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In vitro synergistic antibacterial activity of *Melissa officinalis* L. and some preservatives

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Abstract

The aim of this study was to investigate the antibacterial activity of aqueous, ethanol and ethyl acetate extracts of the species *Melissa officinalis* L. and their *in vitro* synergistic action with preservatives, namely: sodium nitrite, sodium benzoate and potassium sorbate against selected food spoiling bacteria, for a potential use in food industry. Synergistic action was noticed in almost every combination between plant extracts and preservatives. This work showed that the active compounds from ethanol, ethyl acetate and aqueous extracts of *Melissa officinalis* L. significantly enhanced the effectiveness of tested preservatives. Synergism was established at plant extract and preservative concentrations corresponding to 1/4 and 1/8 minimal inhibitory concentration values, which indicated the possibility of avoiding the use of higher concentrations of tested preservatives.

Additional key words: minimal inhibitory concentration, plant extracts, synergism.

Resumen

Actividad antibacteriana sinergística *in vitro* y conservantes de *Melissa officinalis* L.

El objetivo de este estudio fue investigar, para un uso potencial en la industria alimentaria, la actividad antibacteriana de extractos en agua, en etanol y en acetato de etilo de *Melissa officinalis* L. y su acción sinergística *in vitro* con los conservantes nitrito sódico, benzoato sódico y sorbato potásico sobre una selección de bacterias que dañan los alimentos. Se detectó una acción sinergística en casi todas las combinaciones entre los extractos de las plantas y los conservantes. Este trabajo muestra que los componentes activos de los extractos en etanol, acetato de etilo y agua de *Melissa officinalis* L. elevaron significativamente la eficacia de los conservantes analizados. El sinergismo fue establecido a concentraciones de los extractos de plantas y de los conservantes correspondientes a 1/4 y 1/8 de los valores de concentración inhibitoria mínima, lo que indica que se puede evitar utilizar concentraciones más elevadas de estos conservantes.

Palabras clave adicionales: concentración inhibitoria mínima, extractos de planta, sinergia.

Introduction

Melissa officinalis L. (fam. Lamiaceae) is a perennial, aromatic herb native to southern Europe. Due to intense lemon aroma and flavor of leaves, *M. officinalis* is used widely in food and cosmetics (Enjalbert *et al*., 1983). It can be used as a herbal tea for its aromatic, digestive and antispasmodic properties in gastrointestinal disorders (Auf'mkolk *et al*., 1985). Beneficial effects of ethanol, ethyl acetate and aqueous extracts of *M. officinalis* are attributed mainly to the presence of the phenol compounds (rosemary, protocatecuic, coffee acid and their methyl esters) and are related to their antioxidant activity (Hener *et al*., 1995; Tagashira *et al*., 1995; Canadanović Brunet *et al*., 2008).

Various medicinal properties have been attributed to principal constituents. Rosemary acid, a derivative of coffee acid, is the most abundant component of the *M. officinalis* leaf extract, which is known to have antiviral and antioxidant activity (Koch Heitzmann and Schultze, 1984), while the essential oil has antibacterial, antifungal and antihistaminic activities (Burt, 2004).

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Abbreviations used: CFU (colony-forming units), DMSO (dimethyl sulfoxide), EFSA (European Food Safety Authority), FIC (fractional inhibitory index), MIC (minimal inhibitory concentrations).

There is a major drive towards natural rather than chemical or synthetic additives in food, as they are perceived by consumers to be safe and better (Bagamboula *et al*., 2003). The traditional herbal medicines offer a novel source of antioxidants for the food industry, for boosting both the shelf-life and nutritional content of food (Hinneburg *et al*., 2006). Use of plant extracts as natural preservatives have been especially highlighted since The European Food Safety Authority (EFSA, 2008) said the rosemary extract is safe for use as an antioxidant in food. Essential oils are becoming increasingly popular as natural antimicrobial agents to be used for food preservation (Tzortzakis and Economakis, 2007; Pazos *et al*., 2008). Potential use of *M. officinalis* as natural antimicrobial agents have been less explored (Cosentino *et al.,* 2003).

