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Phytochemical Analysis and *in vitro* Antibacterial Activity of the Essential Oil of *Origanum vulgare* from Morocco

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Abstract: The present study was conducted to evaluate the antibacterial activity and phytochemistry of essential oils obtained from *Origanum vulgare*. *Origanum* species from the Lamiaceae family are widely distributed in Morocco. The essential oils of *Origanum vulgare* collected in Tazouta region of Morocco were obtained by hydro-distillation of the aerial parts and analysed by gas chromatography equipped with flame ionisation detector (GC-FID) and gas chromatography coupled to a mass spectrometry (GC/MS) for their chemical composition. Their antibacterial activity was studied in vitro on nine bacterial strains: *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Campylobacter jejuni* and *Vibrio cholerae*. Thirty three constituents were identified in leaves oil representing 76.62% of the total oil composition. The yield of essential oil of *Origanum vulgare* was 1.15% and the major compound in aerial parts was carvacrol (18.06%) followed by thymol (7.36%), γ-Terpinene (5.25%), *p*-cymene (5.02%), Limonene (4.68%), Caryophylene (4.12%), cymene (3.56%), ledene (3.41%), linalool (2.47%), α-Pinene (2.15%), γ-Terpineol (2.10%) and Germacrene-D (2.08%). The bacterial strains tested were found to be sensitive to essential oils studied and showed a very effective bactericidal activity with minimum inhibitory concentrations (MIC) ranging from 0.10 to 4.02 mg/mL.

Key words: *Origanum vulgare* · Chemical composition · Carvacrol · Antibacterial activity

INTRODUCTION

Actually, however, essential oils and their components are gaining increasing interest because of their relatively safe status, their wide acceptance by consumers and their exploitation for potential multi-purpose functional use [1-3]. Essential oils and their components are widely used in medicine as constituents of different medical products, in the food industry as flavouring additives and also in cosmetics as fragrances [4] and pharmaceutical industries [5]. Essential oils are generally used in the cosmetic, medical and food industries. Antibiotic resistance has become a global concern [6]. Medicinal plant and their essential oils have been used extensively for many years in food products, perfumery, dental and oral products due to their different medicinal properties [7-8]. Moreover volatile compounds obtained from plants, have known antimicrobial, antifungal and insecticidal activities [9-11].

Essential oils have many therapeutic and they aid the distribution of drugs and antiseptics [12]. Their most important characteristics are their anti-infection, antimicrobial, antifungal and antioxidative effects [13-15]. Moreover, the screening of such plant extracts for antimicrobial activity has always been of great interest to scientists looking for new sources for drugs for the treatment of various diseases [16,17].

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Origanum vulgare, a member of the family Lamiaceae has been a valuable source of natural products for maintaining human health for a long period of time, especially in last decade, with more intensive studies for natural therapies [18]. Origanum species grow abundantly on stony slopes and in rocky mountain areas at a wide range of altitudes (0–4000 m) [19]. The leaves and dried herb of oregano as well as its essential oil are used medicinally [20]. The essential oil of *Origanum vulgare* has been the object of several studies antifungal [21-9], antibacterial [22-25], antiinflammatory [26], antiaggregant [27], antioxidant [28-29], antihyperglycaemic [30], Antithrombin [31] and cytotoxial activity [32]. Multiple studies have been reported on the chemical composition of the essential oils of *Origanum vulgare* belonging to different regions in the world [33-35]. Morocco is blessed with a rich source of aromatic plants, many of which have not been previously investigated for their chemical constituents and biological potentials. *Origanum vulgare* is a plant belongs to the family Lamiaceae, which grows in Morocco region.

The aim of the current investigation was to analyze the chemical composition of essential oil of *Origanum vulgare* collected in Tazouta, a mountainous region from Morocco in order to be determined the antimicrobial activity.

MATERIALS AND METHODS

Plant Material: The leaves of *Origanum vulgare* were collected in June 2009 at Tazouta; 50 km in the south east of Sefrou. The coordinates: latitude: 35°42 '21" longitude: 4°32' 31"). The climate is semi-humid with strong continental influence with an annual average temperature of 20°C. The leaves were then isolated from the other specimen and conserved for extraction.

