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ANTIBACTERIAL ACTIVITY OF ESSENTIAL OILS OF SOME LAMIACEAE FAMILY SPECIES ISOLATED BY DIFFERENT METHODS*

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In the present study, isolation of essential oils from leaves of rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) by supercritical carbon dioxide as well as hydrodistillation was investigated from the point of yield and antimicrobial activity of obtained extracts against *Geobacillus stearotermophillus*, *Bacillus cereus*, *Bacillus subtilis* var. *niger*, *Enterococcus faecium*, *Salmonella enteritidis* and *E.coli* strains. Thyme isolates had the highest antibacterial efficiency against tested foodborne bacteria strains (MIC=40-640 μg/cm^3) followed by rosemary (MIC=320-1280 μg/cm^3) and sage (MIC=160-2560 μg/cm^3) isolates. Among tested bacteria, *Bacillus* species were the most sensitive to presence of all tested supercritical extracts and hydrodistillates.

Key words: supercritical fluid extraction, essential oils, antibacterial activity

INTRODUCTION

Herbs of the Lamiaceae family, like rosemary, sage and thyme are well-known for their essential oil content as well as for the content of phenolic compounds to which the antioxidant activity was attributed. Besides steam distillation, essential oils of the Lamiaceae family species could be obtained as the main products in the process of SFE, or as by-products in the extraction of antioxidant fractions using supercritical

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fluid [1]. Essential oils from plants of *Rosmarinus*, *Salvia* and *Thymus* species (Lamiaceae family) derived by hydrodistillation have been found to posses significant antibacterial activities [2-7]. On the other hand, there is not very much data on the antibacterial activity of supercritical extracts from herbs and there are only few reports on antimicrobial effects of rosemary and sage supercritical extracts. Santoyo et al. [8] investigated antimicrobial activity of rosemary essential oil reach fractions obtained by supercritical carbon dioxide against gram-positive bacteria (*Staphylococcus aureus* and *Bacilus subtilis*) as well as gram-negative bacteria (*Escheriscia coli* and *Pseudomonas aeruginosa*). *S. aureus* was found to be the most sensitive bacteria. Recently, the strong influence of rosemary supercritical extracts on the growth and viability of *Listeria monocytogeneses* in laboratory medium and broccoli juice was reported [9]. Menaker et al. [10] observed substantional difference in antimicrobial activity between sage essential oil obtained by hydrodistillation and supercritical extract against tested bacteria including *E. coli* and *S. albus*. Supercritical extracts of sage and rosemary were also recently screened [11] for their antimicrobial activity against bacteria and yeasts with dermatological relevance including *Staphylococcus aureus* methicillin-resistant strains. Rosemary extract showed stronger antibacterial activity against tested bacteria than the sage extract. Yet, to the best of our knowledge, there is no data available in the open literature on the antibacterial activity of thyme extracts obtained with supercritical carbon dioxide.

In the present study, the isolation of essential oils from leaves of rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) by supercritical carbon dioxide as well as hydrodistillation was investigated from the point of yield and antimicrobial activity of obtained extracts against *Geobacillus stearotermophillus*, *Bacillus cereus*, *Bacillus subtilis* var. *niger*, *Enterococcus faecium*, *Salmonella enteritidis* and *E.coli* strains.

EXPERIMENTAL

Dried leaves of selected herbs belonging to the Lamiaceae family: rosemary (*Rosmarinus officinalis*), sage (*Salvia officinalis*) and thyme (*Thymus vulgaris*) originated from the southern Balkan region were used for the study.

Commercial carbon dioxide (99% purity, Tehno-gas, Novi Sad, Serbia) was used for the SFE. Thymol (ph. grade, SIGMA) and *p*-cymene standard (FLUKA) were used for the investigation of antibacterial effects of pure compounds.

Essential oil rich fractions were obtained from selected plants in process of fractional supercritical extraction with carbon dioxide. Essential oil fraction was extracted first at the pressure of 11.5 MPa and temperature of 40 °C. Extraction of antioxidant fraction followed at pressure of 35 MPa and temperature of 100 °C. Plant material was milled and sieved using laboratory sieves. The fraction of the average particle diameter of 0.400 mm was used for the further study. Extractions with supercritical carbon dioxide (SC CO_2) were performed in the Autoclave Engineers Screening System previously described [12]. In order to minimize energy consumption, optimal pretreatment of herbaceous matrix which included exposure of milled plant material to SC CO_2 for one

hour before the continuous SFE was applied [13].The flow rate of SC-CO_2 was 0.3 kg/h in all experiments. The initially used mass of the plant samples was 56 g for rosemary, 56.5 g for sage, and 54 g for thyme.

Hydrodisstillation of essential oils from the selected plants was performed in a Clevenger-type apparatus for 4 hours, up to the point at which the oil contained in the herbaceous matrix was exhausted.

