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Full Length Research Paper

Antimicrobial activity and constituents of the hexane extracts from leaf and stem of *Origanum vulgare* L. ssp. *Viride* (Boiss.) Hayek. growing wild in Northwest Iran

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Antimicrobial effects of various hexane extract and fatty acids have been extensively studied. Fatty acids with nonpolar compounds have been found to have a broad spectrum of microbicidal activity. The hexane extracts from leaf and stem of *Origanum vulgare* L. ssp. *Viride* which were collected from Northwestern Iran, were obtained by Soxhlet apparatus. The fatty acids in hexane extracts were derived to their methyl esters and determined by gas chromatography/flame ionization detector (GC/FID) and gas chromatography/mass spectrometry (GC/MS) systems. The extracts from the leaf and stem were characterized by a high amount of unsaturated fatty acids (UFA) and long-chain hydrocarbons. The main components of the leaf and stem extracts were tridecane (14.6 and 16.1%), 9, 12, 15-octadecatrienoic acid (ω-3) (14.7 and 1.2%), tetradecane (8.7 and 10.2%), hexadecanoic acid (1.6 and 6.7%) and pentadecane (5.7 and 4.1%), respectively. The hexane extract from *O. vulgare* leaf was detected as an important source of unsaturated fatty acid compounds. The hexane extract from stem of *O. vulgare* consisted mainly of aliphatic compounds; while in leaf extract of the plant, unsaturated fatty acids predominated over aliphatic components. The antimicrobial activity of the extracts of those samples were determined against seven Gram-positive and Gram-negative bacteria (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), as well as three fungi (*Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus niger*). The bioassay showed that the both oils exhibited a moderate antimicrobial activity.

Key words: *Origanum vulgare* ssp. *viride*, Lamiaceae, antimicrobial activity, unsaturated fatty acid, ω- 6.

INTRODUCTION

Organic fatty acids are naturally found in vegetables and fruits and may be formed during processes like fermentation or may be added into food during the manufacturing process. Numerous species of the plants which are rich fatty acids especially in seeds are of great importance as herbs and spices. During recent years, plant compounds have come more into the focus of phytomedicine (Buckle, 1999; Sylvestre et al., 2006).Their widespread use has raised the interest of scientists in basic research of fatty acids. Especially, the antimicrobial and antioxidant activities of fatty acids have been investigated in recent years (Karlova et al., 2010; Shafaghat, 2011). The genus *Origanum* ((Family: Lamiaceae) (MARZANGOOSH in Persian) is represented in the flora of Iran by one species with 3 subspecies: *Origanum vulgare* L. subsp. *Gracile*; *O. vulgare* L. subsp. *viride* and *O. vulgare* L. subsp. *vulgare* (Mozaffarian, 2007). Many different species, commonly known as *Oregano* or *Origanum*, are of economic interest, although they belong to different botanical families and genera. *O. vulgare* L. ssp. *viride* is an aromatic, herbaceous and perennial plant growing wild in the North West Iran. In folk medicine, *Origanum* species are used as powerful disinfectants, flavouring agents, in perfumes and in scenting soaps (Gunther, 1949; Chiej, 1984 and Kotb, 1985). The essential oils and the constituents of many *Origanum* species have been studied (Sendra and Cunat, 1980; Ravid and Putievsky, 1983; Akgul and Bayrak,

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1987; Harvala et al., 1987; Halim et al., 1991; and Shafaghat, 2011) It was reported that thymol and carvacrol represent the major constituents of the essential oils of *Origanum* species (Sarer et al., 1982). The oil of *O. vulgare* ssp. *viride* aerial parts from Iran was reported to be predominated by linalyl acetate, sabinene, gamma-terpinene, trans-ocimene, and cis-ocimene as the major constituents (Afsharypuor et al., 1997). From Egyptian majorana (*O. vulgare*), arbutin and methyl arbutin have been already isolated and quantitatively estimated (Assaf et al., 1987).

