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Chemical Composition and Antibacterial Activity of Essential Oils and Extracts from *Rosmarinus officinalis*, *Zataria multiflora*, *Anethum graveolens* and *Eucalyptus globulus*

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Abstract: The objective of the study was to characterize the chemical constituents and antibacterial properties of *Rosmarinus officinalis*(rosemary), *Zataria multiflora* (oregano), *Anethum graveolens* (Dill) and *Eucalyptus globulus* (Eucalyptus) essential oils (EO) and extracts against *Lactococcus garvieae*. Lactococcosis is one of the infectious diseases with significant economic and sanitary repercussions for trout farms in Iran during the summer months. Four EOs were initially screened against Lactococus garvieae using agar disc diffusion and broth dilution methods. The highest efficacy against the tested strain was shown when testing the Orogano EO. The chemical components of selected EOs were also analyzed by GC/MS. The most important constituents of the Rosemary, Oregano, Dilland Eucalyptus were 1,8-cineole (78.6 %), carvacrol (71.1 %), D-carvacrol (36.09 %) and 1,8-eucalypol (72.71 %), respectively. Of the 4 plants tested, *Z.multiflora* essential oil was the most active showing a Minimum Inhibitory Concentration (MIC) of 7.8 µg/mL and a Minimum Bactericidal Concentration (MBC) of 15.6 µg/mL. The diameters of the zone of inhibition adjacent to essential oils were estimated 24, 32, 18.5 and 16 mm, respectively. The extracts of different plant resulted in variable zone of inhibition (28–14.8 mm) for *L.garvieae*. The research results showed that the essential oil from of *Z.multiflora* has a great potential for application as a natural antimicrobial agent to preserve food.

Key words: *Lactococcus garvieae* · Antibacterial activity · Foodborne pathogens · Minimum Inhibitory Concentration (MIC) · Minimum Bactericidal Concentration (MBC)

INTRODUCTION

Today's there is a significant consumer demand for foods that are minimally processed and free from synthetic chemical preservatives with the perception of being ''natural". As a result the food industry is facing great challenges to produce naturally occurring food antimicrobials and antioxidants to reduce the use of synthetic chemical preservatives and still produce safe foods that are also regarded as healthy. Also, there are increasing trends to use herbal life style and dietary choices for human welfare and to improve the productivity and health of farm animals these natural products can help the whole body and improve the immunological status [1]. Spices and herbs are well known for their antimicrobial and antioxidant properties and have the ability to produce multidimensional flavors in food. Aromatic plants have been used traditionally in the therapy of some diseases for a long time. Many Plants contain extensive variety of phytochemical compounds with antimicrobial activity [2]. The Clove, Cinnamon, Oregano, Rosemary and Dill are considered as the most common spices and herbs with strong antimicrobial activity. Their essential oils containing chemical compounds such as Carvacrol, Cinnamaldhyde, Eugenol and Camphor are identified as the major chemical components responsible for exerting antimicrobial activity [3]. They are used as culinary herb a beverage drink as well as cosmetics. In folk medicine it is used as analgesic, antirheumatict, carminative, diuretic, expectorant, anti-epileptic, anti spasmodic in renal colic, improve human fertility and stimulate hair growth. Also some of this material added as an antioxidant to food [4].

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Many high risk pathogens that cause diseases in humans are transmitted through various food items. Due to increased morbidity and mortality leading to time lost in the work place and decreased reproductivity, food borne diseases across the world cost billions of dollars annually [4].

Antimicrobial agents, including food preservatives have been used to inhibit food borne bacteria and extend the shelf life of processed food [5]. Many naturally compound extracted like essential oils from edible and medicinal plants, herbs and spices have been shown to possess antimicrobial functions and could be as a source for antimicrobial agents against food spoilage and pathogens. More particularly, essential oils and their components are known effective agent against a wide variety of microorganisms, including Gram-positive and Gram-negative bacteria [5].

Spices and medicinal plants are widely used as raw materials for pharmaceutical preparations as a supplement for dietetic products, especially for "self medications" in public spices having essential oils which exhibit antimicrobial effects, generally show the lowest microbial populations [6].

Several studies have demonstrated the antibacterial properties of these plants, mainly used in vitro assays [7-9]. Moreover, some researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the plants and their above mentioned functional properties. Because these compounds are likely to interact with their environment and the composition could vary according to regional conditions, the investigation of their activity in a range of food systems is still needed to successfully apply them into meat products [10].

