Chemical Composition and Antibacterial Effect of Medicinal Plants against Some Food-Borne Pathogens

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Abstract
Background: Pulicaria gnaphalodes, Ducrosia anethifolia, Trachyspermum copticum, Foeniculum vulgare Mill and Majorana hortensis Minch are widely used as herbal plants in traditional medicine and they have been reported to have a variety of therapeutic effects. This study was carried out to evaluate the antimicrobial effects of essential oils (EOs) extracted from these medicinal herbs against six species of food-borne microorganisms.

Materials and Methods: The EOs were analyzed by gas chromatography mass spectrometry (GC/MS). The detection of inhibitory effect of the EOs on the tested bacteria was carried out by agar disk-diffusion method and then MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) of the EOs against six bacteria were determined.

Results: The analysis of the components of the essential oils (EOs) extracted by gas chromatography spectrometry allowed the identification of 63 compounds of the five tested EOs. All of these five tested EOs indicated an antimicrobial effect against strains of Bacillus sp and Listeria Monocytogenes ATCC1297. Essential oils from T. copticum, M. hortensis and F. vulgare possessed a wide spectrum of antibacterial activity against the growth of the six bacteria with zone diameter of inhibition (ZDI) between 14-32 mm, depending on the susceptibility of the tested organism.

Conclusion: Antibacterial efficacy shown by these plants provides a scientific basis and thus validates their use as medicinal remedies. Isolation and purification of different phytochemicals may further yield significant antibacterial agents.

Keywords: Essential oils; Antimicrobial activity; Chemical composition; Medicinal herb

Introduction
In modern societies, the safety of meat and its products is widely recognized as meat consumption is important for human growth, development and maintenance of health (1). A major issue related to meat consumption is the presence of pathogens and among them the causative agents of food-borne diseases are the leading causes of illness and death (2). Food processors, food safety researchers, and regulatory agencies have been increasingly concerned with the growing number of food-borne illnesses outbreaks caused by the pathogens like Staphylococcus aureus, Salmonella sp, Clostridium perfringens, Campylobacter, Listeria monocytogenes, Vibrio parahaemolyticus, Bacillus sp, and enteropathogenic Escherichia coli (1,3). These bacteria cause over 90% of all cases of food poisoning (4). Listeria monocytogenes is a ubiquitous gram-positive pathogen responsible for a serious disease named listeriosis and the overall mortality rate associated with this disease is 30%. Antibiotic use has been banned in the European Union since January 2006. For this reason, scientists
have become interested in evaluating other alternatives to control specific microbial populations to modulate rumen fermentation (5). The antibacterial activities of spices and their essential oils have been known for a long time and a number of researches have reported the antibacterial effect of these spices, their essential oils as well as their derivatives (6). Plant essential oils are the potentially useful sources of antimicrobial compounds. Numerous studies have been published on the antimicrobial activities of plant compounds against many different types of microorganisms including food-borne pathogens (7). For a long time, plants from the Apiaceae family have been used as spices or drugs particularly due to their essential oils. A dozen important herbal medicinal products from this botanic family are used in some pharmacopoeias, antiseptic, expectorant, diuretic, carminative, vasodilator or spasmytic actions (8). The members of this family are well known as vegetables, culinary and medicinal plants such as *Foeniculum vulgare*, *Ducrosia anethifolia* and *Trachyspermum copticum* Minch. 

**Materials and methods**

*Isolation of essential oil portions*

Portions (100 g) of each prepared plant material were hydro distilled for 3h in a Clevenger type apparatus to isolate the essential oils. The obtained essential oils were dried over anhydrous sodium sulfate. After filtration, they were stored in the dark glass bottles at -4 °C until used for further analyses. The following oils were used: *Pulicaria gnaphalodes*, *Ducrosia anethifolia*, *Trachyspermum copticum*, *Foeniculum vulgare* Mill and *Majorana hortensis* Minch.

