Volatile Constituents Analysis and Antimicrobial Activity of Two Subspecies Ballota nigra L. From Iran

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Research article

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Abstract

Background *Ballota nigra* L. is a perennial herb of the Lamiaceae family and it's native to the Mediterranean region and central Asia, also it can found throughout Europe. during this research, the volatile oil composition and antimicrobial activity of the methanol extract from flowering aerial parts of two subspecies of *B. nigra* (*B. nigra* ssp. *foetida* and *B. nigra* ssp. *anatolica*) were evaluated. Plant extraction was prepared by the maceration method and the antibacterial activity of methanolic extracts against 12 microorganisms was investigated by disc diffusion and minimal inhibitory concentration (MIC) methods.

Results: GC/MS analysis of the volatile oil confirms the presence of 25 constituents in *B. nigra* ssp. *foetida* and 27 constituents in *B. nigra* ssp. *anatolica*. the most constituents in these two subspecies were α- Terpineol, Eicosane, Hexadecanoic acid, Longipinene epoxide, and Ethyl linoleate, which had slight differences within the amount of total volatile oil. within the antibacterial and MIC tests, indicated that methanolic extract of two subspecies *Ballota nigra* L. had acceptable antibacterial activity and therefore the *B. nigra* ssp. *anatolica* had more antibacterial activity in compression to *B. nigra* ssp. *foetida*.

Conclusion: Today the tendency of natural products due to antioxidant and antimicrobial properties is increasing, and plants are the main source of these safe materials. During this respect, our study is often considered because of the first report on the volatile oil composition and antimicrobial activity of methanol extract of various subspecies of .*B. nigra*.

1. Introduction

Secondary metabolites and essential oils have wide applications in dietary regimens, food flavoring and preservation, folk medicine, and the fragrance industry (Huang et al., 2005; Kalemba and Kunicka, 2003). The application of plant products as dietary regimens and preservatives is especially due to their antioxidant, antimicrobial, and other biological potentials.

Tease natural antioxidants can inhibit or delay the oxidation of oxidizable substrates and this appears to be vital within the prevention of oxidative stress which is usually recommended because of the leading explanation for a good deal of oxidation-related diseases (Halliwell et al., 1992; Sarker et al., 2006). Recently, thanks to undesirable side effects like toxicity and carcinogenicity of synthetic additives, interest has considerably increased for locating present antioxidant and antimicrobial compounds suitable to be used in food and medicine (Losso et al., 2007; Sachetti et al., 2005).

In this regard, a growing rate of research was conducted on many plant species so as to seek out new natural bioactive compounds in them. *Ballota nigra* L. (Lamiaceae) may be a perennial herb which is usually distributed in Western Europe where flowering aerial parts are utilized in medicine for treating cough and more especially for neurobiological activities (Sokmen et al., 1999). Two subspecies of *Ballota nigra* were collected in 2019 from the natural habitat of Isfahan and Mazandaran province in Iran, subspecies of this plant species identified in Kashan arboretum (Isfahan/Iran). This study reported the

chemical composition of the volatile oil and antimicrobial activity of the methanolic extract from flowering aerial parts of those two subspecies of *Ballota nigra* L.

2. Materials And Methods

2.1. Plant materials

The flowering aerial parts of *B. nigra* ssp. *foetida* were collected during the flowering period in (April to May) 2019, from Qamsar, around Kashan (Isfahan province, Iran), and therefore the flowering aerial parts of *B. nigra* ssp. *anatolica* were collected during the flowering period in (May to June) 2019, from Kajoor, (Mazandaran province, Iran).