Lactococcosis is an emerging disease which has been particularly devastating in the freshwater culture of salmonid fish and marine culture species of Eel and yellow tail fishes and causes important economic losses both in marine and freshwater aquaculture all over the world, especially when water temperature increases over 15°C [11, 12].

The objectives of this study were investigation (I) the antibacterial activities of commercially available natural extracts and essential oils, (II) the ability of these natural extracts and essential oil for inhibiting the growth of *Lactococcus garvieae.*

MATERIALS AND METHODS

Preparation of Essential Oils and Extracts: R.officinalis, E.globulus and A.graveolens collected from suburbs of Karaj city (Alborz Province of Iran) and *Z. multiflora* Boiss collected from Shiraz province of Iran in June 2011 and were identified by Institute of Medicinal Plants, Tehran, Iran. Air-dried aerial parts of *R. officinalis*, *Z. multiflora* and *E. globulus* and seed of *A. graveolens* were subjected to steam distillation for 2h using Clevenger-type apparatus. The essential oils were analyzed on an Agilent 6890 gas chromatograph interfaced to an Agilent 5973 N mass selective detector (Agilent Technologies, Palo Alto, USA). A vaporization injector operating in the split mode (1:50) at 250°C was used, into which a fused silica capillary column (30m length × 0.32 mm internal diameter × 0.25µm film thickness; HP-5MS; 5% diphenyl, 95% dimethyl polydimethylsiloxane, Agilent Technologies) was installed. Briefly, the essential oils were collected from the air-dried material for 3 h using a Clevenger-type apparatus (Hanil Labtech Ltd., Incheon, Korea) and was dried over anhydrous sodium sulfate for 24 h, measured and stored in hermetically sealed dark-glass containers at -4°C until it was tested and analyzed by gas chromatography/mass spectrometry (GC/MS). The data were acquired under the following conditions: initial temperature 50°C; program rate 2.5°C; final temperature 300°C and injector temperature 290°C. The carrier gas was helium and the split ratio was 0.8 ml/min. The MS was run in the electron ionization mode, using ionization energy of 70 eV.

Thirty grams of the dried and powdered plant materials were extracted with methanol by using Soxhlet apparatus at 60°C for 12 h. These extracts were filtered and concentrated under vacuum at 40°C by using a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany), yielding a waxy material (2.25 g, 7.5% w/w). This extract was suspended in water and extracted with chloroform (4 × 100 ml) to obtain 1.35 g (4.5%) polar and 0.84 (2.8%) nonpolar extracts. It was stored in darkness at 4°C until used within a maximum period of one week.

Bacterial Strains and Culture Conditions: Lyophilized culture of L.garvieae GQ850376 obtained from Department of Aquatic Animals Health, Faculty of Veterinary Medicine, University of Tehran, Iran, was used in this study. it was grown in the tubes containing 10 ml of BHI broth(Merck KGaA, Darmstadt, Germany) incubated at 30°C for 18 h. Then it was followed by streaking on BHI agar(Merck KGaA) slant and incubated at 30°C for 18 h. The culture was stored at 4°C as working culture and subcultured at monthly intervals. The bacterial cells of *L. garvieae* grown in the broth were adjusted to an

optical density (OD) of 0.02 at 600 nm using the Spectronic 20 spectrophotometer (Milton Roy Company, USA). This adjustment give a cell concentration of 1×10^5 cfu / ml [13].

Antibacterial Activity

Paper Disc Diffusion Method: The disc diffusion method was applied for the determination of antimicrobial activities of both extracts and essential oils [14]. Extracts and essential oils were dissolved in dimethyl sulfoxide (DMSO). *L.garvieae* was cultured in a nutrient broth (Merck, Germany) for 24 h and diluted with sterilised peptone water. Then, 0.1 µl of the culture (10^5 CFU) was spread on to the surface of Mueller-Hinton agar (Oxoid, England) to create a bacterial lawn. Sterile blank filter paper discs of 6 mm in diameter(Oxoid, England) were wetted with 20 µl crude extract of filtered *R.officinalis, Z. multiflora*, *E. globulus* and *A. graveolens* (100 mg/ml) and left to dry before being placed on the microbial lawn. The plates were incubated at 37°C for 24 h. Antimicrobial activity was determined based on the diameter of the clear zone surrounding the paper discs [15]. Three replicate discs were prepared for each extracts and essential oil.

