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Chemical Composition and Antimicrobial Activity of Essential Oils of *Ballota nigra* Subsp. *kurdica* From Iran

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Abstract

Background: Medicinal plants have been considered a good source for finding new pharmaceutical chemicals, and identification of chemical composition is the first step towards uncovering the nature of bioactive compounds.
Objectives: The aim of this study was to analyze the composition of the essential oil of *Ballota nigra* subsp. *kurdica* from Iran for the first time and to test its in vitro antibacterial activity against some bacteria.
Methods: The flowers of *Ballota nigra* subsp. *kurdica* were subjected to a Clevenger extractor for essential oil extraction, and gas chromatography mass spectrometry analysis was performed for its analysis. *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Bacillus subtilis*, *Klebsiella pneumonia* and *Pseudomonas aeruginosa* bacteria were used to test antibacterial activity of the essential oil using the disk diffusion method.
Results: GC-MS analysis detected 22 components in the extracted essential oil which constituted more than 98% of total essential oil. The main compounds of the oil were caryophyllene oxide (39.43%), trans-caryophyllene (24.88%), germacrene D (7.64%), 1-undecene (4.20%), isoaromadendrene epoxide (3.25%), and tridecane-1 (2.81%). The essential oil showed moderate to high antimicrobial activity against all tested strains.
Conclusions: The data of this study suggests that the essential oil of *B. nigra* subsp. *kurdica* could be considered a natural antimicrobial agent to preserve food and treat infections in the near future.

Keywords: *Ballota nigra* subsp. *kurdica*, Antimicrobial, Essential Oil, Terpenes, Medicinal Plant

1. Background

Recently, there has been growing interest in the area of research on natural compounds of plants displaying antioxidant, antimicrobial, and pharmaceutical activities which are used for humans and animals as food components or as specific pharmaceutics (1, 2). Today demands for less use of synthetic drugs and food preservatives/additives have increased around the world. Synthetic compounds are sometimes associated with adverse effects on the host, including allergic reactions, hypersensitivity, and immunity suppression, hence using alternative natural compounds with antimicrobial, antifungal, and antioxidant activities, such as essential oils and extracts from different plant species, has become an interesting solution for this problem (3, 4). The *Ballota* genus belongs to the Lamiaceae family (5). It has been reported that 33 to 35 species exist in the genus *Ballota* that are mainly distributed around the Mediterranean and Eurasia (6, 7). *Ballota* species have been widely used in traditional medicine as sedative, antispasmodic, diuretic, choleretic, and antihemorrhoidal agents (8). Several compounds have been reported in *B. nigra*, including terpenes, flavonoids, phenylpropanoids, and tannins (9). *Ballota nigra* (black horehound) is a perennial herb distributed in most areas of the world consisting of several subspecies, e.g., *B. nigra* subsp. *nigra*, *B. nigra* subsp. *foetida*, *B. nigra* subsp. *uncinata*, *B. nigra* subsp. anatolica, and *B. nigra* subsp. *kurdica* (10, 11). *B. nigra* has antiseptic, anti-inflammatory, antirheumatic, antioxidant, and antimicrobial effects. The main significance of *B. nigra* is its neurosedative activity. According to a review of the literature, the essential oil composition of *B. nigra* subsp. *kurdica* (Figure 1) as an endemic medicinal plant from Iran has not yet been determined, hence the first re-

port of its chemical composition and antimicrobial activity has been presented in the present work to evaluate its potential as an antimicrobial agent in the future.

Figure 1. Photograph of *Ballota nigra* subsp. *kurdica* Taken at the Time of Collection

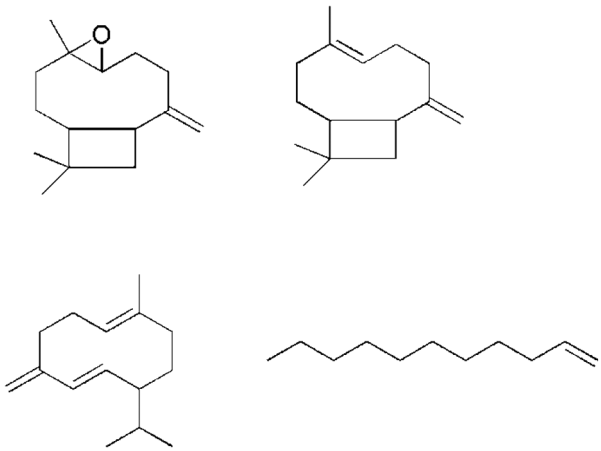

Figure 2. The Structure of Major Compounds of the Essential Oil of *Ballota nigra* subsp. *Kurdica*

2. Objectives

The purpose of this study was to determine the chemical composition and antimicrobial properties of essential oils isolated from *Ballota nigra* subsp. *kurdica*.

