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Comparison of Chemical Components and Antibacterial Activity of Rosemary Essential Oil grown in Various Regions of Iran against Foodborne Pathogenic Bacteria

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Abstract

Aim: Although the discovery and development of antibiotics have undeniable role to control bacterial infections, but increasing concerns about resistance of microorganisms to antibiotics, leading to increasing demand for usage of antimicrobial as natural alternatives. The aim of this study was to investigate the effect of habitat on active components and antibacterial properties of rosemary essential oil on a group of the most common pathogenic bacteria.

Methods: This study was conducted from May to June at the University of Amol in 2015 year. In present study, rosemary plants were collected from regions of Kerman and Tehran and then essential oil extraction were performed by hydrodistillation. Essential oil compounds were identified by gas chromatography analysis. Standard disk diffusion, well diffusion and determination of minimum inhibitory concentration methods were used to study the antibacterial properties.

Results: The most important chemical compounds of rosemary essential oil were α-Pinene and 1,8-Cineole. The antimicrobial activity of essential oils on pathogenic bacteria revealed that the minimum inhibitory concentration of rosemary essential oils from Tehran region for *E. coli*, *S. typhimurium*, *S. aureus*, *L. monocytogenes* and *B. cereus* were 625, 1250, 312.5, 156.25 and 156.25 ppm respectively, and were 625, 625, 156.25 and 78.1 ppm for rosemary essential oils of Kerman, respectively.

Conclusion: The rosemary essential oil in relatively low concentrations is very effective on growth of pathogenic bacteria. Therefore, it can be used as a natural antimicrobial compound.

Keywords: Essential oils, Rosemary, minimum inhibitory concentration.

INTRODUCTION

Infectious diseases are the most common diseases in the world that impose huge financial burden to society. Although synthetic antibiotics have been able to play an important role in treatment of infectious diseases in recent decades, the existence of problems in relation to the incidence of microbial resistance to antibiotics, has led to increase the tendency of consuming the natural ingredients such as essential oils from medicinal plants [1]. Essential oils are a mixture of plant secondary metabolites that obtained from volatile parts of plant. Terpenoids (monoterpenoids and sesquiterpene terpenoids) and phenylpropanoids are two groups of the most important active chemical compounds in the essential oil [2]. Essential oil has positive impacts on cardiovascular diseases, some kinds of tumors, inflammatory reactions and very diseases that are spread by free radicals [3,4]. However, the most important activities of these compounds are their disinfectant and antimicrobial properties. Antimicrobial activity of essential oil is made by their effect on the cell membrane. At least, part of this activity due to the hydrophobic nature of aromatic hydrocarbons (terpenoids), which allow them to affect the bacterial cell membrane and accumulate in their lipid layers through situate in chain fatty acids. This function causes changes in the bacterial membrane structure, reducing the membrane stability, causing leakage of ions from membrane to outside and reduction in ion exchanges [5,6,7]. Among medicinal plants, rosemary plant belonging to the family *Lamiaceae* with more than one percent of essential oil and having antibacterial, antifungal and antioxidant activity is known as a valuable medicinal plant. This plant includes three genus such as *officinalis*, *Eryocalix* and *Tomentosus* widely grown in the western Mediterranean regions and it spread throughout the world from there [8].

Various studies have been conducted on the chemical components and antimicrobial activity of rosemary essential oils, including Zaouali et al (2016) in a study revealed that the most component of two different varieties of *Rosmarinus officinalis* consisted of camphor and 1,8-cineole, and essential oil of both varieties had acceptable antimicrobial activity [9]. In another study, Malakootian and Hatami could identify 20 compounds of rosemary essential oils which maximum amount belonged to α-Pinene, 1,8-cineole and linalool. They also determined that the minimum inhibitory concentration and minimum bactericidal concentration of investigated essential oils on *Escherichia coli* were 3000 and 3200 ppm [10].

