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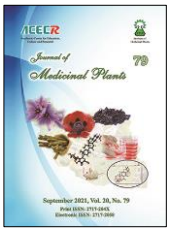

Research Article

Phytochemical constituents of the fruits of *Kelussia odoratissima* Mozaff., an aromatic plant endemic to Iran

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ABSTRACT

Background: *Kelussia odoratissima* Mozaff. from Apiaceae family is a perennial herbaceous plant endemic to the west of Iran. The aromatic aerial parts of this species are traditionally used by indigenous people to flavor some local foods, as well as for various therapeutic purposes. **Objective:** The present study was designed to analyze phenolic compounds and essential oil constituents of *K. odoratissima* fruits. **Methods:** The *n*-butanol fraction obtained from hydroalcoholic extract of *K. odoratissima* fruits was investigated by chromatography on normal phase and Sephadex LH-20 columns. Chemical structures of the isolated compounds were clarified by ^{1}H-NMR and ^{13}C-NMR spectral analyses. Essential oil constituents of the fruits were also analyzed using GC-MS. **Results:** Phytochemical investigation of the *K. odoratissima* fruits resulted in the isolation of five flavonol glycosides; isorhamnetin 3-O-glucoside (**1**), quercetin 3-O-glucoside (isoquercetin) (**2**), isorhamnetin 3-O-rutinoside (narcissin) (**3**), isorhamnetin 3-O-glucuronide (**4**) and quercetin 3-O-glucuronide (mequilianin) (**5**). GC-MS analysis of the fruits essential oil led to the identification of the thirty six compounds, of which (*Z*)-ligustilide (15.93 %), *δ*-cadinene (12.26 %) and germacrene D (12.18 %) were the main compounds. **Conclusion:** The results of this study introduce *K. odoratissima* fruits as a source of flavonoid glycosides and phthalate derivatives. The presence of these compounds with known biological properties and health beneficial effects provides more medicinal potentials for the fruits of *K. odoratissima* and suggest it an appropriate option for further studies.

1. Introduction

The genus *Kelussia* from Apiaceae family is represented by only one member, *Kelussia odoratissima* Mozaff., which is found in central Zagros mountains, west of Iran [1]. The aerial parts of this aromatic species are used by indigenous people under the local names of "Kellos" and "Karafse-Bakhtiari" as vegetable, as flavoring agent in yogurt, for the treatment of indigestion, rheumatism, gastric ulcer, cough,

Abbreviations: NMR, Nuclear magnetic resonance; GC-MS, Gas Chromatography-Mass Spectrometry; TLC, Thin Layer Chromatography

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pain, diabetes and as a sedative agent [2, 3]. A review on literature shows that extracts and essential oils obtained from different parts of *K. odoratissima* have been reported for their biological and pharmacological activities such as antioxidant [4, 5], antibacterial [6], larvicidal [7], antileishmanial [8], cytotoxic [9], spasmolytic [10], anti-inflammatory [11], antihypertensive [12], sedative and anxiolytic [13] effects. One study reported the isolation of two steroids, stigmasterol and *β*-sitosterol and one phthalide, 3-butyliden-4,5-dihydrophthalide from the *n*-hexane extract of *K. odoratissima* fruits [14]. Furthermore, they identified thirty eight compounds in the fruits essential oil, of which (*Z*)-ligustilide (29.2 %), germacrene B (15.9 %) and germacrene D (15.5 %) were the major compounds [14]. Another study reported the analysis of fatty acids in the fruits of *K. odoratissima* and showed that among the five fatty acids identified in the fruits oil, petroselinic acid (72.35 %) and linoleic acid (19.14 %) were the main acids [15]. In the mentioned study, *K. odoratissima* fruits were also reported as a source of phenolic compounds by a total phenolic content of 218.15 milligrams gallic acid equivalents (GAE) per gram of dry fruit weight [15]. Beside the high phenolic content, there is not any report on phenolic principles of *K. odoratissima* fruits. Therefore, the present study was designed to isolation and structural elucidation of the phenolic compounds present in the fruits of this valuable medicinal plant. The essential oil composition of the fruits was also identified and compared by related data previously reported for *K. odoratissima*.

2. Materials and Methods

2.1. Plant material

The fruits of *Kelussia odoratissima* Mozaff. were purchased from Pakan-Bazr Co., Isfahan, Iran (Plant source: Fereydunshahr region, Isfahan, Iran.; Collection date: July 2017). The identity of the fruits was authenticated by botanist Dr. Yousef Ajani (Research Institute of Forest and Rangelands, Tehran, Iran) and the code of PMP-2694 was assigned for it at the herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

2.2. Extraction of Phenolics

The dried and comminuted fruits of *K. odoratissima* (1.4 kg) were subjected to extraction using maceration method with dichloromethane and methanol-water (70:30), successively (4 × 5 L, each). The obtained extracts were concentrated using a rotary evaporator at 45 °C under the reduced pressure and then dried in vacuum oven. Hydroalcoholic extract (250 g) was suspended in water (0.5 L) and fractionated by equal volume of *n*-butanol (×3) to extract its phenolic compounds.

2.3. Isolation and purification of the compounds

A portion of the *n*-butanol fraction (20 g) was chromatographed on a normal phase silica gel column (Mesh 230-400, Merck) using a gradient solvent mixture of $CHCl_3$-MeOH (100:0 to 50:50) to get eighteen subfractions (B1-B18). Thin layer chromatography (Pre-coated silica gel GF_{254} plates, Merck) was applied to monitor column chromatography and fractions giving similar spots under UV (254 and 366 nm) followed by the exposure to ammonia vapor were combined. Based on the TLC analysis, subfractions B6, B10, B11, B14 and B16 were chosen for further isolation. Column chromatography of these subfractions (B6, B10, B11, B14 and B16) on a Sephadex LH-20 column using methanol as mobile phase resulted in the isolation of compounds **1** (19.0 mg), **2**

(13.5 mg), **3** (35.4 mg), **4** (23.8 mg) and **5** (18.1), respectively. The structures of the compounds were elucidated by ^{1}H-NMR and ^{13}C-NMR (Varian- INOVA, 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR) spectral analysis.

2.4. Essential oils extraction

The comminuted fruits (100 g) were subjected to hydrodistillation for 3 hours, using a Clevenger apparatus. The obtained oils were dried over anhydrous sodium sulfate and keep at 4 °C until analysis.

2.5. GC-MS analysis

The essential oil extracted from the fruits of *K. odoratissima* were analyzed on a HP-6890 gas chromatograph with a BPX5 column (30 m × 0.25 mm id, 0.25μm film thickness), equipped with HP-5973 mass detector (Ionization energy: 70 eV) under the following conditions; 5 min after injection, oven temperature was increased from 50 °C to 240 °C at a rate of 3 °C/min and then reached to 300 °C at the rate of 15 °C/min and hold 3 min in this temperature. Injector temperature: 250 °C, detector temperature: 220 °C, injection volume: 1.0 μl, split ratio: 1:35, carrier gas: helium (99.999 %, Flow rate: 0.5 ml/min). The retention indices (RIs) were calculated for all identified compounds using a homologous series of *n*-alkanes (C_8-C_{24}) injected under the same conditions described for the analyzed essential oil. Identification of the compounds were carried out based on computer matching with the Wiley 275.L library, as well as by comparison of RIs and mass fragmentation patterns with those published for standard compounds [16].

3. Results

3.1. Isolation and structural elucidation

Phytochemical analysis of the fruits of *K. odoratissima* using chromatography on normal phase silica gel and Sephadex LH-20 columns resulted in the isolation of five compounds (**1**-**5**) from *n*-butanol fraction of hydroalcoholic extract. The structures of the isolated compounds were characterized as isorhamnetin 3-O-*β*-D-glucopyranoside (**1**), quercetin 3-O-*β*-D-glucopyranoside (isoquercetin) (**2**), isorhamnetin 3-O-β-D-glucopyranosyl-(6→1)-*α*-L-rhamnopyranside (isorhamnetin 3-O-*β*-D-rutinoside, narcissin) (**3**), isorhamnetin 3-O-*β*-D-glucuronide (**4**) and quercetin 3-O-*β*-D-glucuronide (mequilianin) (**5**) (Fig. 1) using ^{1}H-NMR and ^{13}C-NMR spectral analyses, as well as by comparison with published data [17-21].

3.1.1. Spectroscopic data of the isolated compounds

Compound **1**; *Isorhamnetin 3-O-β-D-glucopyranoside* ($C_{22}H_{22}O_{12}$); Yellow amorphous solid; R_f = 0.6 ($CHCl_3$-MeOH, 8:2); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ_H 7.93 (1H, *d*, *J* = 2.0 Hz , H2′), 7.50 (1H, *dd*, *J* = 8.5 and 2.0 Hz, H6′), 6.93 (1H, *d*, *J* = 8.5 Hz, H5′), 6.46 (1H, *d*, *J* = 2.0 Hz , H8), 6.22 (1H, *d*, *J* = 2.0 Hz , H6), 5.56 (1H, *d*, *J* = 7.4 Hz, H1″), 3.83 (3H, *s*, OCH_3), 3.1-3.7 (6H, *overlapped signals*, H2″-H6″). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ_C 177.78 (C4), 164.55 (C7), 161.35 (C5), 156.87 (C9),156.73 (C2), 149.63 (C3′), 147.28 (C4′), 133.46 (C3), 122.51 (C6′), 121.58 (C1′), 115.57 (C5′), 113.87 (C2′), 104.43 (C10) , 101.18 (C1″), 99.10 (C6), 94.22 (C8), 77.76 (C5″), 76.62 (C3″), 74.63 (C2″), 70.12 (C4″) , 60.93 (C6″), 56.15 (OCH_3) [17].

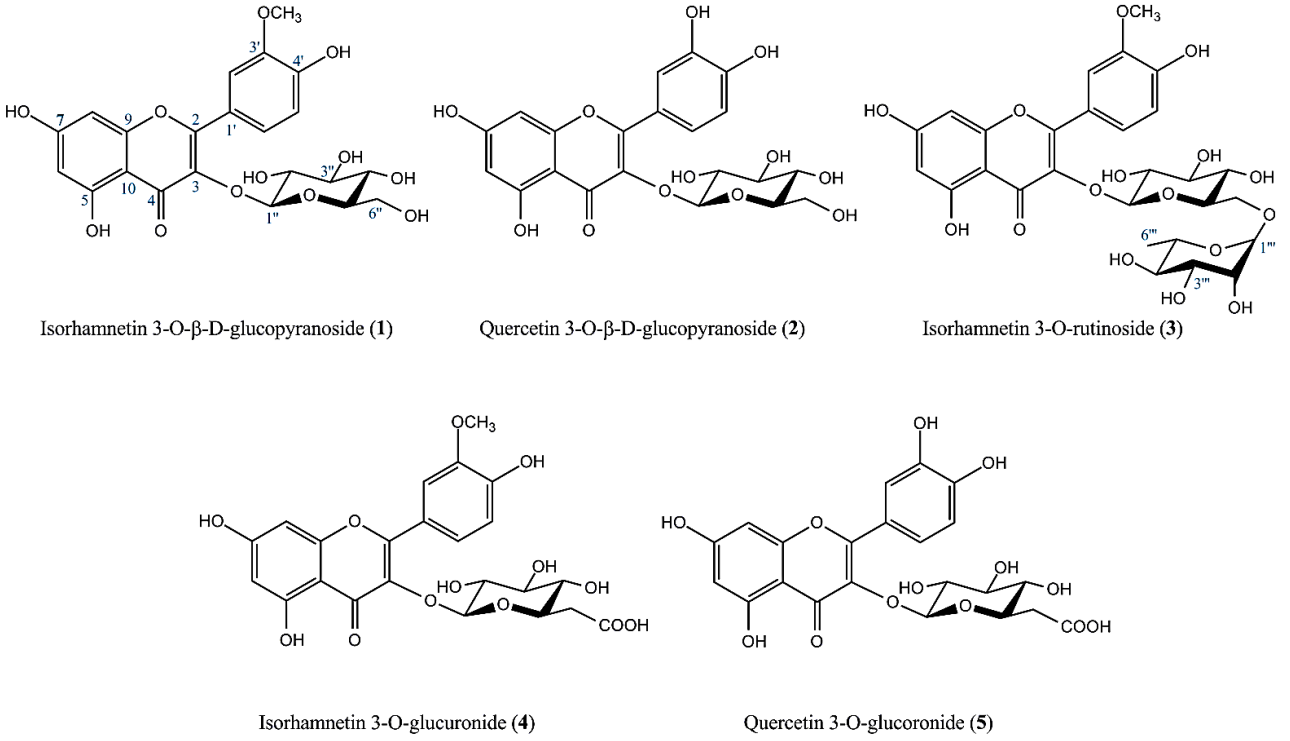

Fig. 1. The structures of isolated compounds (**1**-**5**) from *K. odoratissima* fruits

Compound **2**; *Quercetin 3-O-β-D-glucopyranoside* (*isoquercetin*) ($C_{21}H_{20}O_{12}$); Yellow amorphous solid; R_f = 0.5 ($CHCl_3$-MeOH, 8:2); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ_H 7.58 (1H, *d*, *J* = 2.0 Hz, H2′), 7.56 (1H, *dd*, *J* = 9.0 and 2.0 Hz, H6′), 6.86 (1H, *d*, *J* = 9.0 Hz, H5′), 6.42 (1H, *d*, *J* = 2.0 Hz, H8), 6.21 (1H, *d*, *J* = 2.0 Hz, H6), 5.45 (1H, *d*, *J* = 7.5 Hz, H1″), 3.1-3.7 (6H, *overlapped signals*, H2″-H6″). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ_C 177.82 (C4), 164.52 (C7), 161.37 (C5), 156.90 (C9),156.60 (C2), 148.69 (C4′), 145.07 (C3′), 133.80 (C3), 122.09 (C6′), 121.63 (C1′), 116.55 (C5′), 115.60 (C2′), 104.36 (C10), 101.29 (C1″), 99.05 (C6), 94.02 (C8), 77.88 (C5″), 76.73 (C3″), 74.42 (C2″), 70.21 (C4″) , 61.26 (C6″) [18].

Compound **3**; *Isorhamnetin 3-O-β-D-rutinoside* (*narcissin*) ($C_{28}H_{32}O_{16}$); Yellow amorphous solid; R_f = 0.3 ($CHCl_3$-MeOH, 8:2); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ_H 7.83 (1H, *d*, *J* = 1.8 Hz, H2′), 7.51 (1H, *dd*, *J* = 8.4 and 1.8 Hz, H6′), 6.91 (1H, *d*, *J* = 8.4 Hz, H5′), 6.44 (1H, *d*, *J* = 2.0 Hz, H8), 6.21 (1H, *d*, *J* = 2.0 Hz, H6), 5.40 (1H, *d*, *J* = 7.4 Hz, H1″), 4.40 (1H, *br s*, H1‴), 3.83 (3H, *s*, OCH_3), 3.0-3.7 (10H, *overlapped signals*, H2″-H6″ and H2‴-H-5‴), 0.94 (3H, *d*, *J* = 6.2 Hz, H6‴); ^{13}C-NMR (DMSO-d_6, 125 MHz): δ_C 177.71 (C4), 164.34 (C7), 161.26 (C5), 157.01 (C9), 156.94 (C2), 149.57 (C4′), 147.26 (C3′), 133.48 (C3), 122.77 (C6′), 121.54 (C1′), 115.57 (C5′), 113.63 (C2′), 104.44 (C10), 101.54 (C1″), 101.25 (C1‴), 99.08 (C6), 94.31 (C8), 76.54 (C5″), 76.20 (C3″), 74.52 (C2″), 72.03 (C4‴), 70.82 (C3‴), 70.57 (C2‴), 70.39 (C4″), 68.68 (C5‴), 67.37 (C6″), 56.12 (OCH_3), 17.99 (C6‴) [19].

Compound **4**; *Isorhamnetin 3-O-β-D-glucuronide* ($C_{22}H_{20}O_{13}$); Yellow amorphous solid; R_f = 0.5 ($CHCl_3$-MeOH, 8:2); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ_H 8.06 (1H, *br s*, H2′),

7.36 (1H, *br d*, *J* = 8.4, H6′), 6.87 (1H, *d*, *J* = 8.4 Hz, H5′), 6.16 (1H, *br s*, H8), 5.99 (1H, *br s*, H6), 5.57 (1H, *d*, *J* = 7.7 Hz, H1″), 3.80 (3H, *s*, OCH_3), 3.21-3.39 (4H, *overlapped signals*, H2″-H5″). ^{13}C-NMR (DMSO-d_6, 125 MHz): δ_C 177.55 (C4), 173.13 (C6″), 165.44 (C7), 161.21 (C5), 156.81 (C9), 156.81 (C2), 149.38 (C4′), 147.14 (C3′), 133.23 (C3), 122.24 (C6′), 121.68 (C1′), 115.31 (C5′), 114.48 (C2′), 103.68 (C10), 101.28 (C1″), 99.02 (C6), 94.28 (C8), 76.42 (C3″), 76.61 (C5″), 74.35 (C2″), 72.44 (C4″), 56.13 (OCH_3) [20].

Compound **5**; *Quercetin 3-O-β-D-glucuronide* (*mequilianin*) ($C_{21}H_{18}O_{13}$); Yellow amorphous solid; R_f = 0.4 ($CHCl_3$-MeOH, 8:2); ^{1}H-NMR (DMSO-d_6, 500 MHz): δ_H 8.23 (1H, *br s*, H2′), 7.33 (1H, *dd*, *J* = 8.4 and 1.2 Hz, H6′), 6.83 (1H, *d*, *J* = 8.4 Hz, H5′), 6.24 (1H, *br s*, H8), 6.06 (1H, *br s*, H6), 5.15 (1H, *d*, *J* = 7.3 Hz, H1″), 3.1-3.5 (4H, *overlapped signals*, H2″-H6″).^{13}C-NMR (DMSO-d_6, 125 MHz): δ_C 177.27 (C-4), 172.95 (C6″), 165.06 (C7), 160.99 (C5), 157.25 (C9), 157.25 (C2), 148.62 (C4′), 145.04 (C3′), 134.35 (C3), 121.05 (C6), 121.05 (C1′), 118.10 (C-5′), 115.81 (C-2′), 103.52 (C10), 103.52 (C1″), 100.27 (C-6), 94.79 (C-8), 76.92 (C-3″), 74.54 (C-5″), 74.41 (C-2″), 72.05 (C-4″) [21].

3.2. Essential oil composition

Hydrodistillation of the fruits of *K. odoratissima* led to the extraction of a pale yellowish oil with the yield of 1.5 % (v/w). GC-MS analysis of the obtained essential oil resulted in the identification of the thirty six compounds, representing 90.74 % of the oil (Table 1). Among the identified compounds, (*Z*)-ligustilide (15.93 %), *δ*-cadinene (12.26 %) and germacrene D (12.18 %) were the main compounds (Fig. 2) and oxygenated sesquiterpenes with relative percentage of 53.68 % were the main group of constituents identified in essential oil of *K. odoratissima* fruits.

Table 1. Chemical composition of the essential oil of *K. odoratissima* fruits obtained by hydrodistillation method

No.	Compound name[a]	%	RI[b]
1	*α*-Pinene	0.09	939
2	*β*-Pinene	0.35	978
3	2-Ethyl-2-hexenal	0.71	981
4	*β*-Phellandrene	3.23	1027
5	5-Pentylcyclohexa-1,3-diene	1.43	1161
6	Lavandulol	0.14	1170
7	Acetic acid octyl ester	0.24	1215
8	Citronellol	0.27	1227
9	Lavandulyl acetate	0.75	1292
10	Ylangene	0.62	1374
11	*α*-Copaene	2.92	1378
12	*β*-Cubebene	0.48	1388
13	*β*-Elemene	2.04	1391
14	*α*-Barbatene	0.42	1415
15	Lavandulyl isobutyrate	3.80	1417
16	*γ*-Elemene	4.34	1435
17	Sativene	1.36	1437
18	*α*-Humulene	1.09	1453
19	*γ*-Muurolene	0.49	1481
20	Germacrene D	12.18	1483

Table 1. Chemical composition of the essential oil of *K. odoratissima* fruits obtained by hydrodistillation method (Continued)

No.	Compound name[a]	%	RI[b]
21	Alloaromadendrene	1.81	1487
22	*γ*-Amorphene	1.07	1492
23	*α*-Selinene	1.84	1498
24	*α*-Muurolene	1.78	1504
25	*γ*-Cadinene	2.80	1512
26	*δ*-Cadinene	12.26	1521
27	Selina-3,7(11)-diene	2.16	1532
28	Neryl 2-methyl butanoate	4.81	1571
29	Germacrene B	4.02	1556
30	Spatulenol	0.56	1582
31	Salvial-4(14)-en-1-one	0.27	1599
32	*α*-epi-Muurolol	0.47	1648
33	3-Butylphthalide	0.61	1658
34	(*Z*)-Butylidene phthalide	3.05	1678
35	(*Z*)-Ligustilide	15.93	1797
36	(*E*)-Ligustilide	0.35	1741
	Monoterpene hydrocarbons	3.67	
	Oxygenated monoterpenes	9.77	
	Sesquiterpene hydrocarbons	53.68	
	Oxygenated sesquiterpenes	1.30	
	Non-terpenes	22.32	
	Total identified	90.74	

[a] Compounds listed in order of elution from BPX5 column; [b] Retention indices to C_8-C_{24} *n*-alkanes on BPX5 column.

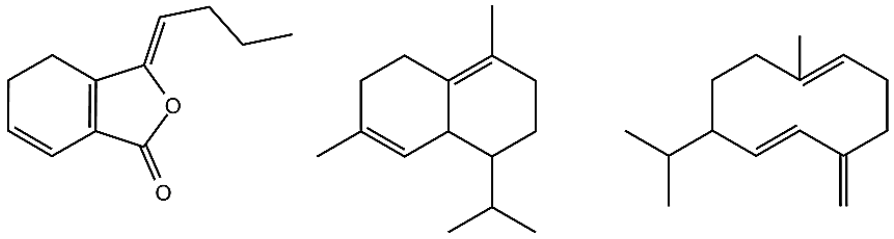

Fig. 2. The structures of main compounds identified in essential oil of *K. odoratissima* fruits

4. Discussion

Five flavonol 3-O-glycosides, namely, isorhamnetin 3-O-*β*-D-glucopyranoside (**1**), quercetin 3-O-*β*-D-glucopyranoside (isoquercetin) (**2**), isorhamnetin 3-O-*β*-D-rutinoside (narcissin) (**3**), isorhamnetin 3-O-*β*-D-glucuronide (**4**) and quercetin 3-O-*β*-D-glucuronide (mequilianin) (**5**) were isolated from the fruits of *K. odoratissima* in present study for the first time.

Previously, two steroid derivatives, stigmasterol and *β*-sitosterol and one phthalide, 3-butyliden-4,5-dihydrophthalide reported from *n*-hexane extract of *K. odoratissima* fruits [14]. Flavonoids are phenolic compounds with well-known free radical scavenging activity which makes them appropriate food supplements to prevent oxidative stress related diseases such as cancers, diabetes, cardiovascular, inflammatory

and neurodegenerative diseases (such as Alzheimer and Parkinson) [22].

The potent antioxidant activity of isorhamnetin 3-O-*β*-D-rutinoside, a major flavonol 3-O-glycoside isolated in present study, has been reported in DPPH (IC_{50}; 9.01 µM, IC_{50} value of ascorbic acid as positive control; 11.93 µM) and $ONOO^-$ (IC_{50}; 2.56 µM, IC_{50} value of DL-penicillamin as positive control; 5.1 µM) methods [23]. This compound (isorhamnetin 3-O-*β*-D-rutinoside) has also been reported as one of antimicrobial principles of *Atriplex halimus* aerial parts with considerable antibacterial activity against *Streptococcus pyogenes* (inhibition zone; 17 ± 0.09 mm), *Escherichia coli* (inhibition zone; 16 ± 0.09 mm) and *Acinetobacter baumanii* (inhibition zone; 16 ± 0.13 mm), having a low cytotoxicity on PMNCs (IC_{50}; 450 µg ml^{-1}) [24].

Isorhamnetin 3-O-*β*-D-glucopyranoside and quercetin 3-O-*β*-D-glucopyranoside (isoquercetin), two other flavonol glycosides isolated from *K. odoratissima* fruits in present study, have been documented for its antioxidant [25], antidiabetic [26] and hepatoprotective [27] activities. Furthermore, in a bioassay-guided fractionation study quercetin 3-O-glucoside was isolated as active compounds of *Sambucus ebulus* L. leaves with rremarkable wound healing activity [28].

Isorhamnetin and quercetin were also isolated as their 3-O-*β*-D-glucuronide derivatives in the present study. A bioactivity-guided isolation study reported the isolation of isorhamnetin 3-O-*β*-D-glucuronide as one of active compounds of *Sanguisorba officinalis* L. (Rosaceae) with considerable lipid accumulation inhibition on 3T3-L1 cells (IC_{30}; 18.43 µM) [29]. *Chuquiraga spinosa* (Asteraceae), *Cichorium spinosum* L. (Asteraceae), *Foeniculum vulgare* L. (Apiaceae) and some cultivars of *Vitis vinifera* L. (Vitaceae) are examples of plants reported to contain isorhamnetin 3-O-*β*-D-glucuronide [30-34].

Quercetin 3-O-*β*-D-glucuronide has been found in some plant species such as *Calligonum comosum* L. (Polygonaceae), *Rubus ulmifolius* Schott. (Rosaceae) and *Phaseolus vulgaris* L. (Fabaceae), as well as one of major human metabolites of quercetin [35-38]. One study suggested that supplementation of quercetin 3-O-*β*-D-glucuronide can reduce the relative risk for developing Alzheimer's disease (AD) dementia [39]. They showed that quercetin 3-O-*β*-D-glucuronide can significantly reduce the generation of *β*-amyloid (A*β*) peptides and improves AD-type deficits in hippocampal formation basal synaptic transmission and long-term potentiation, possibly through mechanisms involving the activation of the c-Jun N-terminal kinases and the mitogen-activated protein kinase signaling pathways [39]. In another study, quercetin 3-O-*β*-D-glucuronide showed to possess anti-neuroinflammatory activity in lipopolysaccharide (LPS)-induced inflammation in BV2 cells by inhibition of the production of NO and PGE2 and reducing the levels of some pro-inflammatory cytokines such as TNF-*α* and interlukin-1*β* [40]. Moreover, beneficial effects of quercetin 3-O-*β*-D-glucuronide in arteriosclerosis prevention has been reported via elevating plasma HDLC followed by induction of ABCA1 (ATP-binding cassette, subfamily A, member 1) expression, a crucial cholesterol transporter involved in reverse cholesterol transport [41].

In the present study, GC-MS analysis of the essential oil extracted from the fruits of *K. odoratissima* resulted in the identification of thirty six compounds, of which (*Z*)-ligustilide (15.93 %), *δ*-cadinene (12.26 %) and germacrene D (12.18 %) were the main compounds (Fig. 2). One study reported *α*-caryophyllene (22.60 %), *α*-humulene (20.0 %) and cyclopropane (11.54 %) as the main compounds of the essential oil of *K. odoratissima* fruits, gathered from Zardkooh

Mountain, (Charmahal-Bakhtiari, Iran) [42]. In another study on the fruits of this plant collected from Samsami region (Chaharmahal-Bakhtiari, Iran) (*Z*)-Ligustilide (86.0 %), (2*E*)-decen-1-ol (8.0 %), pentyl cyclohexa-1,3-diene (4.4 %) were characterized as main compounds of its essential oil [43]. The results of previous reports on essential oil composition of different parts of *K. odoratissima* have been summarized in Table 2 [5, 7, 8, 13, 15, 43-47]. Beside possible genetic diversity, variation observed in essential oil constituents may be raised from some geographical differences between the populations of *K. odoratissima* [48].

Table 2. The results of essential oil analysis of *K. odoratissima* from the previous and present studies.

Location of collection	Date	Method	Part	Main compounds (%)	Ref.
Fereydunshahr, Isfahan, Iran	Dec 2018	HD[a]	Fruit	(*Z*)-Ligustilide (15.9 %), *δ*-Cadinene (12.3 %), Germacrene D (12.2 %)	Present study
Dishmook, Kohgiluye-Buyer Ahmad, Iran	May 2014	HD	Leaf	(*Z*)-Ligustilide (58.7%), Carvacrol (7.8%), *trans*-3-Butylidene phthalide (4.9%), Thymol (4.5%),	5
Keloseh region, Isfahan, Iran				(*Z*)-Ligustilide (53.5%), Thymol (7.9%), *trans*-3-Butylidene phthalide (3.3%)	
Fereydunshahr, Isfahan, Iran				(*Z*)-Ligustilide (51.3%), Thymol (8.7%), Carvacrol (3.2%)	
Central Zagros Mountain, Chaharmahal-Bakhtiari, Iran	Apr 2009	HD	Leaf	(*Z*)-Ligustilide (77.7%), 2-Octen-1-ol acetate (6.3%), (*E*)-Ligustilide (2.3%)	7
Kohgiluye-Buyer Ahmad, Iran	Apr-May 2012	HD	Aerial part	(*Z*)-Ligustilide (34.5%), (*E*)-Ligustilide (11.8%), 3-(*Z*)-Butylidenephthalide (8.8%), Dec-9-en-1-ol (5.9%)	8
Central Zagros mountain, Iran	Mar 2006	HD	Aerial part	(*Z*)-Ligustilide (85.9%), *α*-Copaene (1.4%), *δ*-Cadinene (0.7%)	13
Fereydunshahr, Isfahan, Iran	Aug 2009	HD	Fruit	*Z*-ligustilide (51.0%), *δ*-Terpinen-7-al (10.3%), *δ*-terpinene (5.3%), Cumin aldehyde (5.2%)	15
Zardkooh Mountain, Charmahal-Bakhtiari, Iran	Jul-Aug 2011	HD	Fruits	*α*-Caryophyllene (22.6%), *α*-Humulene (20.0%), Cyclopropane (11.5%)	42
Samsami region, Chaharmahal-Bakhtiari, Iran	Jun-Jul 2014	HD	Root	(*Z*)-Ligustilide (54.0%), (2*E*)-Decen-1-ol (10.7%), Pentyl cyclohexa-1,3-diene (6.4%), (3*Z*)-Butylidene phthalide (5.8%)	43
			Stem	(*Z*)-Ligustilide (58.7%), (2*E*)-Decen-1-ol (11.6%), Pentyl cyclohexa-1,3-diene (4.4%)	
			Leaf	(*Z*)-Ligustilide (66.8%), (2*E*)-Decen-1-ol (12.3%), Pentyl cyclohexa-1,3-diene (3.8%)	
			Flower	(*Z*)-Ligustilide (62.4%), Geranyl butyrate (9.0%), *trans*-Muurola-4(14) 5-diene (5.5%)	
			Fruit	(*Z*)-Ligustilide (86.0%), (2*E*)-Decen-1-ol (8.0%), Pentyl cyclohexa-1,3-diene (4.4%)	

Table 2. The results of essential oil analysis of *K. odoratissima* from the previous and present studies (Continued)

Location of collection	Date	Method	Part	Main compounds (%)	Ref.
Keloseh region, Fereydunshahr, Isfahan, Iran	Aug 2007	HD	Stem	Borneol (36.9 %), Bornyl acetate (14.0 %), 1,8-Cineol (13.6 %), Camphor (9.5 %)	44
			Flower	1,8-Cineol (22.0%), Camphor (20.1%), *α*-Pinene (19.0%), Camphene (12.0%), Bornyl acetate (5.8 %)	
			Leaf	*β*-Terpinene (23.0%), Sabinene (9.0%), *α*-Thujene (8.4%)	
Fereydunshahr, Isfahan, Iran	Jul 2007	HD	Aerial part	(*Z*)-Ligustilide (87.6%), (*E*)-Ligustilide (3.2 %), Piperitone epoxide (3.1%)	45
Bazoft region, Chaharmahal-Bakhtiari, Iran	April 2008	HD	Aerial part	(*Z*)-Ligustilide (47.3%), (3*E*)-Butyldiene phthalide (17.38%), (*E*)-Ligustilide (6.3%), 2-Octen-1-ol acetate (5.4%)	46
Koohrang region, Chaharmahal-Bakhtiari, Iran				(*Z*)-Ligustilide (33.7%), (3*E*)-Butyldiene phthalide (20.1%), (*E*)-Ligustilide (6.6%), 2-Octen-1-ol acetate (5.2%)	
Samsami region, Chaharmahal-Bakhtiari, Iran				(*Z*)-Ligustilide (37.6%), (3*E*)-Butyldiene phthalide (19.9%), (*E*)-Ligustilide (7.0%), Kessane (5.3%)	
Zagros mountain, Iran	May-Jun 2012	HD	Aerial part	*α*-Pinene (20.1%), 1,8-Cineole (18.2%), (*Z*)-Ligustilide (15.5%)	47

(*Z*)-Ligustilide, a phthalide derivative identified as major constituent in most of previously studied *K. odoratissima* essential oils, has received attention for its interesting pharmacological and biological effects such as neuroprotective, anti-oxidation, anti-inflammatory, analgesic and anticancer effects [49].

5. Conclusion

The presence of flavonoid glycosides (**1**-**5**) and phthalide derivatives with known health beneficial effects make the fruits of *K. odoratissima* as a natural remedy with valuable therapeutic potentials and suggest it an appropriate option for further studies. Meanwhile, restricted distribution of *K. odoratissima* underline the importance of an appropriate conservation approach for the uses of this species for medicinal and food purposes.