3. Methods

3.1. Plant Material

In the present survey, the flowering aerial parts of *B. nigra* subsp. *kurdica* were collected in June 2013 from the Saral region in Divandarreh, Kurdistan province, located in western Iran. Voucher specimens were deposited at the Herbarium of the Research Institute of Forests and Rangeland Research by Hossein Maroufi, Sanadaj, Iran, under voucher no. (8515).

The flowers were air-dried in darkness at room temperature.

3.2. Essential Oil Isolation

The essential oil was extracted by hydrodistillation of air-dried flowers using a Clevenger-type apparatus for 3 hours. The essential oil was subsequently dehydrated via anhydrous sodium sulfate and stored in tightly closed dark vials at +4°C until injection.

3.3. Analysis of the Essential Oils

GC analysis was performed using a Thermoquest gas chromatograph with a flame ionization detector (FID). The analysis was performed using a fused silica capillary DB-5 column (60 m $\times$ 0.25 mm i.d.; film thickness 0.25 μm). Detector and injector temperatures were 250°C and 300°C respectively, and the carrier gas was nitrogen with a flow rate of 1 ml/min^{-1}. The oven temperature was programmed from 60°C - 250°C at the rate of 5°C per minutes, and then was held isothermally for 10 minutes. The split ratio was 1/50. GC-MS analysis was performed using a Thermoquest-Finnigan gas chromatograph equipped with the above-mentioned column and coupled to a TRACE mass quadrapole analyzer. The analysis was carried out using a fused silica capillary DB-5 column (60 m $\times$ 0.25 mm i.d.; film thickness 0.25 μm). Helium was used as the carrier gas with an ionization voltage of 70 eV. Temperature programming conditions were as given for GC. Ion source and interface temperatures were 200°C and 250°C, respectively. Mass range was from m/z 43 - 456. The constituents of the essential oil were identified by calculation of their retention indices under the same chromatographic conditions for n-alkanes (C6 - C24) and the oil on a DB-5 column. Compounds were identified by comparing their mass spectra with those of the library or with authentic compounds, and for confirmed compounds, their GC retention indices were compared with authentic compounds or with those reported in the literature (12, 13). For quantification purposes, the relative area percentages obtained by GC-FID were used without the use of correction factors.

3.4. Antimicrobial Activity Assay

The antibacterial activity of the essential oil was determined by the disk diffusion method. Briefly, 0.1 ml of a suspension of the test microorganisms (108 cells ml^{-1}) was spread on Mueller-Hinton Agar plates. Sterile 6 mm disks, each containing 10 μl of the essential oil, were placed on

the microbial lawns. The plates were incubated at 37°C for 24 hours. The diameters of the zones of inhibition were measured and are reported in mm. Triplicate tests were performed in all experiments. The MIC values were determined using a broth microdilution assay. Serial two-fold dilutions of the essential oil were made in a Mueller–Hinton Broth containing 0.5% Tween 80. Fresh microbial suspensions prepared from overnight-grown cultures in the same media were added to give a final concentration of 5 × 105 organisms ml^{-1}. Controls of medium with microorganisms or the essential oil alone were included. Tetracycline and gentamicin were used as positive controls for Gram-positive and Gram-negative bacteria, respectively. The MIC of the essential oil and powdered antibiotics was defined as the lowest concentration that inhibited growth of microorganisms detected visually.

4. Results

4.1. Chemical Composition of the Essential Oil

Analytical results of the essential oil are shown in Table 1, along with the retention indices of the identified compounds, where all constituents are listed in order of their elution from the DB-5 column.