Essential Oil Extraction: The leaves of examined plants were dried in shadow at room temperature and immediately hydro-distilled (30g) for 2.5 h using a modified Clevenger-type apparatus. The yellowish oil (1 ml) for leaves was dissolved in hexane and then dried over anhydrous sodium sulfate. After filtration, the solvent was removed by distillation under reduced pressure in a rotary evaporator at 35°C and the pure oil kept at 4°C in the dark, until the moment of analysis.

Chromatographic (GC/MS and GC-FID) Analysis: The quantitative analysis was done with the help of a chromatographer in gas phase (GC ULTRA S/N 20063009, Thermo-Fischer) equipped with flame ionisation detector (FID), Varian capillary column (5% poly diphenyl 95% dimethylsiloxane, TR-50MS- SPB-50; 60m length, 0.32mm of diameter and Film thickness 0.25 μm). The column temperature was programmed from 40 to 280°C for 5°C/min and finally held at that temperature for 10 min. The temperature of the injector was fixed to 250°C and the one of the detector (FID) to 260°C. The debit of gas vector (nitrogen) was fixed to 1mL/min and split injection with split ratio 1:40.The volume of injected was 0.5μL of diluted oil in hexane solution (10%). The percentage of each constituent in the oil was determined by area peaks.

The identification of different chemical constituents was done by gas phase chromatography (GC ULTRA S/N 20062969, Thermo-Fischer) coupled with spectrometer (PolarisQ, S/N 210729, Thermo-Fischer); equipped with TRIPLUS AS S/N 20063460 Ionisation energy of 70ev. The utilised column was; Varian capillary column (TR-50MS- SPB-50; 60m length, 0.32mm of diameter and Film thickness 0.25 μm). The column temperature was programmed from 40 to 280°C for 3°C/min. The temperature of the injector was fixed to 260°C and the one of the detector (PolarisQ) to 200°C. The debit of gas vector (Helium) was fixed to 1.5mL/min.The volume of injected specimen was 1.5μL of diluted oil in hexane. The constituents of essential oils were identified in comparison with their Kovats Index, calculated in relation to the retention time of a series of lineary alkanes (C_4- C_{28}) with those of reference products and in comparison with their kovats index with those of the chemical constituents gathered by Adams [36] and in comparison with their spectres of mass with those gathered in a library of (NIST-MS) type and with those reported in the literature [37-38].

Antibacterial Tests: The selected essential oils were screened against nine bacteria: *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Campylobacter jejuni and Vibrio cholerae.* The minimal inhibition concentration (MIC) values were evaluated according to published procedures [39-41]. The minimal inhibitory concentration (MIC) was determined only with micro-organisms that displayed inhibitory zones. MIC was determined by dilution of the essential oils in dimethyl sulfoxide (DMSO) and pipetting 0.01 mL of each dilution into a filter paper disc. Dilutions of the oils within a concentration range of 0.10-4.02 mg/mL were also carried out. MIC was defined as the lowest concentration that inhibited the visible bacterial growth [42]. The bacterial plates were incubated at 37°C and the zone of inhibition

measured in mm after 24h, 48h and 72h of growth. A control experiment was set up by using an equal amount of sterile distilled water in place of different extract concentrations. Many screening reports, using disc diffusion and dilution techniques, have established an antimicrobial activity of *Origanum vulgare* extracts from various species against a number of pathogens including [22] (*Aeromonas hydrophila, Bacillus amyloliquefaciens, B. brevis, B. cereus, B. subtilis, Corynebacterium xerosis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Micrococcus luteus. Mycobacterium smegmatis, Proteus vulgaris, Staphylococcus aureus* and *Yersinia enterocolitica),* [25] *(Lactobacillus curvatus, Lactobacillus sakei, Staphylococcus carnosus* and *Staphylococcus xylosus*) and [39] (*Escherichia coli, Staphylococcus aureus, Staphylococcus intermedius, Klebsiella pneumonia, Pseudomonas aeruginosa, Bacillus subtilis, Streptococcus mutans, Micrococcus luteus* and *Proteus mirabilis*).In a previous studies on the antibacterial activity: [43] *(Proteus vulgaris, Salmonella typhimurium, Enterobacter cloacae, Klebsiella pneumonia, Escherichia coli, Serratia marcescens* and *Pseudomonas aeruginosa)* and [44] (*Aeromonas hydrophila, Citrobacter* sp., *Enterobacter aerogenese, Escherichia coli, Flavobacterium* sp., *Klebsiella ozaenae, K. pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhi, S. paratyphi B, Serratia marcescens* and *Shigella dysenteriae*).