Determination of the Minimal Inhibitory and Minimal Bacterial Concentrations: The estimation of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration(MBC) were measured by the broth micro dilution method [16]. The essential oils were individually dissolved in sterilized physiological saline solution (0.9% w/v) supplemented with Tween 80(Sigma) at a final concentration of 0.5% (v/v). Serial doubling dilutions of the oils were prepared in a 96-well microtiter plate in the range of 1000 to 7.8 µg/mL.. The final concentration of each strain was adjusted to 10^5-10^6 cfu / ml. All microtiter plates were incubated at 37°C for 24 h. After incubation, the wells were examined for growth of microorganisms and the MIC were determined. The MIC was defined as the lowest concentration of the essential oil that microorganism does not demonstrate visible growth in it. The MBC is defined as the lowest concentration of the essential oil that incubated microorganisms are completely killed in it. Each experiment was repeated three times [17].

Statistical Analysis: The data were analyzed with Student's t-test or one-way ANOVA followed by Bonferroni test (GraphPad Prism 5.01; GraphPad Software Inc., San Diego, USA). The criterion for statistical significance was taken as $p < 0.05$.

RESULTS AND DISCUTION

Chemical Composition of Essential Oils: The relative quantitative values of R.officinalis essential oils were presented in Table 1. The most important constituents of the Rosemary were 1,8-cineole(78.6%), alpha-pinene (5.87%), toluene(12.26%), camphor(8.22%) and berbonone (7.75%). Table 1 shows the identified compounds and percentage obtained by GC/MS, as well as the retention time listed in order of their elution from the DB-5 capillary column.

Volatiles of A.graveolens essential oils revealed 21 different compounds accounting for 99.34 % of the essential oil composition that are identified in Table 2.

Table 1: Essential oil composition of *R.officinalis* identified by GC-MS

Compound	Retention time (min)	Percentage
1-Hexanol	8.32	0.35
Alpha-pinene	11.39	15.87
Camphene	12.01	4.20
Verbenene	12.26	0.57
Beta-pinene	13.36	0.48
3-Octanone	13.98	2.76
Beta-myrcene	14.23	2.38
Benzene	15.85	1.15
1,8-cineole	16.24	78.60
Gamma-terpinene	17.60	0.40
1-octanol	18.33	0.39
Linalool	19.80	2.20
Camphor	21.92	8.22
Propanoic acid	22.19	0.82
Berbonone	22.64	7.75
Bicycloheptane	28.69	2.34
Acetic acid	25.27	0.97
Toluene	4.90	12.26
Dodecane	24.80	0.60
Ethanol	8.23	0.04
Naphthalenone	74.33	0.12
Eicosadiene	72.55	1.17
9-Octadecenoic acid	60.24	1.89
Octadecanoic acid	60.76	0.65
Phosphoric acid	68.29	0.47
1,4,7,10,13,16-Hexaoxacycloocta	73.87	2.53
Hexadecanoic acid	54.63	1.39
Alpha- Fenchyl acetate	40.75	1.65
Heptadecene	60.11	2.18
Borneol	23.07	5.45
Butanoic acid	24.36	5.70
15-Nonylphenyl	74.80	0.40
Benzaldehyde	12.64	1.11
1,19-Eicosadiene	72.55	1.17
Gamma-sitosterol	73.95	1.51
Benzaldehyde	12.64	1.11

Table 2: Essential oil composition of *A. graveolens* identified by GC-MS

Compound	Retention index[a]	Percentage
β- Pinene	933	0.31
Sabinene	977	tr[b]
ß-Myrcene	989	0.25
β- Phellandren	1004	0.75
Limonene	1030	19.89
γ-Terpinene	1059	0.34
ρ-Cymene	1090	0.41
Linalool	1096	1.68
E-Limonene Oxid	1139	tr
Estragole	1180	0.94
β- Terpineol	1188	0.17
Z-Dihydrocarvone	1202	6.59
E-Dihydrocarvone	1211	7.36
Cumin aldehyde	1235	0.6
D-Carvone	1243	36.09
Thymol	1294	6.5
Carvacrol	1303	0.21
β-Caryophyllen	1429	0.32
D-Germacrene	1490	0.1
Myristiscin	1527	tr
Dill apiole	1634	16.83

