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INFLUENCE OF ETHEREAL OILS EXTRACTED FROM LAMIACEAE FAMILY PLANTS ON SOME PATHOGEN MICROORGANISMS

ABSTRACT: As pathogen microorganisms can be found in different kinds of food, using of natural antimicrobial compounds, like ethereal oils, could be important in the preservation of different groceries. To evaluate antimicrobial activity of ethereal oils extracted from *Lamiaceae* family plants — *Rosmarinus officinalis* L., *Thymus vulgaris* L., *Majorana hortensis* Moench, and *Salvia officinalis* L screening of their effects against food borne bacteria *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and yeasts *Candida albicans* and *Saccharomyces cerevisiae* were applied. All investigated concentrations and pure *Majorana hortensis* and *Thymus vulgaris* ethereal oils showed microbicidal effect on majority of tested microorganisms.

KEY WORDS: ethereal oils, marjoram, rosemary, sage, thyme, antimicrobial effect

INTRODUCTION

We investigated antimicrobial activity of ethereal oils on some strains of gram-negative bacteria: *Proteus mirabilis*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Escherichia coli* O157:H7; gram-positive bacteria: *Enterococcus faecalis*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria monocytogene*; yeasts *Saccharomyces cerevisiae* and *Candida albicans*.

Medicinal, aromatic and herb spices from family *Lamiaceae* such as sage (*Salvia officinalis* L.), rosemary (*Rosmarinus officinalis* L.), marjoram (*Majorana hortensis* Moench.), and thyme (*Thymus vulgaris* L.) are widely distributed in Serbia growing as wild or cultivated species (Sarić, 1989). These

plants are used as stomachic, spasmolytic, carminative, and expectorant agents in folk medicine and in official medicine. Ethereal oils extracted from *Lamiaceae* family plants can contribute the quality of food with better odor and flavor what is consider as very important quality parameter in food manufacturing (K o v a č e v i ć, 2001). Other benefits could be application of ethereal oils in therapeutics purposes due to their antimicrobial (bactericidal and fungicidal) effects on some pathogen microorganisms (S t e f a n i n i et al., 2001, K l a u s et al., 2007).

The folium of sage (*Salvia officinalis* L.) contains 1% to 2.8% of ethereal oil. Monoterpenes are the major ingredients of this oil: tujon (30%—60%) as predominant constituent, cineol (15%) and camphor. The ethereal oils of sage have spasmolytic, astringent and carminative effects (B a r a t t a et al., 1998).

The folium of rosemary (*Rosmarinus officinalis* L.) contains up to 2% of ethereal oil. The major ingredients of ethereal oil of *Rosmarini aetheroleum* are cineol (35%), pinene, camphor and borneol. It is used in the food processing industry as a spice and aroma enhancer (A n g i o n i et al., 2004).

The herb of thyme (*Thymus vulgaris* L.) contains 1%—2% of ethereal oil. Monoterpenes are the major ingredients of this oil with thymol (40%) as predominant constituent and carvacrol, which are well known like antimicrobial substances. The ethereal oils of thyme have spasmolytic and expectorant effects (M a r i n o at al., 1999).

The herb of marjoram (*Majorana hortensis* Moench.) contains 1—2% of ethereal oil (terpinen-4-ol, borneol). It is also used in folk medicine as stomachic and as a spice and aroma enhancer in food processing industry (B i o n d i at al., 1993).

MATERIALS AND METHODS

Antimicrobial effects of ethereal oils extracted from *Lamiaceae* family plants on certain strains of microorganisms were investigated.

Test organisms

Antimicrobial activity was tested on gram-negative bacterial strains *Proteus mirabilis* ATCC 17576, *Salmonella enteritidis* ATCC 31806, *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *E. coli* O157:H7 ATCC 35150, *E. coli* O157:H7 ATCC 12900; on gram-positive bacterial strains *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11788, *Bacillus subtilis* BS-30B-0403001, *Listeria monocytogenes* ATCC 19115, *Listeria monocytogenes* ATCC 19112; and on yeasts *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 24433 and *Candida albicans* ATCC 10259.

