

Chemical Composition, Antimicrobial and Antioxidant Properties of Essential Oil of *Origanum vulgare* ssp. *Gracile*

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ABSTRACT

BACKGROUND AND OBJECTIVE: Nowadays, in order to improve the health and reduce the risks of chemical preservatives in the food industry, researchers have focused on the use of natural compounds, especially plants essential oils that have antioxidant and antimicrobial properties, due to the increased risk of bacteria *Escherichia coli* and *Listeria monocytogenes* in foods, in this study the antimicrobial and antioxidant properties of *Origanum vulgare* ssp. *Gracile* essential oil were investigated in vitro.

METHODS: In this experimental study, *Origanum vulgare* ssp. *Gracile* plant was prepared from Kurdistan and after drying and extraction of essential oils, components were identified using GC-MS device. Strains were prepared from the bacteriology laboratory of the Faculty of Veterinary Medicine, Tabriz University. In order to determine the minimum inhibitory and fatality concentration, various concentrations of extract were used (0.312, 0.625, 1.25, 2.5, 5 and 10 %), the minimum inhibitory and fatality concentration were determined using microdilution method. The antioxidant activity was performed by using the DPPH method and Total phenolic material was measured by Folin Ciocalteu reagent.

FINDING: Based on the results of GC-MS analysis, carvacrol (49.33%) had the most component of the extract composition. The MIC and MBC of essential oils on *Escherichia coli* were 1.25 and 2.5%, respectively, and 0.625 and 1.25% for *Listeria monocytogenes*, respectively. In addition, the antioxidant activity 12.45 ± 0.03 µg/ml, and the content of phenol 39.13 ± 4.13 mg of gallic acid in grams of essence was calculated.

CONCLUSION: The results of this study showed that *Origanum vulgare* ssp. *Gracile* essential oil had antioxidant properties, and the essence had an inhibitory effect on both bacteria and *Listeria monocytogenes* was less resistant than *Escherichia coli* to the essential oil.

KEY WORDS: *Origanum Vulgare* Essential Oil, Antioxidant Activity, Anti-Microbial Activity, Minimum Inhibitory Concentration.

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Introduction

The use of essential oils in the pharmaceutical and food industries, traditional medicine and herbal remedies is based on the antioxidant and antimicrobial properties of the essential oil (1). The presence of chemical compounds such as cineole, camphor, Linalool, alfa-pinene, borneol, carvone, limonene, carvacrol, cymene, camphene and α -Terpineol in the essential oils of the different organs of plant produce antioxidant and antimicrobial effects of the essential oil (2). Essential oils and extracts from medicinal herbs with anti-microbial, anti-cancer, anti-oxidant and free radical remover compounds are extremely powerful for use as new and natural preservatives (4,3).

Among the chemical compounds of essential oils, phenolic compounds have a higher contribution than other compounds, these compounds have antioxidant properties that are due to their regenerative capacity and their chemical structure, which neutralizes free radicals, formation of complex with ions metal and disable them and prevent oxidation (5). The genus *Origanum vulgare* belongs to the Labiatae family and includes many species that are commonly found widely in the Mediterranean (6) and have a high morphological and chemical diversity in the world (7). *Origanum vulgare* L. has six subspecies (ssp. *Hirtum* ssp. *Vulgare*, ssp. *Virens*, ssp. *Viride*, ssp. *Gracile* and ssp. *Glandulosum*) all over the world. In Iran, only three *viride*, *vulgare* and *gracile* subtypes have been identified. (8).

The active ingredient of *Origanum vulgare* is essential oil, which is mainly carvacrol and thymol, but also has other compounds, such as gamma tryphenene, paracymene, linalool and sabinene (9). Moradi et al. reported the major components of *Origanum vulgare* essential oil as carvacrol, and after carvacrol, they introduced the most essential component of gamma tryphenene and paracymene, respectively (10). *Origanum* herbs are widely used as spices, due to the specific compounds of the essential oil (11). *Origanum vulgare* L. in addition to traditional medicine as a medicine, is a diuretic and antiseptic for the treatment of gastrointestinal, intestinal and constipated diseases (12). Recently, spice herbs have attracted the attention of many consumers due to antimicrobial, antifungal, insecticidal and antioxidant effects (13). Today, *Origanum vulgare* plant parts and its biochemical extracts, including whole plant, leaf, essential oil, etc.

are commonly used in the food industry as spices and antioxidants of lipids. *Origanum vulgare* is rich in phenolic antioxidants and an important source of food additives (14). Studies have shown that taking antioxidants in the diet reduces the risk of cancer and delays Alzheimer's disease (15).

