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Anti-Probiotic Effects of Essential Oils from Some Turkish Endemic Thyme Species

BULENT CETIN, SONGUL CAKMAKCI and MUSTAFA GURSES*

Department of Food Engineering, Faculty of Agriculture Atatürk University, 25240 Erzurum, Turkey

*Corresponding author: Fax: +90 442 3150689; Tel: +90 442 2312488; E-mail: mgurses@atauni.edu.tr

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Antimicrobial effects of essential oils from endemic thyme species in Turkey were examined *in vitro* on some probiotic microorganisms. Three thyme species (*Origanumacutidens, Origanumrotundifolium, Thymus sipyleus subsp. sipyleus* var. *rosulans*) and six probiotic bacteria (*Bifidobacterium bifidum, Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus reuteri and Streptococcus thermophilus*) were tested. The disc diffusion and microwell dilution assay methods were used to determine antimicrobial effects of the essential oils. Disc diffusion zone diameters and MIC values of the samples were found as 16-48 mm and 7.80-500 µg/mL, respectively. The carvacrol was detected as the main component by GC-MS analyses. L*actobacillus bulgaricus* and *Streptococcus thermophilus* were the most sensitive strains to the essential oils. *Lactobacillus acidophilus* and *Lactobacillus reuteri* were more resistance than the other strains. It was concluded that the high level of thyme can negatively influence the intestinal microflora and particularly the quality of fermented foods.

Key Words: Probiotic, Antimicrobial effect, Thyme, Essential oil.

INTRODUCTION

Since ancient periods, aromatic plants have been used for flavour and conservation in foods and treatment of several diseases. The synthetic products are used intensively for last many years. The use of natural products and their preparations increase in last years. The food industry now is facing an increased pressure from consumers for using chemical preservatives to prevent the growth of food borne pathogen microorganisms. To reduce or eliminate synthetic additives from foods is a current demand worldwide. A new approach to prevent the proliferation of microorganisms or protect foods from oxidation is the use of essential oils as preservatives. In this context, aromatic plants have emerged as effective agents to provide microbiological safety of foods[1-5]. However, enhancement of probiotic properties in foods is another important consumer demand. Because probiotics are useful microorganisms for human health, when sufficiently being intake ($\geq 10^6$ cfu/g).

Many *Thymus* and *Origanum* species are known as kekik which has a very rich flora in Turkey. They are used in herbal tea, condiments and folk medicine[6]. In addition, the essential oils of some *Thymus* species are characterized by the presence of a high concentration of the isomeric phenolic monoterpens thymol and/or carvacrol[7,8]. *Origanumacutidensis* an endemic, herbaceous and perennial plant growing mainly in calcareous and non-calcareous rocks, slopes and appears in the Central Anatolia region of Turkey[9]. The extract or the essential oil of *Origanum acutidens* has antagonistic activity against food-borne pathogenic bacteria, fungi and yeasts[2,3,10-12]. There is no report available in literature about the effect on the lactic acid bacteria of the essential oils from *Origanum* and *Thymus* species. It is also known that the antimicrobial effects of many aromatic plant essential oils may change depending on origin, locality, climate conditions and the harvest time of the collected plant material[1,2,8,13].

The objective of our research was to determine the anti-microbial activity against different probiotic lactic acid bacteria species of the essential oils from *Origanum acutidens*, *Origanum rotundifolium* and *Thymus sipyleus* subsp. *Sipyleus* var. *rosulans*.

EXPERIMENTAL

The aerial parts of *T. sipyleussubsp. rosulans, O. acutidens* (endemic in Turkey) and *O. rotundifolium* were collected from the province of Ispir-Erzurum in the northeastern region of Turkey in July 2006 during the flowering stage. The plant samples were identified by Prof. Dr. Ramazan Cakmakci and collected plants were stored in the Biotechnology Research and Application Centre at Atatürk University, Erzurum, Turkey.