ATCC cultures were taken from American Type Culture Collection, Rockville, Maryland. *B. subtilis* were taken from Dept. of Food Technology and Biochemistry, Faculty of Agriculture, University of Belgrade. The cultures of

bacteria were maintained in Mueller-Hinton agar and for *Listeria monocytogenes* TSA-YE slants at 4°C throughout the study and used as stock cultures.

Preparing of ethereal oils

Ethereal oils were obtained from sage (*Salvia officinalis* L.), rosemary (*Rosmarinus officinalis* L.), marjoram (*Majorana hortensis* Moench.), and thyme (*Thymus vulgaris* L.). The folium of sage and rosemary and herbs of marjoram and thyme represent a domestic raw material from Serbia. The folia of sage (variety Primorska), rosemary (domestic variety), thyme (variety N-19), and marjoram (variety Holand olfactory) were used in this experiment (Anonymous, 2005). Medicinal herbs were provided by the Institute for Medicinal Plant Research "Dr Josif Pančić" from Belgrade. Materials were determined at the Faculty of Agriculture in Belgrade. Ethereal oils were extracted from dry folia and herbs of sage, rosemary, thyme and marjoram by distillation and vaporization with Clevenger according to the methods of Pharmacopeia Yugoslavica V (Ph. Jug. V, 2000).

Screening of ethereal oils for antimicrobial activity was done by the disk diffusion method with three different concentrations (pure oil, 1:1, and 2:1) (Klaus et al., 2007). Ethereal oils were dissolved in 96% C₂H₅OH in ratio 2:1 (2 ml ethereal oil: 1 ml 96% C₂H₅OH) and in ratio 1:1 (1 ml ethereal oil: 1 ml 96% C₂H₅OH). In cases of ethereal oils obtained from *Majorana hortensis* and *Thymus vulgaris* ethereal oils were additionally dissolved in 96% C₂H₅OH in ratio 1:2 (1 ml ethereal oil:2 ml 96% C₂H₅OH) and 1:3 (1 ml ethereal oil:3 ml 96% C₂H₅OH).

Preparing of microorganisms

Bacterial strains were inoculated in Mueller-Hinton broth except *Listeria monocytogenes* which were inoculated in TSB-YE and incubated at 37°C for 24 h, to reach concentration 10⁶ cells/ml. Yeasts were inoculated in malt broth and incubated at 30°C for 48 h, to reach concentration 10⁶ cells/ml. The inocula of microorganisms were prepared from 24 h and 48 h-old culture and suspensions were adjusted to 0.5 McFarland standard turbidity (~ 10⁶ CFU/ml).

Influence of ethereal oils on microorganisms

Screening of essential oils for antimicrobial activity was done by the disk diffusion method. The cultures were adjusted to approximately 10⁶CFU/ml with sterile saline solution. Petri dishes (100 mm in diameter) were inoculated with 0.2 ml suspension of certain strains of microorganisms and overlaid with 20 ml of the medium. For most of bacteria Mueller Hinton agar was used, for *Listeria monocytogenes* TSA-YE were used and for yeasts malt agar were used. Three filter disks (Sigma-Aldrich's Whatman® Schleicher & Schuell, 6 mm in diameter) were placed on every agar and diffusion method was per-

formed by adding 10ml of appropriate suspension on every disk. Every test was done in triplicate. Blind probe contained only 96% C₂H₅OH, without any ethereal oils. Bacteria were incubated at 37°C for 24h and yeasts were incubated at 30°C for 48h. After the incubation period, the zone of inhibition was measured. Small sectors from the zone of inhibition were taken and inoculated in nutrient broth for cultivating bacteria, TSB-YE for cultivating *Listeria monocytogenes* and in malt broth for yeasts. Sectors in nutrient broth and TSB-YE were incubated at 37°C for 24h and sectors in malt broth were incubated at 30°C for 48h to see if the effect of ethereal oils were microbicide or microbistatic (Klaus et al., 2007).