Different kinds of preservatives are used to prevent bio-deterioration of food products. In our test we choose three, commonly used in food industry: sodium benzoate, sodium nitrite, potassium sorbate. Sodium benzoate has proved a controversial additive, as recent studies have highlighted health concerns from its use (Haws *et al*., 2007; McCann *et al*., 2007). Also the commonly used preservative sodium nitrite has been under the spotlight since 2007 (Jiang *et al*., 2007).

Therefore, the aim of this work was to establish antibacterial activity of *M. officinalis* grown up in Serbia and to estimate the efficiency of combined action of the plant extracts and preservatives (sodium benzoate, sodium nitrite and potassium sorbate) against selected food spoilers and thereby expand the possibilities for more effective and safe preservation of food.

Material and methods

Plant material

Melissa officinalis L. was collected during the summer of 2006 on Mt. Suvobor (Serbia). Identification and classification of the plant material was performed at the Faculty of Science of the University of Kragujevac. The voucher specimen of the plant is deposited in the herbarium of the Faculty of Science.

Preparation of the plant extracts

The aqueous extracts were obtained by cooking dry ground plant material (leaves) (50 g for each solvent) in a water bath at 80°C. The ethanol and ethyl acetate extracts were obtained in a Soxhlet apparatus. Following filtration, the aqueous extract was evaporated in a water bath, while the ethanol and ethyl acetate extracts were evaporated under a vacuum at 40°C. Solutions of different concentrations of all dried extracts were re-suspended in 5% DMSO.

Preparation of preservatives

The preservatives used in the experiment were as follows: sodium benzoate (C Product, Belgrade, 2007); sodium nitrite (Biochemistry Laboratory, Faculty of Science, University of Kragujevac); and potassium sorbate (C Product, Belgrade, 2007). Different concentrations of preservatives were created by dissolving them in liquid Mueller-Hinton broth (Torlak, Belgrade). Before testing, preservatives were heat-treated at 80°C for 15 min.

Test microorganisms

The antibacterial activity of the aqueous, ethanol and ethyl acetate extracts of *M. officinalis* leaves were tested *in vitro* against the Gram-positive bacteria: *Bacillus mycoides* (PMFKg-B1), *Bacillus subtilis* (PMFKg-B2) and *Staphylococcus aureus* (PMFKg-B30); and the Gram-negative bacteria: *Agrobacterium radiobacter* var. *tumefaciens* (PMFKg-B11), *Enterobacter cloacae* (PMFKg-B22), *Erwinia carotovora* (PMFKg-B31), *Escherichia coli* (PMFKg-B26), *Pseudomonas fluorescens* (PMFKg-B28), *Proteus* sp.(PMFKg-B20). All microorganisms were obtained from the stock cultures of the Microbiology Laboratory (Faculty of Science, University of Kragujevac).

The minimal inhibitory concentrations

The MIC was determined by the tube macro-dilution method (NCCLS, 1997). Solution of each extract was serially diluted twofold in Mueller-Hinton broth so the final concentrations of the extracts in the medium were ranged from 40 to 0.15 mg mL^{-1}. Initial inoculants were prepared by suspending growth in a sterile saline and turbidity was adjusted to yield 0.5 McFarland standard and then diluted to 1:10 ratio. Prepared inoculum (0.1 mL) was added into each tube to obtain the final

turbidity (approximately 10^4 colony-forming units (CFU) mL^{-1}).

The MIC was defined as the lowest concentration of the plant extract at which visible growth is inhibited. The test tubes were incubated at 24°C/ 24 h. Each test included control, consisting of the substrate with the solvent. MIC of preservatives was determined the same way, final concentrations of the preservatives ranged from 10 to 0.03 mg mL^{-1}.