The voucher specimen of the plant was deposited within the herbarium of Research Institute of Forests and Rangeland, Kashan, Iran. The aerial parts of the plant were spread uniformly during a thin layer within the shade during a dry place with well air ventilation for drying, when the moisture content fell to a minimum suitable for grinding, the material was ground with a steel mill (Retschmühle, GmbH, 5657 HAAN, Germany), stored in well-closed glass containers, kept during a refrigerator and used within a couple of days. The aerial parts (50 g) of the crushed material were individually subjected to hydrodistillation for 3.5 hours using the modified simultaneous distillation and extraction (SDE) method. (Likens and Nickerson, 1997; Anonymous, 1996; GRIN, 2009). After decanting and drying over anhydrous sodium sulfate, yellow-colored oils were recovered and stored at coldness (4 C °) under a nitrogen atmosphere in amber vials and were used for analyses within a couple of days. so as to gauge the antimicrobial effects, the methanolic extract (Methanol 70%), from aerial parts of the plant was prepared by maceration method (Anonymous, 1996; GRIN, 2009; Bamoniri et al., 2010).

2.2. Gas chromatographic (GC/MS) analysis

Volatile oil samples obtained from the flowering aerial parts of *B. nigra* L. were analyzed using an Agilent HP-6890 gas chromatograph (GC), (Agilent Technologies, Palo Alto, CA, USA) equipped with an FID detector using an HP-5MS 5% phenylmethyl siloxane capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Restek, Bellefonte, PA). Oven temperatures were programmed as follows: 50 C (2 min), 50–130 C (5 C/min), 130 C ° (2 min), and 130–200 C ° (3 C/min). Injector and detector temperatures were set at 220 C and 290 C °, respectively. Ultra-high purity helium (flow rate: 1 ml/min), hydrogen (flow rate: 40 ml/min), and nitrogen (flow rate: 50 ml/min) were used as a carrier, fuel, and makeup gases respectively and compressed gas (flow rate: 450 ml/min) was used for combustion. Diluted samples (1/1000 in n-pentane, v/v) of 1.0 µl were injected manually within the splitless mode. Peak area percent of every compound relative to the world percent of the whole spectrum (100%) were used for obtaining its quantitative data. The injection was repeated 3 times and therefore the peak area percent were reported as means ± SD of triplicates. Co-injection of selected commercially available components of the volatile oil were also administered and led to the enrichment of the respected picks within the spectrum and further confirmation of their identities (Bamoniri et al., 2010).

GC/MS analysis of the oil was administered on an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent HP-5973 mass selective detector within the electron impact mode (ionization energy: 70 eV), operating under equivalent conditions as described above, using an HP-5MS 5% phenyl methyl siloxane capillary column (30 m × 0.25 mm, 0.25 µm film thickness; Restek, Bellefonte, PA). Retention indices were calculated for all components employing a homologous series of n-alkanes injected in conditions adequate to the sample one. Identification of components of volatile oil was supported retention indices (RI) relative to n-alkanes and computer matching with the Wiley275.L and Wiley7n.L libraries, also as comparisons of the fragmentation pattern of the mass spectra with data published within the literature (Adams, 2001). Some commercially available components of the volatile oil were also Co-injected for further confirmation of their identification.

2.3. Antimicrobial activity

The methanolic extracts of two subspecies of *B. nigra* L. were individually tested against a panel of 12 microorganisms. Following microbial strains were provided by the Iranian Research Organization for Science and Technology (IROST) and utilized in this research: *Pseudomonas aeruginosa* (ATCC 27853), *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 C ° in agar (NA) and fungi were cultured overnight at 30 C ° in sabouraud dextrose agar (SDA).To prepare the methanolic extract was used the soaking (Maceration) method. After drying the aerial parts of the plant (flowers, leaves, and stems) under shade conditions and proximity to the air, samples powdered by an electrical mill, then the quantity of 15 g of the sample mixed with 200 ml of methanol 70%. After each day the organic phase was removed and methanol was added again. This action was repeated 3 times, finally, the entire plant extract was collected and used a rotary evaporator to evaporate the solvent, also to full water removal and prepare the plant's powder the freeze dryer was used. To investigate, the antibacterial activity of the extract, 0.2 g of methanolic extracts were weighed and dissolved using 10 ml DMSO. Finally, this stoke was wont to prepare different concentrations of extract within the disc diffusion and MIC methods (Bamoniri et al., 2010).

