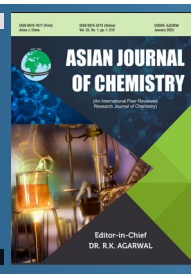
Asian Journal of Chemistry; Vol. 35, No. 8 (2023), 1879-1883

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2023.27948



Chemical Composition and *in vitro* Biological Evaluation of Essential Oil of *Euphorbia larica* Boiss from Northern Oman

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Received: 4 May 2023; *Accepted*: 21 June 2023; *Published online*: 31 July 2023; AJC-21320

Euphorbia larica Boiss is a wild plant of Oman that grows in gravel plains, mountain areas, desert and forest edges. Traditionally, Omani herbalists use the *Euphorbia larica* plant extract, resins, latex and juice to treat a wide range of ailments such as wounds, burns, insect bites, intestinal parasites, gonorrhea, eye infections, migraines and warts. In an attempt to scientifically validate the traditional uses and to unlock its hidden therapeutic potential, in this work, the chemical composition of *E. larica* essential oil (ELEO) along with its potential antioxidant, antimicrobial and hemolytic activities is studied. In a GC-MS analysis of the extracted ELEO, 29 chemicals were found, with α-pinene (27.36%) and limonene (11.5%) being the two most abundant volatile components. ELEO exhibited moderate inhibition of DPPH radicals (17.74-40.96%; IC_{50} = 48.97 µg/mL). It showed antimicrobial activity against *S. aureus, B. subtilis*, *S. pyogenes*, *E. coli*, *P. aeruginosa* and *P. vulgaris* but was ineffective against *K. pneumoniae* bacterial strain however, exhibited the maximum antibacterial activity against *S. au*reus (11 ± 1.3 and 15 ± 2.3 mm at 5 and 10 µL concentrations, respectively) comparable to positive antibiotic ampicillin (15 mm at 5 µg). Moreover, ELEO exhibited a very low hemolytic activity (0.12 to 1.1% at doses of 50-500 µg) suggesting it to be non-toxic to human or animal cells. Thus, it can be concluded that *E. larica* plant holds significant potential as a source of bioactive agents for the development of novel therapeutics.

Keywords: *Euphorbia larica* Boiss, Antioxidant, GC-MS, Antimicrobial, Volatile oil, Hemolytic activity.

INTRODUCTION

Euphorbia larica Boiss (Synonym: *Tirucalia larica)* a member of Euphorbiaceae family, is an evergreen perennial herb that can reach a height of 1.0-1.5 m. It is native to the Himalayas, specifically in Pakistan, India, Nepal and Bhutan, the Horn of Africa and parts of Arabian Peninsula. This plant is an important and commonly occurring component of the native desert flora of Northern Oman [1]. It typically grows in gravel plains, mountain areas, desert and forest edges at the elevations ranging from 2,000 to 3,500 m above sea level. In Oman, *E. larica* has been found to grow on bare limestone rocks in the Hajar Mountain and Ru'us al-Jibal region [2]. The plant is commonly known as Spurge tree, Milky Euphorbia, Laric's spurge or the Laric's milkweed in English and locally it is known as Isbaq. It is a deciduous photoautotroph having thick stem, small smooth leaves and yellow/green flowers. Honeybees use its flower nectar to produce honey. Lower stems are smooth, woody and brown in colour, while the upper stems are light green. When the stems are broken into pieces, they exude milky sap, which is toxic and can irritate skin, although, a few grazing animal species feed on this plant [3].

E. larica plant has a rich history of traditional uses by the local Omani population. The dried stalks of the plant were used for building roofs and as a source of firewood, while the sticky latex was utilized as an anti-parasitic treatment for camels and for catching birds and fish. The plant extract, resins, latex and juice were commonly used by Omani herbalists to treat a wide range of ailments such as wounds, burns, insect bites, intestinal parasites, gonorrhea, eye infections, migraines and warts [4-7]. Recent scientific studies have confirmed that *E. larica* and other species of Euphorbia have potent pharmacological properties, including antifungal, anticancer and antimicrobial activities [8,9]. These findings have expanded the

potential therapeutic uses of *E. larica* beyond traditional medicine. Similar to Omani practices, the sap of *E. larica* has been used for treating skin and respiratory diseases in some African countries, while Yemeni healers primarily used it for gastrointestinal ailments such as stomachache and diarrhea.