Extraction of essential oils: Air-dried plant materials (500 g) were subjected to hydro-distillation for 4 h using a Clevenger type apparatus (Ildam Ltd., Ankara, Turkey). The essential oils

were extracted with $CHCl_3$, dried over anhydrous Na_2SO_4 and then stored at 20 ºC under N_2 atmosphere until analyzed.

Antimicrobial activity: The thyme essential oil extracts were tested individually against 6 different probiotic bacteria strains (*Bifidobacterium bifidum* ATCC 29521, *Lactobacillus acidophilus* BC 3219, *Lactobacillus bulgaricus* BC 2418, *Lactobacillus plantarum* BC 7321, *Lactobacillus reuteri* BC 7218 and *Streptococcus thermophilus* BC 6453). The bacteria were provided by the Food Microbiology Laboratory, Department of Food Engineering, Faculty of Agriculture, Atatürk University. The identification of the microorganisms used in the present study was confirmed by the Microbial Identification System (Sherlock Microbial Identification System version 4.0, MIDI Inc., Newark, DE, USA), API (Bio Merieux, Craponne, France), BIOLOG (Micro Station_ID System, Biolog Inc., Hayward, CA, USA) and classical identification test from Bergey's Manual of Determinative Bacteriology[14]. The bacteria strains were stored in lauryl broth of 20 % glycerol at - 86 ºC and activated using MRS and M17 agar before analyzed.

Disk diffusion assay: Antimicrobial tests were carried out by the disk diffusion method[15] using 100 µL of suspension containing 10^8 colony forming units (CFU)/mL bacteria spread on nutrient agar and MRS agar (Merck). The disks (6 mm in diameter) impregnated with 10 µL of the sample solutions were placed on the inoculated agar. The essential oils were dissolved in dimethyl sulfoxide. Negative control was DMSO without essential oil. cephalothin (30 µg per disk), azithromycin (15 µg per disk), tetracycline (30 µg per disk), clindamycin (2 µg per disk) and novobiocin (5 µg per disk) were used as positive reference standards (Oxoid) to determine the sensitivity of one strain in each bacterial species tested. The inoculated plates were incubated at 37 ºC for 24-48 h[16]. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. Each assay was repeated three times.

Microwell dilution assay: The MIC values were determined for the bacteria species that were sensitive to the essential oil in the disk diffusion assay. The strains were inoculated from 12 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

The essential oils were dissolved in DMSO and first diluted to the highest concentration (500 µg/mL) to be tested and then serial twofold dilutions were made to obtain a concentration range from 7.8 to 500 µg/mL in 10 mL sterile test tubes containing MRS broth. The MIC values of essential oil extracts against bacteria strains were determined on the basis of a micro well dilution method[12]. The 96-well plates were prepared by dispensing 95 µL of broth and 5 mL of the inoculum into each well. 100 mL from the stock solutions of the oils prepared at the 500 µg/mL concentration was added into the first wells. Then, 100 µL from the serial dilutions was transferred into the six consecutive wells. The last well containing 95 µL of MRS broth without compound and 5 µL of the inoculant on each strip was used as a negative control. The final volume in each well was 100 µL. Clarithromycin at a concentration range of 500-7.8 µg/mL was prepared in MRS broth and used as a standard drug for positive control. The plate was covered with a sterile plate sealer. The contents of each well were mixed on a plate shaker (MS2-Minishaker, IKA, Labortechnik, Staufen, Germany) at 300 rpm for 20 sec and then incubated at appropriate temperatures for 24 h.

Microbial growth in each medium was determined by reading the respective absorbance at 600 nm using the ELx800 universal micro plate reader (Bio-Tek Instrument Inc., Highland Park, VT, USA) and confirmed by plating 5 µL samples from clear wells on MRS broth medium. The oil tested in this study was screened three times against each organism.