The essential oils from three chemotypes of *O. vulgare* L. ssp. *hirtum* (Link) Ietswaart growing wild in Campania (Southern Italy) have been investigated by GC, GC/MS and three chemotypes were found: The first, with a prevalence of carvacrol/thymol; the second, characterized by the prevalence of thymol/α-terpineol and the third, featuring a prevalence of linalyl acetate and linalool (Laura et al., 2009). Chemical composition of the ethanolic extract from *O. syriacum* aerial part has been investigated and five monoterpene glucosides were reported as carvacrol and thymol derivatives (Kamela et al., 2001). The essential oil of *O. vulgare* L. ssp. *vulgare* growing wild in Vilnius district (Lithuania) was characterized by a higher amount of β-ocimene, germacrene- D, β-caryophyllene and sabinene (Danute et al., 2001). Essential oils of the same subspecies in India contained 62.0% of γ-muurolene (Pande and Mathela, 2000). Thymol (61.0 to 69.1%) was the main constituent in the oil of *O. vulgare* ssp. *viridulum* from Greece (Arnold et al., 2000). Some essential oils in Finland from wild *O. vulgare* L. contained carvacrol as a dominant constituent (Nykanen, 1986). Essential oils of *O. vulgare* ssp. *virens* cultivated (seeds of different origin) in Italy were grouped into two chemotypes: Linalool (3 samples) and terpineol (3 samples) (Melegari et al., 1995), linalool dominated in the oil of the previous subspecies in Portugal (Alves-Pereira and Fernandes-Ferreira, 1998), sabinene and germacrene D in France (Chalchat and Pasquier, 1998). The oil of *O. vulgare* L. also contained linalool as a main constituent in India (Kaul et al., 1996).

The essential oils of four lines of *O. vulgare* subsp. *hirtum* (Link) Ietswaart cultivated in Hungary were found to contain carvacrol, *p*-terpinene and *p*-cymene as main constituents (Veres et al., 2007).

To the best of our knowledge, there is no previous report on the hexane extract composition of the leaf and stem extracts from the *O. vulgare* L. subsp. *viride* and those antimicrobial activities. Therefore, it is important and necessary to investigate further the composition of the leaf and stem hexane extracts and biological activities.

MATERIALS AND METHODS

Plant materials

Leaf and stem of *O. vulgare* were collected separately in the Khalkhal countrysides (Ardabil province in Northwest Iran) area near Lonbar village at an altitude of 2150 m in August 2011. A voucher specimen (O-328) is kept at the Herbarium of Agriculture Research in Ardabil Center (HARAC), Iran.

Preparation of hexane extracts

Dried and powdered materials (leaf and stem) were extracted with hexane using a Soxhlet apparatus (70°C, 4 h) to obtain the fatty acids, aliphatic compounds and the other apolar components. During extraction procedures, hexane (95%) was used. The extracts were concentrated by rotary evaporator under vacuum at 40°C. The extraction yields are presented in Table 2.

Trans-esterification process

After removing hexane using rotary evaporator, the oily mixtures were derived to their methyl esters by the International Olive Oil Council (IOOC) and IUPAC reports by trans-esterification process (Method of Analysis, 2001; Paquat and Hautfenne, 1992). In this process, dried hexane extracts were dissolved in hexane (5 ml) and then extracted with 5 ml of 2 M methanolic KOH at room temperature for 60 s. The upper phases were analyzed by gas chromatography/flame ionization detector (GC/FID) and gas chromatography/mass spectrometry (GC/MS) systems.

Gas chromatography (GC) analysis

GC analysis was performed on a Shimadzu 15A gas chromatograph equipped with a split/splitless injector (250°C) and a flame ionization detector (250°C). N_2 was used as carrier gas (1 mL/min) and the capillary column used was DB-5 (50m × 0.2mm, film thickness 0.32 µm). The column temperature was kept at 60°C for 5 min and then heated to 220°C with a 5°C/min rate and kept constant at 220°C for 5 min. The relative percentages of the characterized components are given in Table 1.

Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis was performed using a Hewlett Packard 5973 with an HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm). The column temperature was kept at 60°C for 5 min and programmed to 220°C at a rate of 5°C/min and kept constant at 220°C for 5 min. The flow rate of helium as carrier gas was 1 ml/min. MS were taken at 70 eV. The fatty acids and terpenoids were identified by comparing their retention times and mass peaks with those of standard compound mixtures and by NIST-Wiley library data search. Relative percentage amounts were calculated from peak area using a Shimadzu C-R4A chromatopac without the use of correction factors.

Antimicrobial activity

The *in vitro* antibacterial and antifungal activities of the extracts were evaluated by the disc diffusion method (DDM) using Mueller-Hinton agar for bacteria and Sabouraud Dextrose agar for fungi (Baron and Finegold, 1990). Discs containing 30 µL of the hexanic extracts were used and growth inhibition zones were measured after 24 and 48 h of incubation at 37 and 24°C for bacteria and fungi, respectively. Gentamicin and tetracycline for bacteria and nystatin for fungi were used as positive controls. The microorganisms used were: *Bacillus subtilis* ATCC 9372, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 15753, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 3583, *Pseudomonas aeruginosa* ATCC 27852,

Table 1. Chemical composition (%) of the hexanic extract from leaf and stem of *Origanum vulgar*.