Only a few studies have been conducted to explore the phytochemistry of *E. larica* plant. These studies on plant aerial part and latex have reported the presence of various classes of bioactive secondary metabolites including terpenes, flavonoids and their glycosides (rutin, 6-methoxyapigenin, kaempferol-3-rutinoside, 3-O glycosides of quercetin and kaempferol), steroids (β-amyrin acetate, lupeol, lupeol acetate, ginnone, ambrein and lupeone), alkaloids, hydrocarbons (nonacosane), hydroxy acids and phenolic compounds [4,10,11]. Recently, Rahman *et al.* [12] isolated eupholaricanone, an anthracene derivative, from the ethyl acetate fraction of the whole plant, which was demonstrated to exhibit anti-α-glucosidase activity. Presence of these bioactive constituents in *E. larica* may contribute to its beneficial medicinal properties. However, limited data is available which shed light on chemistry and bioactivity of *E. larica* essential oil. Therefore, we aimed to study the chemical composition of its essential oil and to investigate its potential antioxidant and antimicrobial activity to unlock its hidden therapeutic potential.

EXPERIMENTAL

Collection of plant material: *E. larica* (400 g) whole plant was collected from Al-Hajar Mountains, Sultanate of Oman in May 2021. It was identified by a biologist of the Natural and Medical Sciences Research Centre, University of Nizwa, Oman. A voucher specimen of *E. larica* (WP/EL/05/2021) was deposited in the herbarium for future reference. The fresh plant material was cleaned under running tap water and then cut into small pieces.

Extraction of *E. larica* essential oil (ELEO): Hydrodistillation of 400 g of whole *E. larica* plant for 6 h yielded a light yellow essential oil (1.12 mL, 0.28% v/w). The extracted ELEO was dried over anhydrous Na_2SO_4 and stored at 4 ºC in the refrigerator till further use.

GC/MS analysis: The ELEO was analyzed using GC-MS [(Shimadzu GC-2010 Plus, fitted with a Rtx-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; maximum temperature, 350 ºC)] coupled to GCMS-QP2010 ULTRA-MS. Ultra-high purity helium (99.9999%) was used as carrier gas at a constant flow of 1.0 mL/min. The injection, transfer line and ion source temperatures were 280, 270 and 260 ºC, respectively. The ionizing energy was 70 eV. Electron multiplier (EM) voltage was obtained from auto-tune. All data were obtained by collecting the full-scan mass spectra within the scan range 40-550 amu. The injected sample volume was 1 µL with a split ratio of 50:1. The oven temperature program was 60 ºC at a rate of 4 ºC/min–260 ºC hold for 4 min. The run time was 60 min. The unknown compounds present in the essential oil were identified by comparing the spectra obtained with mass spectrum libraries (NIST 2011 v.2.3 and Wiley MS, 9th edition).

***In vitro* DPPH radical scavenging activity of ELEO:** The ability of ELEO to scavenge DPPH free radicals was evaluated at four concentrations (5, 10, 20 and 40 µg/mL) following a reported method [13]. The experiment was performed in triplicate and the results of antioxidant activity are presented as mean ± SD of % inhibition of DPPH radicals and IC_{50} value.

Antimicrobial activity: Antimicrobial activity of the neat ELEO was tested at two concentrations (5 and 10 µL) against three Gram-positive (*Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes*) and four Gram-negative (*Escherichia coli, Pseudomonas aeruginosa, Proteus vulgaris* and *Klebsiella pneumoniae*) bacterial strains by the disc-diffusion method [14]. A blank sterilized disc of 6 mm diameter was impregnated with test samples. Ampicillin disc of 5 µg was used a positive control. Test samples were spotted alternately on both sides of the discs. The impregnated discs with the test samples were placed on the nutrient agar plate surface. Each plate consisted of 4 discs. One positive control (ampicillin disc), one negative control (an empty sterile disc without any impregnation) and two discs with 5 µL and 10 µL concentrations. All the discs were placed about equidistance to each other under aseptic environment. The plates were then incubated at 37 ºC for 18-24 h depending on the bacterial species. Antimicrobial activity was evaluated by measuring the inhibition zone (in mm) around the discs.