Penalver et al. investigated the effect of several essential oils such as *Origanum vulgare* on *Salmonella* and *Escherichia coli* isolated from chicken and pigs. Among the essential oils, *Origanum vulgare* essential oil showed the highest antimicrobial activity. The researchers reported high levels of carvacrol and thymol as inhibitors (16). In the same study, *Origanum vulgare* essential oil had antimicrobial effects on *Salmonella typhimurium*, which 1% concentration of essential oil inhibited microbial growth (17).

Sarikurkcu et al., evaluated the antioxidant and antimicrobial effects of various essential oils of *Origanum vulgare* subspecies, indicated that thymol and linalool were the main components of antimicrobial (anti-bacterial and antifungal) and antioxidant properties (18). In addition, Imtara et al., showed antimicrobial and antioxidant properties of *Origanum vulgare* essential oils in honey, which depend on the content of phenolic compounds and flavonoids, which vary in different regions (19). Accordingly, the components of the essential oil compounds of the herbs depend on the genetic, climatic and geographical conditions of the collection sites (20).

The effects of these factors, as well as other ecological factors, lead to the formation of distinct metabolites, which leads to the identification of new types (21). Climatic conditions, harvest time, storage time, extraction method and genetic differences of the plant, on the type and amount of compounds in the essential oil of the plant can also affect chemical and antimicrobial properties (22).

Therefore, considering the evidence of the antioxidant and antimicrobial properties of essential oils, and since *Origanum vulgare* ssp. *gracile* is one of the three sub-species of the genus *Origanum vulgare* in Iran and there is in different geographical regions, could be considered as a natural and economical preservative to be used and to avoid undesirable effects of chemical and abnormal preservatives is a suitable alternative. Therefore, the aim of this study was to determine the chemical composition and antioxidant and

antimicrobial effects of *Origanum vulgare* ssp. *gracile* essential oil collected from Mountainous regions of Kurdistan.

Methods

Essential oil preparation: In order to carry out this experimental study, *Origanum vulgare* was collected from mountainous areas of Kurdistan and was kept at Uromiyeh University. The collected *Origanum vulgare* after identification by Herbarium Agricultural College with the registration number 259111 was used for experiments. In order to prepare the essential oil, the plant was crushed and then, using a Clevenger device, the essential oils were extracted by water distillation method (23). The obtained essential oil was digested with dry sodium sulfate, and after passing 0.45 µm micro filters were kept in a dark glass container, away from the sun at 4 ° C until the identification and determination of chemical compounds and antioxidant activity. All chemicals used were purchased from MERCK company.

Bacterial strains: To evaluate the antibacterial effects of *Origanum vulgare* essential oil, standard strains were used. These strains included *Escherichia coli* (ATCC 25922) and *Listeria monocytogenes* (ATCC 19117) from the bacteriology laboratory of the Faculty of Veterinary Medicine, Tabriz University. The strains were identified using culture media and biochemical tests.

Analysis of Essential Compounds Using GC-MS: Identification of essential oil composition was performed using retention indices and mass spectrometry of compounds and comparing them with standard mass spectra and valid references. For this purpose, the prepared essential oil was injected into the gas chromatography system and the most suitable temperature setting for the column was obtained for the complete separation of essential oils.

The essential oil was then injected into the mass spectrometer gas chromatography apparatus and the mass spectrum of the compounds was obtained. In this study, the Agilent6890 GC-MS device was used with a 30 mm long hairpin column, internal diameter of 0.25 mm and internal layer thickness of 0.25 micrometers of type HP-5MS with a column temperature program initially at 70 ° C with a 2 minute-stop, then temperature

was increased to 220 °C, 15 °C per minute, and the column temperature increased to 300 °C for 2 min. (24).

