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Research Article

Essential Oil from Flowers and Leaves of *Elaeagnus Angustifolia* (Elaeagnaceae): Composition, Radical Scavenging and General Toxicity Activities

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Abstract

Purpose: The aim of this work was to identify the chemical composition of the essential oils obtained from the flowers and leaves of *Elaeagnus angostifolia* (Elaeagnaceae) along with evaluate the radical scavenging and general toxicity activities.

Methods: A combination of GC-MS and GC-FID were utilized for analyzing the chemical profile of the essential oils extracted by hydro-distillation from the leaves and flowers of *E. angustifolia*. The essential oils were subjected to general toxicity and radical scavenging assays using brine shrimp lethality test and DPPH method, respectively.

Results: In total, 53 and 25 components were identified and quantified in the essential oils of flowers and leaves, accounting for 96.59% and 98.97% of the oil, respectively. The both oils were observed to be rich in ester compounds. The most abundant components of the oil from flowers were E-ethyl cinnamate (60.00%), hexahydrofarnesyl acetone (9.99%), palmitic acid (5.20%) and phytol (3.29%). The major constituents of the oil from leaves were E-ethyl cinnamate (37.27%), phytol (12.08%), nonanal (10.74%) and Z-3-hexenyl benzoate (7.65%). Both oils showed moderate activity in DPPH assay; however, they exhibited potent tocixity in brine shrimp lethality test.

Conclusion: The remarkable toxicity effects of the oils are worthy to further investigation to find the probable mechanisms of action accountable for the noticeable toxic effect of these essential oils.

Introduction

In many countries, the representatives of the genus *Elaeagnus* have been studied in order to produce natural material for nutrition, agriculture and pharmaceuticals. The genus *Elaeagnus*, an important member of Elaeagnaceae family, is widely distributed from the northern areas of Asia to the Himalayas, as well as Europe.[1,2] Although this genus consists of around 40 species in the world, it is only represented by two species in the flora of Iran including *Elaeagnus angustifolia* L. and *E. orientalis* with their common names of "silver berry, Russian olive, oleaster or oleander".[3,4] *E. angustifolia*, with its Persian name "Senjed", is a perennial deciduous tree or large multi-stemmed shrub with flexible branches that can reach 12 m in height. The leaves are alternate and petiolate and the whole leaves, stems, buds and fruits are densely covered by silvery scales. The flowers are fragrant, 3- to 12-mm long, with four-lobed creamy yellow calyx, in small axillary clusters. The plant also possesses the edible fruits which are berry-like or drupe-, oval-shaped, between 1 and 2 cm long as well as deep or extensive roots, with various well-developed laterals.[1,4-6] *E. angustifolia* have been used for centuries as a herbal remedies for the treatment of various diseases in Iran´s traditional medicine.[7] Some of them were proven to exhibit anti-inflammatory,[1,8] muscle relaxant activity,[9] anti-ulcerogenic,[10] antimicrobial,[11-14] antinociceptive,[15,16] antitumor,[17,18] and antioxidant effects.[11,12,14,19-21] Likewise, the whole fruit and medulla powder of *E. angustifolia* showed positive effect in improving pain, stiffness and physical function in women with osteoarthritis of the knee.[22,23] In Azarbaijan province folk medicine, the fruit and flower preparations have been used for healing jaundice, asthma, flatulence, vomiting and nausea.[24] Phytochemical studies on different extracts from fruits and flowers of *E. angustifolia* indicated the presence of polysaccharides, flavonoids, coumarins, phenolcarboxylic acids, amino acids, saponins, carotenoids, vitamins, and tannins as secondary metabolites.[12,13,25] Besides mentioned secondary metabolites, this plant also contains volatile oils that may be useful as a source of nutrition or pharmaceutical compounds.[26-28] To the best of our knowledge, the essential oil composition of the Iranian *E. angustifolia*

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has not been investigated; therefore, based on the prevalent food and medicinal uses of this plant, the present study was conducted to analyze the chemical composition of the essential oils hydrodistilled from the leaves and flowers of *E. angustifolia* as well as their general toxicity and radical scavenging activities.

Materials and Methods

Plant Material

Flowers and leaves of *E. angustifolia* L. were collected from a garden near Toramin, Ilkhchi, Tabriz, East Azarbaijan province, Iran, in May 2015. The identity of the plant was botanically confirmed by morphological examination in comparison to the herbarium specimens. Voucher specimens (no: Tbz-fph-763) is deposited in the Herbarium of faculty of Pharmacy, Tabriz University of Medical Sciences, Tabriz, Iran.