RESULTS AND DISCUSSION

Chemical Composition: The chemical composition of essential oils, yield and the retention time of the components of *Origanum vulgare* are presented in Figure A and Table 1.

The compounds of leaves essential oil of *Origanum vulgare* from Morocco are listed in order of their elution on the TR-50MS- SPB-50 column, Figure (A).

In this study, thirty three volatiles constituents were identified in the leaves oils, representing 76.62% of the total composition (Table 1). The most abundant components found in the leaves oil were carvacrol (18.06%). Other major components were identified as thymol (7.36%), γ-Terpinene (5.25%), *p*-cymene (5.02%), Limonene (4.68%), Caryophylene (4.12%), cymene (3.56%), ledene (3.41%), linalool (2.47%), α-Pinene (2.15%), γ-Terpineol (2.10%) and Germacrene-D (2.08%). The yields of the oils obtained from the hydro-distillation of the leaves of *Origanum vulgare* was 1.15%, it is relatively higher than other plants industrially exploited as a source of essential oils: *Artemisia herba-alba* (0.59%), *Artemisia absinthium* (0.57%) and *Artemisia pontica* (0.31%) [45], lavender (0.8-1.8%), menthe (0.5-1%), néroli (0.5-1%), Laurel (0.1-0.35%) [46], Thymus (1%) [47] and this yield is relatively lower than other plants: *Eucalyptus microtheca* (2.3%), *Eucalyptus tereticornis* (3.4%) and *Eucalyptus grandis* (4.7%) [48], *Mentha rotundifolia* and *Mentha pulegium* of Morocco, which are very high level (4.33%) and (2.33%) [49].

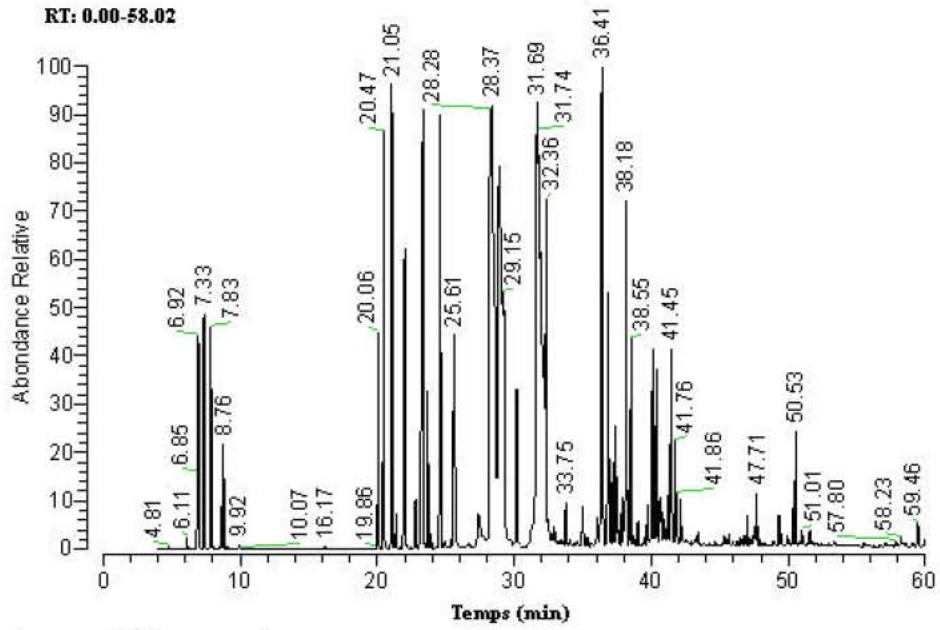

Fig. A: Chromatogram of *Origanum vulgare*

Table 1: Chemical composition of essential oils of *Origanum vulgare* from Morocco