Evaluation of phenolic compounds: Total phenolic materials were evaluated using the Folin-Ciocalteu reagent and Gallic acid as a standard (22). At first, different concentrations of essential oil were prepared and 0.1 ml of each concentration was added to the Erlenmeyer, 46 ml of distilled water and 1 ml of Folin-Ciocalteu reagent were added to the mixture, then the contents of the Erlenmeyer were mixed vigorously and after 3 minutes, 3 ml of 2% sodium carbonate solution was added and the solution was placed on a moderately shaky page for 2 hours and then its absorbance was read by spectrophotometer at 760 nm. The previous steps were carried out for standard Gallic acid solutions (1000-0 µg / 0.1 ml) and plotted to determine the standard curve. Based on that, the concentration of phenolic substances was calculated and expressed as Gallic acid per gram of essential oil (25).

Evaluation of antioxidant activity: Evaluation of the hydrogenation ability of extracts and essential oils is measured by colorless of Purple DPPH methanol solution (26). In this spectroscopic evaluation, stable radical of Diphenyl picrylhydrazyl is used as a reactive agent. The procedure was followed by adding 50 µl of different concentrations of essential oil (from 5 to 40 PPM) to 5 ml of methanol solution of 0.004% DPPH and after 30 minutes of storage at room temperature, absorption at 517 nm compared to the control was read. The DPPH-free radical inhibition based on percent (%) was calculated as follows:

$$\text{I\%} = (\text{A}_{\text{blank}} - \text{A}_{\text{sample}} / \text{A}_{\text{blank}}) \times 100$$

A_{blank} is the absorbance of the control solution (containing all the reagent materials except the essential oil) and A_{sample} is the absorbance of the solution containing various concentrations of the essential oil. Synthetic BHT antioxidants were also used as positive control and the tests were performed in three replicates and their mean values were declared as the desired amount. The antioxidant activity of essential oil is expressed as IC₅₀ value, which indicates the concentration of essential oil, which causes 50% inhibitory activity in radical capacity.

Determination of Minimum inhibitory concentration (MIC): To determine the minimum inhibitory concentration of inhibitor, the consecutive concentrations of essential oil (10, 5, 2.5, 1.25, 0.625

and 0.312 %) were used, initially 100 µl culture medium of the Hinton broth (German marker) was added in the first row to the final concentration of the wells (in each culture medium used in this study, the percentage of salt required for each bacterium was added). Then, the 100 µl of essential oil or extract was added to the first well of each row (previously, the concentrations of essential oil were made with 10% DMSO solvent). After mixing the contents of the first well, 100 µl was added to the next well. The 100 µl of the final well was discarded. For each row of the test (for an essential oil or extract), a control row was considered. The dilution steps of the essential oil were performed in the same way as the test rows. Then 100 µl desired bacterial suspension (with a concentration of $10^6 \times 5$ bacteria per ml) was added into the rows. But bacterial suspension was not added to the control row. The micro dilution method was used to perform this test. After 24 hours' incubation at a suitable temperature for each bacterium, the results were read by the spectrophotometer and the presence of turbidity (compared to the control row) indicates bacterial growth and transparency, indicates lack of bacterial growth (28, 27).

Minimum bactericidal concentration (MBC): To determine the minimum bactericidal concentration, 10 µl of wells at the end of an 18-hour incubation period, was cultured on the Müller Hinton Agar (Merck, Germany). Petri dishes were incubated for 18 hours to determine the growth of bacteria. Finally, the least concentration of essential oil or extract, in which 99.9% of the bacteria did not grow, were reported as MBC. All experiments were repeated three times (28).

Results

Based on the results of essential oil analysis, the essential oil content of *Origanum vulgare* plant is 2% based on the dry weight of the sample. Table 1 shows the major constituents of *Origanum vulgare* essential oil with inhibition time and the percentage of each compound, which contains 93.93% of the essential oils. Most of the essential oil compounds were carvacrol (49.33%), gamma terpinene (15.33%), benzene, ethyl dimethyl (6.4%), carvacrol methyl ether (4.2%), Myrcene (36.3%), thymol (2.79%).