Essential oil Isolation

The air-dried leaves and flowers of *E. angustifolia* L. (65 and 73 g, respectively) were separately submitted to hydro-distillation for 4 h, in a Clevenger type apparatus using hexane (2ml) as collector solvent. The pale yellow colored essential oils were dried over anhydrous sodium sulfate, and then the solvent was evaporated, and the oil stored in sealed vials before chemical analyses.

GC-MS and GC-FID analysis

The analysis of the essential oils were performed on a Shimadzu GCMS-QP5050A gas chromatograph coupled to a Mass Spectrometer detector (GC-MS) equipped with a fused methyl silicon DB-1 column (1% phenylmethylpolysiloxane, 60 m × 0.25 mm i.d., 0.25 µm film thickness), working with the following temperature program: 3 min at 50°C, subsequently at 3°C/min up to 270°C, and held for 4 min; Helium was used as carrier gas at a flow rate of 1.3 mL/min. The injector temperature was 250°C and split ratio was adjusted at 1:24. The injection volume was 1 µL. The transfer line temperature was 280°C. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV with other operating parameters as follow: ion source temperature 280°C; quadrupole temperature 100°C; solvent delay 8.0 min; resolution 2000 amu/s and scan time 78 min; scan range 30-600 amu; EM voltage 3000 volts. Moreover, flame ionization detector (FID) which was operated in ionization potential mode at 70 eV and used the same program reported above, was applied for quantification purpose for calculating the relative area percentage (area %) without the use of correction factors. The mixture of *n*-alkanes (C8-C20) was then injected using the above temperature program in order to calculate the retention indices of each volatile component.

Identification of the compounds

The components of the oils were identified based on GC retention times, retention indices relative to *n*-alkanes and computer matching with the NIST10, NIST 21, NIST 69 and Wiley 229 library data, as well as by comparison of the mass spectra with those reported in the literature.[29,30] Relative area percentages of the volatile constituents were obtained electronically from the GC-FID response without any correction factor.

Free-Radical-Scavenging Activity

The ability of the essential oils to scavenge radicals was assessed by the method based on the reduction of DPPH (molecular formula $C_{18}H_{12}N_5O_6$) solutions in the presence of a hydrogen donating antioxidant. DPPH (8 mg) was dissolved in chloroform (100 ml) to obtain a concentration of 80 µg/ml. The essential oil were dissolved in chloroform to provide a concentration of 1 mg/ml. Dilutions were made to obtain different concentrations of essential oils and then diluted solutions (5 ml each) were mixed with DPPH (5ml). After a 30 minute incubation period at room temperature, the absorbance was read against a blank at 517 nm with a Shimadzu UV/Visible Spectrophotometer 160A (USA). The percentage reduction was plotted against the sample oils concentration in order to calculate RC_{50} values which is the oil concentration providing 50% loss of DPPH activity. Trolox® was used as positive control and all tests were conducted in triplicate.[31,32]

General toxicity assay (Brine shrimp lethality test)

The general toxicity of the essential oils was evaluated by the brine shrimp lethality test presented by Meyer *et al.*[33] with some modifications.[34,35] Brine shrimps were hatched using brine shrimp eggs (*Artemia salina*, Sera brand, Aquarium and Fish shop, Khaghani Avenue, Tabriz, Iran) in a conical shaped vessel, filled with 200 mL filtered sterile seawater (Prepared from commercial sea salt, 38g/L, Aqua Marine, Thailand). The vessel was kept in a water bath (29-30°C) under a bright light and constant aeration for 48 hours. Stock solutions were prepared by dissolving essential oils in DMSO and diluted with seawater so that the final DMSO concentration did not exceed 1%. Seven different concentrations of essential oils were derived through serial dilution. After hatching, ten nauplii (hatched brine shrimp) were transferred to each test and control (containing DMSO and seawater) tubes. Then, the volume was adjusted with sterile seawater and the tubes were left uncovered under the lamp. Three replicates were prepared for each essential oil. After 24 h after introducing the shrimps, the number of dead and surviving nauplii in each tube were counted and recorded. LD_{50} values were determined from the best-fit line plotted concentration versus percentage lethality. Podophyllotoxin was used as a positive control.

Statistical analysis

All experiments were carried out in triplicate measurements and presented as the mean ± standard deviation. Data were analyzed by using Excel 2010 Microsoft. The RC_{50} and LD_{50} values were calculated from linear regression analysis.