Peak	Constituents	*RT (min)	**KI	Area (%)	***Mass range (m/z)
1	Sabinyl acetate	5.85	1224	1.02	(194), 92,91,81,41,134,55,109,79,43,53
2	Pulegone	6.92	1212	1.64	(152),152,81,67,109,82,41,137,69,95,55
3	Caryophyllene oxide	7.33	1506	1.50	(220),43,41,79,93,91,95,69,55,67,81
4	Terpinen-4-ol	7.83	1137	1.32	(154),71,111,93,43,86,41,69,55,68,154
5	Sabinene	8.76	983	1.19	(136),93,41,91,77,79,39,27,69,94,43
6	α-Copaene	9.92	1221	0.69	(204),161,119,105,93,41,91,92,81,120,204
7	Verbenol	16.17	1122	0.41	(152),109,41,94,81,39,69,55,97,43,57
8	Terpinolene	19.84	1052	0.34	(136),93,121,91,136,79,77,105,39,41,107
9	Cymen-8-ol	19.86	1042	0.50	(134),119,134,91,120,117,41,77,39,65,115
10	Ledene	20.06	1419	3.41	(204),107,105,135,93,161,41,91,81,119,204
11	Limonene	20.47	1018	4.68	(136),68,93,39,67,41,27,53,79,94,92
12	Carvacrol	21.05	1262	18.06	(150),135,150,91,136,77,107,117,115,79,105
13	1.8-Cineole	23.75	1059	0.06	(154),43,93,81,71,69,84,68,108,41,55
14	Linalool	25.61	1082	2.47	(136),71,41,43,93,55,69,80,39,121,27
15	Thymol	28.29	1262	7.36	(150),135,150,91,39,115,136,117,77,51,41
16	γ-Terpinene	28.37	998	5.25	(136),93,91,136,121,77,92,79,43,41,105
17	α-Pinene	29.15	948	2.15	(136),93,91,39,121,77,92,79,43,41,105
18	Camphene	30.02	943	0.61	(136),93,79,91,77,41,121,67,27,107,39
19	*p*-cymene	31.69	976	5.02	(136),93,41,79,39,91,77,92,27,80,53
20	Caryophylene	31.74	1494	4.12	(204),93,133,91,41;79,69,105,107,120,77
21	Cymene	32.36	1042	3.56	(134),119,134,91,120,117,41,77,39,65,115
22	Camphor	33.75	943	0.81	(152),95,41,81,39,55,69,108,67,83,27
23	Isosativene	35.41	1339	0.31	(204),94,91,41,105,79,93,204,119,39,77
24	Solanone	36.20	1296	0.23	(194),43,93,136,121,41,79,81,91,77,39
25	Calarene	38.50	1403	0.27	(204),161,41,105,91,119,93,162,107,189,133
26	γ-Terpineol	38.55	1174	2.10	(154),59,93,121,136,81,43,68,95,67,41
27	Germacrene-D	41.45	1515	2.08	(204),161,105,91,41,119,79,81,93,77,27
28	Cadinene	41.76	1440	1.15	(204),161,189,204,41,105,91,119,133,27,55
29	α-Cubebene	41.86	1344	1.09	(204),161,105,119,41,81,91,120,93,55,204
30	Carvone	47.71	1190	1.02	(150),82,54,39,93,108,53,107,41,79,91
31	γ-cardinene	49.07	1430	1.10	(204),161,189,204,105,91,119,133,27,55
32	Bornyl acetate	50.53	1267	0.90	(196), 95,43,93,436,121,41,80,55,108,69
33	Sabina cetone	51.01	1152	0.20	(138),81,96,95,55,41,67,43,39,68,82
Total Identified Constituents				76.62	
Yields (%)				1.15	

*RT: Retention time obtained by chromatogram (Fig. A).

**KI: Kovats Index was determined by GC-FID on a TR-50MS- SPB-50 column.

***Mass range (m/z) was determined by mass spectrometry (PlarisQ).

Table 2: Comparisons of the total oil and major compounds of essential oils of Other *Origanum* Species analyzed in other countries

Plant species	Total Oil (%)	Major compounds	References
Origanum vulgare	76.62	carvacrol (18.06%)	Derwich et al/Morocco
Origanum ehrenbergii	94.9	thymol (19%)	[68]
Origanum glandulosum	99.6	thymol (41.6–81.1%)	[69]
Origanum minutiflorum	98.7	carvacrol (73.9%)	[70]
Origanum compactum	100	carvacrol (30.53%)	[71]
Origanum dictamnus	97.2	carvacrol (84.8%)	[72]
Origanum syriacum	90.6	thymol (24.7%)	[68]
Origanum acutidens	99.0	Carvacrol(87.0%)	[73]