[a]Retention index on D B - 1 column, [b] tr = trace < 0.1 %

Table 3: Essential oil composition of *Z. multiflora* Boiss. identified by GC-MS

Compound	Retention index[a]	Percentage
Thujene	930	0.19
Alpha-pinene	937	4.26
Beta-pinene	976	0.43
Beta-myrcene	985	0.85
Eucaliptol	1024	3.37
Gamma-terpinene	1055	7.34
Linalool	1090	0.68
Thymyl methyl ether	1236	0.47
Carvacrol methyl ether	1243	0.46
Carvacrol	1299	71.12
Trans-caryophyllene	1418	0.41
Globulol	1582	2.32
Sum		91.90

[a]Retention index on DB5 column

Table 4: Essential oil chemical constituents of *E. globulus* identified by GC-MS

Compound	Retention time (min)	Percentage
1,8-eucalypol	20.67	72.71
β- terpineol	17.87	2.54
Terpinen-4-ol	19.44	0.34
linalool	24.56	0.24
β- pinene	14.34	9.22
ß-pinene	15.89	0.40
β- eudesmol	26.54	0.39
-globulol	30.74	2.77
epiglobulol	32.89	0.44
β- terpineol acetate	23.21	3.10
geranyl acetate	32.23	0.71
L-pinocarveol	30.24	0.36
ß-sabinen	19.44	0.25
Terpinolene	22.19	0.19

The essential oil of *A.graveolens* contained a complex mixture consisting mainly of D-Carvacrol (36.09%), Limonene(19.89%), Dill apiole(16.83%), E-Dihydrocarvone(7.36%) and Z-Dihydrocarvone(6.59%).

Table 3 shows, The major compound of *Z. multiflora* Boiss was phenolic monoterpene carvacrol (71.1%).

Table 4 shows that, 1,8-eucalypol(72.71%) was the most abundant individual compound in *E. globulus* oil followed by β-pinene (9.22%), β-terpineol acetae (3.1%) and globulol (2.77%).

Antibacterial Activity: The antimicrobial activity of four commercial essential oils and extracts were evaluated against L.garvieae. According to the results given in Table 5, extracts of *R. officinalis*, *A. graveolens*, *Z. multiflora* and *E. globulus* showed antibacterial effect against *L. garvieae* with zones of inhabitation diameter 20, 16, 28 and 14.8 mm, respectively. While the essential oils shows interesting antibacterial effect with inhibition zones diameter of 24, 18.5, 32 and 16 mm, respectively.

Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations(MBC) of the oils and extracts were determined using a broth microdilution method. As shown in Table 6, the MIC values for the oils and extracts of *R. officinalis*, *A. graveolens, Z. multiflora* and *E. globulus* were found to be in the range of 7.8-250 and 15.6- 500 µg/ml and the MBC values for the oils and extracts were found to be in the range of 15.6-250 and 15.6-500 µg/ml, respectively.

Strong consumer demand for safe and high-quality foods can be attributed in part to the widespread availability and accessibility of quality health data and information. There are also new concerns about food safety due to increasing occurrence of new food-borne disease outbreaks caused by pathogenic micro-organisms [17]. This raises considerable challenges, particularly since there is increasing unease regarding the use of chemical preservatives and artificial antimicrobials to inactivate or inhibit growth of spoilage and pathogenic micro-organisms. As a consequence, natural antimicrobials are receiving a good deal of attention for a number of micro-organism-control issues. Reducing the need for antibiotics, controlling microbial contamination in food, improving shelf-life extension technologies to eliminate undesirable pathogens and/or delay microbial spoilage, decreasing the development of antibiotic resistance by pathogenic microorganisms or strengthening immune cells in humans are some of the benefits [18-20].

Table 5: Inhibition zone diameters (mm)[a] in well diffusion assays of extracts and essential oils of *R. officinalis*, *Z. multiflora*, *A. graveolens* and *E. globulus* against *L. garvieae*

	R. officinalis		*Z. multiflora*		*E. globulus*		*A. graveolens*	
Plant	Essential oil	Extracts	Essential oil	Extracts	Essential oil	Extracts	Essential oil	Extracts
Zone of inhibition	24±1.2	20±0.9	32±1.1	28±0.7	16±1.0	14.8±1.3	18.5±1.1	16±0.8

[a]Each result is the mean ± S.D. of three replicates

Table 6: Minimum Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) (µg / mL) of essential oil and extracts of *R. officinalis*, *A. graveolens, Z. multiflora* and *E. globulus*

Bacterial	*E. globulus*				*A. graveolens*			
L. garvieae	Extract		Essential oil		Extract		Essential oil	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	500	500	250	250	125	125	62.4	125
	R. officinalis				*Z. multiflora*			
	Extract		Essential oil		Extract		Essential oil	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
	31.2	31.2	15.6	31.2	15.6	15.6	7.8	15.6

Various publications have documented the antimicrobial activity of essential oil constituents and plant extracts. In recent years, several researchers have also reported mono- and esquiterpenoids as the major components of essential oils, which are phenolic in nature. It seems reasonable to assume that their antimicrobial mode of action might be related to the phenolic compounds present [21-23].

