IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
February 2017 Vol.:8, Issue:3

Antimicrobial Activities and Chemical Composition of the Essential Oil of *Origanum majorana* L. Growing in Libya

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ISSN 2349-7203
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Submission: 27 January 2017
Accepted: 1 February 2017
Published: 25 February 2017

Keywords: *Origanum majorana*; Essential oils; Antimicrobial; GC/MS

ABSTRACT

The antimicrobial activity and chemical composition of Libyan *Origanum majorana* L. essential oil were investigated. The findings showed an important antimicrobial activity against bacterial strains. In fact, the inhibition zone, MIC and MLC values recorded for the bacterial strains were in the range of 13±1.4 to 22±1.4 mm, ***1.56*** to ***6.25*** and ***1.56*** to ***12.5***0 mg/mL, respectively. GC/MS analysis showed that essential oil is rich in trans-sabinene hydrate (27.1%), terpinen-4-ol (26.9%) followed by cis-sabinene hydrate (6.3%) and carvacrol (3.6%). Results showed an antibacterial effectiveness of *O. majorana* essential oil and supported the possibility of their use as a source of alternative antimicrobial agent.

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1. INTRODUCTION

Food safety is a fundamental concern of both consumers and the food industry, especially as the number of reported cases of food-associated infections continues to increase and is rapidly changing [1]. The increasing incidence of foodborne diseases, coupled with the resultant social and economic implications, means there is a constant striving to produce safer food and to develop new natural antimicrobial agents [2]. Therefore, new methods of reducing or eliminating foodborne pathogens and spoilage microorganisms, possibly in combination with existing methods are still needed. Thus, the food industry at present uses chemical preservatives to prevent the growth of foodborne and spoiling microbes. It has been suggested that some synthetic preservatives convert some ingested materials into toxic substances or carcinogens by increasing the activity of microsomal enzymes [3]. In recent years, there has been a considerable pressure from consumers to reduce or eliminate chemically synthesized additives in their foods. As a consequence, natural antimicrobials are receiving a good deal of attention as a potential antimicrobial agent to bacterial contamination and to extend shelf-life of food products [2, 4]. Most plants produce antimicrobial secondary metabolites, either as part of their normal program of growth and development or in response to pathogens attack or stress. A novel way to eliminate or reduce the proliferation of microorganisms is the use of essential oils. Essential oils are natural products extracted from plant materials, which because of their antibacterial, antifungal, antioxidant properties can be used as natural additives in many foods [3, 5, 6]. Essential oils of herbs such as *Origanum majorana*, rosemary, thyme, sage, basil, have been proven to be inhibitory against a wide range of food-spoiling microbes including Gram-positive and Gram-negative bacteria [7-14]. Also, large range of pathogenic microorganisms such as *Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Vibrio parahaemolyticus, Salmonella typhimurium* and *Escherichia coli* were inhibited by several essential oils [15]. *Origanum majorana* L. (marjoram) essential oil belonging to the family *Lamiaceae* possesses antimicrobial properties against food spoilage and foodborne bacteria and therefore, it may have the greatest potential for use in industrial applications [11, 16]. *Origanum majorana* L. is a hardy perennial and herbaceous plant which grows wild in its natural areas: Egypt and eastern Mediterranean countries and it can be grown in Northern European areas [17]. It is commonly used in Libyan folk medicine for treating common cold or as spasmolytic and as an antirheumatic [18]. Al-Jabel Al- Akhdar province which is located in the eastern part of Libya has many widely predominant species of medical plants such as *Origanum majorana* and thyme which represent a rich source of potential foodborne pathogens

control agents. Therefore, it is of great interest to carry out an antimicrobial screening of Libyan *Origanum majorana* essential oil and to identify its chemical composition to validate its use as natural preservative.

2. MATERIALS AND METHODS

2.1 Plant materials

The aerial parts of plant samples were collected at the flowering stages (June) from Derna region which is located in the eastern part of Al-Jabel Al-Akhdar province –Libya. Samples were identified according to the Flora of Libya [19]. *Origanum majorana* samples were dried in darken and well-ventilated area at room temperature (~28ºC) for 15 days. The leaves used for the extraction of essential oils were separated from the rest of the plant, ground in a mortar and kept in clean container until use.

