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Inhibitory Effects of Endemic *Thymus vulgaris* and *Mentha piperita* Essential Oils on *Escherichia coli* O157:H7

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Abstract: The present study, was conducted to evaluate *in vitro* antibacterial properties of Essential Oil (EO) from endemic plants, thyme (*Thymus vulgaris*) and mint (*Mentha piperita*) ageist *Escherichia coli* O157:H7. Antibacterial screening was done by Disk Diffusion (DD), Minimal Inhibitory Concentration (MIC) and Minimum Bactericide Concentration (MBC) methods. About 3.9 And 7.8 μg mL^{-1}concentrations of thyme EO were required in order to achieving the MIC and MBC. These indexes for mint EO was 15.6 and 31.2 μg mL^{-1}in *E. coli* O157:H7. In DD assay (500 μg $disk^{-1}$ concentration) thyme and mint were shown 35±1.4 and 13±1.5 mm inhibition zone. The chemical composition of hydro distilled EOs of thyme and mint was analyzed by Gas Chromatography-Mass Spectrometry (GC/MS). A total of 36 compounds of thyme and 23 compounds of mint, representing 93.62 and 90.69% of the oils were identified: Thymol (18.12%), Carvacrol (12.11%), p-Cymene (15.12%) in thyme and α-Terpinene (20.11%), Pipertitinone oxaide (17.10%) and *trans*-Carveol (19.48%) in mint, were the main components of the oils. Results here show that the EOs of thyme and mint possess antibacterial activity and therefore, it could be used as a natural preservative ingredient in food and/or pharmaceutical industries against *E.coli* O157:H7.

Key words: *Thymus vulgaris*, *Mentha piperita*, *Escherichia coli*, O157:H7, GC/MS

INTRODUCTION

In spite of modern improvements in slaughter hygiene and food production techniques, food safety is an increasingly important public health issue. It has been estimated that as many as 30% of people in industrialized countries suffer from a food borne disease each year and in 2000 at least 2 million people died from diarrhoeal disease worldwide (Burt, 2004). Among the reported outbreaks in the United States during 1993-1997 periods for which the etiology was determined, bacterial pathogens caused the largest percentage of outbreaks (75%) and the largest percentage of cases (86%) (Oussalah *et al*., 2007). There is still a need for new methods of reducing or eliminating food borne pathogens, possibly in combination with existing methods (Burt, 2004; Lee *et al*., 2005). All diarrhoeagenic strains of *E. coli* were initially termed Enteropathogenic *E. coli* (EPEC) but as their pathogenic mechanisms were known, they were grouped into seven classes (Duarte *et al*., 2007). Infections with Shiga Toxin-producing *E. coli* (STEC) serotype O157:H7 have been associated with a variety of sources, such as minced beef, dairy products, surface water and drinking water (Reinders *et al*., 2001). *E. coli* O157: H7 was recognized first in 1982 as a human pathogen during the investigation of 2 outbreaks of bloody diarrhoea in Oregon and Michigan. The relatively recent emergence of *E.coli* O157:H7 as a food borne pathogen has a significant impact on the food industry (Simsek *et al*., 2007).

Spices are used as culinary ingredients for taste and tang (Simsek *et al*., 2007; Seydim and Sarikus, 2006). They are also, used in oriental medicine (Eteghad *et al*., 2008). Many spices are used for curing minor illnesses like colds and stomach upsets. Research on this problem aimed to understand the natural defence mechanisms of plants and seeds against microorganisms (Simsek *et al*., 2007). Thyme, a member of the Lamiaceae family, is an aromatic and medicinal plant of increasing economic importance for North America, Europe and North Africa (Bisset and Wichtl, 2001). At present time, this plant is cultivated in large scale in Iran. Thyme volatile phenolic oil has been reported to be among the top 10 EOs, showing antibacterial, antimycotic, antioxidative, natural food preservative and mammalian age delaying properties (Badi *et al*., 2004). Mint is an herb used extensively in Indian cuisine and also for curing several common ailments. Mint extract did not show any antibacterial activity, though EOs of some *Mentha* species have been reported to have antibacterial activity (Kanatt *et al*., 2008; Marino *et al*., 2001).