The phytochemistry revealed that this leaves had compositions similar to those of other *Origanum vulgare* essential oils analyzed by [32], which the major compounds was carvacrol, thymol, γ-terpinene and *p*-cymene representing 73.7% of the total oil. As it has been reported previously, the essential oils of *Origanum vulgare* are rich in limonene, ó-cariofilene, rho-cymenene, canfor, linalool, α-pinene, carvacol and thymol [50]. [51] Reported carvacrol, thymol, γ-terpinene and *p*-cymene as main constituents of *Origanum vulgare* essential oil. Also this composition is relatively similar to the composition of essential oil of leaves of *Origanum vulgare* study in Italy which the major constituents were thymol and carvacrol, *p*-cymene and γ-terpinene [52]. Previously, it was reported that *Origanum vulgare* growing in Brazil contained 4-terpineol (47.95%), carvacrol (9.42%), thymol (8.42%) and α-terpineol (7.57%) as major components [9]. Contrary to the composition of essential oils of *Origanum vulgare* study in Lithuania which main constituents were: β-ocimene (14.9–21.6%), germacrene D (10.0–16.2), β-caryophyllene (10.8–15.7%) and sabinene (6.6–14.2%) [53]. On the other hand, while essential oil from *Origanum vulgare* from Greece contained 4-terpineol (37%) as a major component [35]. The essential oil from the same plant from Turkey contained only Caryophyllene (14.4%), spathulenol (11.6%), germacrene-D (8.1%) and aterpineol (7.5%) as the main constituent [28]. Intensive research on the chemical characteristics has been conducted on this species [54-60]. Several species of the genus *Origanum* have carvacrol and thymol among their main constituents; these are accompanied by other compounds such as ρ-cimene, α-terpinolene, α-terpinene, α-terpineol, linalool, 4-terpinol, germacrene-D and α-pinene, which are present in lower concentrations and also show antimicrobial activity [22,61-63]. According to some studies, the composition, quality and content of essential oils present in plants are subject to great variation and are influenced by diverse factors such as the geographical and climatic conditions as well as the conditions used for culture, drying and storage[64-54]. Also, the yield and chemical composition of the leaf oil vary widely between species, individual trees as well as with the growing environment [65-66]. A wide chemical diversity is found even within a single Origanum species e.g. the widely used *Origanum vulgare* where the pattern of variation of quantitative and qualitative essential oils depends on geographical distribution or on the time of plant collection [67] (Table 2).

The essential oil content shows variations in plants of different geographical origin and also in different part of the tree: [74]; studied the composition of *Juniperus phoenicea* oil collected from the Portugal, Spain and Greece, they reported that the yields and the total oil obtained were (0.41% and 98.3%), (0.66% and 99%) and (0.58% and 88%) respectively and the composition is characterized by a high content of α-pinene (34.1%, 53.5% and 41.8%), β-phellandrene (19.2%, 5.9% and 3.5%) and β-caryophyllene (0.22%, 1.0% and 0.5%). In previous studies on the chemistry of Morocco [45], considerable differences were observed in the essential oil composition between *Artemisia herba-alba, Artemisia absinthium and Artemisia pontica*, which the total constituents identified, is 83.10%, 80.72% and 43.95% respectively. Furthermore, the essential oils, obtained from flower, leaves and stems from basil (*Ocimum basilicum L.)* from Mersin province (Bu¨yu¨keceli-Gu¨ lnar) in Turkey contained: estragole (58.26%, 52.60% and 15.91%), limonene (19.41%, 13.64% and 2.40%) and p-cymene (0.38%, 2.32% and 2.40%) [75]. In a previous studies on the chemistry of *Juniperus phoenicea* [76], considerable differences were observed in the essential oil composition between leaves and berries: α-pinene (38.22% and 39.30%), (α -cedrol 31.23% and sabinene 24.29%) respectively.

Antibacterial Activity: The essential oil extracted from the leaves of *Origanum vulgare* was used in the present study to investigate their antibacterial potential. Staphylococcus *aureus, Bacillus subtilis, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Campylobacter jejuni and Vibrio cholerae* were used. The results obtained and screening of antibacterial activity of essential oil of *Origanum vulgare* are summarized in (Table 3).