Hemolytic assay: To determine its toxicity and non-biocompatibility, an ELEO hemolytic assay was performed in triplicate using the procedure suggested by the WHO for testing therapeutic plant products [15,16]. Hemolytic assay of the ELEO sample was determined by comparison with a standard which has 100% hemolytic activity. Stock solution of the test sample was prepared by dilution using phosphate buffer saline and incubated at room temperature. Fresh erythrocytes were collected from the human blood by centrifugation procedure at 2500 rpm for 5 min. Erythrocytes were washed thrice by 150 mM NaCl and then suspended in a measured volume (2 mL in 20 mL) of 100 mM Na_3PO_4 buffer to maintain the physiological environment. This diluted suspension was used for further experiment. The ELEO sample in different (50-500 µg) concentrations were added to 200 µL of erythrocyte suspension and final reaction mixture volume was made up to 1 mL with Na_3PO_4 buffer. The resulting mixture was incubated for 30 min at 37 ºC. The reaction mixture was centrifuged again at 2500 rpm for 5 min to check for hemolysis. The absorbance of the supernatant was measured at 492 nm using a microplate reader. The Na_3PO_4 buffer was used as negative control and Triton X (known hemolytic agent) in RBC suspension was used as positive control. This test is mainly used in toxicological analysis as it gives a quantitative estimation of percentage of RBC lysis and cytotoxic effect [15]. The percentage of hemolysis was calculated using the following formula:

$$\text{Hemolysis (\%)} = \frac{A_{TS} - A_{NC}}{A_{PC}} \times 100$$

where A_{TS} is the absorbance of test sample; A_{NC} is the absorbance of a negative control and A_{PC} is the absorbance of a positive control.

RESULTS AND DISCUSSION

Chemical composition of ELEO: A light yellow oil was obtained in a yield of 0.28% (v/w) following hydrodistillation of *E. larica* fresh plant for 6 h with the help of a Clevenger apparatus. Although percentage yield of ELEO is little low but it is in agreement with the results obtained by Shah *et al.* [17]. The percentage yield of essential oil depends on several factors and may vary significantly depending on harvesting time, environmental conditions, method of extraction, *etc*. [18]. The GC-MS analysis of the extracted ELEO showed presence of 29 compounds in gas chromatogram (Fig. 1) but only 26 volatile chemical compounds could be identified after matching their mass spectra with databases (NIST, Wiley MS library) representing 89.66% of the total oil (Table-1).

Nearly one-third of the identified volatile constituents belong to the sesquiterpenes (34.48%) class of compounds while monoterpenes (24.14%) and monoterpenoids (31.03%) accounted for rest of the compounds. Essential oils from other species of Euphorbia have been shown to contain higher proportion of oxygenated and non-oxygenated sesquiterpenes [19]. α-Pinene (27.36%) and limonene (11.5%) accounting to nearly 40% of total identified constituents were observed to be the major chemical constituents followed by *trans*-verbenol (monoterpenoid, 4.94%), β-caryophyllene (sesquiterpene, 4.41%), β-elemene (sesquiterpene, 4.21%) and β-eudesmene (sesquiterpene, 4.04%). Other bioactive volatile terpenoids of pharmaceutical significance including camphene, β-pinene, cymene, L-pinocarveol, α-terpinene-4-ol, L-verbenol, bornyl acetate, β-bourbonene, humulene, aromandendrene, patchoulane, δ-selinene and