Azizi *et al*. [24] reported that differences in EO constituents indicated possible existence of various chemotypes in the species that may be ascribed to both genetic variation (existence of chemotypes) and environmentally determined fluctuation.

Our results (Tables 5-6) showed significant inhibitory effect in EO on *L. garvieae.* This inhibitory action was explained by the effect of EO on cell membrane *L. garvieae*. As can be seen in Table 6, the essential oil with the highest antibacterial properties was *Z. multiflora* followed by *R. officinalis* while *E. globulus* was the least active essential oil. These result are similar to the findings of Yamazaki *et al*. [25] who found significant effect of carvacrol and thymol on *Listeria monocytogenes.* These results are also in agreement with the findings of Oussalah *et al*. [5] which showed the effect of Spanish Oregano and Savory essential oils with high content of phenolic compounds, i.e. carvacrol and thymol on cell membrane disruption of *Escherichia coli* O157 H7 and *Listeria monocytogenes* and significant increase of cell constituents release in treated cells in comparison with untreated cells.

Rhayour *et al*. [26] also found the envelope damaging of *B. subtilis* and *E. coli* by Oregano essential oil (containing 49.1% carvacrol) and significant increase of cell constituents' release in comparison with untreated cells. Govaris *et al*. [27] showed that carvacrol (80.15%) and thymol (4.82%) were the predominant components of *Z. multiflora.* Saei-Dehkordi *et al*. [28] showed that thymol is the most abundant compound among all constituents. These differences in chemical compositions of the oils could be attributed to environmental effects on the plants.

The antimicrobial activity of essential oils would be related to the respective composition and structural configuration of the plant volatile oils, their functional groups and possible synergistic interactions between components [29]. EO exhibited varying levels of antibacterial activity against *L.garvieae*. The MIC of the EO and extracts were within concentration ranges 7.8-250 and 15.6-500 µg/ml and the respective MBC were 15.6-250 and 15.6-500 µg/ml. The diameter of the zone of inhibition adjacent to essential oils were estimated 32, 24, 18.5 and 16 mm, respectively (Tabel 5). The extracts of different plant resulted in variable zone of inhibition (28-14.8 mm) for *L.garvieae*. The results showed that these essential oils can strongly prevent the growth of *L.garvieae* GQ850376.

The most efficient antibacterial activity was essential oils of *Z. multiflora,* with MIC = 7.8 µg/ml, followed by *R. officinalis* and *A. graveolens* with 15.6 and 62.4 µg/ml, respectively.

Streptococcosis / lactococcosis was described as a hyper acute systemic disease that can occur in marine and fresh waters of many species of fish including rainbow, tilapia, sea bass, eel and yellow tail. Iran is now one of the leading countries in trout production in freshwater with a total production of about 60000 tons in 2008. Since the first reports of a presumptive streptococcosis, *S. iniae* and *L. garvieae* were identified as causative agents of the disease during 2005-2008 [29]. Such training is nowadays; very important particularly in the case of streptococcosis/ lactococcosis that is a human and terrestrial animal zoonotic disease, providing it easy transportation to the fish farms through sewage of terrestrial animals. Lv *et al*. (30-31) recommended elimination or reduction of the *L. garvieae* on the surface of the fish prior to smoking to control foodborne pathogens. They also found *L. garvieae* in smoked fish bought from the fish market. It might be due to the contamination of the raw fish used for this product and processing or post processing contamination of this product [30-32].

This result of this work indicated that essential oils and extracts from *Z. multiflora* and *R. officinalis* can be used as natural preservatives in food against the well-known causal agents of food-borne diseases and food spoilage. Therefore, essential oils and plant extracts are considered as potential alternatives to synthetic bactericides or as leading compounds for new classes of natural bactericides.

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