2.2 Preparation of Essential Oil

The essential oils were extracted by hydrodistillation according to the method recommended by British Pharmacopoeia [20, 21] with some modification. For this, the dried and ground plant samples (100 g) of *Origanum majorana* leaves were subjected to hydrodistillation for 4 hours. The oils were extracted with petroleum ether (40-60ºC; sigma,) and dried over anhydrous sodium sulfate and filtered. The solvent was allowed to evaporate and the collected oil was then stored in dark bottles at 5ºC until use.

2.3 Test microorganisms

For assaying the antibacterial potential of *Origanum majorana* essential oil, control microorganisms including food-borne pathogens namely *Staphylococcus aureus* (ATCC 25923), *Listeria monocytogenes* (ATCC 19115), *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6633), *Shigella sonnei* (ATCC 25931), *Aeromonas hydrophila* (ATCC 35654) and food spoilage bacteria namely *Pseudomonas fluorescens* (ATCC 49838), *Micrococcus luteus* (ATCC 7468), *Enterococcus faecalis* (ATCC 19433), *Alcaligenes faecalis* (ATCC 35655) were used to investigate the antimicrobial activities. In addition, local clinical isolates of *Escherichia coli* and *Salmonella typhimurium* obtained from Biotechnological Researches Center, Tripoli-Libya were used in this study. All test strains were maintained on nutrient agar slants at 4°C and subcultured on nutrient broth for 24 h prior to testing.

2.4 Antimicrobial activity

To evaluate the antimicrobial activity of *Origanum majorana* essential oil against 12 microorganisms of significant importance, the disc diffusion techniques were carried out as described by Murray *et al*. [22] with a slight modification. Briefly, the microorganisms were cultured overnight at appropriate temperature and diluted in sterile saline solution (0.85 g 100 mL^{-1}) to have a final concentration of approximately 10^8 colony forming units per mL (CFU/ ml) adjusted according to the turbidity of 0.5 McFarland standard. 100µl of the culture was spread by a sterile swab on Mueller-Hinton agar (Fluka®, India) medium. Sterile filter paper discs (Whatman No. 1; 6 mm), individually impregnated with 10µl of the isolated essential oil were deposited in the center of the inoculated agar surfaces. Afterward, the plates were incubated at appropriate temperatures for 24 hrs. The diameters of the distinctly clear zones around the discs were measured (in millimeters) including disc diameter. The antimicrobial activity was expressed as the mean of inhibition diameters produced. All tests were performed in triplicate.

2.5 Estimation of the minimal inhibitory concentration and the minimal lethal concentration

The minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) of *Origanum majorana* EO against tested bacteria were evaluated by the broth dilution method [23] with some modifications. The suspensions of the bacterial strains were prepared from 12 hrs broth cultures in nutritive broth and were adjusted to 0.5 McFarland standard turbidity (1 $x10^8$ CFU/ml). Two-fold dilutions of *Origanum majorana* oil ranged from 0.781 to 25.00 mg/ml were prepared aseptically in nutrient broth. Each dilution was seeded with bacterial suspension in test tube and incubated for 24 hrs at appropriate temperatures. The microorganism growth was indicated by the turbidity of the culture. The lowest concentrations of the test samples where no turbidity was observed was determined as the MIC value and expressed in mg/ml. To determine the minimum lethal concentration (MLC), Mueller-Hinton agar plates and broth were inoculated with one loopful of all the tubes with no growth. After incubated at appropriate temperatures overnight, the lowest concentration among the negative tubes at which no growth was observed was recorded as the MLC. Each test was performed in triplicate.

2.6 GC/MS Analyses

The *Origanum majorana* essential oil was analyzed by gas chromatography coupled to mass spectrometry (GC/MS) (Hawlett-Packard computerized system comprising a 6890 gas chromatography coupled to a 5973A mass spectrometer with electron impact ionization 70eV) using a fused silica capillary column with a polar stationary phase HP-5MS (30m×0.32 mm; film thickness 0.25 µm). Analytical conditions were: 60ºC for 8 minutes, ramp of 2°C/min up to 250°C, and then held isothermal for 30 min; injector temperature 250ºC; injector mode, split; split ratio 1:30; interface temperature 250°C; injection volume, 1µL; carrier gas He, 1.5mL/min; Scan time and mass range were one second and 30-600 *uma*, respectively.

2.7 Compound Identification:

Essential oil component identification was assigned by comparison of their retention indices relative to (C_6-C_{28}) *n*-alkanes with those in the literature [24, 25] and by matching their recorded mass spectra with those stored in the Wiley7N and NIST mass spectral library and other published spectral data [24]. The quantitative data were obtained by peak area normalization, the response factor for each component was supposed to equal to one.