Author contributions

MK: Study supervision and data interpretation; SG: Experimental analysis and preparation of manuscript draft; GS: Experimental analysis; MD: Original idea presentation, study design, study supervision, data interpretation and revision of the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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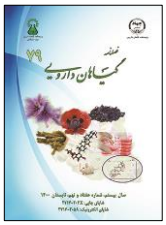

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مقاله تحقیقاتی

ترکیبات فیتوشیمیایی میوه کلوس (*Kelussia odoratissima* Mozaff.)، گیاهی معطر و انحصاری ایران

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اطلاعات مقاله

گل‌واژگان:
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(Z)- لیگوستیلید

چکیده

مقدمه: گیاه کلوس (*Kelussia odoratissima* Mozaff.) از تیره چتریان، گیاهی است علفی و پایا که به صورت انحصاری در غرب ایران می‌روید. مردم بومی از بخش‌های هوایی معطر این گیاه به صورت سنتی به عنوان عطر و طعم‌دهنده در تهیه بعضی غذاهای محلی و همچنین در درمان بیماری‌های مختلف استفاده می‌کنند. **هدف:** مطالعه حاضر با هدف جداسازی و شناسایی ترکیبات فنولی و همچنین آنالیز ترکیبات اسانس میوه‌های گیاه کلوس طراحی شده است. **روش بررسی:** ترکیبات فراکسیون بوتانولی نرمال بدست آمده از عصاره هیدروالکلی میوه گیاه با استفاده از کروماتوگرافی روی ستون‌های فاز نرمال و سفادکس ال اچ-۲۰ مورد جداسازی و خالص‌سازی قرار گرفت. ترکیبات اسانس بدست آمده از نمونه نیز با استفاده از تکنیک کروماتوگرافی گازی متصل به طیف‌سنج جرمی آنالیز شد. **نتایج:** مطالعه فیتوشیمیایی روی میوه‌های گیاه کلوس به جداسازی و شناسایی پنج گلیکوزید فلاونولی با نام‌های ایزورامنتین ۳-اُ- گلوکوزید (۱)، کوئرستین ۳-اُ- گلوکوزید (ایزوکوئرستین) (۲)، ایزورامنتین ۳-اُ- روتینوزید (نارسیسین) (۳)، ایزورامنتین ۳-اُ- گلوکورونید (۴) و کوئرستین ۳-اُ- گلوکورونید (مکوئیلینانین) (۵) انجامید. تعداد سی و شش ترکیب نیز در نتیجه آنالیز اسانس حاصل از میوه‌های گیاه شناسایی شد که از این میان ترکیبات (Z)- لیگوستیلید (۱۵/۹۳ درصد)، دلتا- کادینن (۱۲/۲۶ درصد) و جرماکرن دی (۱۲/۱۸ درصد) ترکیبات عمده بودند. **نتیجه‌گیری:** نتایج این مطالعه، میوه‌های گیاه کلوس را به عنوان منبعی از گلیکوزیدهای فلاونوئیدی و مشتقات فتالات معرفی می‌کند. حضور این ترکیبات که خواص بیولوژیک و اثرات سودمند آنها بر سلامتی در مطالعات پیشین نشان داده شده است، میوه‌های این گیاه را به عنوان گزینه مناسبی برای مطالعات مرتبط بیشتر مطرح می‌کند.

مخففها: NMR، رزونانس مغناطیسی هسته‌ای؛ GC-MS، کروماتوگرافی گازی متصل به طیف‌سنج جرمی؛ TLC، کروماتوگرافی لایه نازک

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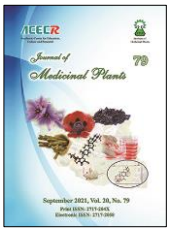

Research Article

Identification of isoflavonoids in antioxidant effective fraction of *Arum rupicola* Boiss. leaves

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ABSTRACT

Background: *Arum rupicola* Boiss. (Araceae Family) is used by the native people of southern areas of Iran as a soup called "kardeh soup". Several flavonoids and phenol compounds have been identified from *Arum* species. **Objective:** The aim of this study was to evaluate antioxidant effect and total phenol contents as well as cytotoxic activity of the leaves of *A. rupicola*. **Methods:** Antioxidant activity of total methanol extract and fractions including *n*-hexane, chloroform, ethyl acetate and water residue were evaluated using FRAP and DPPH methods. Total phenol content was measured using Folin-Ciocalteu method. Cytotoxic activity of the extract and fractions were investigated against human breast cancer MCF-7, MDA-MB-231, and T47D cell lines by MTT assay. Further phytochemical isolation was done on the water residue using column chromatography. **Results:** According to the results, water residue showed the lowest IC_{50} value (186.7 µg/ml) and the total methanol extract showed the most antioxidant power (163.62 mmol FeSO4/100 g extract) and phenol content (135 µmol Gallic acid/g extract). The hexane fraction also showed the highest cytotoxic effect against MCF-7 breast cancer cell line with IC_{50} equal to 118.9 µg/ml. Phytochemical analysis of the water residue resulted in isolation and identification of three isoflavonoids named orobol, genistein and genistein 8-*c*-glucoside. **Conclusion:** Based on the identification of isoflavonoid compounds in this plant, its ability to be used as a phytoestrogenic supplement can be considered in future studies.

Abbreviations: FRAP, Ferric Reducing Ability of Plasma; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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1. Introduction

Arum is a genus of flowering plants belonging to family Araceae, represented by 26 species and distributed in Northern Africa, Mediterranean Region, Western Asia, and Europe [1]. Several *Arum* species are growing in Lebanon, Cyprus, Syria and Palestine and consumed in folk medicine for treatment of cancer, cough, hemorrhoids, worms, constipation and urinary tract infections. The fresh leaves of *Arum* used in cooked or roasted form after washing several times with salted water [2]. *Arum rupicola* Boiss (Syn. *Arum conophalloides* Kotschy ex Schott) is used by the native people of southern areas of Iran as a soup called "kardeh soup" [3]. Also in some areas of Turkey the aerial parts of *A. rupicola* with local name of "kahri" were used as a rice-vegetable dish [4]. Various pharmacological effects of *Arum* species have been reported such as antioxidant, and antimicrobial effects [5-7]

A variety of flavonoids including, vitexin, caffeic acid, luteolin, vicenin and 3,6,8-trimethoxy, 5,7,3',4'-tetrahydroxy flavone [8] and the alkaloid (S)-3,4,5-trihydroxy-1 H-pyrrol-2(5H)-one were isolated from *A. palaestinum* [9]. Also several flavonoids such as apigenin, luteolin, quercetin, vitexin, isoorientin, esculin, quercetin-3-O-β-glucoside, caffeic acid, and ferulic acid have been identified from *A. dioscoridis* [10, 11]. Flavonoids have antioxidant, anti-cancer, anti-allergic, anti-inflammatory and anti-viral phytochemical compounds [12]. Since several flavonoids and phenolic compounds reported in the genus *Arum*, it can be expected that the aerial parts of the plant contain interesting biological active metabolites. The aim of this study was to evaluate phytochemicals, antioxidant effect and total phenolic contents of the leaves of *Arum rupicola* collected from southern areas of Iran.

2. Materials and Methods

2.1. Chemicals

2,2-Diphenyl 1-picrylhydrazyl (DPPH; Fluka, Switzerland); butylatedhydroxylanizole (BHA), methanol, hexane, chloroform, ethyl acetate (Merck, Germany) were purchased. ^{1}H and ^{13}C-NMR spectroscopy of compounds A, B and C were performed in DMSO-d_6 on a Bruker Avance DPX 400 spectrometer (Karlsruhe, Germany) [400 MHz, tetramethylsilane (TMS) as internal standard]. Column chromatography (CC) was done by Sephadex LH-20 (lipophilic Sephadex, 25–100 μm; Sigma, Dorset, UK) columns.

2.2. Plant material

The leaves of *Arum rupicola* were collected from their growing habitats in Yasuj area, Iran, April 2016. The plant identified by Dr. Farideh Attar. A voucher specimen of the plant (46054 TUH) is deposited in the herbarium of the faculty of Sciences, Tehran University.

2.3. Extraction

The leaves of *A. rupicola* were air dried and grounded in the laboratory, and the obtained powder (1000 g) was extracted five times at room temperature with 80 % methanol for an interval of three days. The aqueous-methanol extract was evaporated under reduced pressure and temperature to obtain a residue of 398.63 g. The total methanol extract was suspended in water and then fractionated using hexane (21.52 g), chloroform (3.03 g), and ethyl acetate (1.67 g) according to their polarity also water residue was 160.14 g. The excessive solvents were evaporated with a rotary vacuum evaporator (60 rpm at 40 °C). All extracts were stored at 4 ºC until use.

2.4. Total phenol contents assay

Total Phenol was determined colorimetrically using Folin-Ciocalteu reagent as described by Velioglu et al. 1998 and according to previous study [13]. The experiments were repeated three times. The phenol content calibration curves were plotted by measuring the absorption of certain concentraitions (25-150 mg/L) of gallic acid as a standard and the results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry matter (total extracts and fractions) as means ± SEM [13].

2.5. Antioxidant determination of DPPH assay

The total methanol extract was evaluated for its free radical scavenging activities using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) method according to Brand Williams et al. (1995) [14]. Different concentrations (25, 50, 100 µg/ml) of sample solutions (1 ml) in methanol were added to DPPH methanol solution (2 ml, 40 µg/ml). Butylated hydroxyanisole (BHA) (100 µg/ml) was used as positive control. After 30 min, the absorbance was measured at 517 nm. All tests were carried out in triple replicate. Percentage of radical scavenging activity of sample was calculated according to the following equation: Inhibition % = [(A0–As)/A0] × 100 that A0 is the absorbance of the control and as is the absorbance of the sample. Half maximal inhibitory concentration (IC_{50}) value (indicate the concentration of the sample (mg/ml), required to scavenge 50 % of DPPH) was calculated from the plotted graph of scavenging activity versus the concentration of extract, using linear regression analysis.

2.6. FRAP assay

Ferric reducing antioxidant power assay is based on reduction of a ferric-tripyridyl triazine complex to its ferrous colored form in the presence of antioxidants and was performed according to previous studies [15].

2.7. Cell culture

Human breast cancer cell lines including MCF-7 (human breast cancer cell line with estrogen, progesterone and glucocorticoid receptors), T47D (breast ductal carcinoma), and MDA-MB-231 (human breast adenocarcinoma), were supplied from the National Cell Bank of Iran Pasteur Institute, Tehran, Iran. Cancer cell lines were developed in RPMI-1640 medium accompanied with 10 % heat-inactivated fetal calf serum, 100 µg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humidified atmosphere with 5 % CO_2.

2.8. Determination of cell viability by MTT assay

The in-vitro cytotoxic activity of all samples were assessed against three human breast cancer cell lines including MCF-7, T47D, and MDA-MB- 231 cell lines using MTT colorimetric assay according to previous work [16].

Concisely, the concentration of 50, 100, 200, 400 and 500 µg/ml from all samples were prepared for each cell line. At first, samples were dissolved in DMSO (Dimethyl Sulfoxide) and further diluted with cell culture medium. The final DMSO concentration used was 0.1 % of total volume of the medium in all treatments, including the control group. In each plate, there were three control wells (cells without test extracts) and three blank wells (the medium with 0.1 % DMSO) for cell viability. Etoposide was used as a positive control for cytotoxicity. The plates were incubated for 72 h. At the end of the incubation period, the medium was removed and 200 µl phenol red free medium containing MTT (1 mg/ml), was added to wells, and followed by 4 h incubation. After incubation, the culture medium was then exchanged with 100 µl of

DMSO and the absorbance of each well was measured by using a micro plate reader at 492 nm. For each sample, the concentration causing 50 % cell growth inhibition (IC_{50}) compared with the control was calculated from concentration response curves by regression analysis [16].

2.9. Purification and isolation of flavonoid compounds

In order to identify the main compounds in the plant, water residue was selected for purification due to proper weight and antioxidant effects. Fifteen grams of the water residue were loaded on a Sephadex LH-20 (25 × 5 cm) column and eluted with aqueous methanol (80 %). Fractions with a volume of 30 ml were collected from this column. The obtained fractions were compared using TLC method and the same fractions were combined. To gain more weight for each fraction, this step was performed several times, each time with about 5 g of water residue, and the same fractions were combined to afford 4 fractions. Compound **B** was isolated from the second column and for the final purification, Sephadex LH-20 column (80 × 1 cm) was used. The weight of compound **B** was about 20 mg. The R_f of compound **B** with the solvent system of formic acid/ acetic acid/water/ethyl acetate (11/11/26/100) on TLC sillicagel 60 F_{254} was about 0.7.

To separate compound **A**, the fractions No. 24-27 of the first column, the fractions 20-23 of the second column and the fractions 31-34 of the third column were combined, and first was loaded on the Sephadex LH-20 (70 × 2 cm) column and washed with aqueous methanol (80 %). Then, the fraction No. 17-24 of this column was dried and for final purification, it was loaded on a Sephadex LH-20 (80 × 1 cm) column eluted with methanol (100 %) to afford compound **A** (30 mg). The Rf of compound **A** with the solvent system of formic acid/ acetic acid/water/ethyl acetate (11/11/26/100) on TLC sillicagel 60 F_{254} was about 0.8. To separate compound **C**, the fractions No. 35-37 of the first column, the fractions 28-33 of the second column and the fractions 37-39 of the third column were merged, and then was loaded twice on a Sephadex LH-20 (80×1 cm) column eluted with methanol (100 %) to afford compound **C** (3 mg). The R_f of compound **C** with the solvent system of formic acid/ acetic acid/water/ethyl acetate (11/11/26/100) on TLC sillicagel 60 F_{254} was about 0.6.

2.10. Chemical analysis

^{1}H and ^{13}C-NMR spectroscopy of compounds **A, B** and **C** were recorded on a Brucker Avance 500 DRX (500 MHz) spectrometer. Chemical shifts are given in (ppm) DMSO using TMS as internal standard. Column chromatography (CC) was performed using Sephadex LH-20 (lipophilic Sephadex, 25–100 μm; Sigma, Dorset, UK) columns.

Compound A (Orobol): $C_{15}H_{10}O_6$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ = 8.47 (1H, *s*, H-2), 7.34 (2H, *m*, H-5',6'), 6.83 (1H, *d*, *J* = 7.6 Hz, H-2'), 6.54 (1H, *s*, H-8), 6.37 (1H, *s*, H-6)

Compound B (Genistein): $C_{15}H_{10}O_5$. ^{1}H-NMR (400 MHz, DMSO-d_6): δ =8.07 (1H, *s*, H-2), 7.77 (2H, *d*, *J* = 7.2 Hz, H-2', 6'), 6.8 (2H, *d*, *J* = 7.2 Hz, H-3', 5'), 6.57 (1H, *s*, H-8), 6.46 (1H, *s*, H-6)

Compound C (Genistein 8-*C*-glucoside): $C_{21}H_{21}O_{10}$. UV (MeOH, λ_{max}, nm):265. ^{1}H-NMR (400 MHz, DMSO-d_6): δ =8.00 (1H, *s*, H-2), 7.93 (2H, *d*, *J*= 7.2 Hz, H-2',6'), 6.88 (2H, *d*, *J*= 7.2 Hz, H-3',5'), 6.59 (1H, *s*, H-6), 4.18 (1H, *d*, *J*=10 Hz, H-1")

2.11. Statistical analyses

The values were reported as mean ± SD by SPSS and Excel 2010.

3. Results

3.1. DPPH radical scavenging activity

Free radical scavenging effects of total extract and different fractions from *Arum rupicola* leaves were assessed with 2, 2-diphenyl-1-picryl-hydrazyl (DPPH). IC_{50} values were displayed in Table 1.

3.2. FRAP assay

Results of antioxidant effects of different fractions of the leaves extract using FRAP, are reported based on mmol Fe II/ 100 g of extract or fraction. Ferric reducing antioxidant power of the extracts calculated using the calibration curve and regression equation of ferrous sulfate (R^2 = 0.996, y = 0.0008x-0.015). According to table 1, it was presented that antioxidant property of 100 grams of hexane, chloroform, ethyl acetate, and water residue as well as total methanol extract, had antioxidant activity equivalent to 140.17 ± 4.03, 140.02 ± 4.37, 110.01 ± 3.9, 108.64 ± 4.07 and 163.62 milimol $FeSO_4$, respectively.

3.3. Total phenol content

Total phenol content (µmol of GAE/g of sample) as represented in table 2 varied from 116.55 to 135.00 µmol of GAE/g sample using the standard curve of gallic acid (R^2 = 0.9919, y = 0.0067x - 0.0194).

Table 1. DPPH radical scavenging activity (IC_{50} values)**,** total phenolic content (TPC) and antioxidant power (mmol Fe/100 g sample) of different fractions from *Arum rupicola* leaves.

Sample	IC_{50} (µg/ml)[a]	TPC (µmol Gallic acid/g sample)	Antioxidant power (mmol Fe/100 g sample)
Hexane fraction	481.5 ± 2.26[a]	120.6 ± 12.04	140.17 ± 4.03
Chloroform fraction	637.8 ± 11.3	133.2 ± 13.5	140.02 ± 4.37
Ethyl acetate fraction	441.2 ± 4.77	122.4 ± 12.38	110.01 ± 3.9
Water residue	186.7 ± 5.8	116.5 ± 9.7	108.64 ± 4.07
Total methanol extract	467.3 ± 2.19	135 ± 11.8	163.62 ± 4.42
BHA	7.9 ± 0.06	-	
Vitamin A	14.2 ± 1.23	-	

[a] IC_{50} values represent an average of three independent experiments (mean ± SD); BHA: Butylated hydroxyanisole.

3.4. In vitro cytotoxic assay

The cytotoxicity analysis of total methanolic extract, hexane, chloroform, ethyl acetate and water residue in breast cancer cell lines including MCF-7, MDA-MB, and T47D cell line was performed and the results were shown in Table 2.

Results showed that in MCF-7 cell line, hexane, chloroform fractions and total methanol extract showed cytotoxicity with IC_{50} equal to 118.9 ± 0.38, 258.5 ± 0.25, 392.7 ± 0.25 µg/ml, respectively. In this cell line, etoposide showed cytotoxicity with IC_{50} of 18.53 ± 0.24 µg/ml.

In the MDA-MB-231 cell line, hexane, chloroform fractions and total methanol extract have shown cytotoxicity with IC_{50} of 137.2 ± 0.22, 239.8 ± 0.12, 357.3 ± 0.11 µg/ml, respectively. Etoposide also showed cytotoxicity with IC_{50} of 19.9 ± 0.006 µg/ml.

In the T47D cell line, the fractions of chloroform, hexane, and total methanol extract as well as water residue showed cytotoxicity with IC_{50} of 113.8 ± 1.25, 122.8 ± 0.2, 249.08 ± 0.14, 399.3 ± 0.6 µg/ml), respectively. In this cell line, etoposide showed cytotoxicity with IC_{50} of 23.3 ± 0.096 µg/ml.

3.5. Isolation of isoflavonoid compounds

Isolated compounds **A, B,** and **C** from the methanolic fraction of the total methanolic extract of leaves of *Arum rupicola* were identified by comparison of their NMR (^{1}H- & ^{13}C-NMR) with those reported in the literatures [17-19]. Compounds **A, B** and **C** (Fig. 1) were identified as Orobol, Genistein and Genistein 8-C-glucoside, respectively.

Table 2. In vitro cytotoxic activity (IC_{50}, µg/ml) of total extract and fractions of *Arum rupicola* leaves against cancer and normal cell lines

Sample	IC_{50} (µg/ml)[a] MCF-7	IC_{50} (µg/ml) MDA-MB-231	IC_{50} (µg/ml) T-47D
Total methanol extract	392.7 ± 0.25	357.3 ± 0.11	249.08 ± 0.14
Hexane fraction	118.9 ± 0.38	137.2 ± 0.22	122.8 ± 0.2
Chloroform fraction	258.5 ± 0.25	239.8 ± 0.12	113.8 ± 1.25
Ethyl acetate fraction	> 500	> 500	> 500
Water residue	> 500	> 500	399.3± 0.6
Etoposide	18.53 ± 0.24	19.9 ± 0.006	23.3± 0.096

[a] IC_{50} values represent an average of three independent experiments (mean ± SD).

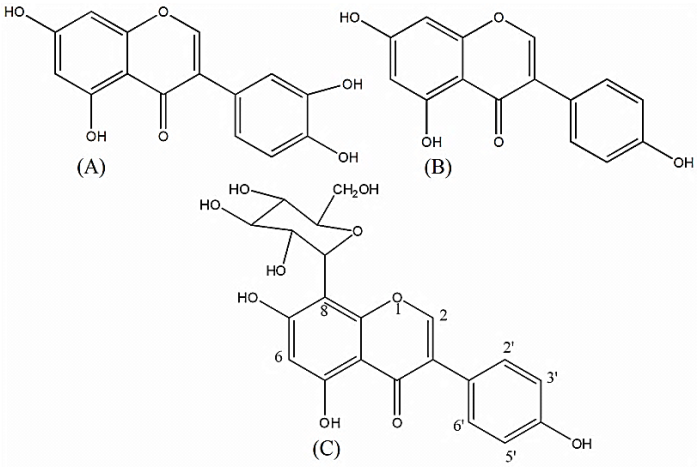

Fig. 1. Chemical structure of isoflavonos isolated from Arum rupicola leaves. A: Orobol, B: Genistein, and C: Genistein 8-C-glucoside

4. Discussion

Various flavonoids and phenolic compounds have been identified and reported in different species of *Arum* [20, 21], but no phytochemical studies have been performed on *A. rupicola*. In this study, antioxidant effects, cytotoxicity as well as purification and identification of secondary metabolites of *A. rupicola* leaves were investigated using different chromatographic and spectroscopic methods.

According to the results, methanolic fraction showed higher radical scavenging effect and the total methanol extract of the plant exhibited higher antioxidant power and total phenol content than other fractions. In a study, antioxidant and antitumor activities of the

ethanol extracts from different parts of six plants growing in Palestine, including *A. palaestinum* were evaluated and concluded that the studied plants showed different antioxidant abilities which were strongly associated with their phenolic contents [22].

The results of cytotoxicity study of plant samples on breast cancer cell lines showed that hexane fraction due to more toxicity in breast cancer cell lines could be considered for future studies. One study reported *in vitro* cytotoxic activity of different fractions and four flavonoid compounds isolated (luteolin, chrysoeriol, isoorientin, isovitexin) from the diethyl ether and ethyl acetate fraction of *Arum palaestinum* extract were investigated against four human carcinoma cell lines, epidermal carcinoma of larynx (Hep2), cervix (HeLa), liver (HepG2) and breast (MCF-7). Results showed that the fractionated extract and the isolated compounds showed significant antitumor activity against the four cell lines [1].

Phytochemical study of water residue of *A. rupicola*, using chromatographic methods, led to the isolation and identification of three isoflavonoid compounds, Orobol, Genistein and Genistein 8-C-glucoside. This is the first report on the occurrence of isoflavonoids in the genus *Arum*. Isoflavones, coumestans, stilbenes, and lignans are important subclasses of phytoestrogens. Isoflavones, which are more studied than other phytoestrogens, have estrogen-like properties in mammals and are found in large quantities in soybean and its products [23, 24]. Isoflavonoids have also been reported in several plant families in addition to the leguminous family and attracted the attention of many specialists from phytochemistry and plant physiology to medicine and nutrition [25]. Several beneficial effects of isoflavones have been reported, such as cardioprotection, osteoporosis prevention and antioxidant effects, which may be related to their phytoestrogenic effects [26]. Orobol is an isoflavone that is in small amount in soybean and is structurally similar to genistein, a largest isoflavone in soybean. Orobiol and genistein and their derivatives are known as multifunctional isoflavones with biological activities like neuroprotective, anti-obesity and anti-cancer [27, 28].

The presence of these isoflavones in *A. rupicola* can be introduced it as a good source of phytoestrogens and antioxidant.

5. Conclusion

According to the results of this study, it can be concluded that the stronger antioxidant effect of water residue of *Arum rupicola* could be due to the presence of three isoflavonoid compounds called orobol, genistein and genistein 8-C-glucoside. Considering the biological effects of isoflavons, which are a class of phytoestrogens, it is recommended to study the effects of this plant on diseases related to estrogen deficiency and also to expand its use as a food source containing phytoestrogens.

Author contributions

Substantial contributions to design, analysis and interpretation of data: Z. T., M. V., M. E., and SN. SL; investigation: Y. S., A. T., S. T., and R. A.; drafting the article or revising: Y. S. and SN. SL.; final approval of the version to be published: Z. T. and MR. SA.

Conflict of interest

We declare that there is no conflict of interest.

Acknowledgment

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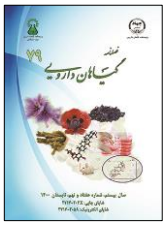

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مقاله تحقیقاتی

شناسایی ایزوفلاونوییدهای فراکسیون موثر آنتی‌اکسیدانی برگ‌های گونه‌ای شیپوری (*Arum rupicola*)

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اطلاعات مقاله

گل‌واژگان:
شیپوری
آنتی‌اکسیدان
اثر سمیت سلولی
جنیستئین
ایزوفلاونویید

چکیده

مقدمه: گیاه *Arum rupicola* Boiss. (خانواده گل شیپوری) گونه‌ای شیپوری است که توسط مردم بومی مناطق جنوبی ایران در آشی به نام "آش کاره" استفاده می‌شود. تاکنون ترکیبات فنلی متعددی از گونه‌های شیپوری شناسایی شده است. **هدف:** هدف از این مطالعه ارزیابی اثر آنتی‌اکسیدانی و تعیین محتوای تام فنلی و بررسی فعالیت سمیت سلولی عصاره برگ *A. rupicola* می‌باشد. **روش بررسی:** فعالیت آنتی‌اکسیدانی عصاره تام متانولی و فراکسیون‌های هگزانی، کلروفرمی، اتیل استاتی و باقیمانده آبی به روش FRAP و DPPH ارزیابی شد. محتوای تام فنلی با استفاده از روش فولین سیوکالتئو مورد سنجش قرار گرفت. اثر سمیت سلولی عصاره بر رده‌های سلولی سرطان پستان انسان MCF-7 و MDA-MB-231 و رده سلولی T47D با روش MTT مورد بررسی قرار گرفت. جداسازی ترکیبات فیتوشیمیایی باقیمانده آبی با استفاده از کروماتوگرافی ستونی انجام شد. **نتایج:** بر اساس نتایج مشاهده شده، باقیمانده آبی کمترین مقدار IC_{50} برابر با ۱۸۶/۷ میکروگرم در میلی‌لیتر را در تست DPPH نشان داد و عصاره متانولی تام نیز بیشترین قدرت آنتی‌اکسیدانی برابر با ۱۶۳/۶۲ میلی‌مول سولفات آهن در صد گرم عصاره و محتوای تام فنلی برابر با ۱۳۵ میکرومول گالیک اسید در گرم عصاره را نشان داد. فراکسیون هگزانی نیز بیشترین اثر سیتوتوکسیک را بر رده سلولی سرطان پستان MCF-7 (IC_{50} برابر با ۱۱۸/۹ میکروگرم در میلی‌لیتر) نشان داد. بررسی فیتوشیمیایی باقیمانده آبی منجر به جداسازی و شناسایی سه ایزوفلاونوئید با نام‌های اوربول، جنیستئین و جنیستئین-۸- سی گلوکوزید شد. **نتیجه‌گیری:** بر اساس شناسایی ترکیبات ایزوفلاونوییدی در این گیاه، می‌توان قابلیت مصرف آن را به عنوان مکمل فیتواستروژنی در مطالعات آینده در نظر گرفت.

مخففها: FRAP، قدرت احیاکنندگی آهن پلاسما؛ DPPH، ۲،۲- دی فنیل -۱- پیکریل هیدرازیل؛ MTT، ۳-(۵،۴- دی متیل تیازول-۲- ایل)-۵،۲- دی فنیل تترازولیوم بروماید

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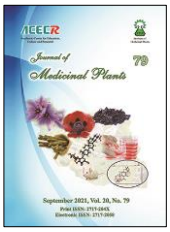

Research Article

Rearranged abietane diterpenoids from roots of *Teucrium hircanicum* L.

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ABSTRACT

Background: Medicinal plants play a significant role in preventing and treating diseases due to their traditional uses. **Objective:** The aim of the study was to isolate, purify and identify phytochemicals from the *n*-hexane extract of *Teucrium hircanicum* L. roots and evaluate the antioxidant activity of the extract and its purified compounds. **Methods:** The *n*-hexane extract (2.27 g) was extracted from the roots of this plant. The extract was fractionated by column chromatography with normal phase by eluting with *n*-hexane-EtOAc and following with EtOAc-Methanol. Fractions with similar phytochemical fingerprints combined to produce 23 main fractions. Final purification was carried out by preparative reversed-phase HPLC-UV. The structures of isolated were secured by different spectroscopic methods such as 1D, 2D NMR, and mass spectroscopy methods and comparing of these data with literature reported values. The antioxidant activity of the *n*-hexane extract of *T. hircanicum* roots and its purified diterpenoids was evaluated in DPPH assay and radical scavenging activity was calculated. **Results:** Extraction and isolation methods were used to purify three rearranged abietane-type diterpenoids villosin A (**1**), teuvincenone B (**2**) and 5, 8, 11, 13, 15-abietapentaen-7-one (**3**), a phenethyl ester namely 4-hydroxyphenethyl pentacosanoate (**4**) and one sterol namely 22-dehydroclerosterol (**5**). **Conclusion:** The *n*-hexane extract from roots of *T. hircanicum* and three rearranged abietane type diterpenoids were showed good antioxidant activities ranged from 3.5-4.3 µg/ml compared with the reference value (BHT = 16.5 µg/ml).

1. Introduction

Herbal products have been essential for the maintenance of human health from time immemorial. The biological activity of plant extracts depends on the type of secondary metabolism in the plant. As a result, these

Abbreviations: DPPH, 2,2-Diphenyl-1-picrylhydrazyl; BHT, Butylated Hydroxytoluene; CC, Column Chromatography; RSA, Radical Scavenging Activity; HMBC, Heteronuclear Multiple Bond Correlation; HSQC, Heteronuclear Single Quantum Coherence

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biological effects are specific to some species or groups of plants [1-3]. The impact of medicinal plants on the body is mostly based on one or a combination of several chemicals. The effects of the drug are caused by the synergy of several secondary metabolites, which act at one or more target locations associated with a physiological process [1]. The essential oils, extracts and purified phytochemicals are essential causes of biological activity such as antibacterial, antimalarial, antipyretic, analgesic, anti-inflammatory, antiviral, anticancer, antifungal and cytotoxic activity [4, 5]. To date, there have been numerous studies on traditional herbal remedies for various diseases that have led to the development of alternative drugs and treatment strategies [6].

Lamiaceae are a large plant family with 236 genera and 6900 to 7200 species. The plants belonging to Lamiaceae are considered an excellent source for discovering new natural products with bioactive capacities [7, 8]. Most of the plants in the Lamiaceae family are used as a perfumery or source of bioactive phytochemicals [9]. A lot of them are cultivated as ornamentals such as *Ajuga, Coleus,* and *Salvia*. Where others are grown as herbal medicines and spices for cooking such as sage (*Salvia*), thyme (*Thymus*), mint (*Mentha*), oregano or marjoram (*Origanum*), rosemary (*Rosmarinus*), lavender (*Lavandula*), and basil (*Ocimum*) are grown [10].

The genus *Teucrium* belongs to the Lamiaceae family, of which about 434 species currently recognized in Southeast Asia, Central and South America, the Mediterranean and the Middle East [11-14]. Plants in this genus are perennial and sometimes shrubby. In Iran, 19 species of the genus have been recorded, mostly in the Iran-Turan region. They are widely distributed in most parts of Iran. *T. persicum* is existing only at highlands in the southern regions of Iran. *T. polium* and *T. orientale* species commonly used in folk medicine are distributed in steppe, arid and semi-arid region. One of the most well-known species of the genus is *T. polium*. In traditional medicine, *T. polium* has been used for a variety of disease disorders such as gastrointestinal disorders, inflammations, diabetes and rheumatism [16, 17]. It has been reported other biological effects such as antioxidants [18], antinociceptive [19], lipid-lowering [20], severe hyperglycemia [21], improved memory [22], blood pressure-lowering [23], and prevention of hypertension [24]. Neo-clerodane diterpenoids are accepted as chemotaxonomic markers for *Teucrium* species, a natural source of many of these compounds. The neo-clerodane diterpenoids were purified in the aerial parts of the *Teucrium* species, but the rearranged abietane diterpenes were obtained from the roots [14]. The species of this genus are used as diuretics, diaphoretic, carminatives, astringents, stimulants and tonics, antiseptics, antipyretics and anti-inflammatory drugs in popular medicine. The species of this genus are used as diuretics, diaphoretic, carminatives, astringents, stimulants and tonics, antiseptics, antipyretics and anti-inflammatory drugs in popular medicine [14].

Teucrium hyrcanicum L. an indigenous plant that grows in the northern part of Iran [15]. This study aimed to isolate, purify and identify phytochemical compounds from *n*-hexane extract from *T. hircanicum* and evaluate the antioxidant activity of *n*-hexane extract and its purified phytochemical.

2. Materials and Methods

2.1. Chemical and reagents

The silica gel 60, TLC plates, solvents and chemical reagents used in the current study were purchased from Merck Chemical Company and Sigma-Aldrich.

2.2. Plant material

Teucrium hircanicum L. was collected in the Hyrcanian forests (Babol, Mazandaran) of northern Iran (south of the city of Babol) in September 2016. The plant material was identified by Dr. Sonboli and voucher specimens (MPH-2434) deposited at Herbarium of Department of Biology, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran.

2.3. Extraction and isolation

After the collection of *T. hircanicum*, the roots were separated from their aerial parts. Roots of *T. hyrcanicum* have been thoroughly dried in a place away from direct sunlight for five days. The plant's dried roots were ground with the laboratory grinder. The powder of the roots (470 g) was extracted by maceration in 3 L of *n*-hexane and repeated 5 times. The *n*-hexane extract obtained concentrated with a 40 °C vacuum rotary evaporator resulted in 2.27 g of gummy extract, the extract stored in the refrigerator for the next step. For the separation of the extract, a glass column of 3.5 cm in diameter and 50 cm long was used. The extract dissolved in dichloromethane has been associated with 10 g of silica gel 60 (0.063-0.200 mm). The resulting mixture was dried by rotary evaporation until a fine powder was obtained. In this study, 100 g of silica gel 60 (0.063 to 0.200 mm) as stationary phase was weighed and packed into the column. The *n*-hexane extract powdered with silica gel 60 was charged on silica gel in the column chromatography. The extract was fractionated to 104 parts by gradient elution with *n*-hexane-EtOAc and in the following EtOAc-Methanol by increasing the polarity of the mobile phase. The volume of collected fractions was 100 ml. All fractions were controlled by TLC under UV 254 nm and anisaldehyde reagent. The fractions with similar phytochemicals fingerprints combined and then 23 major fractions were obtained.