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The aim of present research, is to study *in vitro* antibacterial activities of the EOs of thyme and mint against *E.coli* O157:H7 and to determine the chemical composition of their EOs by GC/MS.

MATERIALS AND METHODS

Plant material: Collective samples of the aerial parts from thyme and mint growing wild in northwest-Iran were collected during the vegetative, the flowering and the post-flowering phases (June and July) from three different localities: Moghan, Misho and Hasanbiglu (in East-Azerbaijan province) and authenticated at Herbarium of Tabriz Faculty of Pharmacy, Tabriz, Iran. The material was dried in the dark at room temperature before extraction.

Isolation of the essential oils: The air-dried and ground herbal part of the plant collected, was submitted for 4 h to water-distillation using a British-type Clevenger apparatus. The obtained EOs were dried over anhydrous sodium sulphate and extracts yield were recorded:

$$\frac{\text{Volume of extract}}{\text{Weight of dry matter}} \times 100$$

and then stored at 4°C in sealed glass vials until tested and analyzed.

Gas chromatography-mass spectrometry: A mass spectrometer with an ion trap detector in full scan mode under electron impact ionization (70 eV) was used. The chromatographic column used for the analysis was HP-5 capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The carrier gas used was helium, at a flow rate of 1 mL min^{-1}. The injections were performed in split less mode at 230°C. One micro liter EO solution in hexane (HPLC grade) was injected and analyzed with the column held initially at 60°C for 2 min and then increased to 260°C with a 5°C min^{-1} heating ramp and subsequently kept at 260°C for 13 min. The relative percentages amounts of the separated compounds were calculated from total ion chromatograms by a computerized integrator.

Preparation of bacterial strains: *E.coli* O157:H7 was obtained from the culture collection of the Department of Food Hygiene and Quality Control, Azad University (Tabriz, Iran). The organism was maintained on tryptic soy agar (TSA, PH 7.0: Difco) slants at 4°C. Cultures were activated by transferring loop inoculating into 10 mL of tryptic soy broth (TSB, PH 7.0: Difco) at 37°C for 20 h. Following 2 consecutive 20 h culture transfers, a 50 mL TSB was inoculated with culture and incubated at 37°C for 20 h. The bacteria was grown overnight at 37°C in sorbitol McConkey Agar (Merck) and inoculum for the assays was prepared by diluting cell mass in 0.85% NaCl solution, adjusted to McFarland scale 0.5 and confirmed by spectrophotometrical reading at 620 nm. Cell suspensions were finally diluted to 10^8 cfu mL^{-1} for being used in the antibacterial assays.

Antibacterial screening: The method of DD was employed for the determination of antibacterial activity of the EOs. The MIC of the samples against the test microorganism was determined by the broth micro dilution method (Sharififar *et al*., 2007). All growth-negative wells were spread on agar media separately for MBC test. All tests were performed in triplicate for each plant.

Disc diffusion assay: The EOs were dissolved in Dimethylsulfoxide (DMSO) (Merk, Schauchardt OHG, Germany) to 5 concentration (3.1, 6.2, 12.5, 25 and 50 mg mL^{-1}) and sterilized by filtration with 0.45 μm Millipore filters. Antibacterial tests were then carried out by DD method, using 100 μL of inculum, spread on nutrient agar. The sterile discs (Whatman No. 1, 6 mm in diameter) were impregnated with 10 μL of each concentration of EOs (31.2, 62.5, 125, 250 and 500 μg $disc^{-1}$) and placed on the inoculated agar. Negative controls were prepared using DMSO. The inoculated plates were incubated at 37°C for 24 h. Antibacterial activity was evaluated by measuring the zone of inhibition against the test organism.

Minimal inhibitory concentration and minimum bactericide concentration test: MIC tests were carried out according to Duarte *et al*. (2007), using Muller-Hinton Broth on a tissue culture test plate (96 wells) for each EO. The stock solutions of the EOs were diluted and transferred into the first well and serial dilutions were performed, so that concentrations in 0.9, 1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250 and 500 μg mL^{-1} were obtained. The inoculum was added to all wells and the plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of oil that inhibited visible growth.