2.8 Statistical Analysis

In order to determine whether there is a statistically significant difference among the results obtained from antibacterial effect of *Origanum majorana* essential oil, variance analyses were carried out using SPSS 20.0 software package. Values of $P \leq 0.05$ were considered as significantly different.

3. RESULTS AND DISCUSSION

3.1 Antimicrobial assay

Origanum majorana essential oil (EO) was screened for antimicrobial activity using the agar diffusion and broth dilution methods against 12 test microorganisms (six Gram-positive and six Gram-negative). Results indicated that *Origanum majorana* oil has a wide spectrum of activity as it was found to be active against all microbial strains but in different degrees (Table 1). The data revealed that the inhibition zone (IZ) was from 13.0±1.4 mm to 22.0±1.4 mm with MIC from 1.562mg/mL to 6.25 mg/mL for tested bacteria. The maximum

antimicrobial activity of essential oil was against *Pseudomonas fluorescens* ATCC 49838 with lowest MIC and MLC (1.562 mg/ml) followed by *Salmonella typhimurium* and *Micrococcus luteus* ATCC 7468 (3.125 mg/ml). The antimicrobial activities of *Origanum majorana* EO against many microorganisms were reported by other researchers [9, 11, 15, 17, 26-28]. In present study, the essential oil of *Origanum majorana* did appear to be equally effective against both Gram-positive and Gram-negative micro-organisms which are in agreement with the results of other studies [26, 29]. The MLC/MIC ratio was used to determine the antimicrobial powers of EO. It has been stated that when this ratio is greater than 4, the oil is considered bacteriostatic whereas it is bactericidal when it is lower or equal to 4 [27, 30]. In this study, the ratio has shown a bactericidal effect against all tested strains with the exception of *Escherichia coli* and *Staphylococcus aureus* ATCC 25923 (Table 1). The antimicrobial properties of *Origanum majorana* EO have purportedly been associated with the high proportion of oxygenated monoterpenes and especially to their major constituents, such as terpinen-4-ol [31], α-terpinol [32], α-pinene, and p-cymene. Other compounds such as γ-terpinene, β-caryophyllene, and sabinene are also known to have efficient antimicrobial properties [33-36].

Table 1. Antibacterial activity for *Origanum majorana* essential oil against human pathogenic and food spoilage bacteria

Bacterial species	***Origanum majorana essential oil***	***Antibiotic** (Chlor)	**MIC**	**MLC**	**MLC / MIC**
Gram negative bacteria	*Inhibition Zone (mm)		mg/ml		
Alcaligens feacalis ATCC 35655	21.0^{ab} ±1.4	23	3.125	6.25	2
Aeromonas hydrophila ATCC 35654	19.0^{abc} ±1.4	23	6.25	12.5	2
Pseudomonas flurosences ATCC 49838	19.0^{abc} ±1.4	15	1.562	1.562	1
Shigella sonni ATCC 25931	17.0^{bcde} ± 0	20	1.562	3.125	2
Escherichia coli (local isolate)	17.5^{bcd} ± 0	30	1.562	12.5	8
Salmonella typhimurium(local isolate)	16.5^{cde} ± 1.4	20	3.125	3.125	1
Gram positive bacteria					
Bacillus subtilis ATCC 6633	22.0^{a} ± 1.4	18	3.125	12.5	4
Listeria monocytogenes ATCC 19115	20.0^{abc} ± 0.0	31	1.562	3.125	2

Bacillus cereus ATCC 10876	$17.0^{bcde} \pm 0.7$	26	3.125	12.5	4
Enterobacter feacalis ATCC 19433	$15.0^{de} \pm 0.4$	25	3.125	6.25	2
Micrococcus luteus ATCC 7468	$13.0^{e} \pm 1.4$	14	3.125	3.125	1
Staphylococcus aureus ATCC 25923	$13.5^{de} \pm 0.7$	31	1.562	12.5	8

Means followed by the same letters are not significantly different at ($P \leq 0.05$). *Inhibition zone (mm ± SD) in diameter around the discs (6 mm) impregnated with 10 µl of essential oil; Inhibition zones in diameter (mm) of *Chlor; Chloramphenicol (30 µg/disc) were used as positive reference standards antibiotic discs; MLC/ MIC ratio.