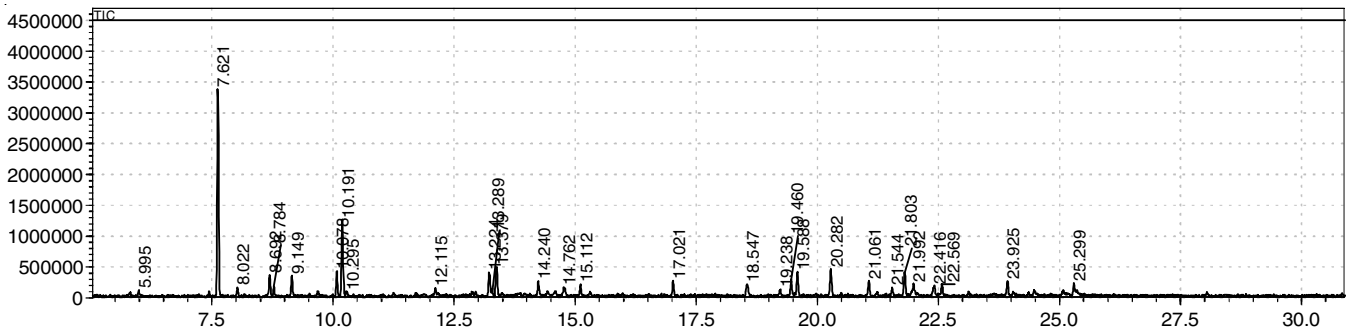

Fig. 1. GC chromatogram of ELEO

TABLE-1
CHEMICAL COMPOSITION OF *Euphorbia larica* VOLATILE OIL (ELEO) OBTAINED THROUGH GC-MS

S. No.	Chemical name	Retention time	Peak area (%)	Type of terpene	KI (calculated)	KI (NIST)
1	α-Pinene	7.621	27.36	Monoterpene	933.6162	931
2	Camphene	8.022	1.20	Monoterpene	948.4133	935
3	4(10)-Thujene	8.692	2.93	Monoterpene	973.1365	964
4	β-Pinene	8.785	1.28	Monoterpene	976.5683	943
5	β-Myrcene	9.149	2.65	Monoterpene	990.0000	981
6	*o*-Cymene	10.078	3.77	Monoterpene	1023.7545	1025
7	Limonene	10.191	11.5	Monoterpene	1027.8339	1020
8.	Cineole	10.295	0.83	Monoterpenoid	1031.5885	1023
9	Isothujol	12.115	0.51	Monoterpenoid	1097.2924	1110
10	L-Pinocarveol	13.224	3.93	Monoterpenoid	1142.8157	1143
11	*cis*-Verbenol	13.289	0.92	Monoterpenoid	1145.5073	1148
12	*trans*-Verbenol	13.379	4.94	Monoterpenoid	1149.2340	1162
13	Terpinen-4-ol	14.240	2.13	Monoterpenoid	1184.8861	1175
14	U.I.	14.762	1.40	–	1206.1496	–
15	*l*-Verbenone	15.112	1.57	Monoterpenoid	1219.8590	1204
16	Bornyl acetate	17.021	2.23	Monoterpenoids	1294.6338	1285
17	α-Terpinyl acetate	18.547	1.98	Monoterpenoids	1357.5871	1330
18.	Copaene	19.238	0.81	Sesquiterpene	1386.2355	1397
19	(-)-β-Bourbonene	19.460	2.26	Sesquiterpene	1395.4395	1386
20	β-Elemene (-)-	19.588	4.21	Sesquiterpene	1400.7463	1387
21.	β-Caryophyllene	20.282	4.41	Sesquiterpene	1430.4534	1421
22	Humulene	21.061	1.74	Sesquiterpene	1463.7725	1456
23	Aromandendrene	21.543	1.45	Sesquiterpene	1484.3884	1455
24	β-Eudesmene	21.803	4.04	Sesquiterpene	1495.5090	1478
25	δ-Selinene	21.992	1.95	Sesquiterpene	1504.0000	1509
26	U.I	22.416	2.21	–	1524.1905	–
27	δ-Cadinene, (+)-	22.569	1.71	Sesquiterpene	1531.4762	1537
28	Patchoulane	23.925	2.18	Sesquiterpene	1596.0473	1618
29	U.I.	25.299	1.28	–	1662.7918	–

Total identified compounds = 89.66%; Retention index was calculated for all volatile constituents using a homologous series of *n*-alkanes (C_8–C_{30}).