RESULTS AND DISCUSSION

After 24-hour incubation at 37°C for bacterial strains and 48-hour incubation at 30°C for yeasts the zone of inhibition around the filter disks were measured and results are presented in Table 1. If nutrient, TSB-YE or malt broth with sectors taken from the zone of inhibition showed turbidity after incubation, influence of applied ethereal oils were regarded as microbistatic. In the cases where nutrient, TSB-YE or malt broth with sectors taken from the zone of inhibition stayed clear after incubation, influence of applied ethereal oil were microbicide. Blind probe showed that 96% C₂H₅OH had no antimicrobial influence on investigated microorganisms.

Pure ethereal oil obtained from *Rosmarinus officinalis* showed microbistatic effect on all investigated bacteria, but had no influence on yeasts *Candida albicans* ATCC 24433, *Candida albicans* ATCC 10259 and *Saccharomyces cerevisiae* ATCC 9763. The most intensive microbistatic influence of pure ethereal oil were in cases of *Bacillus cereus* ATCC 11788 (inhibitory zone 14.67 mm) and *Proteus mirabilis* ATCC 17576 (inhibitory zone 11.67 mm). Suspensions of ethereal oils in 96% C₂H₅OH in ratio 2:1 showed microbistatic effect on all investigated microorganisms, with best results in contact with yeasts *Candida albicans* ATCC 10259 (inhibitory zone 22 mm), *Candida albicans* ATCC 24433 (inhibitory zone 21 mm) and *Saccharomyces cerevisiae* ATCC 9763 (inhibitory zone 20 mm) and bacteria *E. coli* O157:H7 ATCC 35150 (inhibitory zone 10.33 mm). Suspensions of ethereal oils in 96% C₂H₅OH in ratio 1:1 had microbistatic effect on all investigated microorganisms, too, but with best results in contact with *E. coli* O157:H7 ATCC 35150 (inhibitory zone 11 mm) and *Bacillus subtilis* ATCC BS-30B-0403001 (inhibitory zone 9.67 mm).

All concentrations and pure ethereal oils obtained from *Thymus vulgaris* showed microbicide effect on yeasts *Candida albicans* ATCC 24433 (inhibitory zone 29 mm with pure oil), *Candida albicans* ATCC 10259 (microbicide with pure oil and in suspensions 2:1 and 1:1) and *Saccharomyces cerevisiae* ATCC 9763 (microbicide with pure oil-inhibitory zone 30.67 mm, and in suspensions 2:1 and 1:1). Similar results were obtained at bacteria *Staphylococcus aureus* ATCC 25923 (microbicide effect with pure oil-inhibitory zone 25.33 mm and in suspension 2:1-inhibitory zone 25.67 mm), *Listeria monocytogenes*

Tab. 1 — The zone of inhibition procured by presence of pure ethereal oils and in concentrations 2:1 (2 ml ethereal oil:1 ml 96% C₂H₅OH) and 1:1 (1 ml ethereal oil:1 ml 96% C₂H₅OH).