Compound* (related fatty acid)	Rt (min)	Leaf (%)	Stem (%)
2-Propenylcyclohexane	8.2	2.3	--
Undecane, 2,4-dimethyl	8.4	2.8	3.3
Dodecane, 4-methyl	8.5	1.2	1.5
Dodecane, 2-methyl	8.52	--	2.5
Undecane, 3-methyl	8.6	2.2	--
1-Decene, 4-methyl	8.7	4.5	--
Tridecane	9.0	14.6	16.1
2,4,6-Trimethylindane	9.2	1.9	--
2,5-dimethyl,dodecane	9.3	2.1	--
Benzene, 4-(2-butenyl)-1,2-dimethyl	9.4	1.9	4.1
Tetradecane	9.7	8.7	10.2
Tridecane, 4-methyl	9.9	3.2	5.3
Tridecane, 2-methyl	9.92	2.2	3.6
Tridecane, 3-methyl	10.0	1.6	2.9
Dodecane, 2,6,10-trimethyl	10.1	2.8	2.1
5-ethyl-1,3-dimethylindan	10.2	1.2	1.9
Cyclododecane, ethyl	10.3	1.9	1.1
Naphthalene, 2,7-dimethyl	10.6	5.2	4.1
Naphthalene, 2,3-dimethyl	10.8	--	1.3
1-Tetradecene	11.0	2.6	--
Tetradecane, 4-methyl	11.2	0.8	3.9
Tetradecane, 3-methyl	11.3	0.9	1.1
8-Hexadecene	11.5	--	3.7
Pentadecane	11.7	5.7	4.1
Naphthalene, 1-(1-methylethyl)	11.9	0.6	1.3
Naphthalene, 2,3,6-trimethyl	12.1	0.9	1.2
4,6,8-Trimethylazulene	12.2	0.3	2.3
pentadecane, 2-methyl	12.5	0.4	0.5
Pentadecane, 3-methyl	12.6	0.2	0.3
1-Heptadecene	12.7	--	2.2
(-)-Spathulenol	12.8	0.2	--
Hexadecane	12.9	1.1	1.3
Pentadecane, 2,6,10-trimethyl	13.5	0.2	0.3
Heptadecane	14.0	0.2	0.2
Tetradecanoic acid, methyl ester (Tetradecanoic acid)	14.3	0.1	0.4
Octadecane	15.1	0.1	0.1
Nonadecane	16.2	0.2	--
Hexadecanoic acid, methyl ester(Hexadecanoic acid)	16.5	1.6	6.7
9-Octadecenoic acid, methyl ester(9-Octadecenoic acid)	18.0	--	2.0
9,12-Octadecadienoic acid, methyl ester(9,12-Octadecadienoic acid)	18.1	3.2	2.8
9,12,15-Octadecatrienoic acid, methyl ester(9,12,15-Octadecatrienoic acid) ω-3	18.2	14.7	1.2
Octadecanoic acid, methyl ester(Octadecanoic acid)	18.4	0.5	0.4
Eicosane	19.0	0.1	0.4
Eicosanoic acid, methyl ester(Eicosanoic acid)	20.2	0.2	0.2
Docosanoic acid, methyl ester(Docosanoic acid)	21.8	0.1	0.2
Total	**--**	**95.2**	**96.8**

*The composition of the extracts was determined by comparison of the mass spectrum of each component with Wiley GC/MS library data and also from its retention times (Rt). Rt = Retention time.

Table 2. Class compositions and yield of the hexanic extract from leaf and stem of *O. vulgare*.

Class composition	Leaf (%)	Stem (%)
Aliphatic and aromatic compounds	74.8	82.9
Saturated fatty acid (SFAs)	2.5	7.9
Unsaturated fatty acid (UFAs)	17.9	6.0
UFAs/SFAs	7.16	0.76
Yield	5.1	3.3

Table 3. Antimicrobial activity of leaf and stem oils of *"Origanum vulgare"*.