The investigation of the antibacterial effects of rosemary, sage and thyme isolates has been performed on *Geobacillus stearotermophillus*, *Bacillus cereus*, *Bacillus subtilis* var. *niger*, *Enterococcus faecium*, *Salmonella enteritidis* and *E.coli* strains. The investigated *Bacillus*, *Geobacillus* and *Enterococcus* strains were isolated from fresh milk and cheese specimens, *E.coli* strains were isolated from faecal specimens originated from domestic poultry and *Salmonella* strains were isolated from eggshell specimens. The isolation was made from clinical material delivered to the Department of Microbiology of the Faculty of Veterinary Medicine, University of Belgrade.

For the isolation of gram-positive bacteria included in this investigation and for *E.coli*, Columbia agar with the addition of 6% sheep blood (bioMerieux), MacConkey agar (bioMerieux), CNA agar with colistin and nalidixic acid (Becton Dickinson) with the addition of 6% sheep blood and nutrient broth (BioLab) were used. For the isolation of *Salmonella* strains, buffered peptone water (Becton Dickinson) was used and also Mueller Kaufmann tetrathionate broth base with the addition of 4 μg/ cm^3 novobiocin (Difco), Rapport Vassiliadis broth (Biomedics) and XLT4 agar (Difco) were used. An automatic identification system BBL Crystal gram-positive ID kit (Becton Dickinson) was used for typisation of gram-positive bacteria and BBL Crystal Enteric/nonfermenter ID kit (Becton Dickinson) was used for typisation of gram-negative bacteria. Serological determination of *Salmonella* was done by slide agglutination method with the use of specific „O“ and „H“ *Salmonella* antisera (Difco).

For testing the antibacterial activity of plant extracts, cation adjusted Mueller Hinton II broth was used (CAMHB, Becton Dickinson). For the purpose of testing the effect of the extracts on gram-positive bacteria, 1,6% bromcersol purple (Merck) in final concentration at 0,2 cm^3/200 cm^3 was added to CAMHB. For the purpose of testing the effect of the extracts on gram-negative bacteria, 1% phenol red (Merck) in final concentration at 1 cm^3/200 cm^3 was added to CAMHB. Dimethyl sulfoxide, (DMSO, Sigma Aldrich) and 1-2 propanediol (Acros Organics) were used as solvents for the herbal extracts and also for thymol and *p*-cymene.

Conventional microbiological methods were applied for the purposes of isolation and identification of bacterial strains included in the investigation. Isolation of *Salmonella* spp. was done according to ISO 6579 standard. For antibacterial susceptibility testing, broth macrodillution method was applied for determining MIC (minimal inhibitory concentration) values and in accordance with the CLSI prescription (Clinical Laboratory Standards Institute, 2008; Isenberg, 2004). Antimicrobial effects of plant extracts were investigated in concentrations (expressed in μg/ cm^3): 1280; 640; 320; 160; 80; 40 and 20, including the thymol and *p*-cymene 1.9:1 mixture. The extracts were previously dissolved in concentration of 5120 μg/ cm^3, in 50:50 DMSO and 1-2 propanediol solution and then double dilutions of extracts down to the lowest tested

concentration were prepared. The desired innoculum density of $5x10^5$ CFU/ cm^3 was achieved by preparing the suspension of bacteria of approximately $1\text{-}2x10^8$ CFU/ cm^3, which was the density equal to McFarland standard 0,5 (Becton Dickinson). The prepared suspension was diluted 10 times, to obtain final inoculum density of approximately 1-2 x 10^7 CFU/ cm^3 and 50 µl of this suspension was applied to CAMHB, after which the number of bacteria in the media was approximately $5x10^5$/ cm^3. The media were incubated on 37°C for 18 hours. For MIC values the broth with lowest oil concentration, with no visible bacterial growth, was used.

RESULTS AND DISCUSSION

The fractional extraction conditions (pressure and temperature) in this work allowed isolation of essential oil rich fraction separatly from antioxidant fraction. The yields of supercritical extracts (SCE) and essential oils obtained by hydrodistillation (EO) are given in the Table 1.

Table 1 The yields of SC CO_2 extracts and essential oils of rosmary, sage and thyme (mass %)

Plant	SCE	EO
Rosemary (*Rosmarinus officinalis*)	0.52	1.02
Thyme (*Thymus vulgaris*)	1,23	1.09
Sage (*Salvia officinalis*)	2,13	0.86

The investigation on antibacterial efficiency was performed on following representative foodborne aerobic bacteria families: *Enterococcus* (4 strains), *Bacillus* (3 strains), *Escherichia* (4 strains) and *Salmonella* (3 strains). The respective MIC (minimal inhibitory concentration) values are summarized in Table 2. The antibacterial activities of the common antibiotics (chloramphenicol and ciprofloksacin) were used for comparative investigations of obtained MIC values.