Table 1. *Origanum vulgare* essential oil composition analysis using GC/MS

row	Compounds	Inhibition time (Min)	Percentage	row	Compounds	Inhibition time (Min)	Percentage
1	Carvacrol	34.53	49.33	16	alpha-Pinene	20.728	0.56
2	gamma terpinene	22.41	15.53	17	alpha-Pinene	15.998	0.83
3	benzene-1-ethyl-2-4- dimethyl	20.336	6.14	18	beta-Pinene	18.065	0.26
4	Methyl ether carvacrol	31.55	4.2	19	Beta- Bisabolene	44.376	0.27
5	Myrcene	18.736	3.36	20	6,3,1, -octatrien 7,3,-dimethyl	21.685	0.29
6	Thymol	33.668	2.79	21	Phellandrene	19.475	0.26
7	3-Octanol	17.864	2.78	22	D-Limonene	20.785	0.26
8	Alpha- Terpinene	20.145	2.35	23	L-Linalool	24.168	0.23
9	α-Phellandrene	15.636	2.07	24	α-Terpineol	29.048	0.23
10	alpha -Caryophyllene	45.739	1.99	25	Beta-sesquiterpene Phellandrene	44.971	0.2
11	Cis-Ocimene	21.095	1.9	26	2 -hexonal	11.017	0.19
12	7- Octene-4-first, 1- Octene -5-first	17.757	1019	27	Carvacrol acetate	37.322	0.16
13	(Z)-beta- Terpineol	22/578	0.97	28	Camphene	16.652	0.1
14	Beta-Caryophyllen	40.777	0.48	29	3-caron	19.887	0.1
15	Terpineol-4	28.271	0.82	30	Terpinolen	23.881	0.09

Evaluation of antioxidant activity of Essential oil:

The antioxidant activity of the essential oil was evaluated based on the DPPH method, and the IC₅₀ for the essential oil was 12.45±0.33 mg/ml, which compared to the hydroxytoluene butylated (BHT) 7.03±0.01) is weaker. Also, the results showed that by increasing the concentration of essential oils, free radicals were inhibited strongly.

The amount of phenolic compounds of essential oil:

The phenol content of the essential oil was 39.11 ± 4.23 mg of Gallic acid per gram of essential oil using Folin-Ciocalteu method.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC):

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Origanum vulgare* essential oil by broth micro dilution method on the studied microorganisms are presented in Table 2. The results of this study showed that the essential oil has antimicrobial effects. In general, according to the results obtained from this study, it can be stated that gram positive bacteria are more sensitive to essential oils than gram negative bacteria.

Table 2. Min Inhibitory Concentration (MIC) and Min bacteriocidal Concentration (MBC) of *Origanum vulgare* essential oil on the studied bacteria in percent

Bacterium	Type of bacterium	Origanum vulgare essential oil	
		Mean±SD	
		MIC	MBC
Listeria monocytogenesis	+	0.625±0.01	1.25±0.02
E. coli	-	1.25±0.02	2.5±0.01

Discussion

According to the results, *Origanum vulgare* ssp. *Gracile* essential oil had an inhibitory effect on the studied bacteria, so that *Listeria monocytogenesis* was less resistant to essential oils than *Escherichia coli*. The results also showed the antioxidant properties of the essential oil. The results indicate that the antioxidant and antimicrobial properties of the essential oil are influenced by the presence of phenolic compounds in

the essential oil, the compounds obtained from this study are consistent with other studies (30, 29). Andi et al. performed a study on the essential oils of other *Origanum vulgare* species from Southern Chalus, the dominant constituents at the flowering stage were reported Linalool acetate (27.2%), gamma terpinene (16.5%), 3-octanone (10.9%), carvacrol (6.4%) and in seeding stage reported carvacrol (23.2%), Alpha-pinene (15.8%), betapinene (10.7%) and transcaryophyllene (5.3%) which, in terms of compounds, was similar to this study, but differed in terms of the composition percentage (8). In a study by Tahmasebi et al. on the essential oil of *Origanum vulgare*, it was shown that the highest composition of essential oil was sesquiterpene and, in other species, the most important components of the essential oil was tryphenolen-4-L and gammatripen (31). In another study in India on two species of *O. majorana* and *O. vulgare* spp. *Hirtium*, the highest amount of essential oil was reported for trypinolen-4-L (32). Sokmen et al., by analyzing the essential oils of *Origanum vulgare* plant, showed that the highest amount of compound in the essential oil is carvacrol (33) which is similar to that of essential oil analysis of this study. Investigating the antimicrobial and antioxidant properties of edible film containing thyme essential oil, the increase in the concentration of essential oil significantly increased the concentration of phenolic compounds, which also increased the antioxidant and antimicrobial properties [34]. Evaluation of anxiolytic effects of essential oil and methanolic extracts of Shirazi thyme, sage, rosemary and Cinnamon showed that Shirazi thyme essential oil had the highest antioxidant effect (IC₅₀ = 667 µg / ml), which is very high in this study and therefore has the weaker antioxidant property (35). The inhibitory percent is inversely related to anti-radical activity of the compounds. In another study, the inhibitory effect of parsley essential oil was 80.21 mg / ml, indicating low antioxidant properties of parsley (36). Alma et al. investigated the antioxidant effects of the *Origanum vulgare* essential oil and they found that antioxidant properties of essential oil is concentration-dependent and was slightly less than ascorbic acid or BHT, they attributed this effect to a high concentration of phenolic compounds such as carvacrol (26.97%),