The Bacteria strains were grown in Muller-Hinton fluid medium (Merck, Germany). Then a dense and uniform culture of bacteria was prepared with the bacterial suspension like half McFarland by sterile cotton swabs within the surface Müller-Hinton Agar (Merck, Germany). Sterile paper disks with a diameter of 6 mm (Padtan Teb, Iran) and impregnated with 30 µl of methanolic extract and dissolved in DMSO, were placed in agar separately. A disk impregnated with 30 µl DMSO solvent used as a negative control and therefore the antibiotic discs of Rifampin, Gentamicin, and Nystatin, was used as a positive control. Plates incubated for twenty-four to 48 hours at 37 ° C. the antibacterial activity of plant extract was measured by the halo diameter without growth (mm).To determine the minimum inhibitory concentration of growth, the methanolic extracts of the aerial parts of the plant (leaf, flower, and stem) with 20 mg/li

concentration in DMSO solvent was prepared. then was sterilized using the paper, with 0.22 µm in pore diameter, then the sterile extract was added to the LB liquid medium, to be achieved a concentration between 5 to 300 µg/ml. Also, the antibacterial activity of the methanolic extract was compared with Tetracycline antibiotic as a positive control and DMSO as a negative control within the DMSO in similar conditions. Then 0.5 percent of the fresh bacterial culture was added to tubes containing LB liquid medium and plant extract, separately. Pipes were placed at a warm home with Shaker at 37 ° C for twenty-four hours with 100 rounds per minute. Antibacterial activity of the methanolic extract was measured by the minimum inhibitory concentration (MIC) of bacteria growth. (NCCLS, 1997).

3. Results And Discussion

3.1. Chemical composition of the essential oil

Flowering aerial parts of the plant were subjected to simultaneous distillation extraction (SDE) apparatus. GC and GC/MS analysis resulted in the identification of the entire volatile oil percentage (98.65% with 25 components for *B. nigra* ssp. *foetida* and 99.91% with 27 components for *B. nigra* ssp. *anatolica*, for dried flowering aerial parts. consistent with the results presented in (Table 1). α- Terpineol (10.78%), Eicosane (9.11%), and palmitic acid (8.14%) were the most components within the volatile oil obtained from flowering aerial parts of *B. nigra* ssp. *foetida*. Although these compounds were the main compounds within the *B. nigra* ssp. *anatolica* too, but their percentage was less within the volatile oil content. in order that α- Terpineol (9.50%), Eicosane (8.85%), and palmitic acid (7.20%) assigned the quantity of essential o ils.

Table 1
Chemical composition of the flowering aerial parts of volatile oil of *B. nigra* ssp. *foetida*

No	Compound[a]	Composition (%)	RI[b]	RI[c]
1	n-Decane	14.58	989	1000
2	α- Terpineol	**10.78**	1191	1186
3	3-methyl-phenylmethylester, Butanoic acid	5.10	1391	1395
4	Longipinene epoxide	**7.83**	1594	1590
5	Methyl jasmonate	1.45	1633	1644
6	Helifolenol B	1.10	1664	1677
7	(E)-Sesquilavandulyl acetate	1.18	1729	1739
8	(1-butyloctyl)-Benzene	1.29	1735	1731
9	Tetradecanoic acid	1.18	1747	1765
10	p-Cresol octanoate	1.18	1769	1777
11	Conglomerone	5.03	1799	1805
12	Octadecane	1.64	1809	1800
13	Perhydro farnesyl aceton	4.37	1841	1847
14	6,10,14-trimethyl-2-Pentadecanone	3.21	1850	1848
15	Pentadecanoic acid	1.15	1884	1878
16	Methyl palmitate	1.12	1932	1927
17	Hexadecanoic acid	**8.14**	1980	1984
18	Eicosane	**9.11**	2002	2000
19	Methyl linoleate	1.43	2099	2092
20	Heneicosane	2.60	2105	2100
21	Linoleic acid	1.56	2119	2140
22	Ethyl linoleate	**7.08**	2157	2159
23	n-Docosane	2.39	2201	2200

[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.