Piskernik et al (2016) were studied antimicrobial effects of rosemary extract from two commercial varieties (V20 and

V40). They showed that extracts of both varieties could decrease more than 15% sporulation of Alicyclobacillus [5].

Barbosa et al (2016) have determined that the minimum inhibitory concentration of *Rosmarinus officinalis* essential oil for *Listeria monocytogenes* and *Escherichia coli* was 5 ppm and for *Salmonella enteritidis* was 10 ppm [11]. Also, Soltan Dallal et al (2011) and Jafarzadeh and Khalid (2010) approved the antimicrobial activity of *Rosmarinus officinalis* in separate researches [12,13].

Although the antimicrobial activity of rosemary essential oil was investigated by several researchers in different areas, but it is necessary to study the antimicrobial activity of rosemary essential oil in different areas due to variation of factors such as weather, soil pH, type and mineral composition of the soil affecting the composition and antimicrobial activity of essential oils [8]. Pintore et al (2007) Celiktas et al (2007) Keskin et al (2010) in studies on essential oils of medicinal plants such as rosemaries grown in different climate zones emphasized on this important issue that the antimicrobial power will be different by climate change [14,15,16].

The aim of this study was to investigate the chemical components of rosemary essential oil growing in two areas Tehran and Kerman, and antibacterial activity evaluation of them on the bacteria such as *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*.

MATERIALS AND METHODS

Collecting the plants and essential oils extraction

In order to evaluate the antimicrobial effect of rosemary essential oil, mentioned plants were collected from Tehran and Kerman provinces in the spring. Then rosemary plant were dried in favorable condition and were kept in dry and cool conditions until extraction of essential oils. In order to extraction, the mentioned plant was powdered by electric mill. Afterwards, 50 g of dried and powdered leaves of plant along with 700 ml of distilled water was poured in a balloon, and was extracted the essential oils for 3 hours in a Clevenger apparatus with 1 ml per minute distillation speed [17].

Studied bacteria

Microorganisms used in this study included *Escherichia coli* (PTCC 1399), *Salmonella typhimurium* (PTCC 1787), *Staphylococcus aureus* (PTCC 1112), *Listeria monocytogenes* (PTCC 1298) and *Bacillus cereus* (PTCC 1154) that were prepared from microbial collection of Pathobiology Department of Amol University of Special Modern Technologies.

Determination of antimicrobial activity of essential oils

Three standard methods included disk diffusion, microdilution and well diffusion methods were used in order to evaluate the antimicrobial properties of essential oils. Briefly, in disk diffusion method, 100 µL of bacterial suspension containing 5×10^5 CFU/ml were cultured on the plates containing Mueller-Hinton agar culture medium (Merck, Germany) and then 6 mm discs dipped into 15 µL of rosemary essential oil were located with appropriate distances on it. Diameters of inhibitory zone were measured in millimeters after 24 hours incubation at 37 °C [18]. In agar well diffusion, 100 µL of bacterial suspension containing 5×10^5 CFU/ml was cultured on the plates containing Mueller-Hinton agar culture medium. Then, wells were created on culture by sterile Pasteur pipette, and 10 µL of rosemary essential oils were poured into wells, and afterwards plates were incubated for 24 hours at 37 °C. Finally, diameters of inhibitory zone were measured [19].

Microdilution method was used in order to determine the Minimum Inhibitory Concentration (MIC) of rosemary essential oils on the growth of pathogenic bacteria. In this method, 2500 ppm of rosemary essential oils were prepared by using Mueller-Hinton broth medium culture (Merck, Germany) containing 3% DMSO (Dimethyl sulfoxide) and then 6 other concentrations was prepared by two fold serial dilution method. After that, 100 µL of 2500 ppm concentration was added to first well of each row of 96 cell microplate and 100 µL of 1250 ppm concentration was added to second well of each row. This work was continued until the seventh well. Finally, 95 µL of Mueller-Hinton broth culture medium and 5 µL of bacterial suspension containing 5×10^5 CFU/ml was added to each well. The eighth well was containing positive control (culture medium containing the bacteria without essential oils) and the ninth well was containing negative control (culture medium without bacteria). Microplates were incubated for 24 hours at 37 °C. After this period, the turbidity was read by spectrophotometer (Biotech instrument Inc, USA) at 570 nm wavelength and MIC was determined. MIC was considered as minimum concentration of an antimicrobial in which no growth was observed [20].