A total of 22 components were detected in the extracted essential oil, which constituted more than 98% of total essential oil. The 6 major compounds of the oil were caryophyllene oxide (39.43%), trans-caryophyllene (24.88%), germacrene D (7.64%), 1-undecene (4.2%), isoaromadendrene epoxide (3.25%), and tridecane-1 (2.81%). The classification of identified compounds showed that oxygenated sesquieterpene (45.8%) constituted the major part of the oil, followed by sesquiterpens hydrocarbons (41.0%), alkanes (8.8%), oxygenated diterpenes (1.7%), oxygenated monoterpenes (1.13%), and monoterpenes (0.4%).

4.2. Antimicrobial Activity of the Essential Oil

In vitro antibacterial activity of the essential oil was evaluated against six Gram-positive and Gram-negative bacteria (*Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 15753, *Bacillus subtilis* ATCC 9372, *Klebsiella pneumonia* ATCC 3583, *Pseudomonas aeruginosa* ATCC 27852, and *Escherichia coli* ATCC 9763). The results of the antibacterial assay of the essential oil by the disc diffusion method and the MIC values indicated that the essential oil exhibited moderate to high antibacterial activity, especially against *E. faecalis* and *S. aureus* with a MIC value of 3.75mg ml^{-1} (Table 2).

5. Discussion

The essential oil compositions of *B. nigra* subsp. *kurdica* showed a clear difference with what has been reported for *B. nigra* in other studies. The major compounds of the essential oils of *Ballota nigra* subsp. *kurdica* were caryophyllene oxide (39.43%), trans-caryophyllene (24.88%), germacrene D (7.64%), 1-undecene (4.2%), isoaromadendrene epoxide (3.25%), and tridecane-1 (2.81%). In *B. nigra* from northern Iran, the major components of the essential oil were caryophyllene oxide (7.9%), epi-α-muurolol (6.6%), δ-cadinene (6.5%), and α -cadinol (6.3%), which included 35 sesquiterpenoids (89.9%), one diterpenoid (0.1%), and 6 nonterpenoids (5.4%) (14). In *B. nigra* subsp. *foetida*, caryophyllene (25.1%) and germacrene D (24.2%) were the major compounds, while viridoflorol was absent in the essential oil and contained a higher percentage of sesquiterpenes hydrocarbons (60.3%) and two oxygenated sesquiterpenes, including trans-nerolidol (0.3%) and caryophyllene oxide (4.2%) (15). In *B. nigra* subsp. *anatolica*, the major components were germacrene D (18.1%), nerolidol-epoxy-acetate (15.4%), sclareol oxide (12.1%), linalyl acetate (11.5%), and β-caryophyllene (10.5%), which the oil was consisted of oxygenated sesquiterpenes (41.2%), sesquiterpenes hydrocarbons (32.5%), and oxygenated monoterpenes (18.1%) (16). Our results show that the essential oil compositions of *B. nigra* subsp. *kurdica* differ compared with other reported studies for *B. nigra*. The results indicate that caryophyllene oxide (39.43 %) and trans-caryophyllene (24.88 %) are two major compounds of the essential oil of *Ballota nigra* sp. *kurdica*, which constitute about 65% of the total essential oils. Caryophyllene is known for its anti-inflammatory, local anaesthetic, antifungal properties, and also its anticancer activity (17), hence based on the higher concentration of caryophyllene or its derivatives in *Ballota nigra* subsp. *kurdica*, it could be considered a good remedy source for treating related infections in the future.

Our results by the disc diffusion method and the MIC showed in vitro antibacterial activity of the essential oil against both Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and *Escherichia coli*. The essential oil of *Ballota nigra* subsp. *kurdica* exhibited moderate to high antibacterial activity, especially against *E. faecalis* and *S. aureus*, both of which are important for human health (Table 2). *E. faecalis* is a frequent cause of many serious human infections, including urinary tract infections, endocarditis, bacteremia, and wound infections (18). *S. aureus* is a common cause of skin infections (e.g., abscesses), respiratory infections (e.g., sinusitis), and food poisoning. Based on the high antimicro-

Table 1. Composition of the Essential Oil of *Ballota nigra* subsp. *kurdica* From Western Iran (Identification Method: RI, MS)[a]