No	Compound[a]	Composition (%)	RI[b]	RI[c]
24	cis-Ferruginol	1.76	2399	2370
25	Z-12-pentacosene	1.72	2499	2496
Total		98.65		

[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.

Table 2
Chemical composition of the flowering aerial parts of volatile oil of *B. nigra* ssp. *anatolica*

No	Compound[a]	Composition (%)	RI[b]	RI[c]
1	n-Decane	13.90	989	1000
2	α- Terpineol	**9.50**	1191	1186
3	3-methyl-phenylmethylester, Butanoic acid	5.10	1391	1395
4	Longipinene epoxide	**7.03**	1594	1590
5	Humulene oxide	0.56	1629	1625
6	Methyl jasmonate	1.33	1633	1644
7	(2E.6Z)-Farnesol	1.21	1703	1714
8	(E)-Sesquilavandulyl acetate	1.18	1729	1739
9	(1-butyloctyl)-Benzene	1.20	1735	1731
10	Tetradecanoic acid	1.18	1747	1765
11	p-Cresol octanoate	1.18	1769	1777
12	Conglomerone	5.02	1799	1805
13	Octadecane	1.64	1809	1800
14	Perhydro farnesyl aceton	4.37	1841	1847
15	6,10,14-trimethyl-2-Pentadecanone	3.21	1850	1848
16	Pentadecanoic acid	1.10	1884	1878
17	Methyl palmitate	1.23	1932	1927
18	Hexadecanoic acid	**7.20**	1980	1984
19	Eicosane	**8.85**	2002	2000
20	Methyl linoleate	1.57	2099	2092
21	Heneicosane	2.52	2105	2100

[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.

No	Compound[a]	Composition (%)	RI[b]	RI[c]
22	Linoleic acid	1.56	2119	2140
23	Ethyl linoleate	**6.85**	2157	2159
24	n-Docosane	2.31	2201	2200
25	Tricosane	0.89	2300	2300
26	cis-Ferruginol	1.76	2399	2370
27	Z-12-pentacosene	1.72	2499	2496
Total		99.91		
[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.				

The results of biological tests of the volatile oils of those two subspecies indicated that these compounds have an acceptable biological activity separately. Also, (98.65 to 99.91%) of the compounds in essential oils were identified that the foremost important of which is α- Terpineol that's a single-ring monoterpene and is one of the 2 most abundant aroma constituents of lapsang souchong tea; the α-terpineol originates within the pine smoke wont to dry the tea is an anti-cancer compound (Shan et al., 2005). it's also an antiseptic and is additionally utilized in the preparation of vitamins. hexadecanoic acid, or palmitic acid in IUPAC (International Union of Pure and Applied Chemistry) nomenclature, is that the commonest saturated carboxylic acid found in animals, plants, and microorganisms (Beare et al., 2001; Gunstone et al., 2007). Its formula is CH3 (CH2)14COOH, and its C:D is 16:0. As its name indicates, it's a serious component of the oil from the fruit of oil palms (palm oil). hexadecanoic acid also can be found in meats, cheeses, butter, and dairy products. Palmitates are the salts and esters of hexadecanoic acid. The palmitate anion is that the observed sort of hexadecanoic acid at physiologic pH (7.4).

Aluminum salts of hexadecanoic acid and naphthenic acid were combined during war II to supply napalm. The word “napalm” springs from the words naphthenic acid and hexadecanoic acid.