microorganism	Average size of the zone of inhibition in diameter (mm)											
	<i>Rosmarinus officinalis</i>			<i>Thymus vulgaris</i>			<i>Majorana hortensis</i>			<i>Salvia officinalis</i>		
	pure oil	2:1	1:1	pure oil	2:1	1:1	pure oil	2:1	1:1	pure oil	2:1	1:1
<i>Staphylococcus aureus</i>	6.33	6.67	7	25.33	25.67	—	18	23.33	27	7	6.67	8.33
<i>Enterococcus faecalis</i>	8.67	7.67	8.67	69	58	39.67	49.33	32	20.33	3	11	8
<i>Proteus mirabilis</i>	11.67	7.33	7	39.33	25	41.67	37.67	47	52	14	11	7
<i>Salmonella enteritidis</i>	4	9	9	30	31.33	25.33	22	46.33	38	3	9	4
<i>Pseudomonas aeruginosa</i>	5	9	6	57	53.33	45	30.33	32.33	43.33	25	15.66	9.33
<i>E. coli</i>	9	7.67	9.33	54.33	+	36.33	33	47	60	5.67	9.67	8.67
<i>E. coli</i> (O157:H7) 35150	5	10.33	11	15.67	36.33	50	29	24.33	29	3	8.33	4
<i>E. coli</i> (O157:H7) 12900	3	8.33	8.33	29.67	+	+	22	22	31	2.67	7.67	9
<i>Bacillus cereus</i>	14.67	9.33	9	+	+	59	20.67	32	30	15.33	10.33	7.67
<i>Bacillus subtilis</i>	8	7	9.67	47.33	+	27.67	37	50	45	9	8.67	8.33
<i>Listeria monocytogenes</i> 19115	8.67	4.67	5.67	20.67	16	—	38	57	51.67	6.67	4.67	4.67
<i>Listeria monocytogenes</i> 19112	9	4	4	18.67	22.67	—	31	32	36.33	9.67	5.67	5.67
<i>Saccharomyces cerevisiae</i>	—	20	8	30.67	23.67	23	24.67	23	21	56	3.33	5
<i>Candida albicans</i> 24433	—	21	3.67	29	19.33	16	40	43.67	42.33	59	3.33	4
<i>Candida albicans</i> 10259	—	22	4	+	+	+	+	+	71	+	4	5

— With no influence on growth
+ With no growth

ATCC 19115 (inhibitory zone 20.67 mm with pure oil), *Listeria monocytogenes* ATCC 19112 (inhibitory zone 22.67 mm with suspension 2:1) and *E. coli* O157:H7 ATCC 35150 (microbicide in all cases, in suspension 1:1 inhibitory zone was 50 mm). The best microbistatic influence of suspension 2:1 noticed in the cases of *E. coli* ATCC 25922 with no any growth, *Enterococcus faecalis* ATCC 29212 when inhibitory zone was 58 mm (Fig.1) and *Pseudomonas aeruginosa* ATCC 27853 with inhibitory zone of 53.33 mm. In the presence of suspension 1:1, the best microbistatic effect appeared at *E. coli* O157:H7 12900 without growth, at *Bacillus cereus* ATCC 11788 with inhibitory zone 59 mm and at *Proteus mirabilis* ATCC 17576 (inhibitory zone 41.67 mm).

All concentrations and pure ethereal oils obtained from *Majorana hortensis* showed microbicide effect on *Staphylococcus aureus* ATCC 25923 (inhibitory zone 27 mm in suspension 1:1), *Salmonella enteritidis* ATCC 31806 (inhibitory zone 46.33 mm in suspension 2:1 and 38 mm in suspension 1:1), *Saccharomyces cerevisiae* ATCC 9763 (inhibitory zone 24.67 mm in the presence of pure oil), *Candida albicans* ATCC 24433 (inhibitory zone 43.67 mm in suspension 2:1), *Candida albicans* ATCC 10259 (with no growth in the presence of pure oil and suspension 1:1 and with inhibitory zone of 71 mm in the presence of suspension 1:1), *Listeria monocytogenes* ATCC 19115 (inhibitory zone 57 mm in suspension 2:1 and 51.67 mm in suspension 1:1, Fig.2), *Listeria monocytogenes* ATCC 19112 (inhibitory zone 36.33 mm in suspension 1:1), *E. coli* O157:H7 ATCC 35150 (inhibitory zone 29 mm in the presence of pure oil and in suspension 1:1), and *E. coli* O157:H7 ATCC 12900 (inhibitory zone of 31 mm in the presence of suspension 1:1). The most intensive microbistatic influence of suspensions of ethereal oils in 96% C₂H₅OH were in case of *Bacillus subtilis* ATCC BS-30B-0403001 (inhibitory zone 50 mm in suspension 2:1 and 45 mm in suspension 1:1), *E. coli* ATCC 25922 (inhibitory zone 47 mm in suspension 2:1 and 60 mm in suspension 1:1) and *Proteus mirabilis* ATCC 17576 (inhibitory zone 47 mm in suspension 2:1 and 52 mm in suspension 1:1).