**Gas chromatography (GC) and gas chromatography mass spectrometry (GC/MS) analysis**

The composition of the volatile constituents was established by gas chromatography-mass spectrometry (GC-MS) Thermo-UFM (Ultra-fast model, Milan, Italy) equipped with flame ionization detector (FID) and a capillary column Ph-5 (nonpolar) (10 m × 0.1 mm, film thickness 0.4μm, the inner surface covered with stationary phase material, dimethyl siloxane phenyl 5 %). Helium was used as a carrier gas with inlet pressure of 0.5 ml/min. Column temperature was held at 60 °C for 3 min, programmed to 285 °C at the rate of 80 °C/min. Detector and injector chamber temperatures were 280 °C. GC-MS analyses were carried out on a Varian 3400 GC-MS system connected to a mass spectrometer model Saturn II, using an ion trap system having ionization energy of 70 eV, with a semi-polar DB-5 column, with dimensions of 30 m × 0.25 mm, and a film thickness 0.25μm. Gas pressure was 35 pounds per square inch, column temperature was 40 to 250 °C with a rate of increase of 4 °C/min, and the injection chamber temperature and the transfer line were set at 260 °C and 270 °C, respectively. The carrier gas was helium with a linear velocity of 31.5 cm/s. A scan time of 1 s with a mass range of 40-300 amu was used. The components of each oil were identified by comparison of their retention indices (RI) relative to n-alkenes (C7- C25) and confirmed by comparing their mass spectra with those of authentic samples or with data already available in the literature. The quantification of the volatile compounds was performed by GC-MS peak area normalization method using a Shimadzu C-R4AChromatopac without any correction factor (15).

**Antimicrobial assay**

Agar gel disk diffusion test, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were used in this study.

**Microorganisms**

*Bacillus cereus*, *Bacillus sphericus*, *Bacillus antheracoid*, *Bacillus coagulance*, *Bacillus subtilis*
and *Listeria monocytogenes* ATCC 1297 were obtained from the School of Veterinary Science of Shiraz University.

**Disk diffusion susceptibility**

Antibacterial Susceptibility Assay Muller-Hinton Broth (MHB, Merck) medium was used to grow the test isolates for 22 h at 37 °C. Final bacterial numbers were standardized to 1×10⁶ cfu/ml. A total of 0.1 ml of bacterial suspension was poured into each plate containing Muller-Hinton Agar. The surface culture was prepared by sterile L shape pipet Pasteur and allowed to remain in contact for 1 min. Thereafter, a 5% concentration of each plant essential oils was prepared. The sterile filter paper disks (6-mm diameter) were placed on the cultures and 24 h after incubation at 37 °C the zone diameter of inhibition (ZDI) was measured in mm. In order to determine the sensitivity of each bacterial species tested, tetracycline was used as a positive control standard. All the experiments were performed in triplicate (1).

**Determining the MIC and MBC**

For each essential oil, a set of 9 sterile test tubes was used. The stock solutions (500 mg/ml) were further diluted in a 2-fold serial dilution to obtain the following concentrations: 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.91, 1.95, and 0.98 mg/ml. One test tube as a negative control and tetracycline as a positive control was used. An aliquot of 1 ml of the bacterial suspension was inoculated into each tube. The negative control tubes were inoculated with the same quantity of extracts. All tubes were incubated at 37 °C for 24 h. The lowest concentration that did not permit any visible growth when compared with the control was considered as the minimum inhibitory concentration (MIC). The contents of all tubes that showed no visible growth were cultured on Muller Hinton agar and incubated at 37 °C for 24 h. The MIC was considered as the lowest concentration that could not produce a single bacterial colony and the MBC was defined as the lowest concentration of the extract at which 99.9% of the inoculated microorganisms were killed (16).

**Statistical analysis**

In order to determine whether there is a statistically significant difference among the obtained results from zone inhibition assays, MIC and MBC average analyses were carried out using the SPSS V16.0 statistical software package. The differences between any means were tested by the Duncan test and the results were considered significant when P<0.05.