Compounds	RI	%	Compounds	RI	%
n-Decane	999	1.63	trans-β- Farnesene	1458	2.5
Limonene	1024	0.4	Germacrene-D	1484	7.64
1-Undecene	1092	4.20	β-Selinene	1489	0.68
Linalool	1098	0.51	Bicyclogermacrene	1500	0.86
n-Nonanal	1108	0.55	β-Bisabolene	1505	1.01
Camphor	1143	0.2	cis-α-Bisabolene	1532	0.45
Borneol	1165	0.4	Isoaromadendrene epoxide	1579	3.25
Tridecane-1	1291	2.81	Caryophyllene oxide	1582	39.43
β-Bourbonene	1387	0.52	Virdiflorol	1590	0.59
trans-Caryophyllene	1417	24.88	Humulene epoxide	1608	1.69
α-Humulene	1452	2.2	Trans-Phytol	1949	1.66
			Total		98.06

[a] RI; retention indices relative to C8-C28 n-alkanes on DB-5. The components are listed in order of elution from the DB-5 column.

Table 2. Antibacterial Activity of the Essential Oil of *Ballota nigra* subsp. *kurdica*[a]

Microorganism	Essential Oil		Tetracycline, 30 μg disc^{-1}		Gentamicin, 30 μg disc^{-1}	
	IZ[b]	MIC[c]	IZ	MIC[c]	IZ	MIC[c]
S. aureus	22 $\pm$ 0.6	3.75	20 $\pm$ 0.4	3.2	-	nt
E. faecalis	23 $\pm$ 0.4	3.75	9 $\pm$ 0.3	6.5	-	nt
B. subtilis	15 $\pm$ 0.3	15	21 $\pm$ 0.7	3.2	-	nt
K. pneumoniae	10 $\pm$ 0.3	> 10	-	nt	20 $\pm$ 0.8	3.3
P. aeruginosa	13 $\pm$ 0.4	15	-	nt	12 $\pm$ 0.5	6.4
E. coli	17 $\pm$ 0.5	7.5	-	nt	23 $\pm$ 0.6	3.2

[a] Values are expressed as mean $\pm$ SD.
[b] Zone of inhibition in mm includes diameter of the disc 6 mm.
[c] Minimum inhibitory concentration (standard) values as mg ml^{-1}, (-) inactive, (7 - 13) moderately active, (> 14) highly active; nt, not tested.

bial activities of the essential oil of *Ballota nigra* subsp. *kurdica* against these two bacteria, it could be a good candidate for further studies aimed at overcoming related infections in humans or aimed at preserving food. Consistent with our results, the antimicrobial activities of different species of *Ballota* have been reported, e. g., *B. nigra* subsp. *foetida*, *Ballotapseudodictamnus*, and *Ballotasaxatilis* (19-22). Based on the antimicrobial activity of *B. nigra* subsp. *kurdica* essential oil and its new chemical composition, it would be promising to use it as a new source for identification of biologically active compounds. To our knowledge, this study can be considered the first detailed document on the phytochemical study and biological activities of *B. nigra* subsp. *kurdica*. Based on the antimicrobial activity of *B. nigra* subsp. *kurdica* essential oil, it can be considered a natural antimicrobial agent in food preservation.

In conclusion, we evaluated the chemical composition and antimicrobial activity of the essential oils of the flowers in *Ballota nigra* subsp. *kurdica* for the first time. We identified 22 compounds which were more than 98% of the total essential oil. The results show that the chemical composition of *Ballota nigra* subsp. *kurdica* differs from those reported for other *B. nigra* subspecies and showed moderate to high antimicrobial activity. Our results provide evidence for the potential of *B. nigra* subsp. *kurdica* essential oil as a natural antimicrobial agent to treat infections or to preserve food in the near future.

Footnotes

Authors' Contribution: Conceived and designed the experiments, Mohammad Majdi; identified and collected

plants, Hossein Maroofi; performed the experiments, Dara Dastan, Mohammad Majdi; analyzed the data, Dara Dastan; contributed reagents/materials/analysis tools, Dara Dastan, Mohammad Majdi; wrote the paper, Mohammad Majdi.

Conflict of Interest: We declare that we have no conflicts of interest.

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