Compound (**1**), a light yellow powder (5 mg), was isolated from column chromatography (CC) using 6 % EtOAc and 94 % *n*-hexane and was purified from E-fraction by precipitation. Compound (**2**) (8 mg), a yellow powder, and compound (**3**) (7 mg), an orange powder, were extracted from column chromatography in 4% EtOAc and 96 % *n*-hexane and were purified from D fraction by precipitating and preparative HPLC. Compound (**4**) isolated a colorless powder (21 mg), was extracted from column chromatography in 7 % EtOAc and 93 % *n*-hexane, and was purified from F fraction by precipitating. Compound **5**, (84 mg) isolated as a colorless powder in 8 % EtOAc and 92 % *n*-hexane from CC, and additional purified from fraction G by precipitation.

2.4. Antioxidant activity

Investigation of antioxidant activity of *T. hircanicum* root *n*-hexane extract and rearranged abietane type diterpenoids its purified compounds. The antioxidant activity of the *n*-hexane extract of *T. hircanicum* roots and its purified diterpenoid compounds was evaluated by reducing the color reaction between DPPH solution and sample extracts. For this purpose, we used the method described in previous literature [25]. Briefly, the optical absorption rate of the samples and butylated hydroxytoluene (BHT) reference standard recorded in 5 different concentrations 0.1, 0.2, 0.4, 0.8 and 1.6 ppm and in 4 replicates of each concentration after a shake hour in a dark environment by using an ELISA device at a wavelength of 517 nm. A decrease in the DPPH solution absorbance indicates an increase in RSA %.

$$RSA\ \% = [1 - (S - SB) / C] \times 100$$

In this formula, RSA %, S, SB and C, respectively, are the percentage of radical scavenging activity DPPH, the amount of sample adsorption (methanol + sample + DPPH), the amount of standard adsorption (methanol + sample) and the amount of control adsorption (methanol + DPPH). The IC_{50} value is obtained by plotting different values of RSA % according to different concentrations of the sample and calculating the regression line equation.

3. Results

The *n*-hexane extract was obtained from the shade-dried powdered *T. hircanicum* roots. The extract was separated using column chromatography over silica gel and preparative reversed-phase HPLC-UV afforded three rearranged abietane-type diterpenoids and one sterol and phenethyl ester. The structures of compounds were secured by different spectroscopic methods such as 1D, 2D NMR, and mass spectroscopy methods and comparing of these data with literature reported values. The identified compounds were villosin A (**1**) [26], teuvincenone B (**2**) [27], 5, 8, 11, 13, 15-abietapentaen-7-one (**3**) [28] and 4-hydroxyphenethyl pentacosanoate (**4**) and 22-dehydroclerosterol (**5**) (Fig. 1).

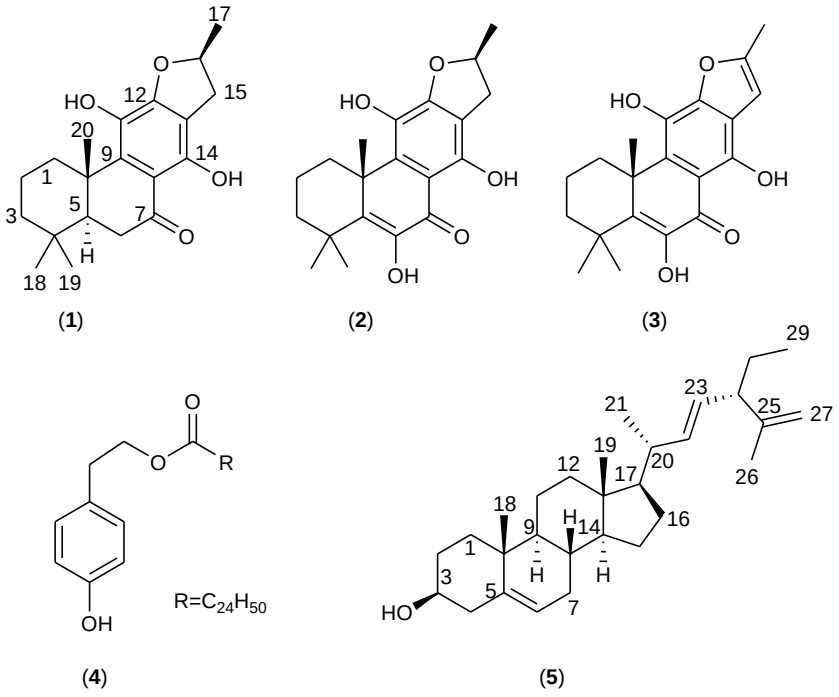

Fig. 1. Structures of purified compounds from *n*-hexane extract roots of *T. hircanicum* L. **1**: Villosin A, **2**: Teuvincenone B, **3**: 5, 8, 11, 13, 15-Abietapentaen-7-one, **4**: 4-Hydroxyphenethyl pentacosanoate and **5**: 22-Dehydroclerosterol.

Compound (**5**), isolated as a colorless powder in 8 % EtOAc and 92 % *n*-hexane from CC, and additional purified from G fraction by precipitating. In the H-H-COSY spectrum we can see that H-4 (δ 2.30, CH_2) is related to H-3 (δ 3.52, (td), CH) and weakly to H-6 (δ 5.36, (m), CH). In the HMBC spectrum, shows the relationship of H-4 with C-2 (δ 31.60), C-10 (δ 36.53), C-3 (δ 71.68), C-5 (δ 140.53) and C-6 (δ 121.35). Five methyl signals there are at 1.03 (s, 3H), 0.72 (s, 3H), 1.04 (d, 3H), 1.66 (s, 3H), 0.86 (3H) in the upfield region. In the ^{1}H-NMR

spectrum, Me-18 signal appear at chemical shift 1.03 ppm. In the HMBC spectrum, shows the relationship of this signal with 37.36 (C-1), 140.53 (C-5), 50.30 (C-9) and 36.53 (C-10). Me-19 (0.72, s) signal has relationship with 39.80 (C-12), 42.50 (C-13), 56.92 (C-14) and 56.12 (C-17) in the HMBC spectrum. Me-21 (1.04, d) signal has relationship with 56.12 (C-17), 40.13 (C-20) and 137.09 (C-22) in the HMBC spectrum. Me-26 (1.66, s) signal has relationship with 51.78 (C-24), 148.18 (C-25) and 109.46 (C-27) in the HMBC spectrum. Me-26 (0.86, dd) signal has relationship with 51.78 (C-24), and 25.60 (C-28) in the HMBC spectrum.

In the H, H-COSY spectrum, H-17 (δ 1.19, CH) signal have cross-peaks with H-16 (δ 1.30 and 1.73, CH_2) and H-20 (δ 2.07, CH), and H-16 signals form cross-peak with H-15 (δ 1.43 and 1.52, CH_2) signals, and so H-15 signals have a cross peak with H-14 (1.04, CH) signal. The signal of H-1 (δ 3.52, td, CH) with H-2 (δ 1.52 and 1.85, CH_2) and H-4 (δ 2.30, CH_2) signals form cross-peaks. The signals of H-12 (δ 2.02, CH_2) and H-11 (δ 1.10 and 1.60, CH_2) have cross-peaks. And so H-11 signals have a relationship with H-9 (δ 0.97, CH). The H-6 (δ 5.36, CH) and H-7 (δ 2.00 and 1.54 CH_2) have cross-peaks in the H-H COSY spectrum. And in the HMBC spectrum H-6 has a relationship with C-4 (δ 42.40), C-5 (δ 140.53), C-7 (δ 32.07), C-8 (δ 32.24) and C-10 (δ 36.53). The sterol structure of 22-dehydroclerosterol (**5**) was confirmed by comparing experimental and literature data.

Compounds teuvincenone B (**2**), a yellow powder, and compound 5,8,11,13,15-abietapentaen-7-one (**3)**, an orange powder, were extracted from column chromatography in 4 % EtOAc and 96 % *n*-hexane and were purified from D fraction by precipitating and preparative HPLC. Compound villosin A (**1**), a light-yellow powder, was extracted from column chromatography in 6 % EtOAc and 94 % *n*-hexane, and was purified from E fraction by precipitating. ^{1}H-NMR and ^{13}C-NMR data of these three compounds were given in tables 1 and 2. Other spectra of compound (**1**) are shown in figures 2 to 5.

Table 1. ^{1}H-NMR data (δH (*J* in Hz) of compounds **1**-**3** (500 MHz, $CDCl_3$)

No	1	2	3
1	1.43 (m, 1H)	1.87 (m, 1H)	1.93 (m, 1H)
	3.29 (dtd, *J* = 13.5, 3.5, 1.5 Hz, 1H)	3.66 (m, 1H)	3.73 (m, 1H)
2	1.60 (m, 1H)	1.75 (m, 1H)	1.67 (m, 1H)
	1.76 (dt, *J* = 13.9, 3.5 Hz, 1H)	1.89 (dt, *J* = 12.3, 3.6 Hz, 1H)	1.88 (m, 1H)
3	1.30 (m, 1H)	1.43 (tdt, *J* = 12.9, 6.0, 2.9 Hz, 1H)	1.40 (m, 1H)
	1.50 (m, 1H)	2.10 (dt, *J* = 15.9, 3.3 Hz, 1H)	2.04 (m, 1H)
5	1.81 (dd, *J* = 11.5, 5.8 Hz, 1H)		
6	2.62 (m, 2H)		
15	3.37 (dd, *J* = 15.3, 9.0 Hz, 1H)	3.34 (ddd, *J* = 15.4, 8.9, 2.8 Hz, 1H)	6.71 (q, *J* = 1.5 Hz, H)
	2.86 (dd, *J* = 15.3, 7.3 Hz, 1H)	2.80 (ddd, *J* = 15.2, 7.8, 2.8 Hz, 1H)	
16	5.12 (m, 1H)	4.94 (m, 1H)	
17	1.52 (d, *J* = 6.3 Hz, 3H)	1.28 1.28 (dd, *J* = 6.4, 2.9 Hz, 3H)	2.25 (d, *J* = 2.5 Hz, 3H)

Table 1. ^{1}H-NMR data (δH (*J* in Hz) of compounds **1**-**3** (500 MHz, $CDCl_3$) (Continued)

No	1	2	3
18	0.96 (s, 3H)	1.66 (d, *J* = 3.2 Hz, 3H)	1.68 (d, *J* = 2.7 Hz, 3H)
19	0.99 (s, 3H)	1.62 (d, *J* = 2.8 Hz, 3H)	1.65 (d, *J* = 2.8 Hz, 3H)
20	1.39 (s, 3H)	1.94 (d, *J* = 2.7 Hz, 3H)	1.98 (d, *J* = 2.8 Hz, 3H)
OH-6		9.13 (s, 1H)	9.29 (s, 1H)
OH-11	4.73 (s, 1H)	4.76 (s, 1H)	4.76 (s, 1H)
OH-14	13.43 (s, 1H)	13.37 (s, 1H)	13.77 (s, 1H)

Table 2. ^{13}C-NMR data of compounds **1**-**3** (125 MHz, $CDCl_3$).

No	1	2	3
1	36.1	29.7	30.1
2	17.5	17.7	17.2
3	41.9	36.1	36.7
4	33.7	27.5	36.3
5	50.0	144.2	145.5
6	35.2	143.5	141.6
7	204.3	182.5	184.1
8	110.5	107.7	107.8
9	140.0	141.2	133.5
10	40.7	41.81	41.6
11	130.9	130.7	130.9
12	155.0	156.5	156.0
13	110.2	111.1	117.1
14	155.7	153.3	150.2
15	34.2	33.9	101.1
16	83.2	81.7	154.6
17	21.9	21.5	13.1
18	33.1	27.9	27.7
19	21.5	27.3	28.0
20	18.2	27.3	27.3

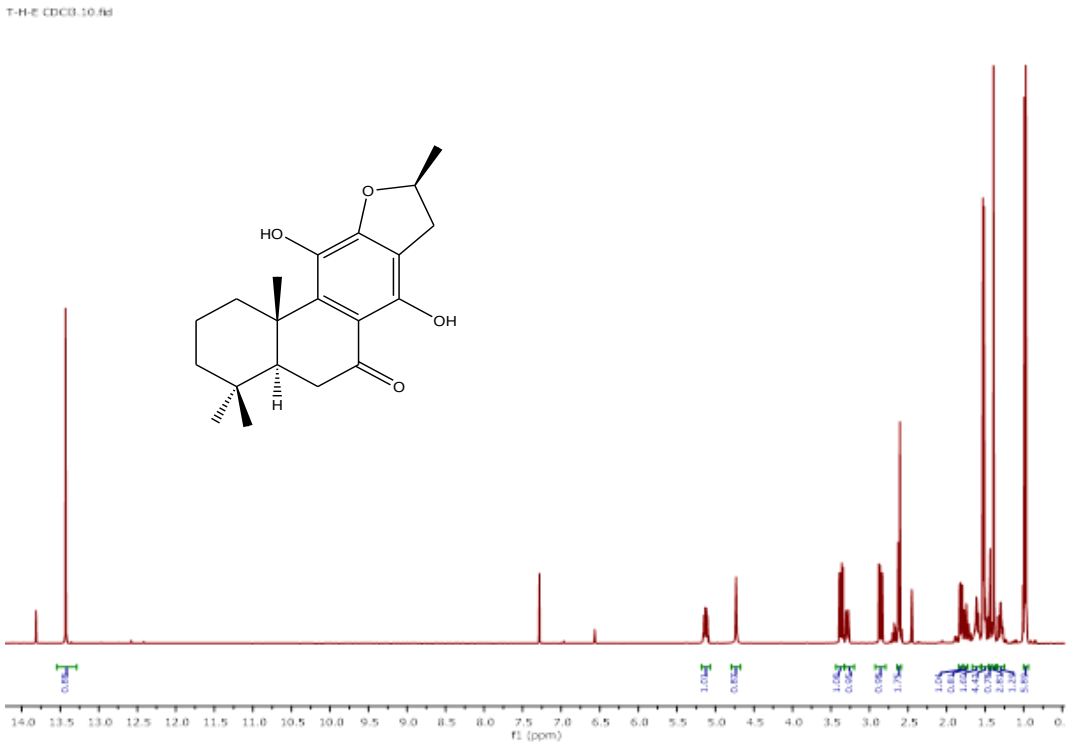

Fig. 2. ^{1}H-NMR spectrum of villosin A (**1**).

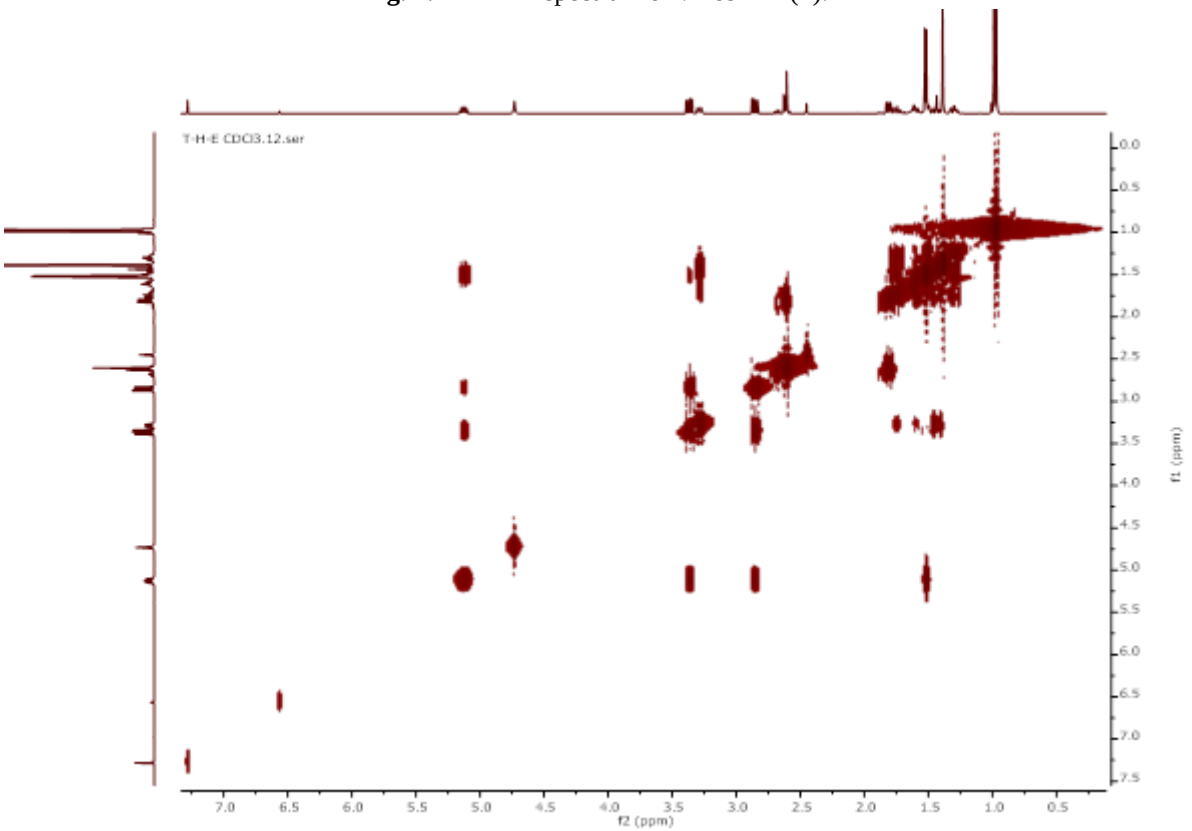

Fig. 3. H, H-cosy spectrum of villosin A (**1**).

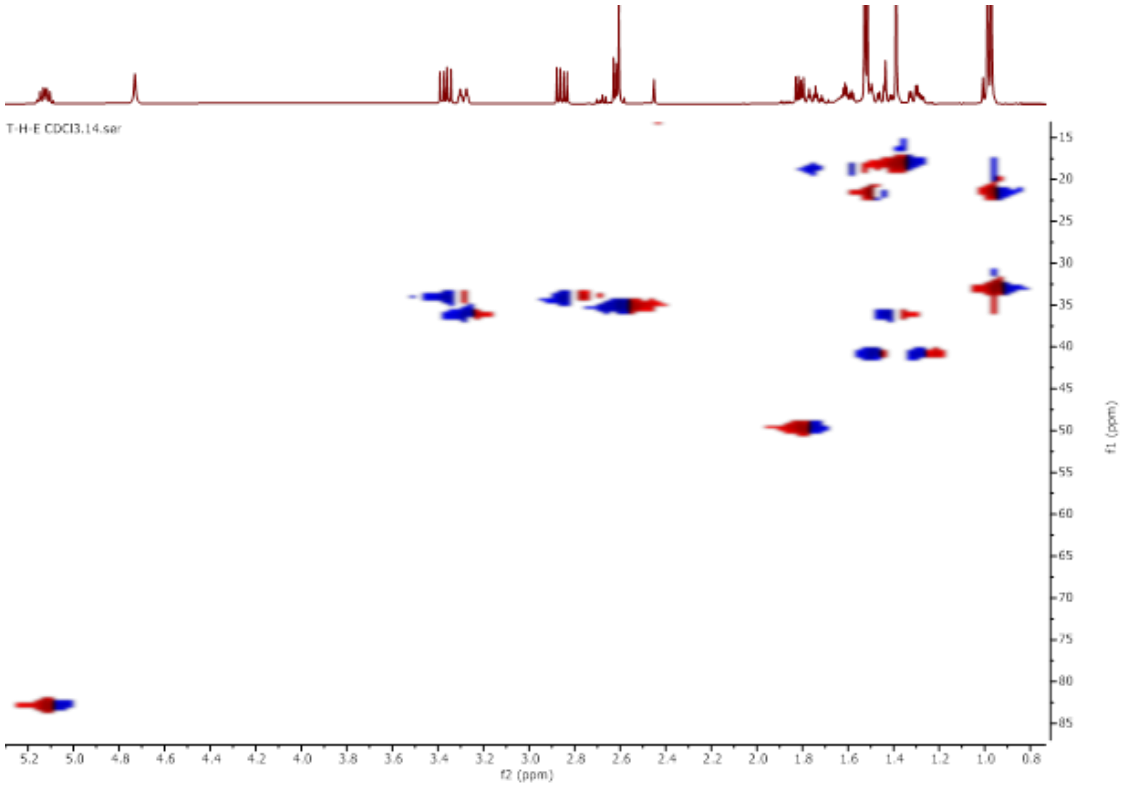

Fig. 4. HSQC spectrum of villosin A (**1**).

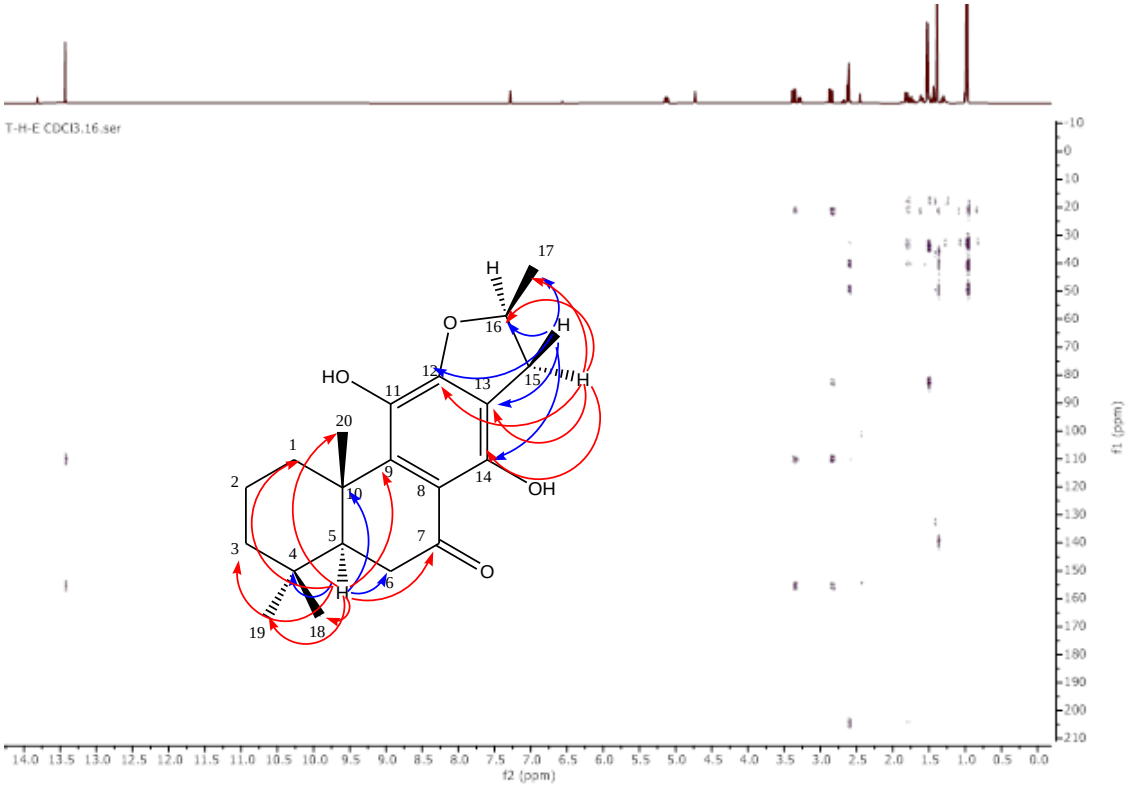

Fig. 5. HMBC spectrum of villosin A (**1**).

Finally, the antioxidant capacity of *n*-hexane extract and three rearranged abietane-like diterpenoids from *T. hircanicum* roots were evaluated by a DPPH scanning assay. Scanning activities for DPPH free radicals of *n*-hexane extract and pure compounds are shown in Table 3. The antioxidant activity was determined with the *in vitro* DPPH reagent and expressed as the required concentration of extracts or purified compounds for 50 % radical scanning (IC_{50}).

Table 3. DPPH radical scavenging capacity of crude *n*-hexane extract and purified compounds

Sample	IC_{50} µg/ml ± SD
n-Hexane extract	83.7 ± 0.1
Villosin A (**1**)	4.3 ± 0.1
Teuvincenone B (**2**)	4.0 ± 0.1
5,8,11,13,15-Abietapentaen-7-one (**3**)	3.5 ± 0.1
BHT	16.5 ± 0.2

4. Discussion

The compounds purified by the phytochemical study of *T. hircanicum* L. are reported for the first time from this plant. The rearranged abietane diterpenes were reported from the roots of genus *Teucrium* [14]. Also, in this study, three rearranged abietane-type diterpenoids were obtained from the roots of this plant. Previously, these compounds were purified and identified from the roots of other species of the genus *Teucrium* [26-28]. Villosin A was isolated for first time from the acetone extract of the *T. divaricatum* subsp. *villosum* roots [26]. Teuvincenone B was reported from the acetone extract of *T. polium* subsp. *vincentinum* roots [27]. And compound 5, 8, 11, 13, 15-abietapentaen-7-one was first time isolated and characterized from EtOAc extract of the *T. polium* roots [28]. The biosynthetic pathway for 4-hydroxyphenethyl pentacosanoate (**4**) and 22-dehydroclerosterol (**5**) are present in many plants, and for this reason, these compounds are popoular in many different plants.

When these antioxidant activities were compared with the reference value (IC_{50} of BHT = 16.5 µg/ml), the DPPH inhibitory activities of rearranged abietane type diterpenoids were high (IC_{50} = 3.5-4.3 µg/ml). The *n*-hexane extract's radical scanning activity (IC_{50} = 83.7 µg/ml) was very small relative to the positive control. These results show that rearranged abietane-type diterpenoids have high antioxidant strength and the ability to recover free radicals.

In this study, the results of the analysis of antioxidants are presented in the form of IC_{50} µg/ml (Table 3). These results show that the adsorption rate of free radicals of three rearranged abietane-type diterpenoids is much better than BHT. However, *n*-hexane extract has less antioxidant activity than BHT. The antioxidant activity of these three rearranging abietane-like diterpenoids is aligned with their structure. Compound 5, 8, 11, 13, 15-abietapentaen-7-one (**3**) has the most potent antioxidant activity in inhibiting free radicals. This compound has more conjugated double bonds than the other two abietane-type diterpenoids. In addition, teuvincenon B, in addition to having double binding than the compound vilosin A, has an additional hydroxyl group at the carbon 6 position. Numerous studies on the antioxidant activity of rearranged abietane diterpenes and rearranged abietane-like diterpenoids have shown that this class of compounds has significant free radical scanning activity [29-36].

5. Conclusion

Phytochemical investigations on genus *Teucrium* showed that some species include

various metabolites such as rearranged neo-clerodane or abietane diterpenes, sesquiterpenes, triterpenes, steroids, flavonoids and aromatic compounds. These metabolites are responsible for different pharmacological effects. This investigation assessed the phytochemical study and antioxidant activity on *n*-hexane extract of *T. hircanicum* roots from the Hyrcanian forests, North Iran. In total, five compounds were reported from the roots of this plant. The roots of *T. hircanicum were* found to be a rich source of rearranged abietane diterpenoids. The biological evolution of *n*-hexane extract and isolated compounds indicated the high antioxidant potential. The beneficial effects of antioxidant compounds are known for human health. Natural bio-antioxidants (biologically active compounds with antioxidant potential) and their synthetic analogs have various applications. They are used as essential drugs, antibiotics, agrochemical substitutes, and food preservatives. Today, many drugs are artificially modified natural substances [37].

The Phytochemical investigation of *T. hircanicum* often focuses on essential oils [38, 39]. However, our study on the *n*-hexane extract of *T. hircanicum* roots shows that rearranged abietane diterpenoids are responsible for their antioxidant activity in this extract. Ultimately, *n*-hexane extract of *T. hircanicum* roots can be introduced as a new antioxidant source.

Author contributions

GV: Experimental part, writing the manuscript, SNE: Supervision, experimental validation in phytochemical part, developing draft of the paper; MG: Analysing HPLC data, developing draft MMT: supervision extraction of plant material and creating the draft of MS HR: Assistance on recording NMR spectra and preparative HPLC. AS: Collection and identification plant materials.

Conflict of interest

The authors declare no conflict of interest.

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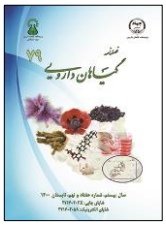

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مقاله تحقیقاتی

دی‌ترپنوئیدهای آبیتانی نوآرایی شده حاصل از ریشه گیاه مریم نخودی خزری

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اطلاعات مقاله

گل‌واژگان:
مریم نخودی خزری
پروفایل فیتوشیمیایی
دی‌ترپنوئیدها
فعالیت آنتی‌اکسیدانی
ویلوسین آ

چکیده

مقدمه: گیاهان دارویی به دلیل استفاده‌های سنتی از آنها در پیشگیری و درمان بیماری‌ها نقش بسزایی دارند. **هدف:** هدف از این مطالعه جداسازی، خالص سازی و شناسایی مواد شیمیایی از عصاره هگزان ریشه گیاه مریم نخودی خزری و ارزیابی فعالیت آنتی‌اکسیدانی این عصاره و اجزای خالص شده آن بود. **روش بررسی:** عصاره هگزانی (۲/۲۷ گرم) از ریشه های این گیاه استخراج شد. عصاره با کروماتوگرافی ستونی با فاز نرمال بوسیله شستشو با هگزان- اتیل استات و سپس با اتیل استات- متانول فراکسیونه گردید، فراکسیون‌ها با اثر انگشت فیتوشیمیایی مشابه با هم ترکیب شده و ۲۳ فراکسیون اصلی بدست آمد. خالص‌سازی نهایی به روش HPLC-UV تهیه‌ای فاز معکوس صورت پذیرفت. ساختارهای ترکیبات جدا شده با روش‌های مختلف طیف‌سنجی مانند رزونانس مغناطیسی هسته‌ای یک بعدی و دو بعدی و طیف سنجی جرمی و مقایسه این داده ها با مقادیر گزارش شده شناسایی شدند. فعالیت آنتی‌اکسیدانی عصاره هگزانی ریشه مریم نخودی خزری و دی‌ترپنوئیدهای خالص از آن با آزمون DPPH مورد ارزیابی قرار گرفت و فعالیت مهار رادیکال آزاد آن محاسبه شد. **نتایج:** روش‌های استخراج و جداسازی منجر به خالص‌سازی سه دی‌ترپنوئید از نوع آبیتان نوآرایی شده ویلوسین آ (**۱**)، تووینسنون بی (**۲**)، ۱۵،۱۳،۱۱،۸،۵- آبیتا پنتائن-۷- اُن (**۳**)، یک فنتیل استر به نام ۴- هیدروکسی فنتیل پنتاکوزانوات (**۴**) و یک استرول به نام ۲۲- دهیدروکلروسترول (**۵**) شد. **نتیجه‌گیری:** عصاره هگزانی ریشه مریم نخودی خزری و سه دی‌ترپنوئید آبیتانی نوآرایی شده آن فعالیت آنتی‌اکسیدانی بالایی در بازه ۳/۵ تا ۴/۳ میکروگرم در میلی‌لیتر در مقایسه با مقدار مرجع (۱۶/۵ میکروگرم در میلی‌لیتر برای BHT) از خود نشان دادند.

مخففها: DPPH، ۲،۲- دی فنیل پیکریل هیدرازیل؛ BHT، دی‌بوتیل هیدروکسی تولوئن ؛ CC، کروماتوگرافی ستونی؛ RSA، فعالیت روبش رادیکال؛ HMBC، طیف‌سنجی همبستگی چند پیوندی ناجور هسته‌ای؛ HSQC، طیف‌سنجی همبستگی تک کوانتومی هسته‌ای

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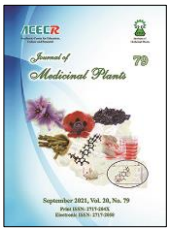

Research Article

Flavonoid, pterocarpans and steroid from *Erythrina fusca* Lour. growing in Bangladesh: isolation, and antimicrobial and free-radical scavenging activity

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BSL assay

ABSTRACT

Background: *Erythrina fusca* Lour. (fam. Fabaceae) is a flowering tree, found extensively in tropical and subtropical Asian countries, and is known for its use in traditional medicine for the treatment of various human ailments, for example, fever, liver complications, infections, and headaches. **Objective:** To carry out phytochemical study, and antimicrobial and free-radical scavenging activity evaluation of *E. fusca*. **Methods:** Ground stem bark of this plant was extracted by maceration with methanol, partitioned with various organic solvents, and compounds were isolated by chromatographic means. Structures of isolated compounds were confirmed by spectroscopic analyses. The antibacterial activity was assessed by the disc diffusion method, and the free-radical scavenging activity was determined by DPPH assay. **Results:** The carbon tetrachloride soluble fraction of the methanol extract of *E. fusca* afforded shinpterocarpin (**1**), lupinifolin (**2**), 3,9-dihydroxy-4-(3,3-dimethylallyl) [6a*R*,11a*R*]-pterocarpan (**3**) and β-sitosterol (**4**). Compounds **1**-**3** showed considerable antimicrobial activity against five Gram-positive and eight Gram–negative bacterial and three fungal strains tested in this study. Compound **1** exhibited the highest zone of inhibition of 19.4 mm against *Bacillus subtilis*. Additionally, compounds showed free-radical scavenging effects in DPPH assay with the IC_{50} values of 8.8, 7.7 and 7.9 µg/mL for compound **1**, **2** and **3**, respectively. However, they displayed some general toxicity in BSL assay. **Conclusion:** The isolation of bioactive compounds **1**-**3** supports some traditional medicinal uses of this plant. However, general toxicities found in the BSL assay might raise concerns regarding its safety, while offering a new avenue of future investigation on cytotoxicity of these compounds against human cancer cell lines.