GC-MS analysis conditions: The analysis of essential oils was performed by using a Thermo Finnigan Trace GC/Trace DSQ/A1300 (E.I. Quadrupole) equipped with a SGE-BPX5MS capillary column (30 m × 0.25 mm i.d., 0.25 µm). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium was used as the carrier gas with a flow rate of 1 mL/min. Injector and MS transfer line temperatures were set at 220 ºC and 290 ºC, respectively. The program used was 50-150 ºC at a rate of 3 ºC/min, isothermal hold for 10 min and finally increased to 250 ºC by 10 ºC/min. Diluted samples (1/100, v/v, in methylene chloride) of 1.0 µL were injected manually in the splitless mode. The components were identified based on the comparison of their relative retention time and mass spectra with those of the standards Wiley 7N library data of the GC-MS system and literature data. The results were also confirmed by the comparison of the compounds elution order to their relative retention indices of non-polar phases reported in the literature[17].

Statistical analysis: In order to determine whether there is a statistically significant difference among the obtained results from inhibition zone assays, variance analyses were carried out using SPSS version 10 software package (SPSS, Chicago, IL, USA). Differences between means were analyzed by the Duncan test and values with $P < 0.05$ were considered significantly different.

RESULTS AND DISCUSSION

The essential oils from thyme species were analyzed by using the GC and GC-MS system and its main components were presented in Table-1. 97.1% of the total components of *Origanum acutidens* were identified. The major components of *O. acutidens* oil were carvacrol (47.5 %), *p*-cymene (22.2 %), borneol (3.4 %), γ-terpinene (2.9 %), β-caryophyllene (2.7 %) and linalool (2.4 %). *Origanum rotundifolium* oil contained mainly carvacrol (54.6 %), *p*-cymene (12.5 %), borneol (5.9 %) and thymol (3.5 %) together with linalool (1.8 %) and terpinene-4-ol (1.5 %). It was identified representing 98.7 % of the oil of *Thymus sipyleussubsp. sipyleusvar. rosulans*. This oil was characterized by the high monoterpene fraction and especially by the presence of the phenolic carvacrol (30.0 %), thymol (14.5 %) and their precursors (*p*-cymene (10.2 %), α-terpinyl acetate (10.4 %), linalool (6.8 %) and γ-terpinene (3.4 %). It is known that major components of thyme and oregano essential oils that exhibit antibacterial properties were carvacrol, thymol *p*-cymene and γ-terpinene[1,18,19].

The antimicrobial activities of the thyme essential oils against some probiotic microorganisms were quantitatively determined by evaluating the presence of inhibition zones and

TABLE-1
BASIC COMPONENTS OF ESSENTIAL OILS EXTRACTED FROM THE THYME SPECIES

Plant species	Main components	Composition (%)
Origanum acutidens	Carvacrol	47.46
	p-Cymene	22.22
	Borneol	3.41
	γ-Terpinene	2.91
	β-Caryophyllene	2.70
	Linalool	2.35
	3-Octanone	1.84
Origanum rotundifolium	Carvacrol	54.56
	p-Cymene	12.53
	Borneol	5.86
	Thymol	3.52
	Linalool	1.77
	Terpinene-4-ol	1.54
	Thymohydroquinone	1.14
	β-Caryophyllene	1.09
	Germacrene D	1.08
	Linlyl acetate	1.07
Thymus sipyleus subsp. *sipyleus* var. *rosulans*	Carvacrol	29.99
	Thymol	14.46
	α-Terpinyl acetate	10.42
	p-Cymene	10.15
	Linalool	6.82
	γ-Terpinene	3.37
	β-Caryophyllene	3.30
	α-Terpineol	3.14
	Geraniol	2.98

MIC values and the results were given in Table-2. The antimicrobial tests showed that all of test microorganisms were highly sensitive against the thyme essential oils. As seen from Table-2, antimicrobial effects of the essential oils were also found higher than test antibiotics.

Contrary to many studies, an important aspect of the present study is the use of some probiotic bacteria as test microorganism. Probiotics-live microbial food supplement that beneficially affect the host by improving its intestinal microbial balance-are quickly gaining interest as functional foods in the current era of self-care and complementary medicine[20,21]. Our findings presented that the probiotic bacteria used as test microorganisms were inhibited by all the essential oils. This is not a good result, because of probiotic bacteria are used for useful aspects in foods. For example, it is known that bifidobacteria constitutes 80-90 % of the total fecal flora in intestinal system and they have an important role in infant health[22]. For this reason, it should be retained that the extensive use of thyme can cause the death of probiotic bacteriain gastrointestinal system.