**Results**

**Chemical composition of the EOs**

The main constituents of the studied essential oils are presented in Table 1.

| Plant       | Compounds            | (%)  | RI   | RT(min) |
|-------------|----------------------|------|------|---------|
| *T. copticum* | β-Pinene             | 0.63 | 989.52 | 1.5     |
|             | Myrcene              | 0.40 | 1010.10 | 1.52    |
|             | p-Cymene             | 22.45| 1049.25 | 1.6     |
|             | 8-terpinen           | 35.45| 1081.93 | 1.67    |
|             | *Thymol*             | 38.97| 1309.99 | 2.11    |
|             | α-Pinene             | 0.76 | 956.99 | 1.42    |
|             | β-Pinene             | 0.55 | 989.52 | 1.5     |
|             | p-Cymene             | 0.34 | 1044.47 | 1.59    |
| *F. vulagre* | Limonene             | 6.06 | 1058.73 | 1.62    |
|             | Fenchone             | 11.15| 1121.99 | 1.76    |
|             | Methyl Chavicol      | 3.79 | 1232.62 | 1.96    |
|             | e-Anethole           | 76.80| 1324.80 | 2.14    |
|             | β-Pinene             | 2.46 | 989.52 | 1.5     |
|             | Limonene             | 0.29 | 1058.73 | 1.62    |
|             | 1,8 cineole          | 0.94 | 1063.43 | 1.63    |
| *M. hortensis* | Linalool             | 86.44| 1113.27 | 1.74    |
|             | geranyl acetate      | 1.17 | 1436.12 | 2.32    |
|             | β-elemene            | 1.60 | 1477.11 | 2.39    |
|             | e-caryophyllene      | 0.61 | 1488.60 | 2.41    |
|             | 8-cadinene           | 1.49 | 1561.04 | 2.54    |
|             | 1,1-di-epi-cubinol   | 0.39 | 1693.50 | 2.72    |
|             | ten-cadinol          | 2.85 | 1712.91 | 2.75    |
The components by gas chromatography and gas chromatography mass spectrometry allowed the identification of 63 compounds, accounting for 94.16%, 97.93%, 97.9%, 99.45% and 98.24% of the composition of volatile substances of Pulicaria gnaphalodes, Drcosia anethifolia, Trachyspermum copticum, Foeniculum vulgare Mill and Majorana hortensis Minch, respectively. The main components of T. ammi EO were thymol (38.97%) and 8-terpinene (35.45%). The components of F. vulgare Mill EO were E-anethole (76.80%) and Fenchone (11.15%). The major components of EO obtained from M. hortensis Minch was linalool (86.44%). The main components of D. anethifolia were Cis-chrysanthenyl acetate (72.28%) and E-anethole (4.33%). 1, 8-Cineole (14.12%) and G-cadinol (13.76%) were the major constituents of P. gnaphalodes EO.

Antimicrobial activity of the tested EOs

For evaluation of bacterial susceptibility to herbal agents three standard tests were carried out, including disk diffusion assay, minimum inhibitory concentration and minimum bactericidal concentration. As can be seen in Tables 2-4, the T. copticum essential oil reveals the potential antibacterial activity against all of the tested bacteria with the mean ZDI ranging from 23.66 to 32.66 mm. The Listeria monocytogenes showed the most susceptibility to the essential oil of T. copticum with the ZDI of 32.66 mm that was more than tetracycline with the ZDI of 30.5 mm (Table 2). As have been shown in Tables 2-4, the lowest effect of essential oil against bacteria was related to D. anethifolia and there was no effect on B. subtilis and B. coagulans. The ZDI of T. copticum essential oil against all of the bacteria was more than control group (tetracycline).
Herbal Plants as Antibacterial Agents

Table 2. The inhibition zone (mm) of selected herbal essential oils against food-borne pathogen bacteria.

| Herbal (EO) | B. cereus | B. sphericus | B. antheracoid | B. coagulance | B. subtilis | L. monocytogenes |
|------------|-----------|-------------|---------------|--------------|-------------|-----------------|
| T. copticum | 31.3±1.15 | 30±2 | 29±1.73 | 27.66±3.78 | 23.66±0.57 | 32.66±6.42 |
| F. vulgare  | 15.66±3.51 | 15.33±3.05 | 14±3.46 | 15.33±2.30 | 16.66±4.16 | 16±3 |
| P. gnaphalodes | 0 | 15.33±1.15 | 14±2 | 0 | 0 | 12±1 |
| D. anethifolia | 13.33±3.05 | 11.33±1.15 | 17±5 | 0 | 0 | 19.66±3.21 |
| M. hortensis  | 15.33±3.05 | 20±4 | 16±2 | 14±3.46 | 20.66±4.16 | 26±2 |
| Tetracycline  | 18±4 | 35.33±6.42 | 29±2 | 26.66±4.61 | 18±3.46 | 30.66±5.13 |

Values are mean ± S.D. of three replicates. Means with different superscripts within the same row are significantly different (p<0.05).