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; NMR, Nuclear Magnetic Resonance; BSL, Brine Shrimp Lethality; PTLC, Preparative Thin Layer Chromatography; CC, Column Chromatography

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1. Introduction

Erythrina fusca Lour (synonyms: *E. atrosanguinea* Ridl., *E. glauca* Willd., *E. ovalifolia* Roxb.; common name: Pannya mandar, Harikakra, Kanta madar; English: Coral bean and purple coral bean) (family: Fabaceae) is a perennial, and medium to large flowering tree with spreading spines (*i.e.*, 1-2 cm long, 10-15 m tall) [1-3]. This medicinal plant grows on the coasts and along rivers in tropical Asia (India, Sri Lanka, Myanmar, Indonesia), Australia, the Mascarene Islands, Madagascar, Africa and southern America. It also grows abundantly in Bangladesh.

In Thai traditional medicine, leaves, root and bark of *E. fusca* are used for its antipyretic potentials [3]. An infusion of the bark is used in the treatment of liver ailments and to induce sleep [4]. Decoctioned extract of the *E. fusca* bark is used in treating intermittent fevers like malaria [5]. The scraped inner bark is applied as a poultice on fresh wounds to prevent infections, and the stem and root bark mixture is used to heal violent and tenacious headaches. Bark and/or root decoctions are used in the treatment of beriberi [1-5]. The root is antirheumatic, sudorific and, in large doses, purgative. The bruised leaves are used for cleansing putrid ulcers, they are applied locally to treat the toothache, and the flowers have antitussive property. Decoctioned extract is used to soothe coughs.

Previous bioactivity studies on *E. fusca* revealed its antibacterial and antimalarial property [2], anti-estrogenic and estrogenic activity [1] and its potential in the treatment of ischemic-reperfusion injury [6]. Antiherpetic activity of the aerial parts of this plant was also reported [7].

A preliminary phytochemical screening of the aerial parts of *E. fusca* identified the presence of alkaloids, flavonoids, triterpenes, steroids, saponins, lactones, coumarins, reducing sugars, carotenoids, amines and cardiac glycosides [7]. Erythratidine was the first alkaloid (and first isolated secondary metabolite) isolated from *E. fusca* [8]. Later, several other compounds including erythrasinate [9] and other alkaloids [10], pterocarpans [11-13] and prenylated flavonoids [12, 14] were isolated from this species. The structure of the alkaloid epierythratidine was reassigned later, and further alkaloids were reported [15].

As a part of continuous explorations of bioactive phytoconstituents from the available medicinal plants of Bangladesh [16, 17], this study was designed to explore the phytochemical components from the stem bark of *E. fusca*, growing in Bangladesh, and evaluate the antimicrobial and free-radical scavenging properties as well as brine shrimp toxicity of the isolated compounds.

2. Materials and Methods

2.1. Plant materials

The stem bark of *E. fusca* was collected from Nandail, Mymensingh District, Bangladesh in September 2011 and identified by the Bangladesh National Herbarium, and a voucher specimen has been deposited there with the accession No. DACB-35902. The stem bark was sun-dried and cut into small pieces followed by pulverization.

2.2. Extraction, fractionation and isolation

The powdered material of *E. fusca* (800 g) was soaked in 2.25 L of methanol in a clean air-tight flat-bottom flask for five days at room

temperature with intermittent shaking. The methanolic extract was filtered initially through a fresh cotton-bed followed by Whatman No. 1 filter paper and subsequently concentrated by utilizing a rotary evaporator at reduced pressure and warm temperature (< 45 ºC).

The crude methanolic extract (30 g) of *E. fusca* was subjected to fractionation through solvent-solvent partitioning process by using the modified Kupchan procedure [18] to obtain *n*-hexane (HxF, 8.25 g), carbon tetrachloride (CTF, 2.5 g), chloroform (CLF, 2 g) and aqueous (AqF, 13.1 g) soluble fractions. An aliquot of the CTF was subjected to further chromatographic separation with silica gel (70-230 mm, E-Merck, Germany)-packed column. The fraction was eluted initially with petroleum ether followed by petroleum ether/ethyl acetate and ethyl acetate/methanol gradients to obtain 250 sub-fractions (20 mL each). Afterwards, based on TLC profiles, the sub-fractions were bulked together to achieve concentrated products. Solvent evaporation from sub-fractions 90-97 afforded a yellowish mass, and further preparative TLC (mobile phase: toluene/ethyl acetate 9.7/0.3) yielded the compounds **1** (6.12 mg) and **2** (8.20 mg). The combined sub-fractions 117-127 were also purified through PTLC and gave compounds **3** (4.5 mg) and **4** (6.87 mg) using the mobile phases toluene/ethyl acetate 9/1 and 8.5 / 1.5, respectively. Both TLC and preparative TLC were run on pre-coated silica gel plates (60 mm, F_{254} aluminum sheets, E-Merck, Germany). The plates were examined under the UV cabinet (at 365 and 254 nm) and vanillin/sulfuric acid reagent (1:100) was sprayed for proper visualization [19].

2.3. NMR analyses and characterization of compounds

^{1}H-NMR spectra were recorded on a Bruker 500 MHz spectrometers, and the chemical shifts were reported with respect to the residual non-deuterated solvent signal. The structures of all compounds (**1**-**4**) were elucidated by the analysis of their ^{1}H NMR data and by direct comparison of these data with the respective published data.

2.4. Bioassays

The brine shrimp lethality [20, 21], antimicrobial [22] and free-radical scavenging activities [23] of the isolated compounds were determined by the established methods. Griseofulvin and Kanamycin were used as the positive controls for antifungal and antibacterial assays, respectively. Ascorbic acid was used as the positive control for DPPH free-radical scavenging assay during the measurement of antioxidant potential of the reported isolated compounds. On the other hand, vincristine sulphate was used as a reference drug for brine shrimp lethality bioassay.

3. Results

3.1. Extraction, isolation and characterization

Four compounds including two pterocarpans shinpterocarpin (**1**) and 3,9-dihydroxy-4-(3,3-dimethylallyl) [6a*R*,11a*R*]-pterocarpan (**3**), a flavonoid lupinifolin (**2**) and a plant sterol β-sitosterol (**4**) (Fig. 1) were isolated from the CTF soluble fraction of the methanolic extract of the stem bark of *E. fusca* using the column chromatography and preparative thin layer chromatography (PTLC) techniques. The structures of the isolated compounds were elucidated by ^{1}H-NMR spectral data analysis (shown below) and comparison with published data.

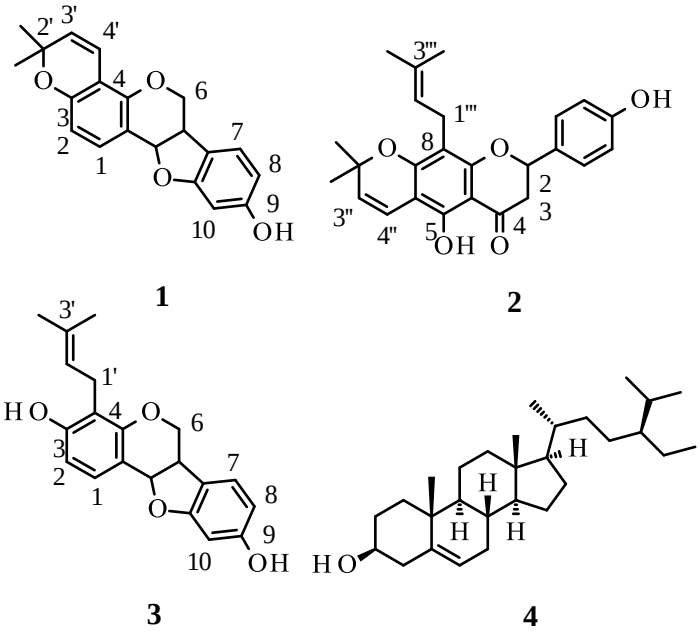

Fig. 1. Structures of the isolated compounds, shinpterocarpin (**1**), lupinifolin (**2**), 3,9-dihydroxy-4-(3,3-dimethylallyl) [6a*R*,11a*R*]-pterocarpan (**3**) and *β*-sitosterol (**4**)

Shinpterocarpin (**1**): Oily transparent liquid; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.43 (1H, d, *J* = 8.0 Hz, H-1), 6.97 (1H, d, *J* = 8.0 Hz, H-7), 6.57 (1H, dd, *J* = 8.0, 2.0 Hz, H-8), 6.44 (1H, d, *J* = 2.0 Hz, H-10), 6.52 (1H, d, *J* = 10.0 Hz, H-4'), 6.36 (1H, d, *J* = 8.0 Hz, H-2), 5.60 (1H, d, *J* = 10.0 Hz, H-3'), 5.52 (1H, d, *J* = 11.0, 2.5 Hz, H-11a), 4.81 (1H, br s, OH), 4.25 (1H, ddd, *J* = 11.0, 5.0, 2.5 Hz, H-6α), 3.63 (1H, dt, *J* = 2.5, 11.0 Hz, H-6β), 3.50 (1H, m, H-6a), 1.45 (3H, s, 6'-CH_3) and 1.41 (3H, s, 5'- CH_3) [24].

Lupinifolin (**2**): Yellow needles; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 12.25 (1H, s, OH-5), 7.33 (2H, d, *J* = 8.5 Hz, H-2', H-6'), 6.87 (2H, d, *J* = 8.5 Hz, H-3', H-5'), 6.63 (1H, d, *J* = 9.5 Hz, H-4"), 5.50 (1H, d, *J* = 9.5 Hz, H-3"), 5.34 (1H, dd, *J* = 12.5, 3.0 Hz, H-2), 5.15 (1H, t, *J* = 7.0 Hz, H-2'"), 3.22 (2H, d, *J* = 7.0 Hz, H-1'"), 3.04 (1H, dd, *J* = 17.1, 12.5 Hz, H-3α), 2.80 (1H, dd, *J* = 17.1, 3.0 Hz, H-3β), 1.66 (3H, s, CH_3-4'"), 1.57 (3H, s, CH_3-5'"), 1.46 (3H, s, CH_3-6"), 1.44 (3H, s, CH_3-5"), [12, 25, 26].

3,9-Dihydroxy-4-(3,3-dimethylallyl) [6a*R*, 11a*R*]-pterocarpan (**3**): Amorphous powder; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.42 (1H, d, *J* = 8.5 Hz, H-1), 6.97 (1H, d, *J* = 8.0 Hz, H-7), 6.57 (1H, m, *J* = 8.0, 2.0 Hz, H-8), 6.43 (1H, d, *J* = 2.0 Hz, H-10), 6.39 (1H, d, *J* = 8.5 Hz, H-2), 5.47 (1H, t like, *J* = 11.5 Hz, H-11a), 5.29 (1H, s, OH-3/9), 5.27 (1H, d, *J* = 8.0 Hz, H-2'), 4.80 (1H, s, OH-9/3), 4.25 (1H, dt, *J* = 5.0, 11.5 Hz, H-6α), 3.65 (1H, dt, *J* = 5.0, 11.5 Hz, H-6β), 3.53 (1H, m, H-6a), 3.36 (2H, m, H-1'), 1.81 (3H, s, CH_3-4'), 1.75 (3H, s, CH_3-5' [27].

3.2. Brine Shrimp Lethality assay

In the Brine Shrimp Lethality assay, Compound **2** showed LC_{50} and LC_{90} values of 3.17 and 62.95 µg/mL, respectively. Compounds **1** and **3** exhibited LC_{50} values of 4.70 and 4.81 µg/mL, respectively, and LC_{90} values of 129.12 and 86.90 µg/mL, respectively. The positive control, vincristine sulphate, had the LC_{50} and LC_{90} values of 0.45 and 10.00 µg/mL, respectively.

3.3. Antimicrobial assay

Compounds **1-3**, at a concentration of 50 µg/disc, when subjected to antibacterial screening on traditional agar disc diffusion method, showed antimicrobial potentials with the zones of inhibition ranging from 14.4 to 19.4 mm against several Gram-positive and Gram-negative bacterial strains (Table 1). The largest zone of inhibition (19.4 mm) was observed for compound **1** against the Gram-positive bacterial strain of *Bacillus subtilis*. All tested compounds gave the zones of inhibition ranging from 11.9 to 15.1 mm in the case of antifungal test (Table 1), indicating comparable antifungal efficacy in contrast to the standard antifungal drug, griseofulvin (18-20 mm).

3.4. DPPH assay

All three compounds (**1-3**) exhibited moderate to high free-radical scavenging activity in the DPPH assay [23]. The IC_{50} (inhibitory concentration 50 %) values of compounds **1-3** were found as 8.8, 7.7 and 7.9 µg/mL, whereas the positive controls, *t*-butyl-1-hydroxytoluene and ascorbic acid, gave the IC_{50} values of 24.35 and 5.80 µg/mL, respectively. The assay showed that compounds **1-3** could be similarly effective in scavenging free-radicals as the positive control ascorbic acid.

Table 1. Antimicrobial activity of compounds **1**, **2** and **3** isolated from *E. fusca.*

Tested bacteria	Diameter of zone of inhibition (mm)			
	Compound 1 (50 µg/disc)	**Compound 2 (50 µg/disc)**	**Compound 3 (50 µg/disc)**	**Kanamycin (30 µg/disc)**
Gram-positive bacteria				
Bacillus cereus	17.8 ± 0.66	16.9 ± 1.10	19.0 ± 0.31	35
Bacillus megaterium	18.5 ± 0.87	19.1 ± 0.60	15.6 ± 0.57	35
Bacillus subtilis	19.4 ± 0.31	18.8 ± 0.35	14.9 ± 0.17	36
Staphylococcus aureus	17.6 ± 0.27	18.1 ± 0.40	16.0 ± 0.42	32
Sarcina lutea	16.9 ± 0.68	16.7 ± 0.60	17.6 ± 0.42	27
Gram-negative bacteria				
Escherichia coli	17.9 ± 0.21	17.8 ± 0.27	17.4 ± 0.42	25
Pseudomonas aeruginosa	14.8 ± 0.82	14.8 ± 0.50	16.6 ± 0.21	20
Salmonella typhi	18.3 ± 0.85	18.8 ± 0.42	17.3 ± 0.60	22
Salmonella paratyphi	17.5 ± 0.32	17.8 ± 0.78	15.2 ± 0.51	27
Shigella dysenteriae	16.8 ± 0.36	17.3 ± 0.59	15.9 ± 0.59	25
Shigella boydii	16.9 ± 0.55	16.8 ± 0.42	17.7 ± 0.66	27
Vibrio parahemolyticus	18.1 ± 0.55	16.9 ± 0.15	17.9 ± 0.15	20
Vibrio mimicus	17.2 ± 0.31	17.3 ± 0.40	16.5 ± 0.32	25

Tested fungi	Diameter of zone of inhibition (mm)			
	Compound 1 (50 µg/disc)	**Compound 2 (50 µg/disc)**	**Compound 3 (50 µg/disc)**	**Griseofulvin (20 µg/disc)**
Aspergillus niger	13.6 ± 0.31	11.9 ± 0.32	13.2 ± 0.31	20
Candida albicans	14.2 ± 0.61	13.6 ± 1.14	15.1 ± 0.35	18
S. cerevisiae	12.6 ± 1.10	14.2 ± 0.60	14.2 ± 0.60	19

4. Discussion

CC and PTLC aided chromatographic separation of the CTF soluble fraction of the methanolic extract of the stem bark of *E. fusca* afforded the isolation of four compounds (**1**-**4**), and the structures of those compounds (except **4**) were deduced primarily based on 1H NMR data analyses, and also comparison with respective published data. The identity of compound 4 was confirmed as b-sitosterol by co-TLC method. The ^{1}H-NMR spectrum of compound **1** showed four proton signals at 5.52 (1H, dd, *J* = 11.0, 2.5 Hz) for H-11a, 4.25 (1H, ddd, *J* = 11.0, 5.0, 2.5 Hz) for H-6α), 3.63 (1H, dt, *J* = 2.5, 11.0 Hz) due to H-6β and 3.50 (1H, m) for H-6 which are indicative for a pterocarpan skeleton [24]. In the ring A, the presence of two doublets of aromatic protons at δ 7.43 (1H, d, *J* = 8.0 Hz) for H-1 and δ 6.36 (1H, d, *J* = 8.0 Hz) for H-2 indicated that C-3 and C-4 of the ring were substituted. In the D-ring, the doublets at δ 6.97 (1H, d, *J* = 8.0 Hz) for H-7 and 6.44 (1H, d, *J* = 2.0 Hz) for H-10 and a double doublet at 6.57 (1H, dd, *J* = 8.0, 2.0 Hz) for H-8 demonstrated that the C-9 was substituted with a hydroxyl (-OH) group. The ^{1}H-NMR spectrum further showed two doublets (*J* = 10.0 Hz) at δ 6.52 (1H) and 5.60 (1H) and two singlets of three proton intensity at δ 1.41 and 1.45 attributable, respectively, to the *cis*-double bond protons (H-4’ and H-3’) and *gem*-dimethyl groups at C-2’ demonstrated the presence of a 2,2-dimethylchromene moiety. These ^{1}H-NMR data of compound **1** were found to be identical with the published values for shinpterocarpin [24]. Thus, compound **1** was identified as shinpterocarpin (**1**), which has never been reported from *E. fusca* before.

The ^{1}H-NMR spectrum of compound **2** revealed a sharp singlet at δ 12.25 (1H, s, OH-5) typical for the chelated hydroxyl group at C-5 in a flavonoid skeleton. The analytical features for a flavanone nucleus were evident from the proton signals at δ 3.04 (1H, dd, *J* = 17.1, 12.5 Hz), 2.80 (1H, dd, *J* = 17.1, 3.0 Hz) and 5.34 (1H, dd, *J* = 12.5, 3.0 Hz), which could be assigned to H-3α, H-3β and H-2, respectively of the flavanone nucleus for compound **2**. The doublet at δ 5.50 (1H, d, *J* = 9.5 Hz, H-3") and 6.63 (1H, d, *J* = 9.5 Hz, H-4") and two singlets at δ 1.44 (3H) and 1.46 (3H) were characteristic for the *cis*-double bond protons and *gem*-dimethyl groups of a 2,2-dimethyl-chromene moiety, respectively [27]. Two *ortho*-coupled doublets (*J* = 8.5 Hz) centered at δ 7.33 (2H) and 6.87 (2H) were assigned to the protons at C-2' & C-6' and C-3' & C-5' of the *para*-disubstituted benzene ring (C ring). The singlets at δ 1.66 (3H, s, CH_3-4'") and 1.57 (3H, s, CH_3-5'"), a doublet at 3.22 (1H, d, *J* = 7.0 Hz, H-1'") and a triplet at 5.15 (1H, t, *J* = 7.0 Hz, H-2'") inferred the presence of an isoprenyl group. These ^{1}H spectral features of compound **2** were comparable with the NMR data of lupinifolin [25, 26] recorded in C_6D_6. Thus, compound **2** was identified lupinifolin (**2**), which has previously been isolated from the bark of *E. fusca* [12].

The ^{1}H-NMR spectrum of compound **3** displayed a set of proton signals at δ 3.65 (1H, dt, *J* = 5.0, 11.5 Hz) and 4.25 (1H, dt, *J* = 5.0, 11.5 Hz) assignable to H-6β and H-6α, respectively another two proton resonances at δ 3.51 (m) due to H-6a and 5.47 (d, *J* = 6.5 Hz) for H-11a. These spectral data suggested the existence of the -O-CH_2-CH-CH-O- moiety connecting the rings B and C of the pterocarpan central skeleton.

Additionally, the ^{1}H-NMR displayed a pair of *ortho*-coupled doublets (J = 8.5 Hz) with one proton intensity at δ 7.42 for H-1 and 6.39 attributable to H-1 and H-2, respectively in ring-A, which demonstrated that C-3 and C-4 of the ring were substituted. The presence of three aromatic protons at δ 6.97 (1H, d, J = 8.0 Hz, H-7), 6.57 (dd, J = 8.0, 2.0 Hz, H-8) and 6.43 (1H, d, J = 2.0 Hz, H-10) indicated an ABX spin system in ring D. Two broad signals were observed at δ 5.29 and 4.80, which could be assigned to the hydroxyl group protons. By comparing the ^{1}H-NMR data of compound **3** with the published values [27], the structure of compound **3** was deduced as 3,9-dihydroxy-4-(3,3-dimethylallyl)[6a*R*,11a*R*]-pterocarpan. To the best of our knowledge, this pterocarpan has not previously isolated from *E. fusca*. It can be note that this compound is biosynthetically related to compound **1**, as the isoprene unit on compound **3** is biosynthetically cyclized to form the pyran ring in compound **1**.

The presence of β-sitosterol (**4**), which is ubiquitously present in many plant species and one of the most common plant sterols, was established in *E. fusca* by running a co-TLC with the known-reference sample.

General toxicity of the compounds was determined by the brine shrimp lethality (BSL) assay [20, 21, 28] and the LC_{50} (lethal concentration 50 %) and LC_{90} (lethal concentration 90 %) values were acquired from the best-fit slope by plotting the concentration (µg/mL) on x-axis and number of brine shrimp nauplii on y-axis for all tested compounds. Among the compounds, compound **2** was found to be the most toxic one (LC_{50} and LC_{90} values of 3.17 and 62.95 µg/mL, respectively). Compounds **1** and **3** were also toxic to brine shrimps to a lesser extent. However, none of the compounds were as toxic as the positive control, vincristine sulphate, which had the LC_{50} and LC_{90} values of 0.45 and 10.00 µg/mL, respectively. Vincristine is a well-known anticancer drug with established cytotoxicity and thus, its toxicity towards brine shrimps is expected to be high as well.

Although the preliminary antimicrobial activity of the crude extract of *E. fusca* was reported earlier [2], there is no previously published report on the isolation of antimicrobial compounds from this plant. Thus, the current finding, revealing considerable antimicrobial activity of compounds **1-3**, provides additional support and clarity to the previously published report, and establishes the compounds responsible, at least partially, for the antimicrobial property of the crude extract of *E. fusca*. It is noteworthy that all tested compounds exhibited significant antifungal property (the zones of inhibition ranging from 11.9 to 15.1 mm) which was comparable to antifungal efficacy in contrast to the standard antifungal drug, griseofulvin (18 - 20 mm).

The DPPH assay provides information on the ability of any test materials for scavenging free-radicals, and thus, reveals the potentials of those test materials as antioxidants. All three compounds (**1-3**) showed some degree of free-radical scavenging activity (IC_{50} = 8.8, 7.7 and 7.9 µg/mL, respectively) in the DPPH assay [23]. However, the IC_{50} value of the positive controls, *t*-butyl-1-hydroxytoluene and ascorbic acid, were 24.35 and 5.80 µg/mL, respectively, which indicated that all three compounds were better than *t*-butyl-1-hydroxytoluene in terms of DPPH-scavenging ability, but were slightly less or similarly potent that ascorbic acid.

5. Conclusion

Among the isolated compounds, pterocarpans **1** and **3** have never been previously reported from *E. fusca*, or from the genus *Erythrina*. The isolation of bioactive compounds **1-3** potentially provides some scientific evidence in support of traditional medicinal uses of this plant. However, general toxicities found in the BSL assay might raise concerns regarding its safety, while offering a new avenue of future investigation on cytotoxicity of these compounds against human cancer cell lines.

Author contributions

AA, MZS, SF and MKI generated data and compiled the first draft. MAR, LN and SDS provided the concept, prepared and edited the final manuscript, and are acting as corresponding authors.

Conflict of interest

The authors declare that there is no conflict of interest.

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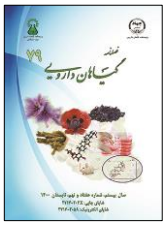

مقاله تحقیقاتی

ترکیبات فلاونوئیدی، پتروکارپانی و استروئیدی گونه‌ای گیاه فردوسی (*Erythrina fusca* Lour.) در حال رشد در بنگلادش: جداسازی و فعالیت ضدمیکروبی و روبش رادیکال‌های آزاد

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اطلاعات مقاله

گل‌واژگان:
گیاه فردوسی
فلاونوئیدها
پتروکارپان‌ها
استرول
فعالیت ضدمیکروبی
سنجش DPPH
سنجش BSL

چکیده

مقدمه: گیاه *Erythrina fusca* گونه‌ای فردوسی است که به‌طور گسترده در کشورهای گرمسیری و نیمه گرمسیری آسیا یافت می‌شود و به دلیل استفاده از آن در طب سنتی برای درمان بیماری‌های مختلف انسان، به عنوان مثال تب، اختلالات کبدی، عفونت‌ها و سردردها مشهور است. **هدف**: هدف از این تحقیق، بررسی فیتوشیمیایی و ارزیابی فعالیت ضدمیکروبی و روبش رادیکال‌های آزاد گیاه *E. fusca* بود. **روش بررسی:** پوست ساقه آسیاب شده این گیاه با استفاده از متانول و روش خیساندن عصاره‌گیری شده و با حلال‌های مختلف آلی تقسیم بندی شده و ترکیبات به روش کروماتوگرافی جدا شدند. ساختار ترکیبات جدا شده با آنالیزهای طیف‌سنجی تأیید شد. فعالیت ضدباکتریایی با روش انتشار دیسک مورد بررسی قرار گرفت و فعالیت روبش رادیکال‌های آزاد با روش DPPH تعیین شد. **نتایج:** فراکسیون تتراکلرید کربنی عصاره متانولی گیاه دارای شینترپوکارپین (۱)، لوپینفولین (۲)، ۹،۳- دی هیدروکسی-۴-(۳،۳- دی متیل آلیل) [Ra۱۱،Ra۶]-پتروکارپان (۳) و بتا- سیتوسترول (۴) بود. ترکیبات ۱ تا۳ فعالیت ضدمیکروبی قابل توجهی در برابر پنج باکتری گرم مثبت و هشت باکتری گرم منفی و سه سویه قارچی آزمایش شده در این مطالعه نشان دادند. ترکیب ۱ بالاترین هاله مهار رشد (۱۹/۴ میلی‌متر) را در برابر باسیلوس سوبتیلیس نشان داد. علاوه بر این، ترکیبات اثرات مهار رادیکال‌های آزاد را در روش DPPH با مقادیر IC_{50} برابر با ۸/۸، ۷/۷ و ۷/۹ میکروگرم در میلی‌لیتر به ترتیب برای ترکیبات ۱، ۲ و ۳ نشان دادند. با این حال، آنها در تست سنجش عمومی سمیت BSL تا حدودی سمیت نشان دادند. **نتیجه‌گیری:** جداسازی ترکیبات فعال زیستی ۱ تا ۳ از برخی کاربردهای دارویی سنتی این گیاه پشتیبانی می‌کند. با این حال، سمیت عمومی یافت شده در روش BSL ممکن است نگرانی‌هایی را در مورد ایمنی آن ایجاد کند، در حالی که این تحقیق راه جدیدی از تحقیقات آینده در مورد سمیت سلولی این ترکیبات در برابر رده‌های سلولی سرطانی انسان ارائه می‌دهد.

مخففها: DPPH، ۱،۱- دی فنیل-۲- پیکریل هیدرازیل؛ NMR، رزونانس مغناطیسی هسته‌ای؛ BSL، کشندگی میگوی آب‌شور؛ PTLC، کروماتوگرافی لایه نازک جداکننده، CC، کروماتوگرافی ستونی

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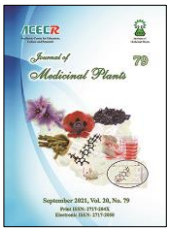

Research Article

Changes in essential oil composition of peppermint (*Mentha x piperita* L.) affected by yeast extract and salicylic acid foliar application

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Medicinal Plant
Menthone
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ABSTRACT

Background: Peppermint (*Mentha x piperita* L.) is one of the most important medicinal plants which used in food, pharmaceutical, perfumery, and flavoring industry. **Objective:** This study was planned to investigate the effects of foliar application of salicylic acid and yeast extract on production of valuable essential oil components in peppermint. **Methods:** A completely randomized design experiment with nine treatments consisting salicylic acid (40, 80, 160 and 320 mg/l), yeast extract (0.25, 0.75, 1 and 1.5 g/l) and distilled water (control) with three replications was carried out under greenhouse conditions. **Results:** In total, forty compounds were identified in the essential oils of the plant aerial parts. Menthone, menthol, piperitone, isopulegol and *γ*-terpinene were the major compounds of the oils studied. Menthone and menthol were 16.69 % and 14.39 % of the essential oils, respectively. Salicylic acid and yeast extract were increased menthone, neomenthol, piperitone, *γ*-terpinene and isomenthol acetate production 42, 60, 39, 59 and 34 % higher than control plants, respectively. Foliar application with 320 mg/l salicylic acid gave the best result in the enhancement of the major essential oil components of treated plants. The results of correlation between essential oil constituents showed that the neomenthol content had a significant positive correlation with menthone ($r = 0.865^{**}$), *γ*-terpinene ($r = 0.848^{**}$) and negative correlation with isopulegol ($r = -0.886^{**}$). **Conclusion:** The quality of essential oil of *M. piperita* were influenced by the foliar application of salicylic acid and yeast extract at the appropriate concentrations. Elicitation by 320 mg/l salicylic acid was the optimum treatment for menthone, neomenthol, *γ*-terpinene and piperitone production.

1. Introduction

Secondary metabolites have complex structures to be manufactured by chemical synthesis and thus frequently extracted from naturally grown or cultivated plants [1]. Essential oils are natural complex volatile secondary metabolites that are often obtained from various aromatic plants using the hydro-distillation technique [2]. Peppermint (*Mentha x piperita* L.), a perennial herbaceous medicinal plant of Lamiaceae family is a natural hybrid from *M. aquatica* × *M. spicata*. The plant is cultivated

Abbreviations: GC-MS, Gas chromatography–mass spectrometry; SE, Standard error; C.V., Coefficient of variation

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in a temperate region of Europe, Asia, United States, India and Mediterranean countries due to their commercial value and distinct aroma [3]. It is an important medicinal and aromatic herb worldwide, in addition to its potential uses as a flavoring agent, in cosmetics, and pharmaceutical products among others [4, 5]. Leaves of *M. piperita* contain 1.2-3.9 % (v/w) of essential oils and more than 300 recognized components. The terpenes with about 52 % of monoterpenes and 9 % of sesquiterpenes are the most important components of peppermint leaves [6]. The composition of the essential oil isolated from aerial parts of *M. piperita* has been a subject of extensive studies [3, 7-11]. Peppermint essential oil containing high concentrations of menthol and menthone are used in traditional medicine to treat various conditions including infections and also as insect repellant. Various *in vitro* and *in vivo* studies have documented the biological properties of menthol such as its analgesic, antibacterial, antifungal, anaesthetic and penetration-enhancing effects as well as chemopreventive and immunomodulating actions [5, 12]. Menthol is one of the most important flavouring additives besides vanilla and citrus. The demand for menthol is high and it was previously estimated that the worldwide use of menthol was 30 - 32,000 metric tonnes per annum [12]. Most of the investigations have shown that the major constituents of essential oils in *M. piperita* were menthol and menthone [7, 9, 10]. The quality of medicinal plants used for the production of pharmacologically useful compounds is usually assessed by the content of biologically active compounds [13]. Several methods such as using of biotic or abiotic elicitor can be a suitable way to increase the production of valuable secondary metabolites in medicinal plants [14], which is currently being implemented extensively owing to its low cost and simplicity of usage [15]. Elicitation is the process of inducing or enhancing synthesis of secondary metabolites by the plants to ensure their survival, persistence and competitiveness [16]. Salicylic acid is a hormone-like substance that plays an important role in the regulation of plant growth and development [7, 17]. In several studies, the effect of salicylic acid on production of many bioactive compounds in medicinal plants was confirmed [13, 18-20]. Yeast extract is one of the biotic elicitors that can result in the improvement of secondary metabolites content. The stimulating influence of yeast extract on secondary metabolites was confirmed in several studies [20-22]. Therefore, in order to economically produce secondary metabolites, it is necessary to use elicitors optimally in medicinal plants. Because of the industrial use of *M. piperita,* it is important to develop an optimal method to obtain standardized plant material with specific quality parameters. Thus, the choice of proper concentration of salicylic acid or yeast extract foliar application can be a suitable strategy to increase the production of the main constituents of *M. piperita* essential oil. Due to the importance of these medicinal compounds, peppermint was therefore studied and explained and the correlation of these compounds was determined.

2. Materials and Methods

2.1. Experimental field

The experiment was conducted under greenhouse conditions from March to July 2017 at the greenhouse of the Malayer Greenhouse Town, Malayer Municipality (latitude: 34° 19´ N, longitude: 48° 51´ E, altitude: 1725 m above sea level), located in the west of Iran and southeast of Hamadan province.

2.2. Plant materials, experimental set up and treatments

In this study, the plant rhizome (Code number: 13723) was obtained from Pakanbazr Company (Isfahan, Iran) (http://www.pakanbazr.com/). For cultivation, 54 clay pots with a height of 25 cm in diameter (radius) of 20 cm were used. All pots were containing a 1:1:1 uniform mixture of field soil, rotten leaf soil and sand. Three rhizomes of peppermint with 3 to 5 cm long were planted in each clay pot in depth of 3 to 5 cm and a thin layer of rotten manure was poured on them and irrigation was carried out immediately. Plants were grown in a naturally-lit greenhouse where relative humidity ranged of 50-55 % and average temperature was 25 ± 3°C during the experimental period. During the experimental period, plants were irrigated two to three times a week as required.