Bader et al., (2013) reported that (α- Terpineol), (Linalool), and (Eicosane) are the most chemical compounds of volatile oil that confirmed this research. Rustaiyan et al., (2006) reported (Germacrene D), (Linalool) and (α-Cadinol) are the most chemical compounds that differentiate this research. So it seems the quantitative and qualitative difference within the combination of the essential oils of various species is thanks to differences in physiological and vegetative characteristics. The results showed although the main essential components in these two subspecies are different, most of the volatile oil components in these two subspecies of *B. nigra* were common.

The reports on the chemical composition of the essential oils of the plants of *B. nigra* is scant within the literature. α- Terpineol, Eicosane, palmitic acid, Longipinene epoxide, and Ethyl linoleate for the stems/leaves were also found within the volatile oil of *B. nigra* ssp. *anatolica* and *B. nigra* ssp. *foetida* as major components (Tepe et al., 2005; Vukovic et al., 2009). Finally, this investigation as compared with other same research shows less accordance with other samples in other regions of the planet. Also in some studies, the most component of *B. nigra* volatile oil Perhydro farnesyl acetone, Pentadecanone, and Conglomerone reported, that different to the present research. This difference regarding plant species, plant's habitat, plant's phenology or volatile oil from other organs of the plant like root (Aruoma, 2003; Foti et al., 2004; Güllüce et al., 2004; Masoudi et al., 2005).

3.2. Antimicrobial activity

Results of antibacterial effects of methanolic extract of two subspecies *Ballota nigra* L. By existence or absence growth inhibition zone (halo diameter of without growth) in Table 3 and minimum inhibition concentration (MIC) showed in Table 4. consistent with Table 3, the very best halo diameter of without growth was observed within the effect of the methanolic extract of *B. nigra* ssp. *anatolica*. on the *B. subtilis* and *E. coli* strains with halo diameters of 11.63 ± 0.52 and 11.20 ± 0.80 respectively. Also, this two *B. nigra* subspecies showed a special reaction against *S. dysenteriae* and *A. niger* strains, that *B. nigra* ssp. *anatolica* had the effect on *S. dysenteriae*, while on the *A. niger* no effect was observed. The results of the MIC test indicated the lowest concentration of the extract regarding inhibiting the expansion of *S. aureus* and *B. subtilis* bacteria with 150 ug/ml concentration (Table 4).

Table 3
Comparison of the halo diameter of without growth (mm) of the methanolic extract of the aerial part two sub-species of *Ballota nigra* L

Microorganisms	*B. nigra* ssp. Foetida	*B. nigra* ssp. anatolica	Antibiotics		
			Rifampin	Gentamicin	Nystatin
P. aeruginosa ATCC 27853	8.33 ± 0.50	9.50 ± 0.20	17.33 ± 0.50	19.33 ± 0.50	15.33 ± 0.50
B. subtilis ATCC 6633	6.63 ± 0.52	11.63 ± 0.52	15.23 ± 0.60	20.75 ± 0.40	NE
E. coli ATCC 10536	9.23 ± 0.36	11.20 ± 0.80	15.30 ± 0.58	18.30 ± 0.44	NE
S. aureus ATCC 29737	8.10 ± 0.20	6.32 ± 0.77	10.22 ± 0.50	13.70 ± 0.33	NE
K. pneumoniae ATCC 10031	NE	NE	NE	NE	10.20 ± 0.11
S. dysenteriae PTCC 1188	NE	5.33 ± 0.88	NE	15.33 ± 0.20	9.43 ± 0.40
S. epidermidis ATCC 12228	6.38 ± 0.65	5.50 ± 0.30	11.38 ± 0.80	15.38 ± 0.30	NE
P. vulgaris PTCC 1182	NE	NE	NE	8.33 ± 0.20	NE
S. paratyphi-A serotype ATCC 5702	NE	NE	NE	NE	NE
C. albicans ATCC 10231	NE	NE	NE	NE	10.33 ± 0.14
A. niger ATCC 16404	4.70 ± 0.25	NE	15.30 ± 0.70	NE	10.25 ± 0.40
A. brasiliensis PTCC 5011	8.25 ± 0.75	10.18 ± 0.65	NE	20.25 ± 0.44	15.36 ± 0.35

Table 4
Minimum inhibitory concentration (MIC) of the methanolic extract of the aerial part two sub-species of *Ballota nigra* L.