MBCs of tests were determined by spreading 100 μL of each clear well on Sorbitol MacConkey agar (SMAC) fallowing incubated at 37°C for 24 h.

Statistical analysis of data: Results from the experiments were analyzed by using the SPSS 13.5 statistical package (SPSS Ltd., Woking, UK). All experiments were accomplished in triplicate and the results reported are averages.

Table 1: MICs, MBCs (μg mL $^{-1}$) and Disk diffusion (millimeters) results against *E. coli* O157:H7

				DD[a]				
Plant sp.	Distilled part	MICs	MBCs	31.2[b]	62.5	125	250	500
Thymus vulgaris	Aerial parts	3.9	7.8	15±1.3	23±1.4	29±1.2	32±1.1	35±1.4
Mentha piperita	Aerial parts	15.6	31.2	na[c]	na	na	10±1.2	13±1.5

[a]Include disk (6 mm in diameter). [b]EO concentration (μg disc^{-1}); [c]Not affect

Table 2: Chemical composition of thyme and mint EOs

	Thymus vulgaris		*Mentha piperita*	
Components	RI	(%)	RI	(%)
Tricyclene	926	0.13	911	1.31
α-Thujene	929	0.58	-	-
α-Pinene	936	6.45	-	-
Camphene	954	4.31	947	1.88
β-Pinene	983	0.76	963	2.20
1-Octen-3-ol	980	2.10	-	-
3-Octanone	985	0.45	-	-
3-Octanol	995	0.12	-	-
Myrcene	997	1.37	-	-
α-Phellandrene	1002	0.10	-	-
α-Terpinene	1017	1.07	1007	20.11
1,8-Cineole	1029	0.54	1020	0.12
p-Cymene	1032	15.12	1027	0.15
Limonene	1038	0.98	1031	1.10
cis-b-Ocimene	1040	0.20	-	-
trans-b-Ocimene	1050	0.31	-	-
γ-Terpinene	1059	1.63	1037	0.58
Terpinolene	1088	1.73	1062	0.14
cis-Thujone	1101	4.28	-	-
trans-Thujone	1114	0.10	-	-
Myrcenol	1105	0.32	-	-
Menthone	-	-	1127	0.80
Isomenthone	-	-	1137	8.92
Camphor	1145	1.64	-	-
Menthol	-	-	1149	3.54
Menthopuran	-	-	1156	2.30
Borneol	1167	2.28	-	-
γ-Terpineol	1185	0.37	1159	1.80
Isomenthol	-	-	1179	0.41
α-Terpineol	1188	0.77	-	-
trans-Carveol	-	-	1208	19.48
Carvone	-	-	1214	0.43
Thymol methyl ether	1235	0.10	-	-
Carvacrol methyl ether	1244	1.42	-	-
Geraniol	1253	9.13	-	-
Geranial	1266	0.12	-	-
Bornyl acetate	1288	0.33	-	-
Thymol	1300	18.12	-	-
Carvacrol	1318	12.11	-	-
Pipertitinone oxide	-	-	1330	17.10
α-Terpynyl acetate	1348	0.75	-	-
p-Menth-1-en-9-ol	-	-	1363	0.13
Geranyl acetate	1380	3.14	1369	0.11
β-Borbonene	-	-	1374	0.97
α-Garjunene	-	-	1395	0.20
β-Caryophyllene	1436	0.51	1407	6.91
Spathulenol	1582	0.18	-	-
Total[a]		93.62		90.69

RI: Kovats Retention Indices. [a]Other components were found at <0.1%

RESULTS

According to the results given in Table 1, the EOs of thyme and mint had great antibacterial activity against *E. coli* O157: H7. Results obtained from DD method, followed by measurements of MICs and MBCs.

The identified components of the EOs, as well as the percentages and Retention Index (RI) of each component are listed in Table 2 GC, GC/MS analysis revealed 36 compounds of thyme and 23 compounds of mint oils, respectively these compounds represent over 90% of the oils. Also, extracts yield were 1.43% for thyme and 1.87% for mint.