2.3. Experimental design and treatments

In this study, a completely randomized design experiment with nine treatments and three replications (two pots in each replication) was carried out under greenhouse conditions to investigate the effect of four salicylic acid doses (40, 80, 160 and 320 mg/l), four yeast extract doses (0.25, 0.75, 1 and 1.5 g/l) and distilled water (control) on major components of peppermint essential oil. For this purpose, yeast extract was dissolved in distilled water. Stock solution of salicylic acid was made by dissolving weighed quantity in minimum quantity of ethanol and final volume made by distilled water. Foliar application of the elicitors on *M. piperita* aerial parts was performed at 40 % flowering stage. In this experiment, distilled water foliar application was used as control. For each treatment, six pots having 3 plants per pot was used. Foliar sprays of the elicitors were done with a portable sprayer, early in the morning and one hour after sunrise. Spraying was done completely on aerial parts of the plants [19]. To evaluate the essential oil composition of *M. piperita*, the sampling was performed 5 days after the foliar application of the elicitors. The samples were dried in the shadow with proper ventilation and normal room temperature (25-30 °C) for 5 days. Each sample was placed in a plastic bag separately and their lids were closed. The characteristics such as amount of essential oil components were measured using gas chromatography-mass spectrometry (GC-MS).

2.4. Essential oil isolation and analysis procedure

Essential oil of each sample was isolated from chopped, dried aerial parts of *M. piperita* by hydro-distillation procedure. Briefly, 50 g of *M. piperita* was transferred into a 1 L round-bottom flask with Clevenger apparatus. Water distillation was performed for 3 h at 100 °C. The collected essential oil was dried over anhydrous Na_2SO_4 and stored in a dark bottle at 4 °C until tested and analyzed. The essential oil components of *M. piperita* affected by salicylic acid and yeast extract were determined by gas chromatography-mass spectrometry (GC-MS) using a Hewlett -Packard 5890 gas chromatograph equipped with a flame ionization detector (HP-5970 mass-selective detector-USA) and a 50 m × 0.20 mm HP-5 (cross-linked Phenyl–Methyl Silicon) column with a 0.25 µm film thickness. The ionization energy of the sample components was set to 70 eV. The flame ionization detector (FID) was maintained at 250 ºC. The temperature program was ranged over 100-250 °C at a rate of 4 °C/min. The carrier gas was helium while the flow through the column and the split ratio were set to 1 ml/min and 100:1, respectively. The individual constituents were identified by their identical

retention index and compared to those in reference books and articles using mass spectra of standard compounds and information contained in a computer library [23].

2.5. Statistical analysis

Data was statistically analyzed by one-way analysis of variance (ANOVA) using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). The mean values were compared using Duncan's multiple range test at $P < 0.01$ significant level. The values are presented as mean ± standard error (SE) of three replications. Correlation coefficient (r) between essential oil components was estimated by Pearson method.

3. Results

Essential oil of *M. piperita* aerial parts, were analyzed and quantified by gas chromatography-mass spectrometry (GC-MS) (Fig. 1). The effects of salicylic acid and yeast extract foliar application on essential oil components of the plant aerial parts were shown in Tables 1 & 2. Results of GC-MS indicated that a total of 40 compounds were identified in the essential oil from the aerial parts of peppermint under salicylic acid and yeast extract treatments. The chemical components are given in Table 2. The results showed that the major components of peppermint essential oil were menthone, menthol, piperitone, isopulegol and *γ*-terpinene, respectively (Totally 44.8 % to 54.3 %). In this study, menthone and menthol were 16.69 % and 14.39 % of the essential oils, respectively. The results of analysis of variance (ANOVA) showed that the effect of different concentrations of salicylic acid and different concentrations of yeast extract on production of 12 major compounds including menthone, neomenthol, isomenthol acetate, piperitone, isopulegol, *γ*-terpinene, *β*-pinene, myrcene, eucalyptol, dihydrocarvone, paramentol, hexyl isovalerate, caryophyllene oxide was highly significant at 1 % probability level ($P < 0.01$), two constituents including *β*-ocimene and dihydro carvyl acetate was significant at 5 % probability level ($P < 0.05$) (Table 1) and on 26 compounds (camphene, sabinene, *β*-pinene, (-)-*β*-pinene, octenol, *α*-phellandrene, *α*-terpinene, isoterpinolene, *p*-cymene, limonene, 1,8-cineole, linalool, *β*-terpineol, isomenthone, menthofuran, lavandulol, l-(+)-menthol, myrtenal, *p*-mentenol, carvone, borneol, dihydro carvyl actate, *β*-bourbonene, spathulenol, *β*-elmene, humulene epoxide) was not significant at 5 % probability level ($P > 0.05$) in *M. piperita* (Data not shown). As shown, salicylic acid and yeast extract significantly altered the amount of 14 constituents of essential oil of *M. piperita* (Table 1). Mean comparison for effects of salicylic acid and yeast extract on essential oil components (%) in *M. piperita* is shown in Table 2. The results of mean comparison showed that the amount of menthone varied from 12.13% to 17.20 %. The highest menthone production 17.20 % was obtained at 320 mg/l salicylic acid treated plants which was 42 % higher compared to control. This was followed by 160 mg/l salicylic acid (15.97 %) treatment. The lowest level of menthone production was observed in control (12.13 %). Also, the results showed that with increasing the amount of yeast extract from 0.75 to 1.5 g/l, the amount of menthone increased. It is observed that with increasing the amount of salicylic acid from 160 to 320 mg/l, the amount of menthone has increased from 15.97 to 17.20 % (Table 2). The highest neomenthol content (4.9 %) was approximately 60 % greater than control level, and was obtained in the plants treated with 320 mg/l salicylic acid. This was followed by 160 mg/l salicylic acid (4.6 %). The lowest level of neomenthol content (3.08 %) was observed in

control plants. The results showed that with increasing the concentration of salicylic acid from 40 to 320 mg/l, the amount of neomenthol increased (3.7, 4.2, 4.6 and 4.9 %, respectively). In other words, with increasing salicylic acid concentration, the amount of neomenthol has increased. The results of mean comparison showed that with increasing the amount of yeast extract from 0.25 to 1.5 g/l, the amount of neomenthol increased from 3.20 to 3.93 %. The results of mean comparison showed that the amount of piperitone varied from 6.40 to 8.90 %. The highest piperitone production (8.90 %) was obtained at 320 mg/l salicylic acid treated plants that was 39 % higher compared to control (6.40 %). This was followed by 160 mg/l salicylic acid (8.30 %). The effect of different concentrations of yeast extract on the amount of piperitone was not significant compared to the control. The results showed that with elicitation by 320 mg/l salicylic acid the accumulation of isopulegol was 27 % fewer than the control plants. The lowest amount of isopulegol production (5.1 %) was obtained at 320 mg/l salicylic acid treated plants and then the treatment of salicylic acid with a concentration of 160 mg/l was 5.4 % of essential oil. It is observed that with increasing salicylic acid concentration, the amount of isopulegol has a decreasing trend. Four levels of yeast extract did not have a significant effect on the production of isopulegol in peppermint in greenhouse conditions, but treatments of 80, 160 and 320 g/l salicylic acid reduced the amount of isopulegol. As shown, the highest γ-terpinene content (6 % of essential oil) was obtained at 320 mg/l salicylic acid treated plants which was 59 % higher compared to control. This was followed by 160 mg/l and 80 mg/l salicylic acid (5.63 and 5.27 %, respectively). These amounts, considering the amount of γ-terpinene in the control plants (3.77 %), could be considered as significant. It is observed that external application of salicylic acid has a positive effect on the production of γ-terpinene peppermint essential oil compared to yeast extract. Low concentrations of yeast extract are not significantly different from the control. It is observed that only 1.5 g/l of yeast extract has significantly different from control. The results of mean comparison for effects of salicylic acid and yeast extract on essential oil components (%) in *M. piperita* showed that the highest isomenthol acetate production 7.30 % was obtained at 40 mg/l salicylic acid treated plants that was 34 % higher compared to control (5.43 %). The results showed that with increasing the concentration of yeast extract, the percentage of isomenthol acetate increased and in contrast, with increasing the concentration of salicylic acid from 40 to 320 mg/l, it was observed that the amount of this essential oil compound decreased. Isomenthol acetate content increased significantly only with 40 mg/l salicylic acid application ($P < 0.01$).

Pearson correlation coefficient was performed by SPSS software to calculate the relationship between the major constituents of peppermint essential oil (Table 3). In the present study, it was found that salicylic acid and yeast extract, while affecting the amount of some of the major constituents of peppermint essential oil, led to a significant reduction in a number of essential oil constituents. The most significant negative correlation coefficients between isopulegol composition and other major essential oil constituents were neomenthol ($r = -0.886^{**}$), γ-terpinene ($r = -0.879^{**}$), menthone ($r = -0.813^{**}$) and piperitone ($r = -0.712^{**}$). In other words, as the amount of major components of the essential oil increased, the amount of isopulegol had an inverse correlation and decreased. The results showed that the menthone content had a

significant positive correlation with *γ*-terpinene (r = 0.881**, at P < 0.01 significant level) and had a significant negative correlation with isopulegol, *β*-pinene, myrcene, eucalyptol and *β*-ocimene. The results of correlation between essential oil constituents showed that the neomenthol content had a significant positive correlation with menthone (r = 0.865**, at P < 0.01 significant level), *γ*-terpinene (r = 0.848**) and had a significant negative correlation with the compounds isopulegol, menthofuran, eucalyptol and *β*-ocimene; So that it had a negative correlation with isopulegol (r = -0.886**). The results showed that the *γ*-terpinene content had a significant positive correlation with menthone (r = 0.881**, at P < 0.01 significant level), neomenthol (r = 0.848**), piperitone (r = 0.713**) and hexyl isovalerate (r = 0.613**) and had a significant negative correlation with isopulegol (r = -0.879**). In this study, the major components of peppermint essential oil often had a positive and significant relationship with each other.

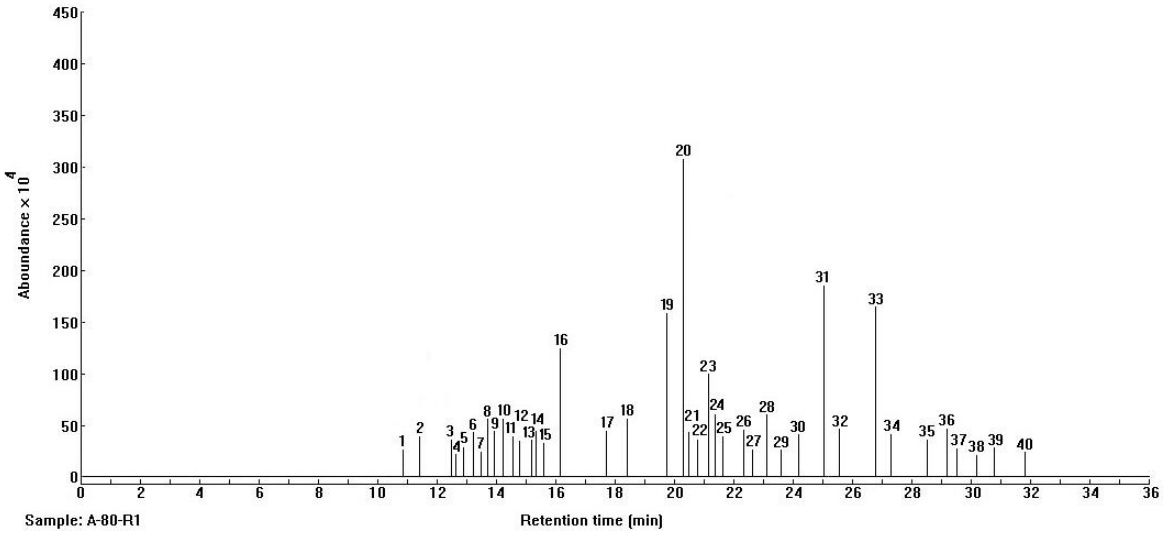

Fig. 1. GC-MS chromatogram of *M. piperita* essential oil treated with 80 mg/l salicylic acid foliar application (horizontal axis is the time diagram and vertical axis is the frequency)

Table 1. Analysis of variance for the effect of salicylic acid and yeast extract foliar application on essential oil components (%) in *M. piperita*

Source of variation (S.O.V)	df	Mean of Squares						
		β-Pinene	Myrcene	Eucalyptol	*γ*-Terpinene	Isopulegol	Menthone	Neomenthol
Treatment	8	0.148**	0.213**	0.328**	1.695**	2.012**	7.302**	1.169**
Error	18	0.036	0.023	0.055	0.098	0.122	0.262	0.067
Total	26							
C.V. (%)		16.47	14.42	19.12	6.75	5.45	3.48	6.73
Source of variation (S.O.V)	**df**	**Dihydrocarvone**	**Hexyl isovalerate**	**Piperitone**	**Isomenthol acetate**	**Caryophyllene oxide**	***β*-Ocimene**	**Dihydrocarvyl actate**
Treatment	8	0.162**	0.519**	2.097**	1.251**	0.141**	0.227*	0.101*
Error	18	0.029	0.024	0.250	0.129	0.034	0.063	0.031
Total	26							
C.V. (%)		9.62	22.85	6.90	19.99	13.64	22.17	18.08

*, ** Significantly different at the 5 and 1 % probability level, respectively

Table 2. Mean comparison for effects of salicylic acid and yeast extract on essential oil components (%) in *M. piperita*

Compounds (%)	RT (min)	Treatment								
		Control	YE-0.25	YE-0.75	YE-1	YE-1.5	SA-40	SA-80	SA-160	SA-320
***α*-Pinene**	10.87	1.40^{a}	1.20^{a}	1.27^{a}	1.07^{a}	1.20^{a}	1.13^{a}	0.97^{a}	0.97^{a}	1.10^{a}
Camphene	11.33	1.83^{a}	1.70^{a}	1.63^{a}	1.60^{a}	1.77^{a}	1.63^{a}	1.63^{a}	1.57^{a}	1.50^{a}
Sabinene	12.43	2.10^{a}	1.73^{a}	1.77^{a}	1.83^{a}	1.63^{a}	1.83^{a}	1.77^{a}	1.73^{a}	1.67^{a}
***β*-Pinene**	12.64	1.63^{a}	1.30^{ab}	1.03^{b}	1.00^{b}	1.13^{b}	1.23^{ab}	1.03^{b}	1.13^{b}	0.87^{b}
(-)-*β*-Pinene	12.96	1.73^{a}	1.83^{a}	1.47^{a}	1.33^{a}	1.63^{a}	1.63^{a}	1.47^{a}	1.37^{a}	1.43^{a}
Octenol	13.27	2.13^{a}	1.80^{a}	1.87^{a}	1.60^{a}	1.67^{a}	1.50^{a}	1.80^{a}	1.67^{a}	1.67^{a}
Myrcene	13.50	1.43^{a}	1.57^{a}	1.07^{b}	0.90^{b}	0.97^{b}	0.80^{b}	0.90^{b}	0.97^{b}	0.87^{b}
***α*-Phellandrene**	13.76	2.47^{a}	2.57^{a}	2.30^{a}	2.77^{a}	2.87^{a}	2.63^{a}	2.30^{a}	2.43^{a}	2.37^{a}
***α*-Terpinene**	13.95	1.83^{a}	1.93^{a}	1.93^{a}	1.90^{a}	1.60^{a}	1.43^{a}	1.93^{a}	1.63^{a}	1.57^{a}
Isoterpinolene	14.26	2.27^{a}	2.20^{a}	2.07^{a}	1.90^{a}	2.10^{a}	2.17^{a}	2.10^{a}	2.03^{a}	2.03^{a}
***p*-Cymene**	14.69	1.90^{a}	2.07^{a}	1.80^{a}	1.67^{a}	1.57^{a}	1.70^{a}	1.63^{a}	2.07^{a}	1.37^{a}
Eucalyptol	14.88	1.90^{a}	1.57^{ab}	0.90^{c}	1.20^{bc}	1.20^{bc}	1.17^{bc}	1.17^{bc}	1.13^{bc}	0.80^{c}
Limonene	15.16	2.03^{a}	1.90^{a}	2.07^{a}	1.97^{a}	1.73^{a}	1.83^{a}	1.73^{a}	1.60^{a}	1.53^{a}
1,8-Cineole	15.30	2.03^{a}	2.00^{a}	1.97^{a}	1.93^{a}	2.00^{a}	1.90^{a}	1.90^{a}	1.90^{a}	1.90^{a}
***β*-Ocimene**	15.66	1.57^{a}	1.47^{a}	1.30^{ab}	1.10^{abc}	0.80^{c}	1.17^{abc}	1.10^{abc}	0.80^{c}	0.90^{bc}
***γ*-Terpinene**	**16.19**	**3.77^{f}**	**4.03^{ef}**	**4.23^{def}**	**4.37^{def}**	**4.70^{cde}**	**4.87^{bcd}**	**5.27^{abc}**	**5.63^{ab}**	**6.00^{a}**
Linalool	17.78	2.00^{a}	1.90^{a}	1.90^{a}	1.90^{a}	1.70^{a}	1.97^{a}	1.77^{a}	1.87^{a}	1.80^{a}
***β*-Terpineol**	18.38	1.93^{a}	1.80^{a}	2.17^{a}	2.00^{a}	1.87^{a}	1.67^{a}	2.10^{a}	1.80^{a}	1.63^{a}
Isopulegol	**19.81**	**7.00^{ab}**	**7.63^{a}**	**7.10^{ab}**	**6.77^{abc}**	**6.40^{bc}**	**6.30^{bc}**	**6.00^{cd}**	**5.40^{de}**	**5.10^{e}**
Menthone	**20.18**	**12.13^{f}**	**13.03^{ef}**	**13.83^{de}**	**14.57^{cd}**	**15.70^{bc}**	**14.70^{bcd}**	**15.40^{bc}**	**15.97^{b}**	**17.20^{a}**
Isomenthone	20.50	2.10^{a}	1.90^{a}	1.93^{a}	2.10^{a}	1.73^{a}	2.10^{a}	1.93^{a}	1.90^{a}	1.97^{a}
Menthofuran	20.86	2.00^{a}	1.90^{a}	2.07^{a}	1.90^{a}	1.83^{a}	1.77^{a}	1.70^{a}	1.93^{a}	1.33^{a}
Neomenthol	21.11	3.07^{e}	3.20^{e}	3.40^{de}	3.60^{cde}	3.93^{cd}	3.70^{cde}	4.20^{bc}	4.60^{ab}	4.90^{a}
Lavandulol	21.40	2.30^{a}	2.53^{a}	2.70^{a}	2.53^{a}	2.53^{a}	2.30^{a}	2.53^{a}	2.53^{a}	2.40^{a}
l-(+)-Menthol	21.73	1.97^{a}	1.87^{a}	1.73^{a}	1.87^{a}	1.67^{a}	1.77^{a}	1.80^{a}	1.67^{a}	1.83^{a}
Myrtenal	22.31	2.070^{a}	1.80^{a}	2.00^{a}	1.93^{a}	1.70^{a}	1.87^{a}	1.87^{a}	1.73^{a}	1.77^{a}
Dihydrocarvone	22.66	1.13^{b}	1.70^{a}	1.17^{b}	1.00^{b}	1.10^{b}	0.93^{b}	1.03^{b}	1.10^{b}	0.93^{b}
***p*-Mentenol**	23.08	2.90^{a}	2.40^{a}	2.63^{a}	2.87^{a}	2.83^{a}	3.00^{a}	2.67^{a}	2.57^{a}	2.87^{a}
Hexyl isovalerate	23.60	0.00^{c}	0.00^{c}	0.70^{ab}	1.03^{a}	0.57^{b}	1.00^{a}	1.00^{a}	0.80^{ab}	1.00^{a}
Carvone	24.11	1.83^{a}	1.93^{a}	1.83^{a}	1.90^{a}	1.93^{a}	1.80^{a}	1.90^{a}	1.87^{a}	1.90^{a}
Piperitone	**25.05**	**6.40^{c}**	**6.60^{c}**	**6.87^{c}**	**6.80^{c}**	**7.50^{bc}**	**6.87^{c}**	**7.00^{c}**	**8.30^{ab}**	**8.90^{a}**
Borneol	25.60	1.93^{a}	1.87^{a}	1.83^{a}	1.80^{a}	1.73^{a}	1.67^{a}	1.73^{a}	1.73^{a}	1.60^{a}
Isomenthol acetate	**26.81**	**5.43^{de}**	**5.23^{e}**	**5.73^{bcde}**	**6.20^{bcd}**	**6.40^{bc}**	**7.30^{a}**	**6.43^{b}**	**5.83^{bcde}**	**5.50^{cde}**
Dihydro carvyl actate	27.21	1.93^{abc}	1.73^{bcd}	2.07^{a}	2.00^{ab}	1.67^{bcd}	1.63^{cd}	1.67^{bcd}	1.70^{bcd}	1.53^{d}
***β*-Bourbonene**	28.52	1.90^{a}	1.83^{a}	1.77^{a}	1.73^{a}	1.83^{a}	1.73^{a}	1.40^{a}	1.33^{a}	1.47^{a}
Spathulenol	29.14	1.77^{a}	1.63^{a}	2.00^{a}	1.90^{a}	1.70^{a}	1.60^{a}	2.00^{a}	1.70^{a}	1.77^{a}
***β*-Elmene**	29.52	0.97^{a}	1.10^{a}	1.33^{a}	1.13^{a}	0.90^{a}	0.77^{a}	0.87^{a}	0.90^{a}	0.87^{a}
Dihydrocarvyl acetate	30.18	1.33^{a}	1.30^{a}	0.87^{a}	0.87^{a}	0.77^{a}	1.03^{a}	1.00^{a}	0.77^{a}	0.83^{a}
Caryophyllene oxide	30.86	1.63^{a}	1.63^{a}	1.27^{ab}	1.00^{b}	1.10^{b}	1.30^{ab}	1.33^{ab}	1.47^{ab}	1.43^{ab}
Humulene epoxide	31.90	1.37^{a}	1.43^{a}	1.27^{a}	1.17^{a}	1.30^{a}	1.30^{a}	0.87^{a}	1.07^{a}	1.00^{a}

Means followed by similar letter (s) in each row are not significantly different by Duncan's multiple range test at $P < 0.01$. The values are mean of three replicates ± standard error (SE). RT: Retention time (min). YE: Yeast extract (g/l). SA: Salicylic acid (mg/l).

Table 3. Pearson's correlation coefficients between some essential oil compositions in peppermint under salicylic acid and yeast extract foliar application

Compounds	Piperitone	Hexyl isovalerate	Neomenthol	Menthofuran	Menthone	Isopulegol	γ-Terpinene	β-Ocimene	Eucalyptol	Myrcene	β-Pinene
β-Pinene	-0.409*	-0.697**	-0.519**	0.421*	-0.531**	0.385*	-0.478*	0.229	0.632**	0.506**	1
Myrcene	-0.405*	-0.826**	-0.640**	0.378	-0.701**	0.636**	-0.600**	0.487**	0.550**	1	-
Eucalyptol	-0.361	-0.633**	-0.576**	0.311	-0.638**	0.460*	-0.545**	0.430*	1	-	-
β-Ocimene	-0.618**	-0.464*	-0.588**	0.090	-0.758**	0.561**	-0.631**	1	-	-	-
γ-Terpinene	0.713**	0.613**	0.848**	-0.482*	0.881**	-0.879**	1	-	-	-	-
Isopulegol	-0.712**	-0.554**	-0.886**	0.425*	-0.813**	1	-	-	-	-	-
Menthone	0.739**	0.683**	0.865**	-0.357	1	-	-	-	-	-	-
Menthofuran	-0.387*	-0.244	-0.493**	1	-	-	-	-	-	-	-
Neomenthol	0.748**	0.578**	1	-	-	-	-	-	-	-	-
Hexyl isovalerate	0.375	1	-	-	-	-	-	-	-	-	-
Piperitone	1	-	-	-	-	-	-	-	-	-	-

*, ** Significantly different at the 5 and 1 % probability level, respectively

4. Discussion

The quality of plants used for production of pharmacological compounds is usually assessed by secondary metabolite content [13]. High-quality oils of *M. piperita* are characterized by a complex compositional balance of monoterpenes with high menthol, moderate menthone, and low pulegone and menthofuran quantities [26]. In this

study, the identified essential oil compounds of *M. piperita* aerial parts are listed in Table 2. Elicitation effects of salicylic acid and yeast extract were investigated and 40 compounds were identified in peppermint essential oil using GC-MS that menthone and menthol were major components (16.69 % and 14.39 % of the essential oils, respectively). To our best knowledge, results are consistent with other studies, wherein menthol and menthone were the most abundant constituents in *M. piperita* essential oils [7-10, 12]. Similarly, it was observed that in the aerial parts of cultivated *M. piperita* plants, a total of 39 compounds were identified in essential oil by hydro-distillation method. The main components in the hydro-distillation procedure were menthol (45.34 %), menthone (16.04 %) and menthofuran (8.91 %) [9]. It was reported that a total of 28 compounds were identified in the essential oil from peppermint aerial parts under salicylic acid treatments and the major constituents (more than 70 %) were menthol, menthone, and isomenthone, respectively [7]. It was observed that a total of 29 compounds were identified in the essential oil from aerial parts of peppermint and the main constituents in the essential oil were camphane (14.01 %), menthone (13.89 %), menthol (12.37 %) *β*-pinene (7.62 %) and pulegone (6.41 %) [8]. The results of an investigation showed that the major components of the oil were menthone, menthol, menthofuran, pulegone, 1,8-cineole, and menthyl acetate [10]. In another experiment, it was found that the major compounds identified were isomenthone (27.4 %), menthol (24.3 %), menthone (9.2 %), limonene (5.8 %), 1,8-cineole (5.6 %), menthofuran (4.4 %) and isomenthol (3.2 %) [12]. In addition, results of an investigation indicated that a total of 33 compounds were detected in the essential oil samples of peppermint treated with different salicylic acid concentrations with menthone (15.8-18.1 %), menthol (46.3-47.4 %), methyl acetate (8.5-9.7 %) and 1,8-cineole (4.3-4.6 %) being the major ones [19]. It is observed that in all researches, menthol and menthone were the main constituents of peppermint essential oil. A comparison of our results with the previous reports suggests some variation in quantities and quality of components within the essential oil of the plants. The phytochemical variability of *M. piperita* oils is may be due to the geographical conditions of the plant sample, agronomic practices, climatic, harvesting time and drying method as well as essential oils extraction procedure [3, 24-25]. The variation in the percentage of some constituents within the essential oil showed trend as a result of the salicylic acid and yeast extract exposure treatment. Our experimental results show that essential oil compounds of *M. piperita* aerial parts can be significantly stimulated by both biotic and abiotic elicitors. Salicylic acid from 40-320 mg/l and yeast extract from 0.25-1.5 g/l exhibited different eliciting effects on major components of *M. piperita*. The lowest level of major components production was observed in control plants that distilled water was used for foliar application. In present study, with increasing salicylic acid concentration, the amount of menthone, neomenthol, piperitone, *γ*-terpinene have increased. The highest percentage of those components were obtained at 320 mg/l salicylic acid treated plants that were 42, 60, 39 and 59 % higher compared to control. Several reports have shown that exogenous application of salicylic acid has a positive effects on production of many bioactive compounds in medicinal plants [7, 13, 18, and 19]. Similarly, it was reported that the foliar application by 10 mM of salicylic acid and 1.5 mg/l indol-3-acetic acid increased major essential oil components of

M. piperita and *Melissa officinalis* aerial parts [7]. In another experiment, it was found that foliar treatment of lemon balm plants with salicylic acid considerably enhanced the monoterpene oxygenated and sesquiterpenes secondary metabolites [18]. It was found that the main oil components were not markedly affected by different salicylic acid treatments. However, the maximum concentration of menthol (47.4 %) was observed when 150 mg/l salicylic acid applied [19] which this is not in agreement with the results obtained in this study. The findings of the present study substantiated the role of salicylic acid as an elicitor that with increasing the amount from 160 to 320 mg/l the content of main constituents of essential oil in aerial parts of *M. piperita* except isopulegol increased. The results of GC-MS analysis of peppermint aerial parts showed that yeast extract significantly altered the amount of some of the main constituents. In present study, the results showed that with increasing the concentrations of yeast extract from 0.75 to 1.5 g/l, the amount of menthone, neomenthol and *γ*-terpinene increased. Also, four levels of yeast extract did not have a significant effect on the production of piperitone and isopulegol compared to the control. The stimulating influence of yeast extract on secondary metabolites was confirmed in several studies [1, 20-22]. The majority of biotic elicitors are recognized by specific receptors bound to the cell membrane. These stimulants are then transferred to the cell by a signal transduction system, producing changes that ultimately lead to the formation of phytoalexins [27]. In general, it was clearly observed from Table 2 that different types of elicitors exhibited distinct influences on the phytochemical accumulation in *M. piperita*. This indicated that the sensitivity of biologically active compounds biosynthesis toward elicitation varied with the elicitors used in *M. piperita*. In this research, correlation coefficient of essential oils compositions in peppermint under salicylic acid and yeast extract foliar application was calculated. In general, often the major constituents of peppermint essential oil in the conditions of this study had a positive and significant relationship with each other. In another experiment, it was found that most of the major constituents of the essential oil of *M. piperita* and *M. officinalis* both had significant positive correlation with each other [7] which was in agreement with the results of this study.

5. Conclusion

In conclusion, the quality of essential oil of *M. piperita* were influenced by the foliar application of salicylic acid and yeast extract at the appropriate concentrations. Forty compounds were identified in peppermint essential oil that the major components were menthone, menthol, piperitone, isopulegol and *γ*-terpinene, respectively. Salicylic acid was effective at high concentration (320 mg/l) for menthone, *γ*-terpinene, neomenthol and piperitone production. In this study, the major components of peppermint essential oil had a positive and significant relationship with each other, generally. The results can be useful to apply in modern agriculture for enhancing the quality of peppermint.

Author contributions

M. M. carried out the experiment and contributed in data gathering. M. A. supervised the research, assisted in data analysis and wrote the manuscript.

Conflict of interest

The authors declare that there is no conflict of interest.

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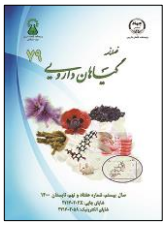

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مقاله تحقیقاتی

تغییرات اجزای اسانس نعناع فلفلی تحت تأثیر محلول پاشی با عصاره مخمر و سالیسیلیک اسید

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اطلاعات مقاله

گل‌واژگان:
نعناع فلفلی
محرک
متابولیت‌های ثانویه
گیاه دارویی
منتون
منتول

چکیده

مقدمه: نعناع فلفلی (*Mentha x piperita* L.) یکی از مهمترین گیاهان دارویی است که در صنایع غذایی، دارویی، عطرسازی و طعم‌دهنده‌ها مورد استفاده قرار می‌گیرد. **هدف:** این پژوهش، با هدف بررسی اثر محلول پاشی سالیسیلیک اسید و عصاره مخمر بر افزایش تولید اجزای ارزشمند اسانس نعناع فلفلی انجام شد. **روش بررسی:** این تحقیق به صورت طرح کاملاً تصادفی با ۹ تیمار شامل سالیسیلیک اسید (۴۰، ۸۰، ۱۶۰ و ۳۲۰ میلی‌گرم در لیتر)، عصاره مخمر (۰/۲۵، ۰/۷۵، ۱ و ۱/۵ گرم در لیتر) و آب مقطر (شاهد) در سه تکرار تحت شرایط گلخانه‌ای انجام شد. **نتایج:** در مجموع، ۴۰ ترکیب در ساختار هوایی نعناع فلفلی شناسایی شد. اجزای اصلی اسانس به ترتیب منتون، منتول، پیپریتون، ایزوپولگول و گاماترپینن بودند. منتون و منتول به ترتیب با ۱۶/۶۹ و ۱۴/۳۹ درصد، ترکیب‌های اصلی اسانس بودند. محرک‌های سالیسیلیک اسید و عصاره مخمر باعث افزایش تولید منتون، نئومنتول، پیپریتون، گاماترپینن و ایزومنتول استات (به ترتیب ۴۲، ۶۰، ۳۹، ۵۹ و ۳۴ درصد) نسبت به گیاهان شاهد شدند. محلول‌پاشی با تیمار ۳۲۰ میلی‌گرم در لیتر سالیسیلیک اسید، بهترین تیمار برای افزایش درصد ترکیبات عمده اسانس گیاهان تیمار شده بود. نتایج همبستگی بین اجزای اسانس نشان داد که میزان نئومنتول همبستگی مثبت معنی‌داری با منتون ($r = ۰/۸۶۵^{**}$)، گاما ترپینن ($r = ۰/۸۴۸^{**}$) و همبستگی منفی با ایزوپولگول ($r = -۰/۸۸۶^{**}$) داشت. **نتیجه‌گیری:** کیفیت اسانس نعناع فلفلی تحت تأثیر غلظت‌های مناسب سالیسیلیک اسید و عصاره مخمر بود. برای تولید منتون، نئومنتول، گاما ترپینن و پیپریتون، ۳۲۰ میلی‌گرم در لیتر سالیسیلیک اسید تیمار مناسبی بود.

مخففها: GC-MS، کروماتوگرافی گازی – طیف سنجی جرمی؛ SE، خطای استاندارد؛ C.V.، ضریب تغییرات

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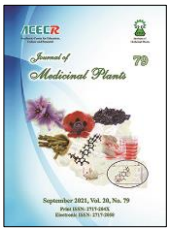

Research Article

The differences between *Froriepia subpinnata* (Ledeb.) Baill. and *Pimpinella anisum* L. commonly named as anarijeh based on major components of the essential oil; a marker for resolve ambiguities

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ABSTRACT

Background: *Froriepia subpinnata* (Ledeb.) Baill. and *Pimpinella anisum* L. are two important medicinal plants belong to the Apiaceae family. Due to the similar Persian name in ethnobotanical studies, namely "anarijeh", these two medicinal plants are mistakenly used instead of each other in the Iranian medicinal plants market and even in scientific reports. **Objective:** In this study, the correct morphological description of studied species were introduced and the chemical composition of their essential oils and their antioxidant activities were determined. **Methods:** The aerial parts of *F. subpinnata* and the seeds of *P. anisum* were crushed separately followed by hydro-distillation method for 3 h using a Clevenger apparatus to obtain essential oils (EOs) and their constituents were analyzed by GC/MS. Also, the potential of antioxidant inhibitory of essential oils were determined using DPPH and FRAP methods. **Results:** *p*-Cymen-8-ol (51.13 %), *α*-terpinolene (7.69 %) and limonene (6.83 %) were the major components of *F. subpinnata* EO while *trans*-anethole (85.65 %) and carvone (5.31 %) were the major components in *P. anisum* EO. The results of antioxidant activities in DPPH and FRAP assays at the concentration of 250 µg/ml were 53.03 and 62.72 % for *F. subpinnata* and 50.27 and 59.91 % for *P. anisum*, respectively. The results of antioxidant activity by DPPH and FRAP methods indicated both essential oils had almost similar potential. **Conclusion:** Type and the amounts of the major components of the essential oils of *F. subpinnata* and *P. anisum* can be regarded as an accurate basis for differential diagnosing the plants. These differences can be used as a good phytochemical marker in correct identification and prevention of mistakes and deceptions in herbal products.