The MIC and MBC values of the leaf essential oils, at different concentrations ranging from 0.98 mg/ml to 250 mg/ml, in comparison with the activity of tetracycline has been shown in Tables 3 and 4.

Table 3. MIC (mg/ml) values of essential oil of the selected plants against isolated bacteria.

| Herbal (EO) | B. cereus | B. sphericus | B. antheracoid | B. coagulance | B. subtilis | L. monocytogenes |
|------------|-----------|-------------|---------------|--------------|-------------|-----------------|
| T. copticum | 1.95 | 1.95 | 1.95 | 1.95 | 3.91 | 0.98 |
| F. Vulgare  | 7.8125 | 7.8125 | 7.8125 | 7.8125 | 7.8125 | 7.8125 |
| P. gnaphalodes | 125 | 7.8125 | 7.8125 | 125 | 125 | 15.625 |
| D. anethifolia | 7.8125 | 15.625 | 7.8125 | 125 | NO | 3.91 |
| M. hortensis  | 7.8125 | 3.91 | 7.8125 | 7.8125 | 3.91 | 1.95 |
| Tetracycline  | 7.8125 | 0.98 | 0.98 | 1.95 | 3.91 | 0.98 |
| Control negative | NO | NO | NO | NO | NO | NO |

NO: No effect
EO: Essential oil

The results of the MBC method (Table 4) are consistent with the results of the disk diffusion test as shown in Table 2. In summary, the essential oil extracted from T. copticum showed the highest antibacterial activity for these bacteria while D. anethifoliae essential oil had the lowest antibacterial activity.

Table 4. MBC (mg/ml) values of essential oil of the selected plants against isolated bacteria.

| Herbal (EO) | B. cereus | B. sphericus | B. antheracoid | B. coagulance | B. subtilis | L. monocytogenes |
|------------|-----------|-------------|---------------|--------------|-------------|-----------------|
| T. copticum | 3.91 | 3.91 | 3.91 | 7.8125 | 7.8125 | 3.91 |
| F. Vulgare  | 15.625 | 15.625 | 31.25 | 15.625 | 15.625 | 15.625 |
| P. gnaphalodes | NO | 31.25 | 62.5 | NO | NO | 31.25 |
| D. anethifolia | 31.25 | 31.25 | 15.625 | NO | NO | 15.625 |
| M. hortensis  | 15.625 | 15.625 | 15.625 | 31.25 | 7.8125 | 3.91 |
| Tetracycline  | 15.625 | 1.95 | 3.91 | 3.91 | 7.8125 | 3.91 |
| Control negative | NO | NO | NO | NO | NO | NO |

NO: No effect
EO: Essential oil
Discussion

The results of the essential oils extracted from different fresh aromatic plants collected from Borazjan, a city located in Bushehr Province in the south of Iran, have been shown in Table 1. To date, a large number of studies have focused on T. copticum EO and some of them have reported thymol as the main compound (17,18). In the present study, the major components of T. copticum EO obtained from Iran were thymol (38.97%) and 8-terpinen (35.45%). Similar to our results, Moazeni et al. (2012) reported that the essential oils extracted from Iran ecotypes consisted of 50.07% thymol (19). It is clear that geographical variation, cultivar differences, the stage of plant growth, preparation process and other factors may influence oil composition both quantitatively and qualitatively (20). Acidic nature of the hydroxyl group in thymol and the involvement of the hydroxyl group in the formation of hydrogen bonds may explain the highest antimicrobial activity (21). Based on current evidence, T. copticum EO can inhibit food-borne pathogenic microorganisms such as Staphylococcus aureus (22), Pseudomonas aeruginosa (23), Salmonella sp, Bacillus subtilis, Aspergillus flaus and Escherichia coli (24). In the present study, T. copticum EO had the strongest antibacterial effect against L. monocytogenes (32.66 mm inhibition zone). Detailed data obtained from a previous study indicated that the growth of pathogenic Bacillus cereus (35 mm inhibition zone) was significantly affected by T. copticum EO (24).