Results and Discussion

The hydro-distillation from the flowers and leaves of *E. angustifolia* L. exuded pale yellow oils with a yield of 0.10% and 0.05% W/W, respectively, based on the dry mass. The list of the components in order of their elution from a DB-1 column, the percentage of the individual compounds and their retention indices are compiled in Table 1. A total of 53 volatile components were identified in the essential oil of the flowers, corresponding to 96.59% of the total oil while 3.41% of the essential oil remained unidentified. Oxygenated compounds especially aromatic esters (65.75%) had the highest contribution and represented 91.90% of the oil (Figure 1). The major components were E-ethyl cinnamate (60.00%), hexahydrofarnesyl acetone or phytone (9.99%), hexadecanoic acid or palmitic acid (5.20%) and phytol (3.29%). The remaining constituents (n=49) present in small quantities, most of them existing at contents lower than 3%. With respect to the leaves, 25 components were identified, accounting for 98.97% of the total oil. Also in this case oxygenated compounds furnished the major contribution of the oil (95.95%), with E-ethyl cinnamate (37.27%), phytol (12.08%), nonanal (10.74%) and Z-3-hexenyl benzoate (7.65%) as the most prevalent. Apart from the major volatiles reported about oil of leaves, only hexadecanoic acid (3.33%) and 9,12,15-octadecatrienal (5.43%) exceeded a content of 3% of the total oil, whilst the remaining volatiles (n=19) were present in low amounts. As depicted in Figure 1, among oxygen-containing components, esters were the most abundant by percentage of 65.75% and 49.12 % of the flowers and leaves oil, respectively. Conversely, the hydrocarbons were relatively poor and constituted 4.69% and 3.02%, respectively. To the best of our knowledge, the essential oil of Iranian *E. angustifolia* has so far never been studied, while there are a few studies about the chemical composition of the same species from other countries.[26-28] With respects to the previous investigation which considered the essential oil from flowers of *E. angustifolia* growing in Romania, limonene, anethol, E-ethyl cinnamate, 2-phenyl ethyl benzoate, 2-phenyl ethyl isovalerate, nerolidole, squalene and acetophenone were identified as the main components.[27] However, the oil studied in China in 2011 represented E-ethyl cinnamate (77.36%), (E)-2-methoxy-4-(1-propenyl) phenol (3.03%), acetal (2.70%), Z-ethyl cinnamate (1.09%) and ethyl benzenacetate (1.06%) as the main components.[28] According to the other report by Zhaolin et al, E-ethyl cinnamte (78.88%) constitutes the principle components of the flowers oil.[26] The comparison of our results with previous literatures shows remarkable similarities and also differences in terms of chemical composition of the flower oil. Presence of E-ethyl cinnamate as the principle constituent is the main similarity of the previous oils[26-28] with our examined flower oil (60.00%). Conversely, anethol, limonene, β-myrcene, squalene and acetophenone were detected at in considerable amounts in previous works,[14,27] whereas it was not found in our tested sample. Moreover, it is notable that hexahydrofarnesyl acetone (phytone), palmitic acid and phytol were found at a relatively high level in our examined flower oil whereas it was not detected in considerable quantities in the oil of previous studies. A variety of factors such as geographical location, climatic condition, altitude, extraction methods and sample collection season might attribute in observed variations in the flower oils composition.[34,35] In regard to leaves, as shown in Table 1 and Figure 1, the essential oil was again characterized by E-ethyl cinnamate (37.27%), however, in this case, aliphatic alcohols and aldehyds reached higher levels in comparison with flowers by percentages of 12.80% and 21.73%, respectively. They were represented by phytol (12.08%) and nonanal (10.74%). The comparison of our results with literature exhibited remarkable differences in terms of chemical composition. According to the report by Incilay, l-limonen, β-myrcene and E-2-hexanal were the main components of leaves essential oil.[14]

The radical scavenging activity of the essential oils from flowers and leaves of *E. angostifolia* was evaluated using DPPH method. From results reported in Table 2, the both essential oils exhibited moderate radical scavenging activity with RC_{50} values of 3.48 ± 0.70 mg/ml (flower oil) and 1.50 ± 0.50 mg/ml (leaves) compared with the values reported for Trolox (0.002 ± 0.20 mg/ml) used as a reference. The more potent activity of the essential oil obtained from leaves can be related to the higher proportion of oxygenated compounds especially alcohols and aldehydes, known to possess antioxidant activity due to their O-atoms. The presence of a hydroxyl moiety on a hydrocarbon skeleton causes that the compound easily oxidizes and shows antioxidant properties; therefore, the possibility that the higher radical scavenging activity by the essential oil of leaves would be due to the presence of higher amount of phytol in leaves (12.08%) could not be excluded. Previous investigations demonstrated that phytol, as a natural linear diterpene alcohol, showed antioxidant activity in different assays[36,37] as well as it is utilized in manufactoring synthetic vitamins E and K.[38]