δ-cadinene were also detected in minor quantities (1-4%). Based on the chemical composition, this *E. larica* species from Northern Oman can be assigned to α-pinene chemotype. A similar study on volatile oil composition of *E. larica* from Southern Oman reported presence of 60 compounds and their results differed significantly. Interesting, they identified camphene (16.41%) and thunbergol (15.33%) as the major chemical components [17]. The qualitative and quantitative variation in chemical composition of essential oil from the same species could be attributed to ecosystem diversity including different habitats, seasons, harvesting time and geographical regions where species grow, *etc*. [20]. Since, the *E. larica* species from Northern Oman is found to be rich in α-pinene and limonene, these two could be used as biomarkers to identify and differentiate this species from other Euphorbia species.

***In vitro* antioxidant activity:** The antioxidant activity of ELEO was investigated through *in vitro* free radical scavenging assay using the standard DPPH method. The principle of assay is based on the reduction of stable radical DPPH by the antioxidant compounds in extract/oil, which results in a colour change from purple to yellow. The degree of discolouration indicates the antioxidant activity of the sample [21]. The results showed that ELEO exhibited moderate but dose-dependent (5-40 μg/mL) inhibition of DPPH radicals (17.74-40.96%) (Table-2). The IC_{50} value, which was computed from the linear equation method, was found to be 48.97 μg/mL, indicating mild antioxidant activity. This could be attributed to the presence of the major volatile constituents of ELEO. A previous study also reported weak antioxidant activity of ELEO, with an IC_{50} value of 133.53 μg/mL [17]. The use of natural products, such as essential oils, has become increasingly popular due to their potential health benefits. The study of ELEO's antioxidant activity is particularly important, as oxidative stress has been implicated in the development of several chronic diseases, such as cancer, diabetes and cardiovascular diseases [22]. The results of this study suggest that ELEO has mild antioxidant activity. However, it is important to observe that the results of *in vitro* studies may not necessarily translate to *in vivo* situations. Therefore, further studies are needed to fully explore the antioxidant potential of ELEO and its effectiveness in preventing chronic diseases.

TABLE-2
In vitro ANTIOXIDANT ACTIVITY OF ELEO AT FOUR DIFFERENT CONCENTRATIONS

Conc. (μg/mL)	Average % inhibition of DPPH (Mean ± SD)
5	17.74 ± 1.35
10	21.56 ± 2.21
20	38.17 ± 2.32
40	40.96 ± 0.98
IC_{50} (μg/mL)	48.97

Antimicrobial activity: The antimicrobial activity of the ELEO was determined by measuring the zone of inhibition (ZOI) against the studied Gram-positive and Gram-negative bacterial strains. ELEO inhibited the growth of *S. aureus, B. subtilis*, *S. pyogenes*, *E. coli* and *P. aeruginosa* at both 5 and 10 μL concentrations (Table-3). However, *P. vulgaris* and *K. pneumoniae* bacterial strains were not affected at 5 μL concentration. At 10 μL concentration, ELEO was able to inhibit *P. vulgaris* but *K. pneumoniae* was found to be resistant even at higher concentration. ELEO exhibited the maximum antibacterial activity against *S. aureus* (11 ± 1.3 and 15 ± 2.3 mm at 5 and 10 μL concentrations, respectively) comparable to positive antibiotic ampicillin (15 mm at 5 μg). A Gram-negative bacterial strain (*S. pyogenes*) showed little sensitivity to ELEO (2 ± 1.2 mm at 5 μL) but its growth was significantly affected (11 ± 0.6 mm; 5.5-fold) at 10 μL concentration Further, it has also been observed that ELEO possesses effective antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. A study carried on ELEO from Southern Oman also reported considerable antimicrobial activity against Gram-positive microorganisms. But in contrast to present results, they demonstrated ELEO to be effective against *K. pneumoniae* [17]. Furthermore, previous studies have reported that terpenoids (oxygenated terpenes) exhibit stronger antimicrobial spectrum than hydrocarbons [23] so the antibacterial activity of ELEO could be due to high content of terpenoids. However, monoterpenes like α-pinene, α-terpineol, limonene and linalool have also been shown to exhibit good antimicrobial activity [24]. Although *E. larica* Boiss species has not been studied extensively for its antimicrobial properties but it could be suggested that the antibacterial activity of ELEO is not because of a single major constituent rather is a function of its complex chemical composition.