Identification of rosemary essential oils components

Components of rosemary essential oils were identified by gas chromatography (Younglin, the Acme 6000, Korea). Device's specifications are given in Table 1 [21].

Statistical analysis

The obtained results were analyzed by SPSS statistical software version 19. One way ANOVA and least significant difference test (LSD) were used to investigate the significant differences in the results, and the differences between mean of data was considered statistically significant at $P< 0.05$.

RESULTS

Identification of rosemary essential oils components by Gas Chromatography

Components of the essential oils were identified according to exit pattern of normal alkanes, retention indices and Kovats coefficients of components and finally, by compare them with reference indices of spectra in relation to each object. The results of compounds forming the rosemary essential oils are presented in Table 2. The results of rosemary essential oils for Tehran showed that the major and important of components included camphor (23.17%), α-Pinene (18.56%), 1,8-Cineole (11.89%), Verbenone (11.23%) and borneol (8.89%) and the main components of rosemary essential oil for Kerman region were included α-Pinene (32.44%), 1,8-Cineole (25.04%), Verbenone (4/15%), limonene (3.97%) and camphene (3.93%).

Table 1: Specifications of gas chromatography using for identify the components of rosemary essential oil.

Components	Specifications
Carrier gas	Helium gas with 1 ml per minute flow
Fuel	Hydrogen gas with 30 ml per minute flow and air flow with 300 ml per minute speed
Column	BP5 capillary column with 30 m length, 0.25 mm diameter and 0.25 µm thickness.
Temperature program	Isothermal with 220 °C temperature during analysis
Detector	FID with 300 °C
Sample injection temperature	290 °C
Retention time	30 minutes
Analysis Method	Normalization the peak of chromatogram

Table 2: Active ingredient compounds of rosemary essential oil grown in Kerman and Tehran regions

Row	Components name	Kovats coefficient	% of components (Rosemary grown in Tehran)	% of components (Rosemary grown in Kerman)
1	Tricyclene	923	0.26	0.17
2	α-Pinene	934	18.56	32.44
3	Camphene	951	5.39	3.93
4	Verbenene	956	0.45	0.8
5	β-Pinene	980	0.24	0.78
6	1-Octen-3-ol	985	0.33	-
7	3-Octanone	991	4.9	-
8	3-Octanol	1002	0.26	-
9	p-Cymene	1030	2.18	2.65
10	Limonene	1033	4.18	3.97
11	1,8-Cineole	1036	11.89	25.04
12	p-Cymenene	1097	0.38	2.65
13	Linalool	1105	3.12	2.44
14	Filifolone	1107	0.6	0.2
15	Endo-Fenchol	1128	0.32	0.16
16	α-Campholenal	1145	0.25	-
17	Camphor	1159	23.17	3.02
18	Camphene hydrate	1166	0.31	-
19	trans-Pinocamphone	1171	1.38	0.56
20	Borneol	1183	8.89	2.62
21	Terpinen-4-ol	1190	1.55	0.56
22	α-Terpineol	1206	2.87	1.69
23	Verbenone	1221	11.32	4.15
24	Trans-Carveol	123	0.15	-
25	Carvotan acetate	1257	0.36	-
26	cis-Myrtanol	1260	1.22	-
27	Isobornyl acetate	1291	1.38	1.7
28	Thymol	1303	0.27	1.98
29	Carvacrol	1311	0.28	0.19
30	Piperitenone	1354	0.31	-
31	α-Terpinyl acetate	1379	0.1	-
32	E-Caryophyllene	1427	0.43	1.39
33	α-Humulene	1464	0.1	0.19
34	Myrcene	991	-	1.95
35	α- Phellendrene	1009	-	0.19
36	α-Terpinene	1020	-	0.46
37	y-Terpinene	1062	-	0.64
38	Terpinolene	1082	-	0.42
39	Citronellol	1234	-	0.11
40	Geraniol	1258	-	1.83
41	Geranyl acetate	1384	-	0.25
42	Viridiflorol	1608	-	0.24
43	Total identified compounds (%)		95.44	97.54