The general toxicity of essential oils was assessed by brine shrimp lethality test which represent a quick, inexpensive and efficient method for evaluating extracts and essential oils toxicity and most of the time correlates fairly well with anti-proliferative and antitumor activities.[34,35] In this assay, compared with the positive control (Podophyllotoxin, LD_{50}= 2.69 ± 0.30 µg/ml) , the essential oils of flowers and leaves showed potent toxicity against brine shrimps with LD_{50} values of 2.25 ± 0.60 and 11.00 ± 5.19 µg/ml, respectively. As can be seen in Figure 2, the toxicity of the oils raised by increasing in the concentration of the essential oil and exposure duration. The general toxicity effects of flower oils were a little more potent than podophyllotoxin. As illustrated above, the essential oil of flowers and leaves are both noticeably rich in ester compounds especially E-ethyl cinnamate; hence, the potent toxicity activity of these oils might be ascribed to this compounds in high

proportion. Preceding studies demonstrated that E-ethyl cinnamate revealed a remarkable insecticidal, nematocidal and antifeedant activities;[39-42] thus, the strong toxicity effect of flowers oil might be attributed to the presence of considerable amount of E-ethyl cinnamate. It is notable that oral and topical administration of extracts containing high amount of E-ethyl cinnamate caused neither fatality nor significant differences or irritation in the body;[40] so, it might be considered as a safe product for human beings or mammals when applied for insecticidal or anti fungal purposes.

Table 1. Composition of the essential oils isolated from the flowers and leaves of *E. angustifolia*