Synergism

The synergism between water, ethanol and ethyl acetate extracts with chosen preservatives was assessed by the checkerboard assay method (Satish *et al*., 2005). The following combinations were tested: ethanol extract with sodium benzoate, sodium nitrite, potassium sorbate, aqueous extract with sodium benzoate, sodium nitrite, potassium sorbate and ethyl acetate extract with sodium benzoate, sodium nitrite, and potassium sorbate. From the first to the sixth horizontal column, each plant extract of the combination was doubly diluted in Mueller-Hinton broth (MIC value of 32), while each of the tested preservatives of the combination was double diluted (MIC values of up to 32) and added in a quantity of 0.1 mL from the first to sixth vertical row. The MIC was defined as the lowest concentration of the plant extract at which visible growth is inhibited. The synergism between plant extracts and preservatives was determined by calculating the fractional inhibitory index (FIC) according to the formula: $\Sigma FIC = FIC_A + FIC_B = [A]/MIC_A + [B]/MIC_B$, where FIC_A is the MIC of drug A in the combination/ MIC of drug A alone, and FIC_B is the MIC of drug B in the combination/ MIC of drug B alone. Types of effects were classified as follows: FIC ≤ 0.5, synergism; FIC 0.5-1, additive effect; FIC 1-4, indifferent effect; and FIC > 4, antagonism (Berenbaum, 1981).

Results

Antibacterial activity of aqueous, ethanol and ethyl acetate extract of *M. officinalis* was quantitatively estimated by MIC values and results are represented in Table 1. The MIC varied, depending on the type and concentration of plant extracts and taxonomic characteristics of the species of microorganism tested. All tested extracts exerted antibacterial effects.

The strongest antibacterial activity was exhibited by ethanol extract. MIC was 5 mg mL^{-1} on all tested bacterial species except *S. aureus* (MIC = 10 mg mL^{-1}), *Proteus* sp. and *E. coli* (MIC = 40 mg mL^{-1}). The MIC of ethyl acetate extract on all tested bacterial species was 10 mg mL^{-1}, while MIC of aqueous extract fluctuated, depending on tested bacterial species from 5 to 20 mg mL^{-1} (Table 1). The most resistant bacterial species to all tested plant extracts and preservatives were *E. coli* and *Proteus* sp.

The MIC values of tested preservatives ranged from 5 to 10 mg mL^{-1} for sodium benzoate, from 0.5 to 2 mg mL^{-1} for sodium nitrite and from 5 to 10 mg mL^{-1} for potassium sorbate (Table 1).

Synergism was established in almost every combination of plant extract with preservatives. By the checkerboard method combinations (ethanol extract/ sodium benzoate, ethanol extract/sodium nitrite and

Table 1. Minimal inhibitory concentration (mg mL^{-1}) values of plant extracts and preservatives

Bacterial species	Plant extracts			Preservatives		
	Aqueous extract	Ethanol extract	Ethyl acetate extract	Sodium benzoate	Sodium nitrite	Potassium sorbate
Agrobacterium tumefaciens	5	5	10	10	1	10
Bacillus mycoides	5	5	10	10	2	10
Bacillus subtilis	5	5	10	10	1	10
Enterobacter cloaceae	10	5	10	10	2	10
Erwinia carotovora	5	5	10	10	2	5
Escherichia coli	20	40	10	5	2	5
Proteus sp.	20	40	10	10	2	10
Pseudomonas fluorescens	10	5	10	5	0.5	10
Staphylococcus aureus	10	10	10	10	1	10

Table 2. Fractional inhibitory index (FIC) showing synergism for combination ethanol extract/preservatives

Bacterial species	MIC (mg mL^{-1})				Ethanol extract+					
	Extr	Sn	Sb	Ps	Sn	FIC values	Sb	FIC values	Ps	FIC values
Agrobacterium tumefaciens	5	1	10	10	1.25+0.125	0.375				
Bacillus mycoides	5	2	10	10			1.25+1.25	0.375	1.25+1.25	0.375
Bacillus subtilis	5	1	10	10	0.625+0.125	0.25	1.25+1.25	0.375		
Enterobacter cloaceae	5	2	10	10	1.25+0.50	0.50				
Erwinia carotovora	5	2	10	5	1.25+0.50	0.50			0.625+1.25	0.375
Escherichia coli	40	2	5	5	5+0.50	0.375	10+1.25	0.50		
Pseudomonas fluorescens	5	0.5	5	10					0.625+2.5	0.375