Tested microorganism	Zone of inhibition (mm)				
	Hexane extracts		Antibiotics		
	leaf oil	stem oil	Gentamicin	Nystatin	Tetracycline
B. subtilis	11.9±0.13	10.1±0.10	NT[b]	NT	22.6±0.1
S. epidermidis	13.1±0.12	11.1±0.21	NT	NT	34.3±0.5
E. faecalis	NA*	NA	NT	NT	9.4±0.2
S. aureus	14.1±0.13	14.1±0.12	NT	NT	21.3±0.3
K. pneumoniae	13.7±0.31	13.2±0.23	20.3±0.2	NT	NT
P. aeruginosa	NA	NA	12.5±0.1	NT	NT
E. coli	10.1±0.12	10.9±0.20	24.1±0.9	NT	NT
A. niger	12.2±0.22	11.7±0.13	NT	16.6±0.5	NT
C.albicans	11.8±0.23	10.9±0.11	NT	18.5±0.3	NT
S. cerevisiae	7.9±0.21	7.8±0.26	NT	18.3±0.4	NT

*NA = Not active.

Escherichia coli ATCC 25922, *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 5027 and *Saccharomyces cerevisiae* ATCC 9763.

RESULTS AND DISCUSSION

Fatty acids profile

The hexane extract composition from leaf and stem of *O. vulgare* was investigated using GC/FID and GC/MS techniques for the first time. Analysis of the fatty acid methyl esters and aliphatic compounds from *O. vulgare* leaf and stem oils showed the presence of fatty acid methyl esters and indirectly the fatty acids. According to the results, the hexane extract yields of the studied *O. vulgare* species leaf and stem were found 5.1 and 3. 3% on the basis of dry weight of the plant materials and the unsaturated fatty acid contents were higher than saturated ones and the highest total percentage was detected in stem (Table 1).

The percentage and retention time of components are given in Table 1. As it is shown, the total contents of hexane extracts varied from 95.2 (in leaf) to 96.8% (in stem). The major unsaturated and saturated fatty acid including linolenic (ω-3) and hexadecanoic acid are shown in the Table. The major polyunsaturated fatty acid (PUFAs) was linolenic (ω-3) acid. Four minor acids were tetradecanoic acid (0.1 to 0.4%), octadecanoic acid (0.4 to 0.5%), eicosanoic acid (0.2%) and docosanoic acid (0.1 to 0.2%).

As can be seen in Table 1, about 40 components of the extract from leaf, and 36 components from stem extract were identified, too. There were some differences in the fatty acid profiles of the different part of this plant. Unsaturated fatty acids (UFAs), saturated fatty acids (SFAs) and some of the aliphatic compounds were observed in both parts of this plant. In fact, both fractions mainly include aliphatic compounds, with a clear predominance of tridecane and tetradecane. One of the essential fatty acids (EFAs), 9, 12, 15- octadecatrienoic acid (linolenic acid or ω-3) was a predominant component in *O. vulgare* leaf, but not found in the stem oil. Linoleic acid is an omega-6 fatty acid, ranging from 2.8 (in stem) to 3.2% (in leaf) that was found a little amount in this work. The main aliphatic compounds in the *O. vulgare* (leaf and stem) extracts samples studied were tridecane (14.6 and 16.1%), respectively. The ratios of unsaturated fatty acid (UFAs)/SFAs (saturated fatty acid) were 7.16 and 0.76 in extract from leaf and stem, respectively (Table 2). The hexanic extract of leaf from this plant had a higher proportion of UFAs compared to stem part (Table 1).

The hexane extracts of leaf and stem from *O. vulgare* was tested against four Gram-positive and three Gram-negative bacteria, as well as three fungi. The results, presented in Table 3, show that the hexane extracts from

leaf and stem exhibited a moderate biological activity against all tested fungi and bacteria except for a resistant Gram-negative bacteria, *K. pneumoniae*, as well as a fungi, *A. niger.* The most sensitive microorganisms against leaf and stem extracts were *S. aureus* with inhibition zones of 14.1 to 14.7 mm, *K. pneumoniae* 13.2 to 13.7 mm and *S. epidermidis* 11.1 to 13.1 mm, respectively. Other microorganisms were found to be less sensitive to the extracts with inhibition zones ranged from 7 to 11 mm. According to our results, the main constituents of hexane extracts were aliphatic and aromatic compounds.

It is clear that there is a significant correlation between the chemical compositions and antimicrobial activity. Thus, it seems that *O. vulgare* leaf may be a moderate dietary source for UFAs and/or effective antimicrobial.

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