Compound name and class [a]	RI	Flowers (%)	Leaves (%)	Identification method [b]	Compound name and class [a]	RI	Flowers (%)	Leaves (%)	Identification method [b]
Heptanal	877	0.41	-	GC/MS, I_b	2-Hexyl-1-octanol	1669	0.19	-	GC/MS, I_s
Benzaldehyde	928	0.06	-	GC/MS, I_s	Hexadecanal(Palmitaldehyde)	1696	0.42	4.09	GC/MS, I_s
Benzeneacetaldehyde(Hyacinthin)	1007	0.25	-	GC/MS, I_s	2-Methylhexadecan-1-ol	1723	0.58	-	GC/MS, I_s
Nonanal	**1083**	1.36	**10.74**	GC/MS, I_s	Tetradecanoic acid (Myristic acid)	1744	0.36	-	GC/MS, I_s
Linalool	1185	-	0.17	GC/MS, I_s	n-Octadecane	1800	0.13	-	GC/MS, I_s
Decanal	1185	0.20	0.45	GC/MS, I_s	2-Phenylethyl benzoate	1819	0.39	1.89	GC/MS, I_s
4-Ethylphenyl acetate	1213	0.40	-	GC/MS, I_s	6,10,14-trimethyl-2-pentadecanone	1831	-	2.01	GC/MS, I_s
(-)-Myrtenyl acetate	1273	-	1.83	GC/MS, I_s	**Hexahydrofarnesyl acetone(phytone)**	**1835**	**9.99**	-	GC/MS, I_s
Undecanal	1287	0.35	-	GC/MS, I_s	1-Octadecene	1864	0.12	0.45	GC/MS, I_s
Theaspirane A	1293	-	0.99	GC/MS, I_s	9,12,15-Octadecatrienal	1869	0.22	5.43	GC/MS, I_s
Theaspirane B	1307	-	0.98	GC/MS, I_s	Farnesyl acetone	1895	0.16	-	GC/MS, I_s
Ethyl dihydrocinnamate	1319	0.08	-	GC/MS, I_s	n-Nonadecane	1900	0.21	0.54	GC/MS, I_s
Methyl cinnamate	1352	1.38	-	GC/MS, I_s	9-Hexadecenoic acid	1921	0.40	-	GC/MS, I_s
E-β-Damascenone	1364	0.17	0.72	GC/MS, I_s	**Hexadecanoic acid (Palmitic acid)**	**1950**	**5.20**	3.33	GC/MS, I_s
n-Decanoic acid	1366	0.07	-	GC/MS, I_s	Hexadecanoic acid, ethyl ester	1978	0.22	-	GC/MS, I_s
n-Dodecanal (Lauraldehyde)	1389	0.23	-	GC/MS, I_s	E-15-Heptadecenal	2104	0.1	-	GC/MS, I_s
Trimethyl-tetrahydronaphthalene	1398	0.4	-	GC/MS, I_s	**Phytol**	**2108**	**3.29**	**12.08**	GC/MS, I_s
β-Caryophyllene	1420	-	2.14	GC/MS, I_s	Methyl linolenate	2117	1.11	-	GC/MS, I_s
Neryl acetone	1430	-	0.73	GC/MS, I_s	9-Octadecenoic acid (Oleic acid)	2146	0.37	-	GC/MS, I_s
E-Ethyl cinnamate	**1435**	**60.00**	**37.27**	GC/MS, I_s	(E)-Ethyl- 9-octadecenoate	2151	1.06	-	GC/MS, I_s
Oxacyclotetradeca-4,11-diyne	1457	0.47	-	GC/MS, I_s	Octadecanoic acid, ethyl ester	2177	0.13	-	GC/MS, I_s
2,3,5,8-tetramethyl-decane	1461	0.16	-	GC/MS, I_s	Docosane	2200	0.18	-	GC/MS, I_b
β- Ionone	1466	-	1.80	GC/MS, I_s	1-Docosene	2271	0.47	-	GC/MS, I_b
4,6-Dimethyl-dodecane	1468	1.77	-	GC/MS, I_s	n-Tricosane	2300	0.37	-	GC/MS, I_b
Germacrene D	1483	0.1	-	GC/MS, I_s	Eicosanoic acid, ethyl ester	2339	0.1	-	GC/MS, I_b
Tridecanal	1493	0.32	-	GC/MS, I_s	1-Docosanol (Behenic alcohol)	2388	0.7	-	GC/MS, I_b
γ-Cadinene	1517	-	0.34	GC/MS, I_s	n-Pentacosane	2500	0.12	-	GC/MS, I_b
Isobutylcinnammate	1540	0.68		GC/MS, I_s	n-Heptacosane	2700	0.51	-	GC/MS, I_b
Z-3-Hexenyl benzoate	**1546**	0.12	**7.65**	GC/MS, I_s	n-Octacosane	2800	0.15	-	GC/MS, I_b
Nerolidole B	1550	0.20	-	GC/MS, I_s					
2-Phenylethyl tiglate	1555	0.28	0.52	GC/MS, I_s	Total compounds		53	25	
(+)- Spathulenol	1568	-	0.55	GC/MS, I_s	Total identified		96.59	98.97	
Caryophyllene oxide	1575	-	1.70	GC/MS, I_s	Not identified		3.41	1.03	
Tetradecanal (Myristaldehyde)	1594	**0.08**	0.57	GC/MS, I_s	Hydrocarbons		4.69	3.02	
Elemicin	1613	0.08	-	GC/MS, I_s	Oxygenated compounds		91.9	95.95	

a) Compounds are listed in order of their elution from a DB-1 column. Their nomenclature is in accordance with Adams [29]. b) Identification Method (Is = Kovats retention indices as determined on DB-1 column using homologous series of C_8-C_{20}, Ib = Kovats retention indices according to Literature published by Adams [29] and/or listed in the NIST08 mass-spectral library [30].