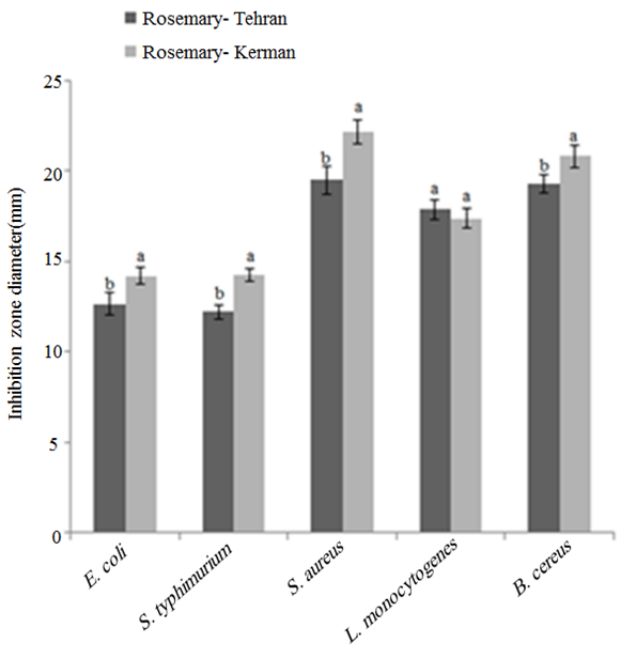

Figure 1: Comparison of antibacterial properties of rosemary essential oils of Tehran region with Kerman region by the formation of inhibition zone (mm) in disk diffusion method.

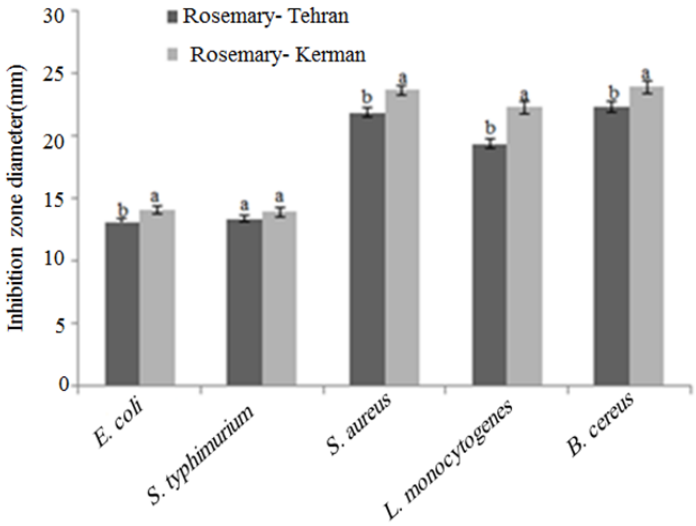

Figure 2: Comparison of antibacterial properties of rosemary essential oils of Tehran region with Kerman region by the formation of inhibition zone (mm) in well diffusion method.