All concentrations and pure ethereal oils obtained from *Salvia officinalis* showed microbicide effect on yeasts *Saccharomyces cerevisiae* ATCC 97639 (inhibitory zone 56 mm in the presence of pure oil) and *Candida albicans* ATCC 24433 (inhibitory zone 59 mm in the presence of pure oil). In the contact with other investigated microorganisms, pure oil and suspensions of ethereal oil in 96% C₂H₅OH in ratio 2:1 and 1:1 showed microbistatic effect. The best microbistatic influence of pure oil noticed in the cases of *Pseudomonas aeruginosa* ATCC 27853 (inhibitory zone 25 mm) and *Bacillus cereus* ATCC 11788 (inhibitory zone 15.33 mm). Suspension 2:1 showed the best results in the contact with *Pseudomonas aeruginosa* ATCC 27853 (inhibitory zone 15.66 mm), *Enterococcus faecalis* ATCC 29212 (inhibitory zone 11 mm) and *Proteus mirabilis* ATCC 17576 (inhibitory zone 11 mm). Suspension 1:1 showed best results in the contact with *Pseudomonas aeruginosa* ATCC 27853 (inhibitory zone 9.33 mm) and *E. coli* ATCC 25922 (inhibitory zone 9.33 mm).

In cases of ethereal oils obtained from *Majorana hortensis* and *Thymus vulgaris* ethereal oils were additionally dissolved in 96% C₂H₅OH in ratio 1:2

(1 ml ethereal oil:2 ml 96% C₂H₅OH) and 1:3 (1 ml ethereal oil:3 ml 96% C₂H₅OH), because previous investigations with dissolving of ethereal oils in 96% C₂H₅OH in ratio 2:1 (2 ml ethereal oil:1 ml 96% C₂H₅OH) and in ratio 1:1 (1 ml ethereal oil:1 ml 96% C₂H₅OH) showed very good results. Results are presented in Table 2.

Tab. 2 — The zone of inhibition procured by presence of ethereal oils in concentrations 1:2 (1 ml ethereal oil:2 ml 96% C₂H₅OH) and 1:3 (1 ml ethereal oil:3 ml 96% C₂H₅OH)

microorganism	Average size of the zone of inhibition in diameter (mm)			
	<i>Thymus vulgaris</i>		<i>Majorana hortensis</i>	
	s u s p e n s i o n			
	1:2	1:3	1:2	1:3
<i>Staphylococcus aureus</i>			11.33	11.67
<i>Salmonella enteritidis</i>	10	9	10	11.67
<i>E. coli</i> (O157:H7) 35150	9.67	9.67	11	12.67
<i>E. coli</i> (O157:H7) 12900			10	11.33
<i>Bacillus cereus</i>			19.33	31.67
<i>Listeria monocytogenes</i> 19115	31	37	36.33	36.33
<i>Listeria monocytogenes</i> 19112	38	34	34	42.33
<i>Saccharomyces cerevisiae</i>	27	30.67	21.67	23.33
<i>Candida albicans</i> 24433	27	22	21	26
<i>Candida albicans</i> 10259	84	75	60	45

Suspensions of *Thymus vulgaris* ethereal oil in 96% C₂H₅OH in ratio 1:2 and 1:3 showed microbicide effect in contact with all investigated microorganisms except *E. coli* O157:H7 ATCC 35150 when it was microbistatic with inhibitory zone 9.67 mm in both concentrations. The best microbicide effect of these two suspensions appeared when applied on *Listeria monocytogenes* ATCC 19112 (inhibitory zone 38 mm in suspension 1:2 and 34 mm in suspension 1:3) and *Listeria monocytogenes* ATCC 19115 (inhibitory zone 31 mm in suspension 1:2 and 37 mm in suspension 1:3).