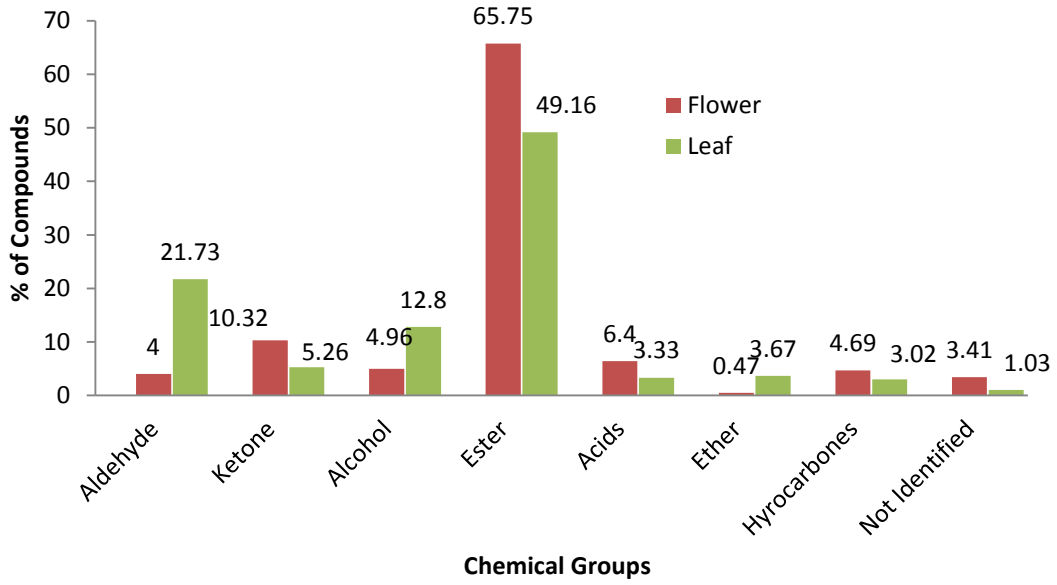

Figure 1. Identified chemical groups from the essential oils of the flowers and leaves of *Elaeagnus angustifolia* L.

Table 2. Radical scavenging and general toxicity activities of the essential oils obtained from leaves and flowers of *E. angustifolia* L.

	DPPH assay (RC_{50}, mg/ml)	General toxicity (LD_{50}, µg/ml)
Essential oil of flowers	3.48 ± 0.70	2.25 ± 0.60
Essential oil of leaves	1.50 ± 0.50	11.00 ± 5.19
Trolox®	0.002 ± 0.20	-
Podophyllotoxin	-	2.69 ± 0.30

RC_{50}, the concentration of compound that affords a 50% reduction in the assay, LD_{50}, the required dose of compound that kills 50% of a population of brine shrimps

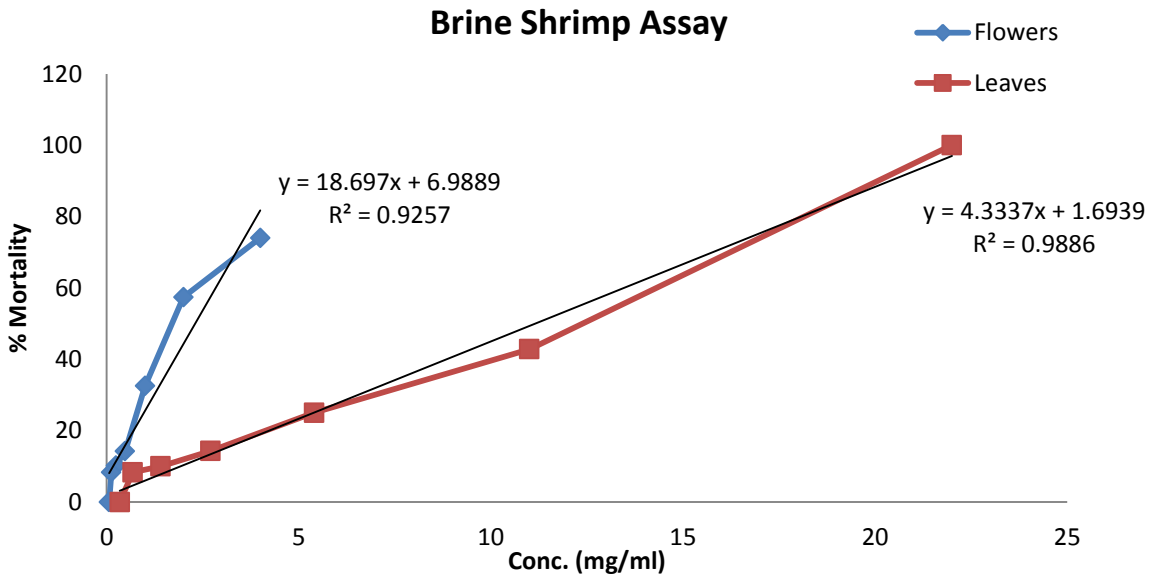

Figure 2. Brine shrimp lethality assay of the essential oils obtained from the flowers and leaves of *Elaeagnus angustifolia* L. against *Artemia salina*

Conclusion

To sum up, the present study reported the chemical profile of the essential oils obtained from the leaves and flowers of Iranian *E. angustifolia* for the first time, and also assessed the radical scavenging and general toxicity activities of the oils. On the basis of the chemical composition and bioactivity results, we can declare that the oils of this plant might be considered as preservative agents in food industry as well as natural insecticides in agriculture.

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Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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