrofarnesyl acetone	00.59	00.96	00.56	-	1848	-
66	Pentadecanoic acid	00.09	00.98	-	-	1882	1881
67	nonadecane	-	-	01.25	00.92	1901	1900
68	Palmitic acid	-	-	01.83	-	1932	1922
69	Hexadecanoic acid	22.09	32.60	11.07	06.04	2011	1991
70	Heneicosane	00.48	-	-	01.55	2099	2100
71	Methyl linoleate	-	-	06.30	-	2103	2089
72	Phytol	01.31	-	01.78	02.24	2118	2110
73	Linoleic acid ethyl ester	04.22	-	-	-	2163	2162
74	Linoleic acid	01.18	03.79	12.36	05.21	2171	2180
75	Tricosane	-	-	-	01.32	2302	2300
76	Tetracosane	-	-	-	02.05	2402	2400
77	Pentacosane	-	-	-	03.80	2502	2500
Compound Classes							
Monoterpene hydrocarbons		-	-	-	0.74		
Monoterpene oxygenated		04.49	00.23	04.96	0.96		
Sesquiterpene hydrocarbons		17.69	01.69	05.12	17.78		
Sesquiterpene oxygenated		29.41	29.94	45.27	34.48		
Diterpene oxygenated		01.31	-	01.78	02.24		
Nonterpene hydrocarbons		4.71	02.78	2.31	09.64		
Nonterpene oxygenated		36.44	56.51	36.03	28.42		
Total		**94.05%**	**91.15%**	**95.47%**	**94.26%**		

a) Retention index of sample
b) Retention index of reference

The highest phenolic content, measured using Folin-ciocalteu reagent was observed at the mature fruit stage (263.3 µg GAE/mg extract), followed by vegetative stage (255.3 µg/mg extract), while it was comparable in immature fruit (189.5 µg/mg extract) and flowering stages (184.5 µg/mg extract) (table 3).

Radical scavenging and cytotoxic activity of the samples have been listed in table 4. Evaluation of antioxidant activity showed that essential oil of the vegetative and the flowering stages possess stronger activity than BHT (with IC_{50} values of 12.06±1.01 and 19.21±1.43 µg/mL, respectively, compared to 19.72±0.71 µg/ml for BHT) but inhibition of lipid peroxidation activity of the oil samples were not considerable. However, the methanol extract showed high inhibition percentages of lipid peroxidation (ranging from 76.76±0.92% for the immature fruit to 86.84±0.66% for mature fruit).

Table 3. Antimicrobial activity of the essential oil and the extract of *Pterocarya fraxinifolia* Lam. stems at different developmental stages

Microorganism	Inhibition zone diameter (minimal inhibition concentrations)							
	Essential oil				Extract			
	Vegetativ	Flowering	Immature Fruit	Mature Fruit	Vegetative	Flowering	Immature Fruit	Mature Fruit
B. subtilis	NE[a]	10[b] (>500[c])	11 (>500)	NE	NE	NE	NE	NE
S. aurerus	NE	10 (>500)	9 (500)	12 (500)	13 (250)	16 (500)	13 (>500)	14 (500)
S. epidermidis	12 (500)	27 (500)	30 (125)	25 (250)	NE	NE	NE	NE
E. coli	11 (500)	NE	12 (>500)	NE	13 (500)	11 (>500)	11 (>500)	13 (>500)
S. paratyphi-A serotype	NE	NE	NE	NE	NE	NE	NE	NE
S. dysenteriae	NE	NE	NE	NE	15 (250)	17 (250)	14 (>500)	12 (500)
P. vulgaris	NE	NE	NE	NE	11 (>500)	13 (500)	14 (500)	12 (>500)
K. pneumonia	NE	NE	NE	NE	NE	NE	NE	NE
C. albicans	NE	NE	NE	NE	NE	NE	NE	NE
A. niger	NE	11 (>500)	10 (>500)	NE	NE	NE	NE	NE

a) Not effective
b) Inhibition zone in diameter (mm) around the impregnated discs
c) Minimal inhibition concentrations as µg/mL

Table 4. Antioxidant and cytotoxic activities of the essential oil and total extract of *Pterocarya fraxinifolia* Lam. stems at different developmental stages. Total phenolic content of the total extracts are also included.

Test	Essential oil				Total Extract			
	Vegetative	**Flowering**	**Immature Fruit**	**mature Fruit**	**Vegetative**	**Flowering**	**Immature Fruit**	**mature Fruit**
DPPH IC_{50} (µg/ml) [a]	12.06±1.01	19.21±1.43	41.93±2.11	38.52±2.54	15.84±0.08	25.40±0.09	16.80±0.20	9.12±0.32
β-carotene/linoleic acid inhibition (%) (BHT=95.32±1.22)	15.40±0.20	22.60±0.20	29.10±0.20	30.20±0.20	86.74±0.81	80.35±0.68	76.76±0.92	86.84±0.66
Total phenolics as GAE[b] (µg/mg)	NA[c]	NA	NA	NA	255.30±1.20	184.50±0.80	189.50±1.20	263.30±1.40
Brine shrimp lethality as LC_{50} (µg/ml)	75±4	123±4	98±3	28±2	561±6	427±5	583±5	371±4

a) BHT IC_{50}=19.72±0.71
b) Gallic acid equivalent
c) Not applicable

Cytotoxic effects of the essential oils and total extracts were assessed using brine shrimp method. This method is used as an indicator for general toxicity and as a guide for detection of antitumor and pesticides [32]. The results demonstrated very high activity for all samples; however, the essential oils showed higher toxicity, with LC_{50}s ranging from 28±2 to 123±4 µg/mL. The most considerable activity was found for the mature fruit stage (table 4). By comparing the results, it seems that the essential oil of the plant is a good candidate for more investigations about determination of cytotoxic components or antitumor properties.

Based on previous studies, the difference between the chemical compositions of the essential oil of the fruits, leaves and barks of *P. fraxinifolia* has been significant, whereas aromadendrene (26.3%) and caryophyllene (15.2%) have been reported as the major components of the leaves and fruits, respectively [8]. However, the developmental stages have not been considered in the mentioned literature. Developmental stages had significant effects, not

only on the quantity and quality of the essential oil, but also on antioxidant and biological activities.

Showing considerable cytotoxic effects in the brine shrimp method, makes the studied plant a suitable candidate for investigations about new anti-cancer drugs, especially in mature fruit state.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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