Microorganisms	Minimum inhibitory concentration (MIC) µg/ml		
	B. nigra ssp. Foetida	*B. nigra* ssp. anatolica	**Tetracycline**
P. aeruginosa ATCC 27853	300	250	50
B. subtilis ATCC 6633	250	150	25
E. coli ATCC 10536	300	250	25
S. aureus ATCC 29737	200	150	10
S. epidermidis ATCC 12228	250	200	25
A. niger ATCC 16404	250	200	10

As the results of Table 4 show, *B. nigra* ssp. *anatolica* has been ready to inhibit the expansion of two of these bacteria strains at lower concentrations. It is often said that the inhibitory power of the extract of those two subspecies was acceptable as compared with Tetracycline that used because of the control.

Dulger et al., (2012) Showed that the methanolic extract of the leaf of *Ballota acetabulosa* features a strong antibacterial effect against *E. coli* acetabulosa and features a poor effect against *S. aeroginosa* that which doesn't match the present research.

This study was done by disc diffusion method that its results and MIC effects showed the antibacterial activity of methanolic extract of those two subspecies on the bacteria strains that utilized in this research were higher compared to other *Ballota species* (Trouilla et al., 2003; Scalbert et al., 2005; Slinkard and Singleton, 2016). The antimicrobial activity methanol extracts of two subspecies of *B. nigra* against a panel of 12 microorganisms were examined and their potencies were assessed both qualitatively and quantitatively by the presence or absence of inhibition zones, the halo diameter of without growth, and MIC values. because the results presented in Table 3 and Table 4, the methanolic extract of flowering aerial parts showed acceptable antimicrobial activities against almost quite half microorganisms, the results of this section of our research confirmed the previous study within the other species of *B. nigra* that reported by (Miraliakbari and Shahidi, 2008; Murray et al., 1999; Omidbaigi, 2005). It should be noted that the essential oils and therefore the antimicrobial effects of those two subspecies haven't been reported so far, and this study has addressed this issue for the primary time.

4. Conclusion

Different plant species and subspecies have differences in composition and percentage within the volatile oil. therefore the explanation of characteristics and perform studies on subspecies levels of the

plants is extremely important and may use by researchers for proper selection of the plant for his or her future and supplementary research. Also, the Growing tendency for replacing synthetic additives with natural ones has emerged an excellent interest within the evaluation of antioxidant and antimicrobial properties of plant products in both research centers and industry. during this respect, our study is often considered because of the first report on the volatile oil composition and antimicrobial activity of methanol extract of various subspecies of *B. nigra*. The rather good antimicrobial activity of the plant extract especially within the MIC test encourages more elaborate investigations in this respect.

List Of Abbreviations

DMSO: Dimethyl sulfoxide

GC/MS:Gas chromatography–mass spectrometry

MIC: Minimal inhibitory concentrations

Na_2SO_4: Sodium sulfate

CLSI: Clinical and Laboratory Standards Institute

SDE: Simultaneous Distillation and Extraction

NCCLS: National Committee for Clinical Laboratory Standards

IUPAC: International Union of Pure and Applied Chemistry

Declarations

Ethics approval and consent to participate:

The author is fully satisfied with this project and its publication.

Consent for publication:

The author agrees to publish this article

Availability of data and material:

Not applicable

Competing interests:

Not applicable

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Conflict of interest:

The author declare that there are no conflicts of interest in this research study.

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