Abbreviations: GC/MS, Gas Chromatography/Mass Spectroscopy; EOs, Essential Oils

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1. Introduction

Nowadays, medicinal plants have a vital role in health and economy. Millions of people around the world are working on different aspects of medicinal plants such as planting, harvesting and processing [1]. Many problems due to misuse of medicinal plants like lacking of proper evaluation and monitoring system for activists in this field have been generated, as a result, we see many mistakes or deceptions in preparation of medicinal plants for public use [2]. Use of unknown or dobtfull plant species in the market of dried herbs and production of herbal drugs is the most important challenges in this issue. Hence, shortage of essential knowledge has caused many mistakes and deceptions in pharmaceutical research and their products [3, 4].

Exact identification of plants begins with direct observation of the morphological characteristics of plants body or with microscopic descriptions of powder of plant tissues and organs. After taxonomic verification, the type and content of specific phytochemical compounds of the plant are evaluated and approved with comparison by the plant's literature review. Essential oil compositions and antioxidant properties of the plant as a suitable marker can be used for phytochemical verification of plants [5-7].

The widespread presence of plants in different societies has led to the formation of different local names for plants. This significantly leads to similarities and differences in the naming of plants. So that there may be different names for one species in different cultures and languages, or the same names may be used for different plant species in different languages [8, 9]. For this reason, the scientific explanation of medicinal plants samples has a very crucial and fundamental role and also it is considerated as the first step for use of traditional medicinal plants [2].

One of the mistakes in naming plants, which is unfortunately common in Iran herbal stors and scientific societies, is related to *Froriepia subpinnata* (Ledeb.) Baill. and *Pimpinella anisum* L. plant species which have been introduced in various sources with the same local name of anarijeh. While this common persian name is used for both plants that different parts of them are used in medicinal plants market. The aerial part of *F. subpinnata* has been used as a carminative, appetizer, antispasmodic, diuretic antiseptic and sedative. This species contains antioxidant and antibacterial properties [10]. *P. anisum* seeds are the used part of plant which heals, stimulates and improves the function of the digestive system and is also beneficial for liver and circulatory system. Essential oil of *P. anisum* seeds has antimicrobial, antifungal, antiviral, antioxidant, analgesic and antispasmodic, anti-epileptic effects and is effective in healing stomach ulcers. Also, in traditional medicine has been used to treat some diseases such as epilepsy [11, 12].

Based on our field and herbarium observations and reports of other researchers, it certainly seems that the vernacular name of anarijeh is related to *F. subpinnata* [9, 10, 13-16], However, there are several researches in the scientific sources that shows the name of anarijeh has been used for the *Pimpinella* genus [17-23].

Due to the mistakes in identification and thus the use of *Froriepia subpinnata* and *Pimpinella anisum* and also the lack of accurate illustrative studies, the present research specificly aimed to correct introduction and identification of these plants and also provide their essential oil composition and antioxidant capacity.

2. Materials and Methods

2.1. Plant material, extraction of essential oils and analysis by GC/MS

The samples of *F. subpinnata* and *P. anisum* were collected in 2018 from Behshahr and Isfahan cities (Mazandaran and Isfahan provinces respectively) in Iran. Voucher specimens (No. 580-SANRU for *F. subpinnata* and No. 601-SANRU for *P. anisum*) were determined by expert authors and deposited in herbarium of the Sari Agricultural Sciences and Natural Resources University, Sari, Iran.

The aerial parts of *F. subpinnata* and seeds of *P. anisum* were air-dried at ambient temperature (25 °C) during a week and then they were powdered separately and essential oils were isolated using Hydrodistillation method for 3 h [24]. 300 g of powdered samples were heated with one liter of distilled water in Clevenger apparatus and finally the obtained essential oils were collected. After dehumidification with anhydrous sodium, the essential oils were stored in glass containers at 4 °C in the refrigerator and away from light.

The essential oils were analysed by gas chromatography/ mass spectroscopy. 1 µl of the oil sample was injected into the GC/MS apparatus. The essential oils were also analyzed by an Agilent 6890 apparatus on capillary column. Mass spectrometry (Agilent 5973N, USA) equipped with a BPX5 fused silica column of (30 m × 0.25 mm i.d. × 0.25 µm film thickness) were done in electronic impact mode (70 eV), split injection ratio (1: 35), Carrier gas helium with 0.5 ml/min flu rate and mass range of 40 to 500 amu. The temperature program of the column was adjusted as follows: The initial temperature of the oven was 50 °C for 5 min, the temperature gradient of 3 °C per min and rises to 240 °C and then at a speed of 15 °C per min, the temperature rises to 300 °C for 3 min. Stopping at this temperature and response time was 75 min. Compounds were identified by comparing retention time (RT) with those reported in the literature and their mass spectrum with Wiley library [25, 26].

2.2. Antioxidant capacity of essential oils

Two common methods of DPPH and FRAP were used to measure the antioxidant activity of essential oils of *F. subpinnata* and *P. anisum* plants at the concentration of 250 µg/mL. In the DPPH method, the potential of the essential oils for free radical scavenging activities were evaluated based on percentage inhibition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity as calculated using previously introduced methods [27, 28]. Then it was expressed as a percentage of inhibition of DPPH which was calculated according to the following Equation:

$$\text{Percent (\%) inhibition of DPPH} = [(A_0 - A_1) / A_0)] \times 100$$

The A_0 was the absorbance value of the blank sample or control reaction and A_1 was the absorbance value of the test sample. All measurements were performed in triplicates. vitamin C and E were separately used as control due to their potential for inhibition of free radicals effect.

In the second method, the ferric reducing antioxidant power (FRAP) of the essential oil from the samples of *F. subpinnata* and *P. anisum* were assayed according to described assay [29]. The percent of free radical inhibition in FRAP assay of the samples was calculated according to the below formula:

$$\text{Antioxidant Activity (\%)} = (A_1A_0)/A_1$$

A_0 is the absorbance of the control (potassium phosphate buffer + FRAP reagent), A_1 is the Absorbance of sample. The vitamin C and E were separately used as the reference antioxidants.

2.3. Botanical illustrations

For Full confidence of coccerct identification of plant species, they were studied and compared with related references in aspects of taxonomy, morphological description and Geographical distribution [9, 30-32].

2.4. Statistical analysis

Statistical analysis of antioxidant activities of essential oils were conducted using SAS version 9.1. All assays were performed in at least three replications. Differences between means were distinguished using F-test and the confidence limits was based on 95 % ($P < 0.05$).

3. Results

3.1. Taxonomy, Geographycal distribution and morphological description of plants samples

Distribution of *F. subpinnata: Froriepia* is a fragrant plant belongs to the Apiaceae family, native to the Caucasus region which has only one species (*F. subpinnata*) in Iran and has been reported from the provinces of Gilan, Mazandaran and Golestan. The species grows on the roadsides and mountainous areas . This plant is known as anarijeh or in the northern regions of the country and its fresh and dried leaves are commonly used in local foods.

Morphological description of *F. subpinnata:* Slender glabrous biennial. Stems up to 10 cm, divaricately branched, purple-striped below, glaucous-green above. Basal leaves unknown. Cauline leaves with a white, membranous, petiolar subauriculate sheath; median leaves with few, filiform, mucronulate segments up to 5 mm long; upper leaves reduced to one segment. Umbels 2-4-rayed, up to 1.5 cm long. Bracts absent. Bracteoles 1-3, narrowly lanceolate, longer than pedicels, with a white margin and long awn, somewhat rigid. Central umbellule subsessile. Central fruit 2-2.5 mm long, outer fruits 1.5-2 mm long, often with rudimentary mericarps. Mericarps subterete, primary ridges 5-6, secondary ridges 4-5. Dorsal vittae 4-5, commissural vittae 2, all small (Fig. 1).

Distribution of *P. anisum:* The genus *Pimpinella* L. from the Apiaceae family have about 21 species of annual or perennial species in Iran. They are generally fragrant plants in Europe, Turkey, Iran, Caucasus, Armenia, Central Asia, Syria and Egypt. *P. anisum* with common name of Anise or Roman anise and only its seeds have medicinal use. This species is widely cultivated in different parts of the Iran and has been reported from the Azerbaijan, Isfahan and Tehran provinces and probably does not originate from Iran while *P. affinis* Ledeb. is widespread species in Iran and therefore it is mostly used in traditional medicine. Due to similarities beteween two species of *P. anisum* and *P. affinis* in seed shape, odor and medicinal properties they were used instead of each other in Iran.

Morphological description of *P. anisum:* Strongly aromatic branched annual with puberulent or pubescent stems up to 70 cm. Basal leaves simple, 2-5 cm, petiolate, reniform or ovate, dentate; lower cauline leaves pinnate with ovate or obovate dentate segments; upper cauline leaves 2-3-pinnate, lobes linear-lanceolate, petioles sheathing. Umbels 7-15rayed; bracts absent or 1; bracteoles absent to few, very narrow. Flowers white, c. 10 per umbellule. Fruit 3-5 mm long, ovoid, shortly adpressed-hairy; stylopodium conical (Fig. 2).

Fig. 1. Used part (vegetative organs = above) and generative organs (inflorescent = below) of *Froriepia subpinnata*

Fig. 2. Used part of used part (Seeds = left) and generative organs (inflorescent = right) of *Pimpinella anisum*

3.2. Composition and antioxidant properties of essential oils

Based on the results of GC/MS analysis, 36 bioactive compounds were identified in the essential oil of *F. subpinnata* constituting 93.21 % of the total oil. The most important of these compounds were *p*-cymen-8-ol, α-terpinolene and limonene by about 51.13, 7.96 and 6.83 %, respectively (Table 1, Fig. 3).

Table 1. Essential oil compounds in *F. subpinnata*

No.	Compounds	RT	KI	%	No.	Compounds	RT	KI	%
1	Hexanal	6.16	809	0.11	19	Terpinen-4-ol	24.78	1192	0.16
2	Heptanal	10.47	911	0.79	20	*m*-Cymen-8-ol	25.30	1203	0.12
3	*α*-Pinene	11.73	936	0.72	21	Citronellol	26.92	1237	0.13
4	Sabinene	13.81	977	1.99	**22**	*E*-Ocimenone	**27.16**	**1242**	**5.54**
5	*β*-Pinene	14.08	983	0.92	23	Anethole	30.02	1303	0.84
6	*β*-Myrcene	14.65	994	0.75	24	Thymol	30.33	1310	0.29
7	2-Pentylfuran	14.78	997	0.24	**25**	***β*-Elemene**	**34.16**	**1396**	**2.42**
8	*trans*-2-(1-Pentenyl) furan	15.28	1006	0.15	26	*(E)*-*β*-Famesene	36.74	1458	0.63
9	*α*-Phellandrene	15.61	1013	1.14	27	*trans*-*β*-Ionone	38.15	1491	0.17
10	*α*-Terpinene	16.15	1023	0.13	28	*β*-Selinene	38.55	1501	0.25
11	*o*-Cymene	16.66	1033	0.36	29	*α*-Selinene	38.80	1507	0.38
12	**Limonene**	**16.81**	**1036**	**6.83**	30	Germacrene A	39.28	1519	0.53
13	*β*-Ocimene	17.68	1052	0.27	**31**	**Neophytadiene**	**51.11**	**1838**	**3.48**
14	*γ*-Terpinene	18.35	1065	1.27	32	Hexahydrofarnesyl acetone	51.46	1849	0.18
15	***α*-Terpinolene**	**19.74**	**1091**	**7.69**	33	Diisobutyl phthalate	52.40	1876	0.11
16	***p*-Cymen-8-ol**	**22.18**	**1140**	**51.13**	34	Hexadecanoic acid - methyl ester	54.31	1934	0.12
17	Anisole	23.79	1172	1.08	35	Dibutyl phthalate	55.59	1973	0.13
18	4-Pyridinol	23.96	1176	0.86	36	Phytol	60.07	2059	1.30
	Total Identified			**93.21**					

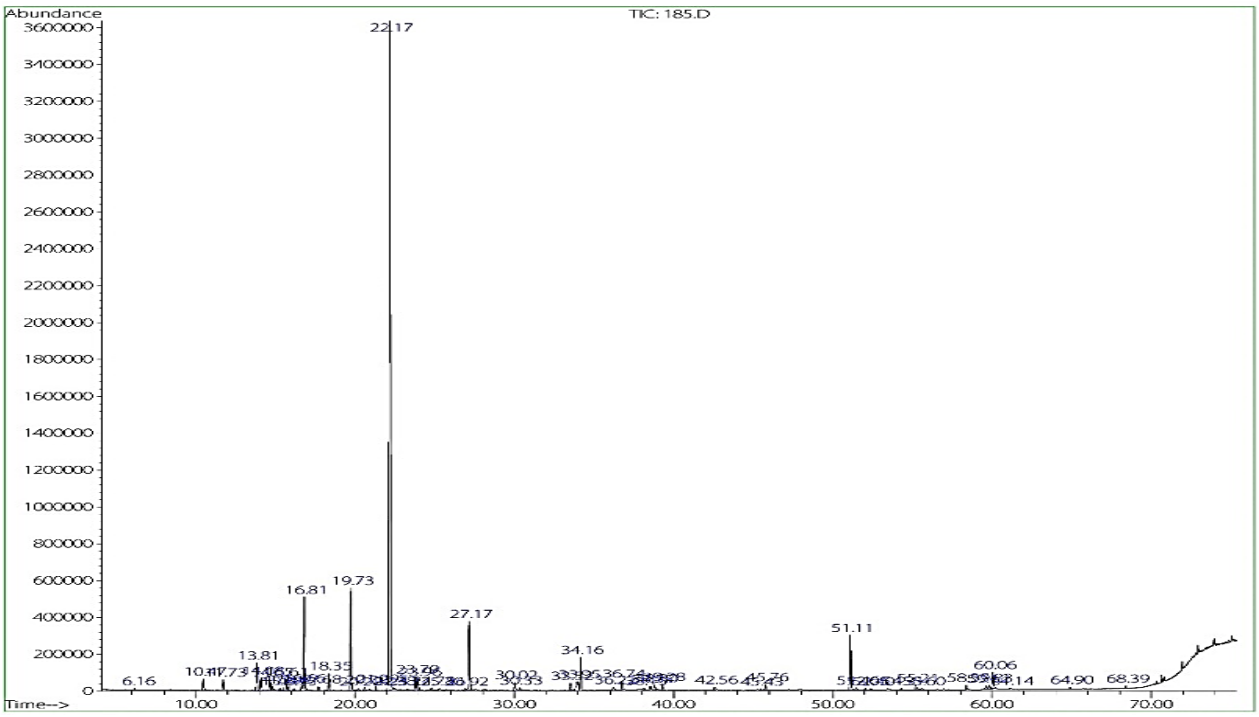

Fig. 3. Chromatogram obtained from GC/MS analysis of *F. subpinnata* essential oil

The amount of hydrogenated monoterpenes by about 22.07 %, oxygenated monoterpenes by about 58.26 %, hydrogenated sesquiterpene with 4.21 %, hydrogenated diterpenes with 1.3 % and other compounds with 7.25 % of total compounds of essential oil were reported.

Assay of antioxidant activity of *F. subpinnata* essential oil in the range of 250 µg/ml showed that the percentage of antioxidant inhibitors for *F. subpinnata* essential oil by DPPH and FRAP methods were about 53.03 and 62.72 %, respectively. Despite, along with rising in essential oil concentration, inhibitory activity was also increased but could not compete with the antioxidant power of vitamins C and E (Table 3).

The results of *P. anisum* essential oil analysis by GC/MS showed 13 biologically active compounds that constituteing 99.3 % of the total oil.

The most important of these compounds were *trans*-anethole (85.65 %), carvone (31.5 %) and Limonen (3.25 %) respectively (Table 2, Fig. 4). The content of hydrogenated monoterpenes about 3.35 %, oxygenated monoterpenes about 93.15 %, hydrogenated sesquiterpene 2.69 and oxygenated sesquiterpene 0.12 % of total compounds of essential oil were reported.

Assay of antioxidant activity of *P. anisum* essential oil in the range of 250 µg/ml showed that the percentage of antioxidant inhibitors for *P. anisum* essential oil by DPPH and FRAP methods were about 50.27 % and 59.91 %, respectively. Despite, along with rising the essential oil concentration, inhibitory activity was also increased but could not compete with the antioxidant power of vitamins C and E (Table 3).

Table 2. Essential oil compounds in *P. anisum*

No.	Compounds	RT	KI	%	No.	Compounds	RT	KI	%
1	*α*-Phellandrene	15.61	1013	0.10	8	*α*-Himachalene	36.86	1460	0.08
2	**Limonene**	**16.81**	**1036**	**3.25**	9	*γ*-Himalachene	38.10	1490	1.65
3	Estragole	25.74	1212	1.38	10	Zingiberene	38.63	1503	0.77
4	*trans*-Dihydro carvone	26.07	1219	0.66	11	*β*-Himachalene	38.96	1511	0.12
5	**Carvone**	**28.03**	**1261**	**5.31**	**12**	*β*-Bisabolene	39.14	1515	0.07
6	Anethole	28.39	1268	0.15	13	Pseudoisoeugenol 2-methylbutanoate	51.53	1580	0.12
7	***trans*-Anethole**	**30.09**	**1304**	**85.65**					
	Total Identified			**99.30**					

Table 3. Evaluation of antioxidant activity in essential oil in *F. subpinnata* and *P. anisum* by DPPH and FRAP methods. Vitamins C and E were considered as positive controls.

Plant essential oil or Positive control (Concentration)	Inhibition (%) DPPH method	Inhibition (%) FRAP method
F. subpinnata (250 µg/ml)	53.03 ± 0.24^{b}	62.72 ± 0.37^{b}
P. anisum (250 µg/ml)	50.27 ± 2.15^{b}	59.91 ± 1.52^{c}
Vitamins C (8 µg/ml)	91.37 ± 3.68^{a}	94.37 ± 1.78^{a}
Vitamins E (5 µg/ml)	95.58 ± 2.63^{a}	96.23 ± 1.89^{a}

Values are mean ± SD from three replicates (n = 3) at the 0.05 probability levels. ^{a,b,c} Superscript lowercase letters are for significance values.

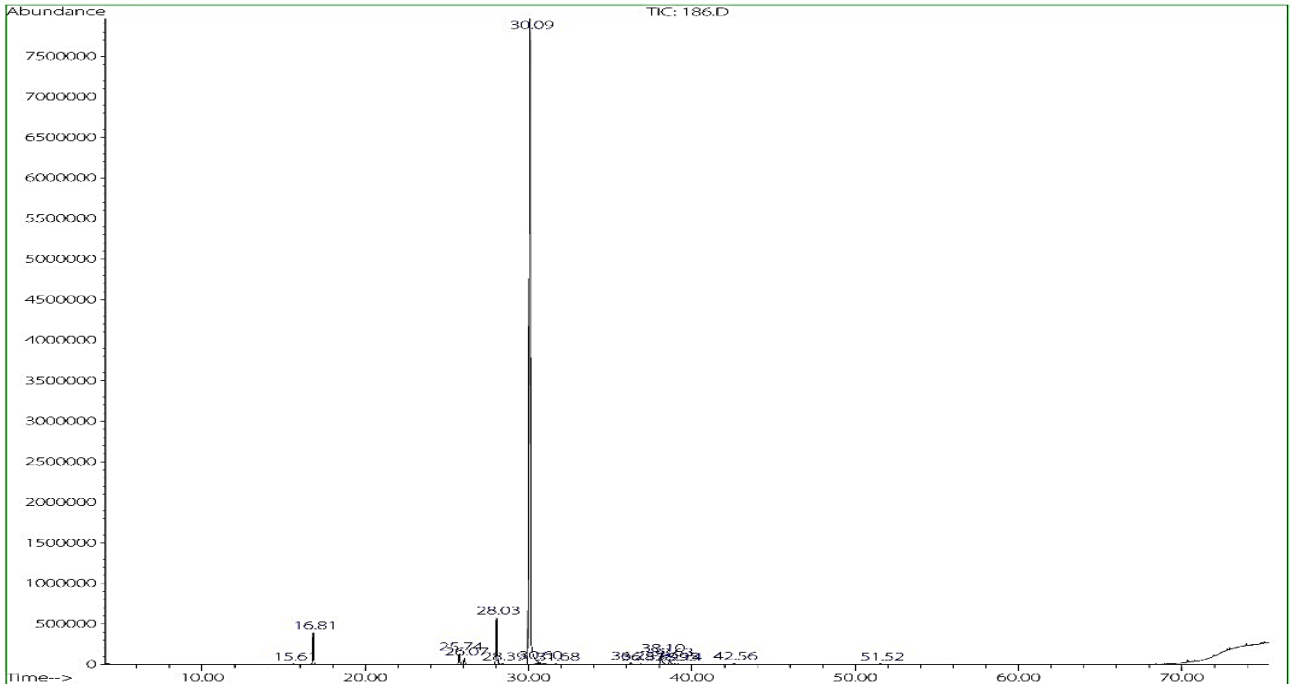

Fig. 4. Chromatogram obtained from GC/MS analysis of *P. anisum* essential oil

4. Discussion

It has been observed that two plant species of *F. subpinnata* and *P. anisum* are known as anarijeh in medicinal plants stores and also in some scientific reports of Iran.

Our results indicated 36 bioactive compounds in essential oil in the aerial part of *F. subpinnata* showed that (Table 1, Fig. 3) constituted 93.21 % of total oil. Contradictory scientific reports can be found regarding the composition of the essential oil of this plant [32, 33, 34]. The compounds of essential oils *F. subpinnata* reported by [33] are in consistent with the results of the present study and therefore it seems that the plant species has been correctly identified and evaluated. In the another study, 36 compounds were identified from the essential oil extracted from flower of *F. subpinnata* and the major compounds were include *p*-cymen-8-ol (34.7 %), Terpinolene (12.5 %) and Limonene (10.5 %). Due to the similarity of these results with the present study, it confirms the plant correctly introduced [34].

Also in another research study, totally 10 compounds were reported 10 compounds in essential oil of *F. subpinnata*. While the most important compounds were β-phellandrene (50.3 %) and Sabinene (25.7 %) [35]. However, the data from GC/MS analysis of this present study showed the compounds that have the highest amount in essential oil were including *p*-cymen-8-ol, *α*-Terpinolene and Limonene by about 51.13 %, 7.96 % and 6.83 %, respectively. It is clearly shown that the type of essential oil compounds in this study [35] are very different from our results and therefore may have been mistaken in the exact identification of the plant species in Rustaiyan et al. report.

In another research two sceintific name of *F. subpinnata* and *Eryngium bungeii* has been used for anarijeh. Application of two simultaneous scientific names for one plant is

basically incorrect. Also, the compounds reported in this report do not match the compounds reported in the present study probably due to wrong plant identification [19].

In two other reports [35, 36], the plant species of *F. subpinnata* was called as Zolang in Persian, which is according to the Mozaffarian [32]. However, based on perevious studies this naming does not agree to the common naming of this plants in the north of Iran and therefore did not approve [9, 15, 16].

Predominant phenolic compounds and their antioxidant effects of *F. subpinnata* were reported previousely [38]. Due to similarity of antioxidant activities of this report with present study, it seems that the plant species is probably correctly used.

Also, some controversial reports had found on *P. anisum*. Our results indicated that 13 bioactive compounds identified in essential oil of the essence *P. anisum* (Table 2, Fig. 4) that showed 99.3 % of total oil.

Some studies were reported results of analysis of seeds essential oil of *P. anisum* from different regions of Turkey. The most common identified compounds were trans-Anethole, methyl chavicol and Alpha-terpineol. In the present study, more than 85% of essential oil was trans-Anethole. Also, it has also been reported that the antioxidant activity of essential oil of *P. anisum* extracted from their seeds was higher than alpha-Tocopherol. Therefore, the results of this study are consistent with all the studies mentioned [11, 39].

One report was found antifungal activity of *P. anisum* essential oil on *Fusarium solani* that were introduced by persian name of anarijeh [17]. Also, another report of improper use of local name of anarijeh were observed on the application of essential oil of *P. affinis* [13]. In both of these studies, the exact identification of plant species was not mentioned and the type of compounds was not introduced. Therefore, the results can not be judged scientifically and it is better to evaluate the plant samples due to their incorrect naming.

Some doubtful researches were carried out on *P. affinis* which introduced by the local name of anarijeh. In these reported studies, mentioned that the plant was collected from the north of Iran and also the extraction was done from the aerial part of the plant [20-22]. The used parts of plants are noticable here. only seeds in *P. affinis* have medicinal uses and the aerial part of the plant is not used while the aerial parts of *F. subpinnata* is known due to its trapeutical properties [15, 16]. So it is concluded that the used plants in this studied were *F. subpinnata* and authors have made a great mistake in introducing the plant.

In another research, the antioxidant effects and phenolic and flavonoid content of extracts and essential oils in *P. affinis* which mistakenly introduced as anarijeh were studied [18]. Due to the lack of exact herbarium specimens and mistakes that occurred in the naming of the plant, the results of this study is questionable and need to be examined more carefully.

The studied medicinal plants have different phytochemical compounds with antioxidant properties. The presence of these compounds has been regarded as biologically effective factors. As a result, the antioxidant power of plant essential oils is considered to be directly related to the presence of biological compounds in the essential oils [28].

Antioxidant properties of studied species in both methods were investigated and shown that the essential oil of the aerial part of *F. subpinnata* and seeds of *P. anisum* were not very different

from each other. Both plants have less antioxidant power than vitamin C and E and therefore, they can be used as a moderately effective agent in the food and pharmaceutical industries.

5. Conclusion

There is a common mistake in Iran in identifying the medicinal plants *F. subpinnata* and *P. anisum*, which they call by the same name of anarijeh. On the other hand in many scientific reports, the plant materials are not correctly identified by an expert botanist. The herbarium samples do not exist and therefore so many mistakes were occured here. In this study, the essential oils of both *F. subpinnata* and *P. anisum* plant species were analyzed and the most prominent compounds and antioxidant potential of each species were introduced. Therefore in order to prevention of mistakes or deception, major compounds and properties of essential oils can be used as a suitable phytochemical indicator to identify plants or derived products of them. Also, due to the antioxidant properties of both plants the consumption of these plants can be useful in everyday human food. As a final conclusion, cooperation or consulting with an expert botanist and presentation of voucher specimen indicating the correct identification of plant is necessary and highly recommended.

Author contributions

P. M. and M. GH N. designed and performed the experiment and participated in writing of manuscript. The analysis of GC/MS were carried out by E. K. and A. A. guided aspects of the research.

Conflict of interest

The authors declare that there is no conflict of interest.

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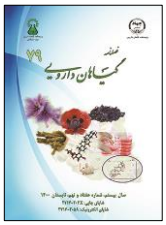

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مقاله تحقیقاتی

تفاوت بین گیاهان دارویی *Froriepia subpinnata* (Ledeb.) Baill. و *Pimpinella anisum* L. با نام عمومی اناریجه بر اساس ترکیبات عمده اسانس؛ شاخصی برای رفع ابهامات

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اطلاعات مقاله

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Froriepia subpinnata
Pimpinella anisum
کروماتوگرافی گازی متصل به طیف‌سنج جرمی

چکیده

مقدمه: گیاهان *Froriepia subpinnata* (Ledeb.) Baill. و *Pimpinella anisum* L. دو گیاه دارویی مهم از تیره چتریان هستند. این دو گیاه به دلیل نام عمومی فارسی مشابه «اناریجه»، در بازار گیاهان دارویی ایران و حتی در گزارش‌های علمی به اشتباه به جای یکدیگر استفاده می‌شوند. **هدف:** معرفی دقیق ریخت‌شناختی و ترکیب اسانس همراه با اثرات آنتی‌اکسیدانی گیاهان *F. subpinnata* و *P. anisum* و جلوگیری از اشتباهات و تقلبات در گیاهان مذکور و محصولات ناشی از آنها می‌باشد. **روش بررسی:** بخش هوایی گیاه *F. subpinnata* و بذر گیاه *P. anisum* پس از خشک شدن، پودر و اسانس آنها با استفاده از روش تقطیر توسط دستگاه کلونجر به مدت ۳ ساعت استخراج و سپس ترکیبات تشکیل‌دهنده اسانس توسط کروماتوگرافی گازی متصل به طیف‌سنج جرمی مورد ارزیابی قرار گرفت. همچنین، با استفاده از روش‌های DPPH و FRAP قدرت مهار آنتی‌اکسیدانی اسانس‌ها تعیین شد. **نتایج:** در اسانس گیاه *F. subpinnata* ترکیبات پارا سیمن –۸– ال (۵۱/۱۳ درصد)، آلفا ترپینولن (۷/۶۹ درصد) و لیمونن (۶/۸۳ درصد) یافت شدند. در اسانس گیاه *P. anisum* نیز ترکیبات ترانس آنتول (۸۵/۶۵ درصد) و کارون (۵/۳۱ درصد) ترکیبات اصلی اسانس را شامل می‌شدند. قدرت مهار آنتی‌اکسیدانی به دو روش DPPH و FRAP در اسانس هر دو گیاه با غلظت ۲۵۰ میکروگرم در میلی لیتر تقریباً مشابه و در گیاه *F. subpinnata* به ترتیب به میزان ۵۳/۰۳ و ۶۲/۷۲ درصد و برای گیاه *P. anisum* به ترتیب به میزان ۵۰/۲۷ و ۵۹/۹۱ درصد اندازه‌گیری شد. **نتیجه‌گیری:** نوع و میزان ترکیبات اصلی اسانس در گیاهان مطالعه شده، می‌تواند به عنوان مبنایی دقیق برای تشخیص افتراقی این گیاهان قلمداد شود. جهت شناسایی صحیح و جلوگیری از اشتباهات و تقلبات در گیاهان و محصولات گیاهی، می‌توان از همه این تفاوت‌ها به عنوان یک نشانگر فیتوشیمیایی مناسب استفاده کرد.

مخففها: GC/MS، کروماتوگرافی گازی متصل به طیف سنج جرمی؛ EOs، روغن‌های فرار

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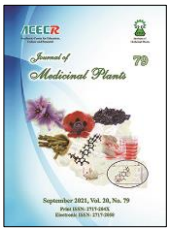

Research Article

Therapeutic potential of ointment containing methanol extract of *Lamium album* L. on cutaneous wound healing in rats

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ABSTRACT

Background: Proper wound management which improves the quality of life and reduces patient costs is required. *Lamium album* L. has been used in traditional medicine to heal skin wounds and recent studies show anti-inflammatory, haemostatic and antimicrobial properties of this plant. **Objective:** The effect of the ointment containing methanol extract of *L. album* aerial parts was evaluated on the full-thickness wound healing in rat model. **Methods:** Thirty-two Wistar rats (250-300 g) were used to be created a square full-thickness wound on the dorsal cervical area and randomly divided into four groups: I; control, II; ointment base, III; 1 % phenytoin cream and IV; 5 % *L. album* ointment. Wound size for determination of the percent of wound healing was measured on days 3, 5, 7 and 12 of the experiment. The excisional biopsies were evaluated histopathologically on the 12th day of treatment according to the Abramov score method. **Results:** The herbal ointment significantly increased fibroblast maturation, collagen deposition and neovascularization compared to the control group. The wound healing rate was significantly increased in the group treated with *L. album* ointment, same as phenytoin group, on days 3, 7 and 12. Based on the spectrophotometric analysis, the extract contains phenol and flavonoid compounds. **Conclusion:** It seems that *L. album* could be considered as a new candidate for further studies in the field of wound healing.

1. Introduction

A wound is defined as a tissue injury with a disruption in anatomical integrity. Physiologically the healing process begins immediately after injury to replace or restore the damaged tissues. The healing process can be

Abbreviations: WHR, Wound Healing Rate; HPF, High Power Field; LAO, *Lamium album* Ointment

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mainly divided into inflammation, proliferation and maturation phases. In the first phase, the blood vessels contract in the area and a clot is formed. Then the blood vessels dilute to allow the white blood cells and other essential elements to reach the wounded area. During this phase, the inflammation is occurred through the cells activities. In the proliferation phase, tissue regeneration, the new collagen and blood vessels development occurs. Maturation is the final phase and occurs once the wound has closed. During this phase cellular activity reduces and the remodeling of collagen is seen [1]. Because impairment or failure of wound healing during the injuries, burns or surgery, can lead to substantial morbidity and even mortality in patients [2], it is important to conduct research to accelerate wound healing. To stimulate the healing process and reduce scar formation, several wound care products and therapies have been developed. The recent therapies comprise grafts, modern dressings, bioengineered skin substitutes, and cell growth factors which exert high medical costs [3]. Despite the recent advances in wound care products, traditional therapies based on natural origin compounds, such as plant extracts are interesting alternatives [4].