Terpenes are thought to be inducing alterations in cell permeability by entering between the fatty acyl chains that make up the membrane lipid bilayers, thus disrupting lipid packing and causing changes to membrane properties and functions [27]. Additionally, sesquiterpenes, caryophyllene, germacrene D, caryophyllene oxide, and spathulenol were also reported among the most potent antimicrobial agents frequently occurring in essential oils [37-39].

3.2 The chemical composition of *Origanum majorana* essential oil

The hydrodistillation of dried flowering plants of *O. majorana* gave a colorless oil with a yield of 1.31%. The results from the GC–MS analysis of *Origanum majorana* EO constituents revealed the presence of a total of 47 components, representing 96.1% of the total oil (Table2). The essential oil is mainly rich in oxygenated monoterpenes especially monoterpene alcoholic such as Trans sabinene hydrate (27.1%), Terpinen-4-ol (26.9%). *Origanum majorana* EO contained a complex mixture dominated by oxygenated monoterpenes (74.7%) and monoterpene hydrocarbons (16.8%) with a small amount of sesquiterpene hydrocarbons (4.4%). Other prominent components were detected including cis-sabinene hydrate (6.3%), alpha-terpineol (6%), γ-terpinene (4.3%) and Carvacrol (3.6%). These results are in accordance with previous studies [17, 27, 40] reporting that sabinene hydrate and terpinen-4-ol were the major components of *O. majorana* EO although there was different in quantities. According to earlier work, there are two main chemotypes. One consists mostly of monoterpene alcohols and the other of phenols. In the first chemo-type, terpinen-4-ol, either alone or together with other monoterpene alcohols such as cis- and trans-sabinene hydrate has been found to be the main volatile components [41]. For the second chemotype marjoram oils rich in phenols consisted mainly of thymol [17] and/or carvacrol [41, 42]. The present study shows that essential oil of *Origanum majorana* L. grown in Derna rejoin with a temperate climate presents characteristics of the first chemotype. Overall, it can be concluded that *Origanum majorana* EO is one of the most promising natural compounds that can be used to develop safer antibacterial agents.

Table 2. Chemical composition of the essential oil extracted from *Origanum majorana* L.

No.	Compound	(RI) HP-5	%
1	α-thujene	920	0.2
2	α-pinene	926	0.4
3	Camphene	939	0.2
4	Benzaldehyde	950	0.1
5	Sabinene	965	1.5
6	1-octen-3-ol	973	0.1
7	β-myrcene	988	0.6
8	α-phellandrene	1002	0.1
9	α-terpinene	1014	2.0
10	p-cymene	1021	1.6
11	β-phellandrene	1024	0.9
12	1,8-cineole	1026	1.0
13	Benzeneacetaldehyde	1039	0.1
14	β-ocimene	1046	TR
15	γ-terpinene	1056	4.3
16	Cissabinene hydrate	1065	**6.3**
17	α-terpinolene	1084	1.1
18	Trans sabinene hydrate	1103	**27.1**
19	Linalool	1099	1.5
20	Cis p-menth-2-en-1ol	1119	**2.2**
21	α-campholenaldehyde	1122	TR
22	Trans pinocarveol	1133	0.1
23	Trans menth-2-en-1ol	1137	1.1
24	Borneol	1163	0.1
25	Terpinene-4-ol	1177	**26.9**
26	α-terpineol	1190	6.0
27	Cis-piperitol	1193	0.6

28	Trans hydrocarvone	1200	0.3
29	Trans piperitol	1205	0.5
30	Trans carveol	1216	TR
31	Nerol	1227	0.1
32	Carvone	1239	TR
33	Geraniol	1255	0.2
34	Trans sabinene hydrate acetate	1257	0.3
35	Bornyl acetate	1282	TR
36	Indole	1287	0.1
37	Thujanol-3-acetate	1297	0.1
38	Carvacrol	1303	**3.6**
39	Neryl acetate	1366	0.1
40	Geranyl acetate	1385	0.2
41	β-caryophyllene	**1410**	**2.0**
42	α-humulene	1443	0.1
43	Bicyclogermacrene	1488	1.9
44	4(4-methoxyphenyl) but-2-one	1493	0.1
45	Spathulenol	1566	0.2
46	Viridiflorol	1571	0.1
47	Muurola 4,10(14) dien 1-β-ol	1628	0.1

(RI): retention index on stationary phase HP5MS
Total identified 96.1%
Oxygenated Monoterpene hydrocarbons 74.7%
Monohydrocarbons 16.8%
Sesquiterpenes 4.4%
Others 0.2 %
TR: trace (< %0.05)

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