thymol methyl ether (1.3%) in plant essential oil (37). In this study, the high level of carvacrol (49.33%) can be attributed to the high antioxidant capacity of the *Origanum vulgare* plant, which this inhibition was weaker than BHT. In other studies, by investigating antioxidant activity of thyme and sweetheart essential oil was found to be 8.9 and 5.8 mg / ml (38%) and methanol extract and essential oil of peppermint with inhibitory concentration of 74.4 µg/ml and 10700 µg/ml, respectively (39) indicate the high antioxidant properties of the essential oil of *Origanum vulgare* ssp. *gracile*. Mahmoudzadeh et al. investigated the antioxidant effect of *Kahlik oti* essential oil, indicating that the IC₅₀ of *Kahlik oti* was 32.35 µg / ml in inhibition of free radicals and its inhibitory power was lower than *Origanum vulgare* essential oil, as well as *Kahlik oti* essential oil compared to BHT, it showed a poorer antioxidant property that is consistent with this study (40). The presence of a different structure in the wall of the gram-positive and negative bacteria causes them to change their resistance to antimicrobial compounds. Due to the fact that a large part of the walls of the gram-negative bacteria is composed of lipoprotein and lipopolysaccharide, as well as the presence of a thin layer of mucopeptide in their walls, it can be attributed the higher resistance of these bacteria to the external phospholipid membrane, which also been reported in the results of other researchers (41). Mahmoudi et al. showed that the antimicrobial effects of *Oregano* essential oil on *Staphylococcus aureus* were 0.015 and 0.03% (42). In a study, the bacteria of the hospital infection agent were highly sensitive to *Origanum vulgare* and could have antibacterial effects depending on the germs and the concentration of essential oil or extract of the plant. In this study, the effects of *Origanum vulgare* essential oil on pathogenic bacteria including *Escherichia coli*, *Bacillus aureus*, *Klebsiella pneumoniae*, *Helicobacter pylori* were studied. *Origanum vulgare* demonstrated all inhibitory effects on all tested organisms. The researchers suggested that *Origanum vulgare* alone or in combination could be effective in the treatment of infections (43), which our study, based on the results of the antimicrobial activity test could prove this. In another

study on antimicrobial effects of Pennyroyal and Peppermint on *E. coli*, the MIC of Pennyroyal on *Bacillus cereus* was 5000 and *Escherichia coli* was 4166 µg / ml, and the MIC of Peppermint essential oil on two bacteria was 100000 µg / ml, which is consistent with our research. Sefidkon et al. also investigated the effects of three species of Sweetheart on *Salmonella paratyphoid*. The results showed that Sweetheart essential oil had a inhibitory effect in 2.5 and 5% concentrations, which has a lower antimicrobial activity than *Origanum vulgare* essential oil (44). One study showed that *Origanum vulgare* essential oil had antimicrobial effects on *Salmonella typhimurium* bacteria, which 1% of essential oil inhibited the microbial growth (45). The results of MIC in this study revealed that the essential oil of *Origanum vulgare* in lower concentration (0.625%) can have inhibitory effects on bacteria. In another study, *Origanum vulgare* essential oil effectively inhibited the growth of microbes produced in food such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli* (46). Finally, it can be said that the differences in the results obtained in various studies can be explained by the variation in the chemical composition of the plants, their different reaction mechanisms and the different kinetics of their inhibitory reactions (under the influence of genetics, water, air, environment, harvesting season ...) in selected methods. There is also a direct relationship between the amount of phenol and the antioxidant activity of medicinal plants (47). According to the results obtained in this study and the need for using natural preservatives, the essential oil in the food industry can be used to increase the shelf-life and protect it from oxidative and microbial agents and control of food-borne microbial diseases. It is suggested that further studies of this essential oil be considered in food models and in combination with other essential oils.

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