Lamium album L. is a perennial flowering plant, which is widespread throughout Asia (including Iran), Europe, and Africa. It is belonging to the Lamiaceae family and commonly known as white nettle [5, 6]. The young shoots, leaves and flowers of this plant are edible and can be consumed raw or cooked for health benefits and nutritional value [7]. Different *Lamium* species have long been used in traditional and folk medicine for trauma [8], fracture [8], hemorrhage [9], gastric ulcer [10], wounds [11], skin [12] and joints swelling [13]. Traditionally, *L. album* is used in skin wound care for many years [5, 9] and recent investigations show anti-inflammatory [14], haemostatic [15], antimicrobial [16], and cell-stimulating properties of the plant [17]. It is reported that phenolic compounds are responsible for these activities [18].

Considering the above properties, there has been no comprehensive study on the wound healing activities of *L. album*. The aim of the present work was to assess the healing properties of 5 % *L. album* ointment (LAO) on the full-thickness wound in Wistar albino rats by determination of wound healing rate (wound closure) and histological evaluation of skin samples. Moreover, the spectrophotometric analysis was performed for determination of the total phenol and flavonoid contents of *L. album* methanol extract.

2. Materials and Methods

2.1. Plant material and extraction

The aerial parts of *L. album* were collected from Tallarposht village, Mazandaran province, Iran, during the flowering stage (May 2019). A voucher specimen (no. E1-36-4171) was deposited at the Herbarium of the Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran. The amount of 100 g of the dried sample was powdered and macerated in methanol for 48 h (four times). The resulted extracts were filtered, then concentrated by a rotary evaporator and defatted with hexane. Finally, the extract was freeze-dried to remove the residual moisture and stored at -20°C for further uses.

2.2. Determination of total phenol and flavonoid contents

Total phenol and flavonoid contents of the extract were determined by Folin-Ciocalteu and aluminum chloride methods, respectively [19,

20]. The standard calibration curves were plotted using different concentrations of gallic acid and quercetin (6.25-200 µg/mL).

2.3. Topical ointment preparation

The *L. album* ointment (5 %) was prepared by mixing 5 g of the extract with 10 g of propylene glycol by mortar and pestle. Finally, the amount of 85 g of Eucerin was added and mixed to form homogeneous ointment, then transferred to the suitable container [21].

2.4. Animals

Thirty-two Wistar albino rats (female, 250-300 g) were used in this study and kept in standard conditions (12 h light/dark cycles at room temperature (20°C) with 50 % humidity) and allowed to consume standard laboratory food and water. All experiments were approved by the Ethics Committee of Mazandaran University of Medical Sciences, Sari, Iran (Approval Code IR.MAZUMS.REC.1397.3191) and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.5. Study design

The wound model was chosen according to our previous study [22]. Rats were anaesthetized using an intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The dorsal skin (neck area) of the rats was depilated and disinfected with 70 % ethanol, a square full-thickness wounds (1.5 × 1.5 cm dimension) was excised under aseptic conditions using a surgical blade. Immediately after the surgery, the animals were randomly divided into 4 experimental groups (n = 8) as follows: (I) control group, without treatment; (II) the ointment base treated group; (III) Phenytoin group, rats treated with 1 % phenytoin cream; (IV) LAO group, rats treated with 5 % W/W *L. album* ointment. The phenytoin cream, ointment base and LAO ointment were applied topically once daily on the wounded area by the 1st to 12th days of the study. The wounds were uncovered during the examination. The rats were maintained in their separate experimental units which were cleaned and dried daily to prevent the secondary infection.

2.6. Determination of the rate of wound healing

On the 3rd, 5th, 7th and 12th days, the wounded area was covered with a transparent sheet to mark the area around the wound. Then using graph paper, the number of squares within the wound area was counted. The rate of the wound healing (WHR) was determined using the following equation:

$$WHR\ (\%) = [(Wo - Wu)/Wo] \times 100$$

Where, Wo and Wu mean the original and unhealed area of the wound, respectively [23].

2.7. Histopathological analysis

To separate the biopsy specimen, about 5 mm of healthy skin with the repaired area was collected from the wound bed. The samples were fixed in 10 % formalin, dehydrated in alcohol, clarified in xylene and embedded in paraffin. The fixed skin tissues were sectioned at 5 µm thickness and stained with hematoxylin and eosin for histopathological assessment. The histological images were analyzed according to the Abramov score method [24].

The following scoring criteria were used for histological assessment of the wound. Acute inflammation grading were scored as 0: none; 1: scant; 2: moderate; 3: abundant. Granulation tissue fibroblast maturation grading were scored as 0: immature; 1: mild maturation; 2: moderate maturation; 3: fully maturate. Collagen

deposition grading were scored as 0: none; 1: scant; 2: moderate; 3: abundant. Epidermis formation grading were scored as 0: none; 1: partial; 2: complete but immature or thin; 3: complete and mature. Neovascularization grading were scored as 0: none; 1: up to five vessels per high-power field (HPF); 2: 6-10 vessels per HPF; 3: more than 10 vessels per HPF. Keratin layer formation grading were scored as 0: none; 1: partial; 2: complete but immature or thin; 3: complete and mature.

2.8. Collagen assessment

12 days after the surgery, the skin samples stained using Light Green staining for assessment of the collagen content. The semi-quantitative evaluation was assessed by densitometry using MacBiophotonics Image J 1.41a software. The collagen fiber intensity was measured by the green color stains. The ratio of the stained area to the entire field was assessed [23].

2.9. Data analysis

Data were analyzed by SPSS software (version 15). All data were expressed as mean ± standard deviation (M ± SD). One-way analysis of variance and Tukey tests were used. $P < 0.05$ was considered statistically significant in all groups.

3. Results

3.1. Total phenolic and flavonoid contents

The concentration of total phenol was calculated 96.25 ± 0.009 mg gallic acid ($y = 0.0012x + 0.0625$, $R^2 = 0.9996$) equivalents per gram of dried extract. The total flavonoid content was calculated 77.887 ± 0.014 mg quercetin ($y = 0.0071x + 0.055$, $R^2 = 0.9989$) equivalents per gram of dried methanol extract.

3.2. Macroscopic evaluation of the wound healing

No obvious infections and complications were observed in any animals during the experiment. The necrotic tissue was not seen in the treated group with the herbal ointment while it was clearly visible in the control group from the early days.

A statistically significant difference ($P < 0.05$) was seen in the epithelization of the wound between the treatment and control groups. New epithelium with the pink color was observed at the edges of the wound in *L. album* and phenytoin groups (Fig. 1).

3.3. Evaluation of the rate of wound healing

Table 1 shows the rate of wound healing in different days of treatment. The wounded area was significantly ($P < 0.05$) decreased during days 3, 7 and 12 in the groups treated with the LAO and phenytoin compared to control (Table 1). On day 12, the wound had contracted down to a minimal area in the treatment groups (Fig. 2). The rate of wound healing was increased gradually during the inflammatory phase in all groups, however it was significant ($P < 0.05$) in the treated with LAO and phenytoin. On day 12, a noticeable increase was observed in the healing rate during the proliferative phase in the LAO and phenytoin groups ($P < 0.05$) compared to the control.

3.4. Microscopic observation

The photomicrographs of the wounded area in different groups are shown in Fig. 3. The wound score was calculated in each group and the average scores of wound healing criteria were analyzed (Fig. 4). Based on the findings, the granulation tissue formation appeared during the repair phase. The keratin filaments, neovascularization and the neoepiderm were

appeared. The proliferation of the fibroblast cells was induced significantly ($P < 0.05$) in the treated group with LAO compared to the control. The granulation tissue increased and covered the wounded area in phenytoin and LAO groups. Moreover, the maturation of the granulation tissue was increased in the LAO group compared to the control and phenytoin groups. The number of new blood vessels per HPF × 40 in LAO and phenytoin groups was increased rapidly.

Based on Fig. 5, the collagen fibers, which appear in light green color were deposited significantly in LAO and phenytoin ($P < 0.05$) groups compared with control. The analysis of ImageJ showed a 50 % increase in the collagen content of LAO group.

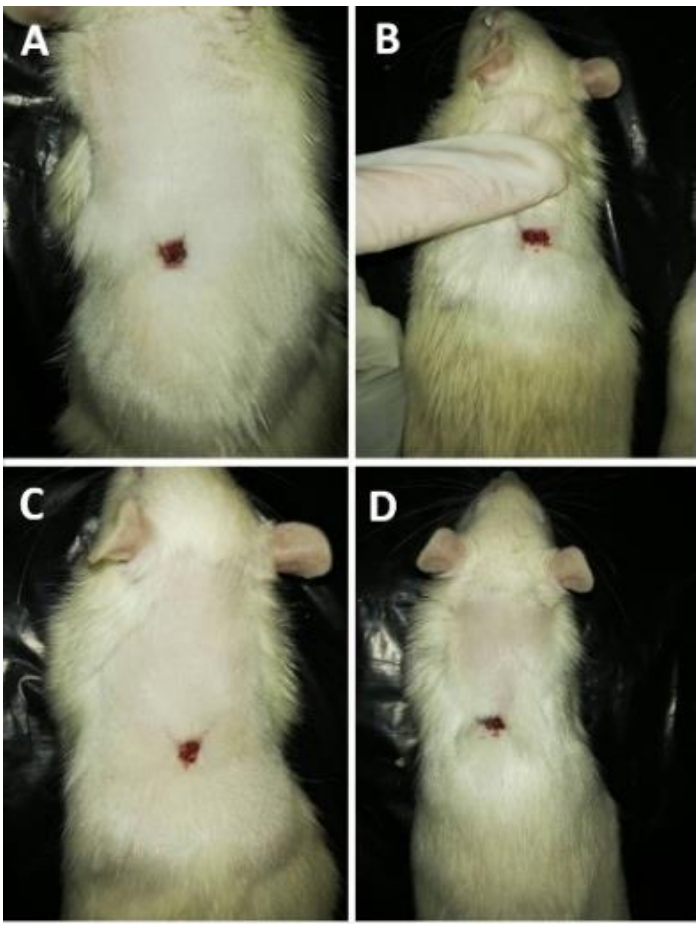

Fig. 1. The morphology of the wound area during the healing processes in different groups on the 12th day of the treatment. A: Control group; B: The ointment base group; C: Phenytoin group; D: *L. album* ointment group. The obvious reduction of the wound size was observed in the treated group with the *L. album* ointment.

Table 1. The rate of wound healing in rats treated with *Lamium album* ointment

Groups	3th day	5th day	7th day	12th day
Control	14 ± 2.6	25 ± 3.5	44 ± 2.16	69 ± 2.3
Ointment base	17 ± 4.32	25 ± 4.16	47 ± 3.74	72 ± 3.86
Phenytoin	20 ± 2.56*	30 ± 3.79	52 ± 3.19*	75 ± 3.04*
5% *L. album* ointment	19 ± 2.76*	28 ± 2.94	50 ± 2.7*	77 ± 1.79*#

The wound healing rate was expressed as mean ± S.D with the surface area in cm^2. (*) = $P < 0.05$, significant against the control group. (#) = $P < 0.05$, significant against the ointment base group.

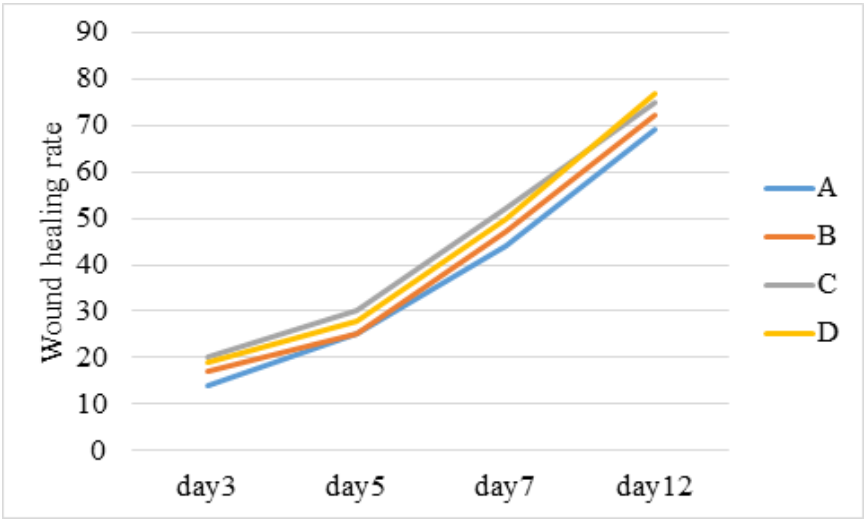

Fig. 2. Changes in the areas of the wounds on days 3, 5, 7, and 12. A: Control group; B: The ointment base group; C: Phenytoin group; D: *L. album* ointment group.

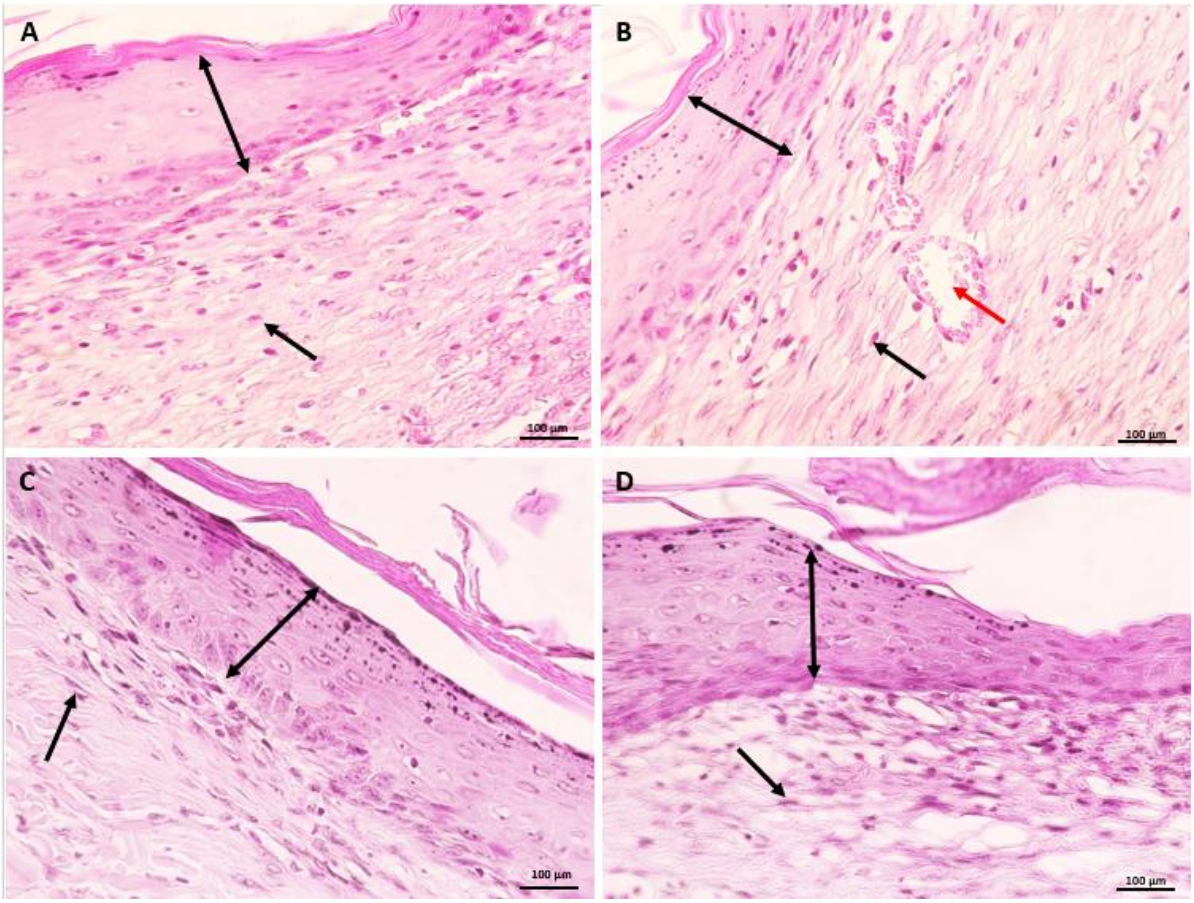

Fig. 3. The photomicrographs of the biopsy specimens from the animals on day 12 of the surgery. A: Control group; B: The ointment base group; C: Phenytoin group; D: *L. album* ointment group. The new epidermis layer (↕ arrow) is seen in LAO and phenytoin groups. The granulation tissue formation (↓ arrow) and neovascularization (↓ arrow) is clearly seen in the treated groups. H & E. Mag; × 40. Scale bar = 100 µm.

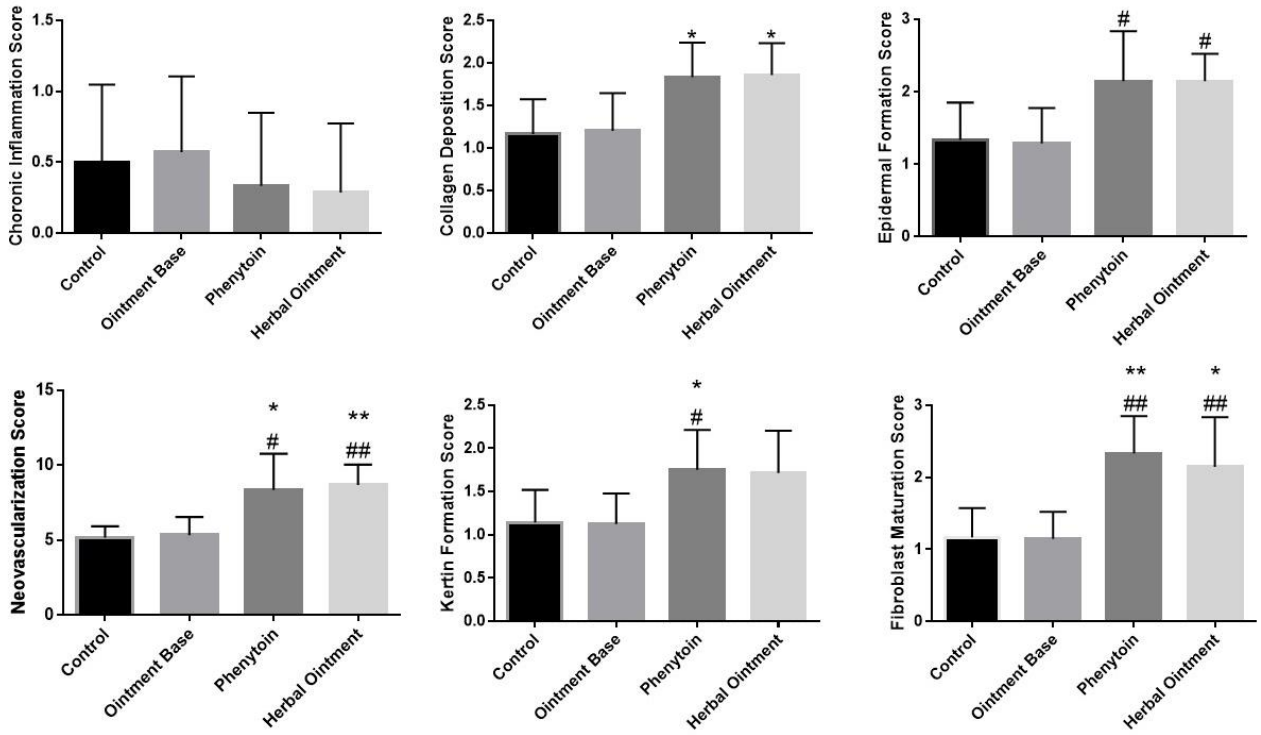

Fig. 4. Effects of *L. album* ointment on the wound healing criteria compared to the control. All values are expressed as mean ± SD. (*) $P < 0.05$ and (**) $P < 0.01$, significant against control group. (#) $P < 0.05$, (##) $P < 0.01$ significant against ointment base group**.**

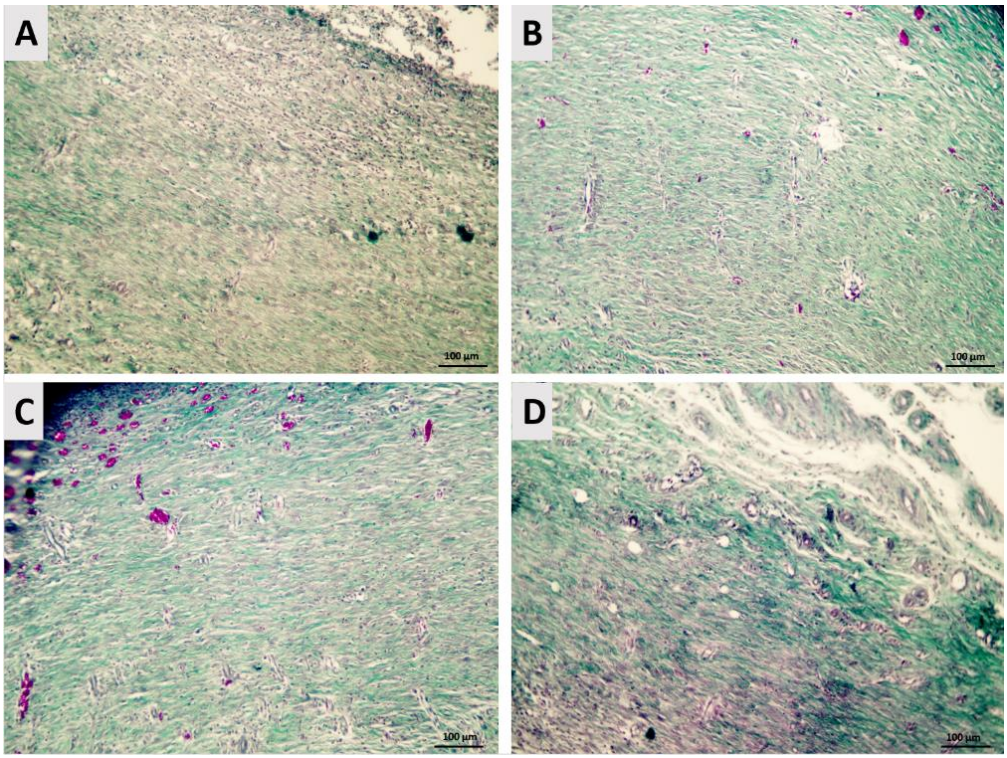

Fig. 5. The amount of collagen deposition in the skin tissue on the 12th day of the study. A: Control group; B: Ointment base group; C: 1 % Phenytoin; D: 5 % *L. album* ointment. Light Green staining, Magnification; × 10. Scale bar = 100 µm.

4. Discussion

The wound healing properties of various herbal formulations have been evaluated in recent years. Medicinal plants cause wound healing by different mechanisms such as wound contraction, promoting epithelization and collagen deposition, increasing granulation tissue and collagen synthesis [25].

The present study showed that the 5 % *L. album* ointment accelerates the wound healing in acute wound model in rats. The topical administration of LAO to animals significantly increased the rate of the wound healing compared to the control group during the days 3, 7 and 12. The results showed the statistically significant differences in histological criteria (collagen deposition, neovascularization and fibroblast maturation) of the groups treated with the LAO and phenytoin compared to the control. The top score fibroblast maturation and proliferation, tissue granulation, dermal and epidermal regeneration, enormous angiogenesis and collagen synthesis were observed in the group treated with LAO.

Based on the recent studies, the topical application of the plant extracts or herbal formulations is effective for the wound healing process [26]. It was reported that the topical application of 5 % *Salvia officinalis* (Lamiaceae) leaf extract ointment in excision and incision wound model, significantly ($P < 0.05$) improves the wound contraction and period of re-epithelialization in Wistar rats via increasing the new vessel formation and Fibroblast distribution. The high levels of phenolic compounds and flavonoids were found in the extract [27]. The wound healing properties of 1 % ointment prepared from *S. kronenburgii* and *S. euphratica* ethanol extracts were evaluated by Güzel et al. [28]. They reported that the topical application of the ointments, could contract the excision wounds (more than 99 %) in rats compared to the standard drug. The re-epithelialization and angiogenesis were significantly ($P < 0.001$) increased in the treated group with the herbal ointments. The spectrophotometric analysis on the extracts revealed the presence of phenolic and flavonoid constituents.

In the present study, the spectrophotometric analysis of the extract from *L. album* aerial parts, revealed the presence of phenols with the total amount of 96.25 ± 0.009 mg gallic acid equivalents per gram of dried extract.

Based on the recent studies, the total amount of phenolic compounds in the methanol extract of *L. album* flowers [29], and other *Lamium* species aerial parts such as *L. amplexicaule*, *L. galactophyllum*, *L. macrodon* [30] and *L. purpureum* [31], were in the range of 94.75 to 192 mg gallic acid per gram of dried extract.

The present result showed that total flavonoid content in the methanol extract of *L. album* aerial part was 77.887 ± 0.014 mg quercetin equivalents per gram of dried extract. Based on two recent studies, the total flavonoid contents of *L. album* aerial parts were reported 79.83 and 82.11 mg quercetin per gram of dried methanol extract [32, 33].

Collagen is functionally one of the key protein in extracellular dermal matrix which mainly produced by fibroblast cells [34]. Collagen deposition in connective tissues can regenerate the skin and heal the wounds [35]. Several plant extracts have been reported to possess wound healing activity through the fibroblast proliferation and collagen deposition [26]. In a

recent study, cytotoxicity and cytostatic activities of the ethyl acetate and methanol extracts from *L. album* flowers against human normal skin fibroblasts were evaluated. Both extracts showed no toxic effects in the range of applied concentrations (25-225 µg/ml). Moreover, the methanol extract exhibit DPPH free radical scavenging activity and was rich in flavonoids and phenolic acids. Therefore, it was suggested that the *L. album* extracts, can be used in preparation of wound healing and skin protective formulations [17].

Control of hemorrhage following the injury is the prime concern in the management of wounds [36]. Various published results indicate that the plant extracts can promote the blood clotting in injuries [26]. Recently, the haemostatic activity of butanol extracts from the aerial parts of two *Lamium* species (*L. album* and *L. purpureum*) have been investigated by tail bleeding time determination and acenocoumarol-carrageenan test. Both of the extracts demonstrated haemostatic activity in the tail bleeding test. Moreover, the *L. album* extract showed the protective effects in acenocoumarol-carrageenan test, compared to vitamin K. They indicated that 8-acetyl shanzshiside, the iridoid glycoside detected in the extracts, might be responsible for the observed haemostatic activity [15].

Several phenol compounds especially phenyl propanoid glycoside (such as verbascoside and lamalboside) and flavonoids (such as apigenin, kaempferol and quercetin glycosides) were detected and isolated from *L. album* aerial parts [37]. Many in vivo studies, including the murine cutaneous inflammation, incisional and excisional wound model on rats, demonstrated the wound healing activities of apigenin, kaempferol and quercetin (topical usage) via collagen synthesis of fibroblasts [38], reepithelialization, angiogenesis [39] and decreasing inflammatory cells in the wounded area [40].

Verbascoside has been widely used in the cosmetics with potential anti-inflammatory, antioxidant and photoprotecting activities [41, 42]. It has been previously shown that the topical application of a semi-synthetic derivative of this compound was able to promote the wound healing in rats [41].

It could be suggested that the wound healing activities of *L. album* extract may be attributed to the presence of phenolic and flavonoid constituents.

5. Conclusion

It is concluded that the 5 % ointment from the *L. album* methanol extract, same as 1 % phenytoin cream, provided considerable wound healing effects on the full-thickness wound in rats via increasing the fibroblast maturation, collagen deposition and neovascularization compared to the control group. The spectrophotometric evaluation of the extract showed considerable phenolic compounds which could be involved in the plant healing activities. It is recommended that the *L. album* extract could be as a candidate for treatment of acute wounds. Although, it is needed to evaluate the different doses of the *L. album* extract in the other wound models and to identify the effective constituents of the plant.

Author contributions

S. Sh. and F. TA. designed the study and analyzed the results. N. H. and F. M. performed

the material preparation and data collection. R. E. supervised the laboratory work (ointment preparation). F. M. wrote the first draft of the manuscript. S. Sh. and F. TA. provided the critical reading and insightful recommendations of the manuscript. All of the authors have read the final manuscript and approved the submission.

Conflict of interest

The authors declare that there is no conflict of interest.

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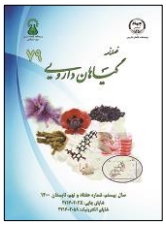

مقاله تحقیقاتی

پتانسیل درمانی پماد حاوی عصاره متانولی گیاه گزنه سفید (*Lamium album* L.) بر ترمیم زخم پوستی در موش‌های صحرایی

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اطلاعات مقاله

گل‌واژگان:
گزنه سفید
پماد
ترمیم زخم
فلاونوئید
موش صحرایی

چکیده

مقدمه: مدیریت مناسب زخم که کیفیت زندگی را بهبود بخشد و هزینه‌های درمانی بیماران را کاهش دهد، مورد نیاز است. گیاه گزنه سفید در طب سنتی جهت التیام زخم‌های پوستی استفاده می‌شود و مطالعات اخیر نشان‌دهنده اثرات ضد التهابی، هموستاتیک و ضد میکروبی این گیاه می‌باشد. **هدف:** اثر پماد حاوی عصاره متانولی از اندام هوایی گیاه گزنه سفید، بر ترمیم زخم تمام ضخامت حاد پوستی در مدل موش صحرایی بررسی شد. **روش بررسی:** از ۳۲ موش صحرایی ویستار (۲۵۰-۳۰۰ گرم) برای ایجاد زخم تمام ضخامت مربعی در ناحیه پشتی گردن استفاده شد و به طور تصادفی به چهار گروه تقسیم شدند: I؛ کنترل، II؛ پایه پماد، III؛ کرم ۱ درصد فنی توئین و IV؛ پماد ۵ درصد گزنه سفید. اندازه زخم جهت تعیین درصد ترمیم زخم در روزهای ۳، ۵، ۷ و ۱۲ از شروع آزمایش اندازه‌گیری شد. نمونه های بافتی برش داده شده از نظر هیستوپاتولوژیکی در روز ۱۲ درمان بر اساس روش Abramov score ارزیابی شدند. **نتایج:** پماد گیاهی به میزان قابل توجهی بلوغ فیبروبلاست، رسوب کلاژن و نورگزایی را در مقایسه با گروه کنترل افزایش داد. میزان ترمیم زخم در گروه تحت درمان با پماد گیاهی مشابه با گروه فنی توئین، به میزان قابل توجهی در روزهای ۳، ۷ و ۱۲ افزایش یافت. بر اساس آنالیز اسپکتروفتومتری، عصاره گیاه حاوی ترکیبات فنولی و فلاونوئیدی می‌باشد. **نتیجه‌گیری:** به نظر می‌رسد گیاه گزنه سفید می‌تواند به عنوان یک کاندیدای جدید برای مطالعات بیشتر در زمینه ترمیم زخم مورد توجه قرار گیرد.

مخففها: WHR، میزان ترمیم زخم؛ HPF، با بزرگنمایی بالا؛ LAO، پماد گزنه سفید

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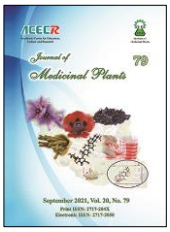

Research Article

Essential oil analysis and biological activities of the aerial parts of *Zygophyllum eichwaldii* C. A. Mey., a native plant from Iran

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ABSTRACT

Background: *Zygophyllum eichwaldii* C. A. Mey. is a medicinal plant from Zygophyllaceae family. This family consists of 27 genera and 285 species. **Objective:** The present study was performed to evaluate the chemical composition of the essential oil of *Z. eichwaldii* and evaluation of biological activities of its extracts. **Methods:** Essential oils extraction of *Z. eichwaldii* aerial parts was performed by three different procedures and its chemical composition were evaluated by GC and GC-MS analyses. Evaluation of the biological activities was carried out by spectrophotometric methods. **Results:** GC-MS analysis showed menthol, thymol and palmitic acid as major components of the plant essential oils. In biological activity evaluation, while methanol extract showed moderate to week potency in antioxidant assessments, the ethyl acetate fraction was strong in these tests (IC_{50} = 178.63 µg/ml in DPPH assay and 70.52 % inhibition in β-carotene/linoleic acid tests). This fraction also showed significant phenol, flavonoid and tannin contents (99.83, 118.29 and 188.05 µg/mg, respectively). Also, plant extracts exhibited considerable antimicrobial activities against most selected bacteria. **Conclusion:** Significant amount of phenol, flavonoid and tannin compounds in the ethyl acetate fraction and high antimicrobial activity against most bacterial strains, candidates this plant as a good case for further studies in this respect.

1. Introduction

Natural products, such as plants extract, either pure compounds or standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity [1]. According to the World Health Organization (WHO), more than 80 % of the world's population relies on

Abbreviations: DPPH, 2,2-Diphenyl-1-PicrylHydrazyl; BHT, Butylated Hydroxyl Toluene; DMSO, Dimethyl Sulphoxide; HD, Hydrodistillation; SDE, Simultaneous Distillation Extraction; SFME, Solvent Free Microwave Extraction; GC, Gas Chromatography; GC/MS, Gas Chromatography/Mass Spectrometry; IROST, Iranian Research Organization for Science and Technology; NA, Nutrient Agar; SDA, Sabouraud Dextrose Agar; PDA, Potato Dextrose agar; BHI, Brain Heart Infusion; SDB, Sabouraud Dextrose Broth; MIC, Minimal Inhibition Concentration; MBC, Minimum Bactericidal Concentration; IC_{50}, Mean Inhibitory Concentration; SD, Standard Deviation; RI, Retention Indices

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traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia represents a long history of human interactions with the environment. Due to the development of adverse effects and microbial resistance to the chemically synthesized drugs, men turned to ethnopharmacognosy. Many beneficial biological activities such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing were reported from the plants. In many cases, people claimed good benefit for certain natural or herbal products. However, clinical trials are necessary to demonstrate the effectiveness of a bioactive compound to verify this traditional claim. The first steps to utilize the biologically active compound from plant resources are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological evaluation and clinical evaluation [2].

The genus *Zygophyllum* belongs to the family Zygophyllaceae. According to some botanical sources, the number of genera belonging to this family is about 27 genera and 285 species, most of which are distributed in arid and semi-arid regions of the world. There are more than 9 species of herbaceous, semi-shrub and shrub plants of this genus in Iran.