Pulicaria gnaphalodes is traditionally used as a flavoring agent in food. The major components of the essential oil of P. gnaphalodes obtained from different areas in Mashhad, a city located in Iran, were completely different from our results (13). Also, B. sphericus was more sensitive to P. gnaphalodes with the ZDI of 15.33 mm in comparison to B. antheracoid with the ZDI of 14 mm. It has further been reported that the MIC values of P. gnaphalodes against Salmonella typhimurium and Staphylococcus aureus were 0.2 and 0.1 v/v, respectively (25).

Verma et al. (2010) showed that M. hortensis has two chemotypes, including terpinen-4-ol and cis-Sabinene hydrate (26). In a research conducted by Verma et al. (2010) the main constituents of M. hortensis EO were Z-Sabinene hydrate (31.81), terpinen-4-ol (22.02) and (E)-Sabinene hydrate (27). The main components of the EO in the present study were Linalool (86.44%), ten-cadinal (2.85%) and β-pinene (2.46%). These differences in chemical compositions of the oils could be attributed to the effect of environment on the plants (28). Investigations showed that M. hortensis EO can inhibit food-borne pathogenic microorganisms such as Staphylococcus aureus (22 mm) and Enterobacter spp (15 mm) (29). M. hortensis showed that EO was more active than P. gnaphalodes against all tested pathogenic bacteria. The higher antibacterial activity of M. hortensis is possibly related to the high amount of Linalool that has been reported to possess antibacterial properties.

The components of F. vulgare EO in our study were e-Anethole (76.80%), Fenchone (11.15%) and Limonene (6.06%) which is relatively similar to F. vulgare composition from Pakistan (30). Dua et al. (2013) reported that the ZDI of F. vulgare against Staphylococcus aureus and Bacillus pumilus were 11.17 and 12.67 mm, respectively (31). The growth ZDI in the presence of F. vulgare EO seeds was 15.66 mm for B. cereus, 15.33 mm for B. pumilus, 14 mm for B. antheracoid, 15.33 mm for B. coagulance, 16.66 mm for B. subtilis and 16 mm for L. monocytogenes. However tetracycline was more efficient with the growth ZDI of 18-35.33 mm. N-decanal (70.1%), α-pinene (12.4%) and dodecanal (5.4%) are vastly utilized in the industrial companies as major components of essential oils. N-Decanal is used in fragrances and flavorings (32). The main components of D. anethifolia in this study were cis-chrysanthenyl acetate (72.28%), β-eudesmol (8.79%), and e-Anethole (4.33%). There is also an interesting similarity between our study and the study conducted by Sohrabi et al. (2016) regarding two main components including 1, 8-cineole 14.12% and G-Cadinol 13.76% (33). Biological activities such as antimicrobial, antibacterial and antianxiety effects have been reported for D. anethifolia (34). The growth ZDI in the presence of D. anethifolia EO seeds was 13.33 mm for B. cereus, 11.33 mm for B. sphericus, 17 mm for B. antheracoid, 0 for B. coagulance, 0 for B. subtilis and 19.66 mm for L. monocytogenes, although tetracycline showed to be more efficient with the growth ZDI of 18-35.33 mm.

Conclusion

In conclusion, the essential oils extracted from medicinal plants in this study have potential antibacterial activities against the bacteria strains. Our results support the use of these plants in traditional medicine and suggest that some of the plant essential oils possess compounds with good antibacterial properties that can be used as antimicrobial agents in the search for new drugs.

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Author Contributions

The present study was funded by HH and NGH. HH and NGH were also involved in the collection of data, statistical analysis and drafting of the manuscript. Gas chromatography (GC) analysis well done by MA K. MA Kand FE read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.
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