According to the results presented in Table 2, thyme isolates had the highest antibacterial efficiency against tested foodborne bacteria strains (MIC=40-640 µg/cm^3) followed by rosemary (MIC=320-1280 µg/cm^3) and sage (MIC=160-2560 µg/cm^3) isolates. Among tested bacteria, *Bacillus* species were the most sensitive to presence of all tested SCEs and EOs, especially to presence of thyme isolates. Thereto, gram-positive bacteria seemed to be more susceptible to all tested SCEs and EOs. The gram-negative bacteria generally have higher tolerance to presence of essential oils due to hydrophilic outer membrane that blocks the penetration of hydrophobic essential oils into target cell membrane [7]. In this study, notable suceptiblity of tested gram-negative bacteria on thyme EO and SCE was recorded (MIC=640 µg/cm^3). The tested bacterial straines were equally suceptible to both thyme SCE and EO except *B. cereus* and *B. stearotermophillus* strains which were more suceptible to supercritical extract. Rosemary isolates seemed to have higher antibacterial efficiency in growth inhibiton of *Enterococcus*, *Bacillus* and *Salmonella* strains compared to sage isolates. Higher antibacterial efficiency of rosemary EO in inhibition *Bacillus subtilis*, *Salmonella*

enteritidis and *E. coli* growth with respect to sage EO was previously reported [5, 11]. SCE and EO of both rosemary and sage had same antibacterial activities against tested bacteria, except in the case of *Bacilus* species. As for rosemary, the *B. cereus* was more sensitive to SCE, whereby *B. subtilis* was more susceptible to EO. In the case of sage, EO had stronger antibacterial activity against *B. stearotermophillus* and *B. subtilis*.

Table 2 Antibacterial activity of essential oil fractions isolated by SFE and essential oil obtained by hydrodistilation from *R. officinalis*, *S. officinalis* and *T. vulgaris*

	plant material	*Thymus vulgaris*		*Salvia officinalis*		*Rosmarinus officinalis*		Control	
	Method of extraction	HD	SFE	HD	SFE	HD	SFE	Chloram-phenicol	Cipro-floksacin
	Bacterial strain	MIC (μg/cm^3)							
gram (+)	*Bacillus cereus*	80	≤40	1280	1280	640	320	-	≤0,5
	Bacillus stearotermophillus	80	≤40	640	1280	640	640	-	≤0,5
	Bacillus subtilis	≤40	≤40	160	2560	320	640	-	≤0,5
	Enterococcus faecium 1	320	320	1280	1280	>1280	>1280	≤4	-
	Enterococcus faecium 2	320	320	1280	1280	1280	1280	≤4	-
	Enterococcus faecium 3	320	320	1280	2560	1280	1280	≤4	-
gram (–)	*E.coli* 1	640	640	>2560	>2560	>1280	>1280	≤4	-
	E.coli 2	640	640	>2560	>2560	>1280	>1280	≤4	-
	E.coli 3	640	640	>2560	>2560	>1280	>1280	≤4	-
	Salmonella enteritidis 1	640	640	>2560	>2560	>1280	>1280	≤4	-
	Salmonella enteritidis 2	640	640	>2560	>2560	>1280	>1280	≤4	-
	Salmonella enteritidis 3	640	640	>2560	>2560	>1280	>1280	≤4	

CONCLUSION

According to the obtained results it could be concluded that thyme isolates had the highest antibacterial efficiency against tested foodborne bacteria strains (MIC=40-640 μg/cm^3) followed by rosemary (MIC=320-1280 μg/cm^3) and sage (MIC=160-2560 μg/cm^3) isolates. Among tested bacteria, *Bacillus* species were the most sensitive to presence of all tested SCEs and EOs, especially to presence of thyme isolates.

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SUMMARY

ANTIBAKTERIJSKA AKTIVNOST ETARSKIH ULJA ODABRANIH BILJAKA FAMILIJE LAMIACEAE IZOLOVANIH RAZLIČITIM METODAMA

(Naučni rad)

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U ovoj studiji je ispitana izolacija etarskih ulja iz lista ruzmarina (*Rosmarinus officinalis*), žalfije (*Salvia officinalis*) i timijana (*Thymus vulgaris*) postupkom natkritične ekstrakcije ugljenik(IV)-oksidom sa aspekta prinosa i antimikrobne aktivnosti dobijenih izolata protiv *Geobacillus stearotermophillus*, *Bacillus cereus*, *Bacillus subtilis* var. *niger*, *Enterococcus faecium*, *Salmonella enteritidis* i *E.coli* sojeva. Izolati timijana su pokazali najjače antibakterijsko dejstvo u odnosu na testirane sojeve (MIC=40-640 μg/cm^3). Zatim slede izolati ruzmarina (MIC=320-1280 μg/cm^3) i žalfije (MIC=160-2560 μg/cm^3). Među testiranim bakterijama, *Bacillus* sojevi su se pokazali kao najosetljiviji na prisustvo kako natkritičnih ekstrakata, tako i hidrodestilata.

Ključne reči: ekstrakcija superkritičnim fluidom, etarska ulja, antibakterijska aktivnost

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