TABLE-3
ANTIMICROBIAL ACTIVITY OF ELEO AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Microorganism	ELEO		Positive ampicillin (5 μg/disc)
	5 μL	10 μL	
S. aureus	11 ± 1.3	15 ± 2.3	15
B. subtilis	10 ± 0.9	13 ± 1.2	17
S. pyogenes	2 ± 1.2	11 ± 0.6	13
E. coli	4 ± 1.1	8 ± 0.5	16
P. aeruginosa	5 ± 1.1	8 ± 0.9	17
P. vulgaris	0	9 ± 0.5	15
K. pneumoniae	0	0	–

Hemolytic activity: To evaluate the toxicity and biocompatibility of ELEO, a hemolytic assay in triplicate, following the standard protocol recommended by the WHO for medicinal plant materials, with slight modifications [15] was performed. Present results showed that ELEO test samples exhibited an almost imperceptible hemolytic effect, with percentages ranging from 0.12 to 1.1% at doses of 50-500 μg. Importantly, the results were independent of concentration (Fig. 2), indicating the biocompatibility and safety of the test compounds. Moreover, the values did not follow an increasing concentration pattern. Specifically, the percentage of hemolysis was less than 1.5%, which is considered safe based on many scientific reports. Hemolysis up to 5% is considered as safe and can be correlated with the nontoxic nature of the test compound [15,16]. Overall, present findings suggested that ELEO has low hemolytic activity and is unlikely to be toxic to human or animal cells. This supports its potential use in various therapeutic applications.

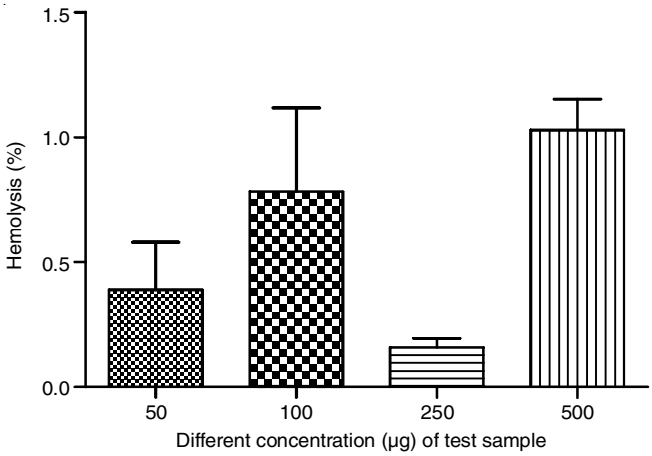

Fig. 2. % Hemolysis of RBCs caused by ELEO at different concentrations [Values represent mean ± SEM (n = 3)]

Conclusion

GC-MS analysis revealed that the essential oil isolated from *Euphorbia larica* Boiss plant grown in Northern Oman belongs to α-pinene chemotype and has different chemical composition than the same species cultivated in Southern Oman and or other parts of the world. Limonene is found to be the second major chemical constituent. Based on the chemical compositions, it could be suggested that α-pinene and limonene content in *E. larica* species may be used as biomarkers in herbal, food and cosmetics industries to identify, to check adulteration and differentiate this species from other Euphorbia species. Although *E. larica* volatile oil showed mild antioxidant activity and hence it should be further explored to study its potential health benefits especially in chronic diseases. The essential oil of *E. larica* demonstrated strong antibacterial activity against Gram-positive pathogenic bacteria and thus it has the potential to be used as a source of antimicrobial agent(s). Alternatively, *E. larica* volatile oil can also be used as a preservative in food industry. However, further studies are needed to fully understand the mechanisms underlying the antibacterial activity of *E. larica* volatile oil and its potential applications in the development of new antimicrobial agents. Findings of this study suggest that *E. larica* volatile oil has low hemolytic activity and is unlikely to be toxic to human or animal cells. This supports its potential use in various therapeutic applications. Overall, the traditional uses and emerging scientific evidence suggest that *E. larica* holds significant potential as a source of bioactive agents for the development of novel therapeutics.

ACKNOWLEDGEMENTS

The authors thank the Dean, College of Pharmacy, National University of Science and Technology, Muscat, Sultanate of Oman for providing facilities to carry out this research work.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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