Table 3: The minimum inhibitory concentration (ppm) of rosemary essential oils of Tehran and Kerman regions against pathogenic bacteria**.**

Rosemary essential oils	*E. coli*	*S. typhimurium*	*S. aureus*	*L. monocytogenes*	*B. cereus*
Tehran	625	1250	312.5	156.25	156.25
Kerman	625	625	156.25	156.25	78.1

Evaluation of antibacterial activity of rosemary essential oils by disc diffusion method

The results derived from evaluation of antibacterial activity of rosemary essential oils by disc diffusion method are presented in Figure 1. The results indicated significant inhibitory effect of essential oils of Tehran and Kerman regions on the growth of evaluated pathogenic bacteria that was observed as inhibitory zone. As can be seen in Figure 1, the diameter of inhibitory zone resulting from the essential oils of Kerman region on *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* were significantly larger than the essential oils of Tehran region, and did not have significant differences on *Listeria monocytogenes* ($P<0.05$). In addition, the

largest inhibition zone arising from both essential oils were formed in order on gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* and smallest inhibition zone were formed on *Salmonella typhimurium* and *Escherichia coli*.

Evaluation of antibacterial activity of rosemary essential oils by well diffusion method

In another part of this research, the antimicrobial activities of essential oils by well diffusion were studied (Figure 2). Based on the observations, the diameter of inhibition zone of rosemary essential oils of Kerman region significantly greater than the rosemary essential oils of Tehran region against *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Escherichia coli*, but the difference of inhibition zone diameter arising from two essential oil was not significant against *Salmonella typhimurium* ($P<0.05$). The results of this section as well as the results of antibacterial activity of rosemary essential oils by disk diffusion method demonstrated that inhibition zone diameter obtained from studied essential oils for the gram-positive bacteria was larger than gram-negative bacteria.

Evaluation of antibacterial activity of rosemary essential oils by microdilution method

Based on the results in Table 3, MIC of rosemary essential oils from Tehran region for *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* were 625, 1250, 312.5, 156.25 and 156.25 ppm respectively, and were 625, 625, 156.25 and 78.1 ppm for rosemary essential oils of Kerman, respectively. The obtained results showed that rosemary essential oil for Kerman had highest inhibitory effect against *Bacillus cereus* and minimum inhibitory effects against *Salmonella typhimurium* and *Escherichia coli*. In addition, the highest inhibitory effect of Tehran rosemary essential oil was evaluated against *Listeria monocytogenes* and *Bacillus cereus* and had lowest effect on *Salmonella typhimurium*. Based on the present results, MIC of rosemary essential oils from Tehran and Kerman regions for gram-positive bacteria (*Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus*) were less than gram-negative bacteria (*Escherichia coli* and *Salmonella typhimurium*). Also, MIC of rosemary essential oil from Kerman for the *Escherichia coli* and *Listeria monocytogenes* were similar to rosemary essential oils from Tehran and in the case of bacteria such as *Salmonella typhimurium*, *Staphylococcus aureus* and *Bacillus cereus* were less than rosemary essential oil from Tehran.

DISCUSSION

In this study, the major components of rosemary essential oils of Tehran were as follows: camphor, α-Pinene, 1,8-Cineole, Verbenone and borneol. Also, the major components of Kerman rosemary essential oils included α-Pinene, 1,8-Cineole, Verbenone, limonene and camphene. The results of inhibition zone measurements in disc and wells methods indicated that both essential oil were more effective on gram-positive bacteria than gram-negative bacteria. Minimum inhibitory concentration of these two essential oils on evaluated gram positive and negative bacteria varied from 78.1 to 312.5 and from 625 to 1250 ppm, respectively. Growth and yield of plants in ecosystems affected by factors such as species, climate, soil condition, altitude and geographical position. Each of these factors can have a significant impact on the quantity and quality of essential oils components derived from plants [22]. The research results showed that the amount of different compounds of studied essential oils were different. For example, camphor (oxygenated monoterpenes) and α-Pinene (hydrocarbons terpene) in rosemary essential oils from Tehran were 23.17 and 18.56 % respectively, but the amount of these compounds in rosemary essential oils from Kerman were 3.02 and 32.44 %, respectively. As can be seen in Table 2, there were some components that existed in one of these essential oils. This could suggest that the type and amount of the compounds in rosemary essential oil will be different by ecosystems differences.