DISCUSSION

The oils were characterized with prominent (>10%) concentrations of Thymol, p-Cymene and Carvacrol in thyme and α-Terpinene, Pipertitinone oxide and trans-Carveol in mint. The thyme oil was also distinctive in its high concentrations (>5%) of α-Pinene and Geraniol, while, β-Caryophyllene, Isomenthone were two compounds in mint oil in these concentrations. The presence of Tricyclene, Camphene, β-Pinene, α-Terpinene, 1, 8-Cineole, p-Cymene, Limonene, γ-Terpinene, Terpinolene, γ-Terpineol, Geranyl acetate and β-Caryophyllene was detected in both oils in different concentrations.

To the best of our knowledge, there are many reports on the chemical composition of the oils isolated from thyme and mint (Dimitrijevi *et al*., 2007; Martinez *et al*., 2005; Jordan *et al*., 2006; Oussalah *et al*., 2007; Rota *et al*., 2007; Kitajima *et al*., 2004; Yadegarinia *et al*., 2006; Gherman *et al*., 2000; Tassou *et al*., 2000; Burt, 2004). Most of these reports indicate that Thymol, p-Cymene and Carvacrol in thyme and α-Terpinene, Pipertitinone oxide and trans-Carveol in mint are the main and/or characteristic constituents of these plant oils.

Based on the results of chemical composition of the EOs, we can conclude that the antibacterial nature of the EOs studied is apparently related to Carvacrol and Thymol in thyme and α-Terpinene, Isomenthone, Piperitone and Carvone in mint (Burt, 2004; Tassou *et al*., 2000). This claim is further supported by our findings; indicating high contents of Thymol, Carvacrol and α-Terpinene (Table 2).

The inherent activity of EOs can be expected to relate to the chemical configuration of the components, the proportions in which they are present and interactions between them. An additive effect is observed when the combined effect is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together

than when individually applied (Burt, 2004). For example in mint, minor compounds such as Carvone and p-Cymene have synergistic antibacterial effects along major compounds such α-Terpinene.

According to Rota *et al.* (2007), ≤0.2 μL mL^{-1} concentration of thyme EO was required in order to achieve the MIC and MBC in *E. coli* O157:H7. In another study that was prepared with Oussalah *et al.* (2007), EO was required in order to achieve the MIC in same bacteria was 50 μL mL^{-1}. In other study, MIC for Enteroinvasive *E. coli* (EIEC) and STEC, 400 and 300 μg mL^{-1} was recorded (Duarte *et al*., 2007). In same condition, MIC and MBC for mint EO 20 and 22 μL $mL^{-1}$was reported (Moreira *et al*., 2005). The previous studies, showed that Iranian mint EO had antibacterial effect against *E. coli* in 2 μL mL^{-1} Concentration (MIC) (Yadegarinia *et al*., 2006).

In current study, the results of MIC and MBC for each plant EOs against test pathogen (*E. coli* O157:H7) are written in Table 1, they are different from previous findings.

In studies done with DD method we seen wide range of concentration in Eos usages. According to Martinez *et al.* (2005), thyme EO inhibits zone for *E. coli* was 18.4 mm (6 μL $disk^{-1}$). In another study that was prepared by Sagdic (2003) inhibits zone for *E. coli* O157:H7, 12 mm was recorded. In same condition, 17 mm inhibit zone was reported for mint EO (Moreira *et al*., 2005). According to Kanatt *et al.* (2008) mint extract (0.1%) did not have any antibacterial activity. But Yadegarinia *et al.* (2006) shown that Iranian mint EO can certain 31.33 mm inhibit zone in *E.coli*. By attention to Table 1, our results are different from previous findings in DD method and same concentration.

The composition of EOs from a particular species of plant can differ between harvesting seasons and between geographical sources. So, EO components have been shown to exhibit antimicrobial activity to different extents (Burt, 2004) in different plants.

By notice to results, thyme and mint essences cultured in area can be used as a natural antibacterial compounds, preservative ingredient in food and/or pharmaceutical industries in said concentrations against *E. coli* O157:H7. Also inhibition in same concentration in thyme is grater than mint.

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