Suspensions of *Majorana hortensis* ethereal oil in 96% C₂H₅OH in ratio 1:2 and 1:3 showed microbicide effect in contact with *Staphylococcus aureus* ATCC 25923 (inhibitory zone 11.67 mm in suspension 1:3), *Salmonella enteritidis* ATCC 31806 (inhibitory zone 11.67 mm in suspension 1:3), *Saccharomyces cerevisiae* ATCC 97639 (inhibitory zone 23.33 mm in suspension 1:3), *Candida albicans* ATCC 24433 (inhibitory zone 26 mm in suspension 1:3), *Candida albicans* ATCC 10259 (inhibitory zone 60 mm in suspension 1:2), *Listeria monocytogenes* ATCC 19115 (inhibitory zone 36.33 mm in suspensions 1:2 and 1:3) and *Listeria monocytogenes* ATCC 19112 (inhibitory zone 42.33 mm in suspension 1:3). These suspensions performed microbistatically when applied on *Bacillus cereus* ATCC 11788 (inhibitory zone 31.67 mm in suspension 1:3), *E. coli* O157:H7 ATCC 35150 (inhibitory zone 12.67 mm in suspension 1:3), and *E. coli* O157:H7 ATCC 12900 (inhibitory zone 11.33 mm in suspension 1:3).



Fig. 1 — Inhibitory effect of *Thymus vulgaris* etheral oil (pure oil) on the growth of *Enterococcus faecalis* ATCC 29212



Fig. 2 — Inhibitory effect of *Majorana hortensis* etheral oil (ratio 1:1) on the growth of *Listeria monocytogenes* ATCC 19115

CONCLUSIONS

There are many demands regarding the food safety in modern food production. Microorganisms can contaminate the food on different points, from raw to final product, so producers have to protect the food. Easy, promising and not harmful way to protect the food could be adding the specific herbs spices or their etheral oils, which could be harmonized with flavor, taste and odor of the particular groceries. Besides, historically it is confirmed that some etheral oils possess antimicrobial activities. This could be very important regarding the fact that microorganisms become resistant on numerous antibiotics. By adding some herbs or their etheral oils in food, it is possible to upgrade the quality of food and to protect the groceries from unwanted microorganisms.

In this work it was shown that medicinal, aromatic and herbs spice from *Lamiaceae* family-sage (*Salvia officinalis* L.), rosemary (*Rosmarinus officinalis* L.), marjoram (*Majorana hortensis* Moench.) and thyme (*Thymus vulgaris* L.) could be used for microbial control of some kinds of food by using pure oils or suspension in alcohol. As some of tested pathogen microorganisms could be presented in large numbers, addition of etheral oils is possibly the good way to inhibit their growth and they can be used as antimicrobial supplement for development of new therapeutic agents. Additional *in vivo* studies and clinical trials would be needed to justify and further evaluate the potential of etheral oils as an antimicrobial agent.

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УТИЦАЈ ЕТАРСКИХ УЉА ЕКСТРАКОВАНИХ ИЗ БИЉАКА ФАМИЛИЈЕ *LAMIACEAE* НА НЕКЕ ПАТОГЕНЕ МИКРООРГАНИЗМЕ

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Резиме

Како патогени микроорганизми могу да се нађу у различитим прехранбеним производима, коришћење природних антимикуробних компонената, као што су етарска уља, може да буде важно за њихово чување. За процењивање антими-

кробне активности етарских уља екстракованих из биљака фамилије *Lamiaceae*-*Rosmarinus officinalis* L., *Thymus vulgaris* L., *Majorana hortensis* Моенсн. и *Salvia officinalis* L. посматран је њихов утицај на микроорганизме контаминенте хране, бактерије *Staphylococcus aureus*, *Enterococcus faecalis*, *Proteus mirabilis*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Escherichia coli* O157:H7, *Listeria monocytogenes* и квасце *Candida albicans* и *Saccharomyces cerevisiae*. Све испитиване концентрације као и чиста етарска уља *Majorana hortensis* и *Thymus vulgaris* показала су микробицидни ефекат на већину тестираних микроорганизама.