On the other hand, the antimicrobial activity rations of the essential oils were different among the microorganism species. This situation may be originated from composition of essential oils or specific resistance of microorganisms. According to the results, *L. bulgaricus* and *Str. Thermophilus* were the most sensitive strains against the thyme essential oils but *L. reuteri* and *L. acidophilus* strains were more resistance than the others (Table-2).

While the essential oils obtained from *Origanum rotundifolium* had the least level of disc diffusion value (16 mm) determining on *L. reuteri*, the highest level of disc diffusion value was found as 48 mm for *Origanum acutidenson Str. Thermophilus* strain. As well as some studies interesting with antimicrobial effects of thyme essential oils on many microorganism groups were done[23,24]. There is no report about its effects on probiotic microorganisms. However, Cetin *et al.*[12] reported that these three thyme species exhibited a high level of antimicrobial activity against to many bacteria, fungi and mold species, particularly gram positive bacteria and the high thyhmol and carvacrol concentrations of the essential oils attributed to antimicrobial effect.

When Table-2 was examined, it was observed that the strains had different sensitivity to the essential oils and different strains had different sensitivity to the same essential oil ($p < 0.05$). *Lb. bulgaricus* and *Str. Thermophilus* were more sensitive to essential oils; on the other hand *Lb. reuteri* and *Lb. acidophilus* were more resistant than the others ($p < 0.05$).

Conclusion

The thyme essential oils used in this study affected on the lactic acid bacteria well-known probiotic characteristics. Probiotic microorganisms were mostly used in foods due to positive effects on consumer health. Our result show that thyme and its products shouldn't use with probiotic products.

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TABLE-2
ANTIMICROBIAL ACTIVITIES OF THE THYME ESSENTIAL OILS AGAINST THE PROBIOTIC BACTERIA

Test microorganisms	Tsr		Oa		Or		Antibiotics	
	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[a]	MIC[b]	DD[c]	MIC[d]
B. bifidum	32.00 ± 1.41Bb	500	40.00 ± 0.71Ba	125	30.15 ± 0.50Cb	250	25.55 ± 1.77Bc	7.8
Lb. acidophilus	18.25 ± 1.06Db	250	25.00 ± 0.00Da	250	26.25 ± 1.06Da	250	15.00 ± 0.00Cc	7.8
Lb. bulgaricus	39,75 ± 0.35Ab	62.5	42.25 ± 0.35Ba	62.5	32.35 ± 1.20Bc	62.5	30.20 ± 0.28Ad	7.8
Lb. plantarum	25.75 ± 1.06Cb	500	32.35 ± 1.20Ca	500	32.25 ± 0.35Ba	500	19.60 ± 1.13Cc	7.8
Lb. reuteri	32.00 ± 2.82Ba	7.8	20.00 ± 0.00Eb	125	16.00 ± 0.00Eb	500	18.05 ± 1.91Cb	15.6
Str. thermophilus	34.00 ± 3.53Bb	250	48.00 ± 2.82Aa	125	35.75 ± 0.35Ab	250	34.00 ± 3.53Ab	7.8

B: *Bifidobacterium*, *Lb*: *Lactobacillus*, *Str*: *Streptococus*. Tsr: *Thymus sipyleus* subsp. *sipyleus*var. *rosulans*, Oa: *Origanumacutidens*, Or: *Origanum rotundifolium.* [a]DD (Disc diffusion), inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 10 µL of essential oil. [b]Minimal inhibitory concentration as µg/mL. [c]DD, inhibition zone in diameter (mm) around the standard antibiotic disc; KF 30 (30 µg cephalothin/disc) (Oxoid). [d]Clarithromycin (µg/mL) was used as reference antibiotic in micro well dilution assay (Oxoid). Duncan test with A-E is differences between column, Duncan test with a-d is differences between line

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