Zygophyllum eichwaldii grows on sand dunes in relatively humid parts of the deserts. Geographical distribution of this plant is the central parts of the Iranian-Turanian zone (central deserts in Isfahan province and Khorasan Razavi province), Central Asia and Afghanistanat an altitude range of 780 to 1200 meters above the sea level. Different species of *Zygophyllum* are used as anti-diabetic, anti-spasmolytic, anti-eczema, antiseptic, anti-diarrheal and anti-inflammatory. *Z. eichwaldii* has antiseptic, anti-eczema and anti-diabetic activities [3].

In literature, there is no report on essential oil composition and biological activities of *Z. eichwaldii*. In the present study, essential oil chemical composition of *Z. eichwaldii* is reported. Meanwhile, extraction of aerial parts of this plant was prepared and evaluated for antioxidant and antimicrobial activity.

2. Materials and Methods

2.1. Plant material

The aerial parts (leaves, stems & flowers/inflorescences) of *Z. eichwaldii* were collected in September 2019 from sand dunes of northern Aran and Bidgol deserts, around Kashan (Isfahan province, Iran) at an altitude of ca. 980 m. The plant was authenticated and its voucher specimen was then deposited in the herbarium of Kashan Botanical Garden, Iran (Voucher No. HKBG: 2016).

2.2. Preparation of the extracts

2.2.1. Isolation of the essential oils

Three portions (150 g each) of the crushed plant material were individually subjected to three different essential oil extraction methods: usual hydrodistillation (HD) for 3.5 h using a Clevenger-type apparatus, simultaneous distillation extraction (SDE, a well-known distillation-extraction method for volatile materials extensively reviewed by Alain Chaintreau [4] with *n*-pentane as solvent for 2.5 h, and solvent free microwave extraction (SFME) in power 850 watts for 20 min. After decanting and drying over anhydrous sodium sulphate, yellow colored oils were recovered and stored at low temperature (4 ºC) under nitrogen atmosphere in amber vials and were used for analyses within a few days.

2.2.2. Extraction and fractionation

Dried powdered aerial parts of *Z. eichwaldii* (2 Kg) were subjected to extraction by maceration with enough volumes of methanol (7 L) for four times, to obtain total methanol extract (444.00 g). Five fractions were obtained from elution of total methanol extract by *n*-hexane (HE; 41.00 g), chloroform (CL; 18.50 g), ethyl acetate (EA; 7.25 g) and *n*-butanol (BU; 91.00 g), respectively and the residue was labeled as methanol extract residue (ME; 160.00 g). The obtained extracts were concentrated using a rotary evaporator under the reduced pressure at 40°C. The concentrated fractions were kept at refrigerator temperature for further investigations.

2.3. Chromatographic analysis

2.3.1. Gas chromatography (GC) analysis

Essential oil samples obtained from aerial parts of *Z. eichwaldii* were analyzed using an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a FID detector using a HP-5MS 5 % phenylmethylsiloxane capillary column (60 m, 0.25 mm, 0.25 µm film thickness; Restek, Bellafonte, PA). Oven temperatures were programmed as follows: 60-246 ºC (3 ºC/min). Injector and detector temperatures were set at 220 ºC and 290 ºC, respectively. Ultra-high pure helium (flow rate: 1 ml/min), hydrogen (flow rate: 40 ml/min) and nitrogen (flow rate: 50 ml/min) were used as carrier, fuel and make up gases respectively and compressed air (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 in the splitless mode. Peak area percent of each compound relative to the area percent of the entire spectrum (100 %) were used for obtaining their quantitative data.

2.3.2. Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis of the oils were carried out on an Agilent HP-6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent HP-5973 mass selective detector in the electron impact mode (ionization energy: 70 eV), operating under the same conditions as described in 2.4.1., using a HP-5MS 5 % phenylmethylsiloxane capillary column (30 m, 0.25 mm, 0.25 µm film thickness; Restek, Bellafonte, PA). Retention indices were calculated for all components using a homologous series of *n*-alkanes injected in conditions equal to the sample one. Identification of components of essential oils were based on retention indices (RI) relative to *n*-alkanes and computer matching with the NIST14.L and W10N14.L libraries, as well as comparisons of the fragmentation pattern of the mass spectra with data published in the literature [5].

2.4. Antioxidant activity

2.4.1. Radical scavenging assay

Radical-scavenging activities of the extracts were determined using a published 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity assay method with minor modifications [6].

Absorbance values were recorded on an ultraviolet and visible (UV–Vis) spectrometer at 517 nm using a blank containing the same concentration of DPPH radicals. Inhibition percentages (I %) of DPPH radicals were calculated using following equation:

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

All tests were carried out in triplicate and IC_{50} values were reported as means ± SD of triplicates.

2.4.2. β-Carotene/linoleic acid bleaching assay

The method described by Miraliakbari and Shahidi [7] was used for the evaluation of *β*-carotene/linoleic acid bleaching ability of the plant samples with slight modifications [8]. The absorbance values were measured at 470 nm onan ultraviolet and visible (UV–Vis) spectrometer. Antioxidant activities (inhibition percentages, I %) of the samples and BHT (positive control) were calculated using the equation:

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

All tests were carried out in triplicate and inhibition percentages were reported as means ± SD of triplicates.

2.5. Quantitative estimation of total phenol, flavonoids and tannin contents

2.5.1. Estimation of total phenol content

Total phenol content of *Z. eichwaldii* extracts were calculated using published colorimeteric procedure involving Folin-Ciocalteu phenol reagent and gallic acid standard [9].

According to this test, total phenol compounds content of each extract, as gallic acid equivalent, were determined using its absorbance measured at 760 nm as input to the obtained standard curve and following equation.

$$\text{Absorbance} = 0.0012 \times \text{gallic acid (µg)} + 0.0033$$

All tests were carried out three times and obtained values as gallic acid equivalents were reported as mean ± SD of three determinations.

2.5.2. Estimation of total flavonoid content

The flavonoids content of the *Z. eichwaldii* extracts were determined by Aluminium chloride method using quercetin as reference compound [10]. The absorbances were measured against the blank at 415 nm using UV-Visible spectrophotometer. A standard graph was plotted using various concentrations of quercetin and their corresponding absorbance with the following equation:

$$\text{Absorbance} = 0.0079 \times \text{Quercetin (µg)} + 0.0206$$

Total flavonoid content of each extract, were determined using its absorbance measured at 415 nm as input to the obtained standard curve and equation and expressed as µg quercetin/mgdried extract. All tests were carried out three times and reported as mean ± SD of three determinations.

2.5.3. Estimation of total tannin content

The tannins were determined by Folin-Ciocalteu method and tannic acid as standard [10] Absorbance of standard solutions was measured against the blank at 725 nm with an UV/Visible spectrophotometer. A standard graph was plotted using various concentrations of tannic acid and their corresponding absorbance with the following equation:

$$\text{Absorbance} = 0.008 \times \text{tannic acid (µg)} + 0.0480$$

Total tannin content of each extract, was determined using its absorbance measured at 725 nm as input to the obtained standard curve and equation and expressed as µg tannic acid/mg dried extract. All tests were carried out three times and reported as mean ± SD of three determinations.

2.6. Antimicrobial activity

2.6.1. Microbial strains

The extracts and isolated compounds of *Z. eichwaldii* were individually tested against a panel of 12 and 6 microorganisms, respectively. Following microbial strains were provided by Iranian Research Organization for Science and Technology (IROST) and used in this research: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC

29737), *Klebsiella pneumoniae* (ATCC 10031), *Staphylococcus epidermidis* (*CIP 81.55*), *Shigella dysenteriae* (PTCC 1188), *Streptococcus pyogenes* (ATCC 19615), *Salmonella paratyphi-A serotype* (ATCC 5702), *Candida albicans* (ATCC 10231) *Aspergillus brasiliensis* (ATCC 16404) and *Aspergillus niger* (ATCC 9029). Bacterial strains were cultured overnight at 37 ºC in nutrient agar (NA) and fungi were cultured overnight at 30 ºC in sabouraud dextrose agar (SDA).

2.6.2. Well diffusion assay

Determination of antimicrobial activity of the extracts and isolated compounds of *Z. eichwaldii* were accomplished by agar well diffusion method [11]. Gentamicin (10 µg/disc) and Rifampin (5 µg/disc) were used as positive controls for bacteria and Nystatin (100 IU) for fungi and yeast. The diameters of inhibition zones were used as a measure of antimicrobial activity and each assay was repeated three times [12].

2.6.3. Micro-well dilution assay

Minimal inhibition concentration (MIC) values of the extracts and isolated compounds of *Z. eichwaldii* were studied using micro-well dilution assay method [13]. Gentamicin and Rifampin for bacteria and Nystatin for yeast were used as standard drugs for positive control in conditions identical to tests materials. The MIC value was defined as the lowest concentration of the plant extracts required for inhibiting the growth of microorganisms. All tests were repeated three times.

2.6.4. MIC agar dilution assay

MIC values of the extracts for the fungi were evaluated based on the agar dilution method [14]. The MIC was defined as the lowest concentration of the compounds needed to inhibit the growth of microorganisms. Each test was repeated three times.

2.6.5. Minimum bactericidal concentration (MBC) assay

To determine the minimum concentration able to kill the bacteria the same microdilution assay described above was used [15]. The MBC was the lowest concentration able to effectively reduce the growth of microorganisms by 99.5 %. Each test was repeated three times.

3. Results

3.1. Chemical composition of the essential oils

The pale yellowish essential oils of dried aerial parts of *Z. eichwaldii* were obtained by hydrodistillation (HD), simultaneous distillation extraction (SDE) and solvent free microwave extraction (SFME) in the yields of 0.0122, 0.0159 and 0.0101 (% w/w on the basis of dried plant material), respectively. Essential oils were analyzed by GC/FID and GC/MS systems and the oils components were identified both quantitatively and qualitatively. Forty components were identified in the essential oils of the plant obtained from three different methods accounting for 96.52 %, 99.98 % and 99.99 % of the oils, respectively. Table 1 shows the percentage of volatile fraction compositions. Thirty two components (98.05 %) were identified in the oil obtained by HD with menthol (12.89 %), thymol (12.02 %), *β*-damascenone (8.21 %), hexadecanoic acid methyl ester (6.69 %) and carvone (4.71 %) as major components. The main constituents of the essential oil extracted using SDE apparatus were palmitic acid (28.36 %), (*Z*)-9,17-octadecadienal (21.22 %), linoleic acid (14.90 %), eicosane (11.28 %), 9,12-octadecadienoic acid methyl ester (9.45 %) and thymol (3.49 %) constituting 99.98 % of the oil.

Table 1. Chemical composition of the essential oils of *Z. eichwaldii*

NO	Compound[a]	Composition (%)			RI[b]	RI[c]	Identification method
		HD	SDE	SFME			
1	Linalool	3.15	-	2.14	1102	1103	d,f
2	Menthone	2.82	-	2.18	1163	1154	d,f
3	Neo-Menthol	2.14	-	-	1178	1165	d,f
4	Menthol	12.89	2.32	10.57	1186	1167	d,f
5	*α*-Terpineol	3.46	1.00	2.69	1203	1195	d,f
6	Citronellol	1.85	-	-	1229	1245	d,f
7	Carvone	4.71	1.01	2.64	1252	1246	d,f
8	Piperitone	0.98	-	1.38	1262	1268	d,f
9	Thymol	12.02	3.49	8.88	1294	1306	d,f
10	Carvacrol	1.39	-	-	1303	1312	d,f
11	(*E, E*)-2,4-Decadienal	1.59	-	-	1322	1326	d,f
12	(6*Z*, 8*E*) -(+)-2,5-Epoxy-6,8-megestigmadiene	1.26	-	-	1340	1312	d,f
13	Ascaridole	1.29	-	-	1351	1257	d,f
14	Geranyl acetate	1.53	-	-	1378	1378	d,f
15	*β*-Damascenone	8.21	1.94	4.27	1387	1383	d,f
16	Tetradecane	1.22	-	-	1400	1400	d,f
17	Dihydrodehydro-β-ionone	4.09	-	-	1417	1424	d,f
18	Butanoic acid, 2-methyl-, octyl ester	3.29	-	1.09	1433	1438	d,f
19	Geranyl acetone	3.03	-	-	1450	1453	d,f
20	*trans-β*-Ionone	1.32	-	-	1485	1487	d,f
21	Pentadecane	0.90	-	-	1500	1500	d,f
22	Dodecanoic acid, methyl ester	1.44	-	-	1523	1526	d,f
23	Caryophyllene oxide	2.46	-	-	1599	1582	d,f
24	Tetracosanoic acid, methyl ester	-	-	8.73	1599	1729	d,f
25	1,9-Tetradecadiene	-	1.12	-	1626	1411	d,f
26	Cyclopropaneoctanal, 2-octyl-	-	-	7.99	1672	2062	d,f
27	Pentadecanal	0.98	-	-	1717	1715	d,f
28	Tetradecanoic acid, methyl ester	1.12	-	-	1724	1722	d,f
29	Tetradecanoic acid, 12-methyl-, methyl ester	0.44	-	-	1795	1786	d,f
30	Eicosane	-	11.28	-	1802	2000	d,f
31	Hexahydrofarnesyl acetone	1.81	-	-	1843	1835	d,f
32	Linoleic acid	-	14.90	10.62	1843	2132	d,f
33	Farnesyl acetone	2.00	-	-	1805	1915	d,f
34	Hexadecanoic acid, methyl ester	6.69	1.65	6.17	1814	1926	d,f
35	Palmitic acid	2.71	28.36	10.88	1841	1959	d,f
36	2-Methyl-*Z,Z*-3,13-octadecadienol	-	2.24	3.46	1850	2104	d,f
37	Hexadecanoic acid, 14-methyl-, methyl ester	1.62	-	-	1866	1983	d,f
38	Oleic Acid	-	-	12.83	1866	2141	d,f
39	(*Z*)-9,17-Octadecadienal,	-	21.22	1.00	1895	2297	d,f
40	9,12-Octadecadienoic acid, methyl ester	2.11	9.45	2.47	1935	2088	d,f
	Total	96.52%	99.98%	99.99%			

[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.
[d] Retention index in HP-5MS column.
[e] GC/MS comparison with NIST08.L, W10N14.L and Adams (2001) libraries.

Finally, in the oil collected by SFME 18 components were identified, which made up 99.99 % of the total oil. The major constituents were oleic acid (12.83 %), palmitic acid (10.88 %), linoleic acid (10.62 %), menthol (10.57 %) and thymol (8.88 %).

3.2. Antioxidant activity

In vitro antioxidant tests are designed to mimic the oxidation–reduction reactions popularly occurring in the live biological systems and are used to estimate the antioxidant potentials of various chemical and biological samples. In this work, two classical antioxidant tests namely DPPH and *β*-carotene/linoleic acid tests were carried out.

In 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical test the capacity of the samples to donate hydrogen atom and/or electron to this blue/purple stable radical and converting it to yellow diphenyl picrylhydrazine molecule was measured [16]. This reaction is used for measuring the ability of the extracts or pure molecules (such as BHT) to scavenge free radicals. The results of this test are presented in Table 2. Compared to synthetic standard antioxidant BHT (IC_{50} = 19.20 ± 0.68 µg/ml), extracts and fractions of the plant exhibited moderate to weak radical-scavenging activities, but the free radical scavenging activity of the ethyl acetate fraction (IC_{50} = 178.63 ± 0.85 µg/ml) was superior to that of other extracts and methanol extract residue showed the weakest activity (IC_{50} = 1098.83 ± 1.18 µg/ml).

Potential ability of the antioxidants to delay lipid peroxidation by reacting with chain propagating peroxyl radicals faster than the reaction of these radicals with proteins or fatty acid side chains is usually evaluated by *β*-carotene/linoleic acid test [17]. In this test, the inhibition percentages of the extract and fractions of *Z. eichwaldii* on the oxidation of β-carotene in the presence of linoleic acid oxidation intermediates are listed in Table 2. The effectiveness of the extract ethyl acetate fraction (70.52 ± 0.99 %) was comparable to that of BHT (89.70 ± 1.05 %), but the inhibition values observed for other extracts were lower than that of BHT, and again methanol extract residue showed the weakest activity (14.64 ± 0.64 %).

Table 2. Antioxidant activities of aerial parts from *Z. eichwaldii*

NO.	Sample	DPPH assay (IC_{50}, µg/mL)	*β*-Carotene assay (% I)
1	Total methanol extract	460.81 ± 0.93	53.20 ± 0.53
2	Hexane fraction	548.15 ± 1.15	50.85 ± 0.42
3	Chloroform fraction	407.94 ± 1.06	59.33 ± 0.65
4	Ethyl acetate fraction	178.63 ± 0.85	70.52 ± 0.99
5	*n*-Butanol fraction	580.16 ± 0.89	61.58± 1.09
6	Methanol extract residue	1098.83 ± 1.18	14.64 ± 0.64
7	BHT	19.20 ± 0.68	89.70 ± 1.05

Values are mean ± SD (n = 3)

Table 3. Quantitative estimation of total phenol, flavonoids and tannin content of *Z. eichwaldii* aerial parts

NO.	Sample	Total phenol contents Gallic acid equivalent (µg/mg)	Total flavonoid content Quercetin equivalent (µg/mg)	Total tannin content Tannic acid equivalent (µg/mg)
1	Total methanol extract	68.33 ± 1.10	58.30 ± 1.03	63.32 ± 1.30
2	Hexane fraction	60.30 ± 1.06	62.13 ± 0.89	60.69 ± 1.11
3	Chloroform fraction	79.33 ± 0.65	78.26 ± 1.30	79.43 ± 1.14
4	Ethyl acetate fraction	99.83 ± 0.92	118.29 ± 0.68	188.05 ± 0.92
5	*n*-Butanol fraction	53.50 ± 0.98	68.48 ± 0.93	67.45 ± 1.13
6	Methanol extract residue	11.42 ± 0.82	12.01 ± 0.72	41.7 ± 1.08

Values are mean ± SD (n = 3)

3.3. Quantitative estimation of total phenol, flavonoids, and tannin

Total phenol content of the plant extracts were determined using a colorimetric assay method based on Folin-Ciocalteu reagent reduction. Results, expressed as gallic acid equivalents, and presented in Table 3. As expected, due to the major contribution of phenol compounds in antioxidant activity, ethyl acetate fraction showed the highest phenol content (99.83 ± 0.92 µg/mg) and methanol extract residue showed the lowest phenol content (11.42 ± 0.82 µg/mg). Other extracts and fractions of the plant showed moderate to weak amounts of phenol compounds content.

The content of total flavonoids in extractsand fractions were determined and reported (Table 3) asµg per mg of the dried extracts based on quercetin. The total flavonoid varied from 12.01 ± 0.72 to 118.29 ± 0.68µg/mg and the in the extracts. Maximum flavonoid content was found in the ethyl acetate fraction (118.29 ± 0.68µg/mg) of the plant. The content of tannin expressed as tannic acid equivalents, varied from 41.7 ± 1.08 to 188.05 ± 0.92 µg tannic acid equivalent/mg extract (Table 3). The ethyl acetate fraction showed the highest amount of tannin contents (188.05 ± 0.92 µg/mg).

3.4. Antimicrobial activity

Antimicrobial activity of *Z. eichwaldii* extracts against a panel of 12 microorganisms was examined and their potencies were assessed both qualitatively and quantitatively by the presence or absence of inhibition zones, zone diameters and MIC & MBC values. The results are given in Table 4. The maximum inhibition zones and MIC values for microbial strains sensitive to the extracts of the plant were in the range of 8-12 mm and 15.63-1000 µg/ml, respectively.

Total methanol extract showed great antimicrobial activities against *Candida albicans*, *Escherichia coli*, *pseudomonas aeruginosa* and *Streptococcus pyogenes* (MIC & MBC < 15.63 µg/ml), Also, ethyl acetate fraction showed considerable antimicrobial activities against *Candida albicans* and *pseudomonas aeruginosa* (MIC & MBC < 15.63 µg/ml).

On the other hand, methanol extract residue of the plant showed weak antimicrobial activities compared to other extracts in both MIC and MBC assay tests. The fungi strains tested *Aspergillus niger* and *Aspergillus brasiliensis* were weakly sensitive to all samples of the plant.

Table 4. Antimicrobial activities of the extracts of *Z. eichwaldii* aerial parts against tested microbial strains

Test Microorganism	Total methanol extract			Hexane fraction			Chloroform fraction			Ethyl acetate fraction		
	ZI [a]	MIC[b]	MBC[c] or MFC[d]	ZI	MIC	MBC or MFC	ZI	MIC	MBC or MFC	ZI	MIC	MBC or MFC
***A. brasiliensis* (ATCC 16404)**	—	—	1000	—	—	1000	—	—	1000	—	—	1000
***A. niger* (ATCC 9029)**	—	—	1000	—	—	1000	—	—	1000	—	—	1000
***B. subtilis* (ATCC 6633)**	—	31.25	62.50	—	31.25	250	—	125	500	—	31.25	500
***C. albicans* (ATCC 10231)**	—	< 15.63	< 15.63	—	< 15.63	< 15.63	—	62.50	62.50	—	< 15.63	< 15.63
***E. coli* (ATCC 25922)**	—	< 15.63	< 15.63	—	62.50	62.50	—	62.50	250	—	62.50	62.50
***K. pneumonia* (ATCC 10031)**	—	< 15.63	62.50	—	31.25	1000	—	62.50	1000	—	62.50	1000
***P. aeruginosa* (ATCC 27853)**	—	< 15.63	< 15.63	—	31.25	62.50	—	< 15.63	< 15.63	—	< 15.63	< 15.63
***S. paratyphi-A serotype* (ATCC 5702)**	—	125	> 1000	—	31.25	500	—	62.50	1000	—	62.50	500
***S. dysenteriae* (PTCC 1188)**	—	125	> 1000	—	31.25	1000	—	62.50	125	—	62.50	125
***S. aureus* (ATCC 29737)**	—	250	1000	—	250	1000	—	250	> 1000	—	500	1000
S. epidermidis (CIP 81.55)	9	125	500	11	62.50	500	—	125	250	8	250	500
***S. pyogenes* ATCC 19615**	—	< 15.63	< 15.63	—	31.25	31.25	—	250	250	12	62.50	62.50

A dash (-) indicate no antimicrobial activity.

[a.] ZI: Zone of inhibition in diameter (mm); [b.] MIC: Minimal inhibition concentrations (µg/ml)

[c] MBC: Minimum Bactericidal Concentrations (µg/ml); [d] MFC: Minimum Fungicidal Concentrations (µg/ml)

Table 4. Antimicrobial activities of the extracts of *Z. eichwaldii* aerial parts against tested microbial strains (Continue)

Test Microorganism	*n*-Butanol fraction			Methanol extract residue			Antibiotics					
							Rifampin		Gentamicin		Nystatin	
	ZI [a]	MIC[b]	MBC[c] or MFC[d]	ZI	MIC	MBC or MFC	ZI	MIC	ZI	MIC	ZI	MIC
***A. brasiliensis* (ATCC 16404)**	—	—	1000	—	—	1000	NA[e]	NA	NA	NA	30	31.2
***A. niger* (ATCC 9029)**	—	—	1000	—	—	1000	NA	NA	NA	NA	27	31.2
***B. subtilis* (ATCC 6633)**	—	62.50	62.50	—	62.50	> 1000	19	31.25	30	3.90	NA	NA
***C. albicans* (ATCC 10231)**	—	< 15.63	62.50	—	< 15.63	< 15.63	NA	NA	NA	NA	33	125
***E. coli* (ATCC 25922)**	—	62.50	62.50	—	125	125	11	3.90	20	3.90	NA	NA
***K. pneumonia* (ATCC 10031)**	—	62.50	1000	—	62.50	250	8	15.63	17	3.90	NA	NA
***P. aeruginosa* (ATCC 27853)**	—	< 15.63	62.50	—	< 15.63	250	-	62.50	22	3.90	NA	NA
***S. paratyphi-A serotype* (ATCC 5702)**	—	62.50	1000	—	62.50	1000	8	15.63	18	3.90	NA	NA
***S. dysenteriae* (PTCC 1188)**	—	62.50	1000	—	62.50	1000	9	15.63	17	3.90	NA	NA
***S. aureus* (ATCC 29737)**	—	500	1000	9	250	1000	21	31.25	27	1.95	NA	NA
S. epidermidis (CIP 81.55)	—	125	250	8	125	250	27	1.95	45	1.95	NA	NA
***S. pyogenes* ATCC 19615**	10	125	125	10	125	125	21	0.975	32	0.975	NA	NA

A dash (-) indicate no antimicrobial activity.

[a.] ZI: Zone of inhibition in diameter (mm); [b.] MIC: Minimal inhibition concentrations (µg/ml)

[c] MBC: Minimum Bactericidal Concentrations (µg/ml); [d] MFC: Minimum Fungicidal Concentrations (µg/ml)

[e] NA: Not Applicable

4. Discussion

Chemical compositions of the essential oils of *Z. eichwaldii* extracted by three different methods HD, SDE and SFME were presented in Table 1. The overall yields of essential oil extraction by all of these methods were low recording a low essential oil production potential for the plant. The plant essential oils mainly consist of alcoholic, ketonic and peroxide monoterpene hydrocarbons and fatty acids and their esters. Due to aqueous media used in the HD and SFME methods, identified components are mainly classical terpene essential oils components leaving behind water insoluble fatty acid of the plant unextracted. On the other hand, using n-pentane as an organic media in the SDE method, led to the extraction of fatty acids of the plant and appearance of them as the major constituents of the oil obtained by this method.

One hundred components have been reported in the essential oil of *Zygophyllum album* aerial parts and (E)-β-Damascenone (11.8 %) was reported as the major component [18]. Kchaou et al [19], reported 19 components in the essential oil obtained from the fresh leaves of *Z. album* and 2,6-Di (tert-butyl) phenol, delta decalactone, cocolactone and carvacrol were detected as major constituents. A study of the sources revealed that there is no report on essential oil composition of *Z. eichwaldii* and the results in this work are reported for the first time.

According to the results of antioxidant activity tests, antioxidant capacity of the ethyl acetate fraction of the plant in DPPH test showed the highest antioxidant activity compared to other extracts may be a consequence of its high phenol compounds content which was reflected in its Folin–Ciocalteu test result. The ethyl acetate fraction of *Z. eichwaldii* was also considerably potent in blocking of the oxidation of β-carotene by the active peroxyl radicals resulted from the oxidation of linoleic acid molecules in the presence of O_2 and an activity comparable to that of BHT was recorded for it (Table 2), which may be a consequence of the presence of molecules with easy hydrogen donating parts such as allylic and/ or benzylic functional groups. Other extracts and fractions of the plant were moderately active in this test and showed inhibition percentage of about two third of that of BHT.

Antioxidant capacity may be associated with high phenol content. Structurally, phenols comprise of anaromatic ring bearing one or more hydroxyl substituents. The antioxidant activity of this type of molecule is due totheir ability to scavenge free radicals, donate hydrogen atoms or electrons or chelate metal cations.

There are few reports on the antioxidant activity of the *Zygophyllum* genus plant species [20] such as *Z. simplex* [21], *Z. Album* [22], *Z. cocceniem* [23], and *Z. cornutum* [24]. But, according to our best knowledge, literature has no report on the antioxidant activity of the *Z. eichwaldii* and this is the first one.

Natural antioxidants such as phenols, flavonoids and tannins are increasingly attention attracting because they are natural disease preventing, health promoting and anti-ageing substances. Phenol are the most abundant widely distributed secondary metabolites of the plants, with about 8000 structures currently identified. Plant phenolics include phenolic acids, flavonoids, tannins, stilbenes and lignans, are a class of antioxidant agents acting as free radical terminators and their bioactivities may be related to their abilities to chelate metals, inhibit lipoxygenase and scavenge free radicals. Several pharmacological activities have been associated with phenolic compounds. These include anti-ulcer, anticancer, antioxidant, anti-inflammatory, antimicrobial and anticholinesterase [25]. Flavonoids are a group of polyphenolic

compounds with known properties including free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Tannins are generally defined as naturally occurring polyphenolic compounds of high molecular weight capable of forming complexes with proteins [26]. Based on the antioxidant activity results (Table 2) and as shown in Table 3, the ethyl acetate fraction of the plant showed the highest amount of total phenol, flavonoid and tannin contents. Literature review shows the presence of different hydroxyl containing and phenolic compounds such as polyphenols, tannins, phenolic acids and flavonoids, in the plants of the genus *Zygophyllum* [20-22, 27]. But, literature has no report on the estimation of total phenol, flavonoids, and tannin of *Z. eichwaldii* and this is the first one.

As can be seen from the antimicrobial test results, total methanol extract and ethyl acetate fraction of the plant showed stronger and broader range of antimicrobial activity than the other samples, compared with positive control drugs Rifampin, Gentamicin and Nystatin. Also, the weakest antimicrobial activity was observed in methanol extract residue of the plant in both MIC and MBC assay tests.

Although, this is the first report on antimicrobial activity of *Z. eichwaldii,* the only report in literature on antimicrobial activity of a plant extract from the genus *Zygophyllum* and shows considerable antimicrobial activity for leaves aqueous extract of this plant [28].

5. Conclusions

This report is on the essential oil composition and biological activities of the plant extracts of *Z. eichwaldii*. The plant was detected as a poor source of essential oil quantitatively, but the monoterpene contents of its essential oil are mainly oxygenated and commercially valuable and candidates the plant as a potential source of aromatic raw material for health, hygiene and cosmetic industries. Ethyl acetate fraction showed both the highest antioxidant activities and phenolic, flavonoid and tannin contents. High antimicrobial activity against most bacterial strains, candidates this plant as a good case for further studies.

Author contributions

All authors contributed toward data analysis, drafting, and revising the paper and agreed to be responsible for all the aspects of this work.

Conflict of interest

The authors declare that there is no conflict of interest.

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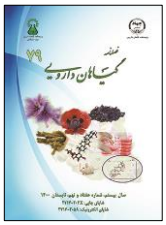

مقاله تحقیقاتی

بررسی ترکیبات شیمیایی اسانس و ارزیابی خواص بیولوژیکی سرشاخه‌های هوایی قیچ کاشانی (*Zygophyllum eichwaldii* C. A. Mey.)، گیاهی بومی ایران

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اطلاعات مقاله

گل‌واژگان:
قیچ کاشانی
خانواده اسفند
اسانس‌ها
کروماتوگرافی گازی
متصل به
طیف‌سنجی جرمی
آنتی‌اکسیدانی
ضدمیکروبی

چکیده

مقدمه: گیاه قیچ کاشانی *Zygophyllum eichwaldii* C. A. Mey. یک گیاه دارویی متعلق به خانواده Zygophyllaceae (اسفند) است. این خانواده مشتمل بر ۲۷ جنس و ۲۸۵ گونه است. **هدف:** مطالعه حاضر برای بررسی ترکیبات شیمیایی اسانس گیاه قیچ کاشانی و ارزیابی فعالیت‌های بیولوژیکی عصاره‌های آن می‌باشد. **روش بررسی:** استخراج اسانس از سر شاخه هوایی گیاه قیچ کاشانی توسط سه روش مختلف انجام و ترکیبات شیمیایی اسانس‌های حاصل با روش کروماتوگرافی گازی و کروماتوگرافی گازی متصل به طیف‌سنجی جرمی شناسایی شد. ارزیابی فعالیت‌های بیولوژیکی با استفاده از روش‌های اسپکتروفتومتری انجام شد. **نتایج:** آنالیز کروماتوگرافی گازی متصل به طیف‌سنجی جرمی نشان داد که منتول، تیمول و پالمیتیک اسید، از اجزای اصلی اسانس‌های استخراج شده از گیاه هستند. در بخش فعالیت‌های بیولوژیکی، در حالی که عصاره متانولی در ارزیابی‌های آنتی‌اکسیدانی قدرت متوسط تا ضعیف را نشان داد، بخش اتیل استاتی در این آنالیزها قوی بود (IC_{50} برابر با ۱۷۸/۶۳ میکروگرم بر میلی لیتر در سنجش DPPH و ۷۰/۵۲ درصد مهار در آزمون بتا-کاروتن/ اسید لینولئیک). این بخش اتیل استاتی همچنین محتوای قابل توجه فنلی، فلاونوئیدی و تانینی را نشان داد (به ترتیب برابر با ۹۹/۸۳، ۱۱۸/۲۹ و ۱۸۸/۰۵ میکروگرم بر میلی‌گرم). همچنین، عصاره‌های گیاه فعالیت‌های ضد میکروبی قابل توجهی را در برابر بیشتر باکتری‌های انتخاب شده نشان دادند. **نتیجه‌گیری:** مقدار قابل توجهی از ترکیبات فنولی، فلاونوئیدی و تانینی در بخش اتیل استات و فعالیت ضد میکروبی بالا در برابر بیشتر سویه‌های باکتریایی، این گیاه را به عنوان یک مورد مناسب برای مطالعات بیشتر در این زمینه معرفی می‌کند.

مخففها: DPPH، ۲،۲- دی فنیل-۱- پیکریل هیدرازیل؛ BHT، بوتیل هیدروکسی تولوئن؛ DMSO، دی متیل سولفوکسید؛ HD، تقطیر با آب؛ SDE، تقطیر همزمان با استخراج؛ SFME، استخراج بدون حلال با ماکروویو؛ GC، کروماتوگرافی گازی؛ GC/MS، کروماتوگرافی گازی متصل به طیف‌سنجی جرمی؛ IROST، سازمان پژوهش‌های علمی و صنعتی ایران؛ NA، نوترینت آگار؛ SDA سابرو دکستروز آگار؛ PDA، پوتیتو دکستروز آگار، BHI، برین هارت اینفیوژن؛ SDB، سابرو دکستروز براث؛ MIC، حداقل غلظت مهارکنندگی رشد؛ MBC، حداقل غلظت کشندگی باکتریایی؛ IC_{50}، غلظت مهارکنندگی میانگین؛ SD انحراف معیار؛ RI، اندیس بازداری

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