With the agar disc diffusion assay, oils were found to be active against *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens* and *Escherichia coli* at a minimal inhibitory concentration (MIC) of 0.10, 0.15, 0.19, 0.98, 1.58 and 1.82mg/mL. Against *Pseudomonas aeruginosa, Salmonella typhimurium, Campylobacter jejuni* and *Vibrio cholerae*, the oil from the leaves was found to be more active; the oils showed MIC values of 2.25, 3.12, 3.84 and 4.02mg/mL respectively. The data indicated that *Staphylococcus aureus, Bacillus subtilis* and *Listeria monocytogenes* were the most sensitive strain tested to the oil of *Origanum vulgare* with the strongest inhibition

Table 3: Antibacterial activity of *Origanum vulgare* ssp. vulgare extract and essential oil against the bacterial strains tested based on MIC and disc diffusion method

Micro-organisms	*MIC (mg/mL)	**Disc diffusion assay (inhibition zone mm)
Staphylococcus aureus	0.10	43
Bacillus subtilis	0.15	38
Listeria monocytogenes	0.19	32
Clostridium botulinum	0.98	28
Clostridium perfringens	1.58	24
Escherichia coli	1.82	22
Pseudomonas aeruginosa	2.25	14
Salmonella typhimurium	3.12	11
Campylobacter jejuni	3.84	10
Vibrio cholerae	4.02	8

*MIC: Minimal Inhibitory Concentration, concentration range: 0.10-4.02 mg/ml.

**Disc diameter 6 mm average of three consecutive trials

zone 43, 38 and 32mm. The *Clostridium botulinum, Clostridium perfringens* and *Escherichia coli* was, in general, found to be more sensitive among bacteria with inhibition zone of 28, 24 and 22mm. Modest activities were observed against *Pseudomonas aeruginosa, Salmonella typhimurium, Campylobacter jejuni* and *Vibrio cholerae*, with inhibition zones of 14, 11, 10 and 8mm (Table 3).

These results are similar to those found by [22-44]. The component of this oil, carvacrol, has been known to exhibit antimicrobial activity against the bacterial strains (*Proteus vulgaris, Salmonella typhimurium, Enterobacter cloacae, Klebsiella pneumonia, Escherichia coli, Serratia marcescens* and *Pseudomonas aeruginosa*) [43]. Carvacrol and Thymol are the main components of the essential oil of *Origanum vulgare,* which are responsible for its antimicrobial [77]. Similarly, the results of present study are in accordance with the reports on oregano oil against GNB viz., *Proteus vulgaris, Aeromonas hydrophila, Klebsiella pneumoniae* and *Escherichia coli* [22] and with the reports on oregano oil against *Staphylococcus saprophyticus, S. aureus, Micrococcus roseus, M. kristinae, M. nishinomiyaensis, M. lylae, M. luteus, M. sedentarius, M. varians, Bacillus megaterium, B. thuringiensis, B. alvei, B. circulans, B. brevis, B. coagulans, B. pumilus, B. laterosporus, B. polymyxa, B. macerans, B. subtilis, B. firmus, B. cereus* and *B. lichiniformis* [78]. The antimicrobial activities, in general have been mainly explained through terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall antimicrobial effect of essential oils [79]. Essential oils rich in phenolic compounds, such as carvacrol, are widely reported to possess high levels of antimicrobial activity [62-22]. On the other hand, it has previously been shown that carvacrol [80] is capable of inhibiting bacteria. It has been suggested that carvacrol exerts its activities by interacting with the cytoplasmic membrane via its own hydroxyl group, thus changing the permeability of membrane for protons and potassium ions [81]. The study demonstrated that *Origanum vulgare* represents a source of natural mixtures of antibacterial constituents that can be as effective as modern medicine to combat pathogenic micro-organisms.

CONCLUSION

Present study was conducted to investigate the chemical composition and antibacterial activity of essential oil extracted from *Origanum vulgare*. The leaves oil obtained from *Origanum vulgare* was characterized by GC-MS, GC-FID and 33 volatiles compounds were identified which made up 76.62% of the total essential oil. The essential oil yields of the studies were 1.15%. The major compounds were carvacrol (18.06%) followed by thymol (7.36%), γ-Terpinene (5.25%), *p*-cymene (5.02%), Limonene (4.68%), Caryophylene (4.12%), cymene (3.56%), ledene (3.41%), linalool (2.47%), α-Pinene (2.15%), γ-Terpineol (2.10%) and Germacrene-D (2.08%). The bacterial strains *Staphylococcus aureus, Bacillus subtilis, Listeria monocytogenes, Clostridium botulinum, Clostridium perfringens, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium, Campylobacter jejuni* and *Vibrio cholerae* tested were found to be sensitive to essential oils studied and showed a very effective bactericidal activity with minimum inhibitory concentrations (MIC) ranging from 0.10 to 4.02 mg/ml.

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