Zaouali et al (2016) Malakootian and Hatami (2013) and Lemos et al (2015) were able to identify 20-25 compounds in the rosemary essential oils. They stated that the highest amount of compounds was belonging to the terpene hydrocarbon and oxygenated monoterpenes. Although the results of this study were consistent with the above studies and showed that the highest percent of Tehran and Kerman rosemary essential oils were belonging to above mentioned groups [9,10,22]. For instance Zaouali and colleagues reported that among the terpene hydrocarbon, 3-carene with 12.05% and α-pinene with 0.14% had the highest and lowest amounts of Moroccan rosemary essential oil, respectively [9]. But in present study, amounts of α-pinene in Tehran and Kerman rosemary essential oils were 18.56 and 32.44%, respectively and these two essential oils were free from 3-carene compound.

Lemos et al (2015) investigated the effect of habitat weather conditions on the type and amount of rosemary essential oils compounds and its biological activity. They demonstrated that by increase in region temperature the camphor essential oils reduced and Carnosic acid increased and inhibitory effect on *Staphylococcus aureus* will increase subsequently [22]. Findings of this research also indicated that the camphor amount of Tehran essential oils as compared to Kerman essential oils were lower and antimicrobial effect of Kerman essential oils were higher than Tehran essential oils. In another study, Celiktas et al investigated the antimicrobial effect of rosemary essential oils collected from three different regions on the bacteria such as *Staphylococcus aureus*, *Vulgaris proteus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Escherichia coli* and *Enterococcus faecalis*. Their research results demonstrated that all tested bacteria were sensitive to all three essential oils, but antimicrobial power of essential oil depended on the region of harvest, and their effects on gram-positive bacteria were more than gram-negative bacteria [15]. Findings of this research also revealed the direct effect of ecosystem conditions on the type and amount of the composition and antimicrobial power of essential oils.

Also findings of this study revealed that the MIC for gram-positive bacteria was 78.1-312.5 ppm and for gram-negative bacteria was 612-1250 that indicated higher

resistance of gram-negative than gram-positive bacteria to rosemary essential oils. In the present study, also revealed that Kerman rosemary essential oil as compared to Tehran rosemary essential oil had higher amounts of α-Pinene and 1,8-Cineole. These compounds have a high potential to react with the bacterial membrane and changes in it and this is why the Kerman rosemary essential oil has higher antimicrobial effect against gram-positive bacteria. These findings are in agreement with the results of various studies [9,10,16,22].

The most important reason of higher resistance of gram-negative bacteria against essential oil can be due to multilayer Lipopolysaccharides of outer membrane of Gram-negative bacteria that make them more resistant to penetration of essential oils. However, the outer membrane of gram-negative bacteria is not completely impermeable to hydrophobic compounds and compounds with low molecular weight can react with water through hydrogen bridges, and by penetrate the lipopolysaccharide or protein layers passes in the cell wall slowly and reacts with lipophilicities layers [23]. Although it seems that the antimicrobial activity of essential oils is through their effects on cell membranes, but it is not the only mechanism of action. In many studies, has been reported the ability of a number of phenolic and non-phenolic compounds of essential oils to react with the chemical groups of proteins and other effective biological molecules such as enzymes. In general, phenols react through hydrogen and ionic bridges or hydrophobic reaction with proteins. Whereas the nonphenolic compounds through other major groups such as carbonyl groups of cinnamyl aldehyde react with proteins and inactivate them. This mechanism is more effective on the gram-positive bacteria [23].

CONCLUSION

Based on this research, considering the presence of compounds such as α-Pinene and 1,8-Cineole in rosemary essential oil of Kerman, this essential oil is highly effective on the growth of pathogenic bacteria in relatively low concentrations. Hence, the use of this essential oil as a natural antimicrobial instead of synthetic preservatives that their negative effects has been determined, seems to be a useful and effective way, and also, mentioned essential oils and their active components can be used as a safe alternative for antibiotics.

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