Sn: sodium nitrite. Sb: sodium benzoate. Ps: potassium sorbate.

ethanol extract/potassium sorbate) showed synergism against *A. radiobacter* var. *tumefaciens, B. mycoides, B. subtilis*, *E. cloaceae, E. carotovora*, *E. coli* and *P. fluorescens*. FIC ranged from 0.25 to 0.50. MIC values of preservatives and ethanol extract in combination were reduced up to 1/8 MIC (Table 2).

Ethyl acetate/sodium benzoate, ethyl acetate/sodium nitrite, ethyl acetate/potassium sorbate combinations manifested synergism in the relation to *B. mycoides, B. subtilis, E. carotovora* and *E. coli.* FIC index fluctuated from 0.375 to 0.50. MIC values of ethyl acetate extract in combination was reduced up to 1/4 MIC value and MIC of preservative up to 1/8 MIC (Table 3).

Aqueous extract manifested synergism with sodium nitrite and potassium sorbate. Synergism was established in relation to *B. mycoides, B. subtilis* and *P. fluorescens*. FIC range was 0.375 to 0.50. MIC of aqueous extract, reduced to 1/4, while MICs of sodium nitrite and potassium sorbate were respectively reduced to 1/4 and 1/8 (Table 4).

Apart from synergism, other types of interaction between plant extracts and preservatives noticed in this study as deduced from calculation of the FIC index were additive and indifferent effects, as represented in Table 5.

Discussion

The antibacterial activity of *M. officinalis* has been reported in previous investigations (Friedman Henika *et al.*, 2004; Mimica Djukic *et al.*, 2004). In this study, the strongest antibacterial activity was manifested by the ethanol extract. Earlier works showed that the ethanol extract of *M. officinalis* can possess an antioxidant (Yanishlieva and Marinova, 2006) and antinociceptive effect (Guginski *et al.*, 2009).

The strongest antibacterial activity among preservatives was exhibited by sodium nitrite.

Canadanović Brunet *et al*. (2008) tested different kinds of *M. officinalis* extracts. They showed that *M. officinalis* can be used as a source of natural antioxidants and as a possible food supplement. The preservative effect of *M. officinalis* was also showed in the work by Akarpat *et al.* (2008). Due to the results of this test, for the significant antibacterial activity of

Table 3. Fractional inhibitory index (FIC) showing synergism for combination ethyl acetate extract/preservatives

Bacterial species	MIC (mg mL^{-1})				Ethanol extract+					
	Extr	Sn	Sb	Ps	Sn	FIC values	Sb	FIC values	Ps	FIC values
Bacillus mycoides	10	2	10	10	2.5+0.5	0.50			2.5+2.5	0.50
Bacillus subtilis	10	1	10	10					2.5+2.5	0.50
Erwinia carotovora	10	2	10	5	2.5+0.5	0.50				
Escherichia coli	10	2	5	5			2.5+0.625	0.375		

Sn: sodium nitrite. Sb: sodium benzoate. Ps: potassium sorbate.

Table 4. Fractional inhibitory index (FIC) showing synergism for combination aqueous extract/preservatives

Bacterial species	MIC (mg mL^{-1})			Aqueous extract+			
	Extract	Sn	Ps	Sn	FIC values	Ps	FIC values
Bacillus mycoides	5	2	10	1.25+0.50	0.50		
Bacillus subtilis	5	1	10			1.25+1.25	0.375
Pseudomonas fluorescens	5	0.5	10			1.25+1.25	0.375

Sn: sodium nitrite. Ps: potassium sorbate.

M. officinalis, along with recent studies which have highlighted health concerns about the use of artificial preservatives, we tested the potential synergism between *M. officinalis* and the preservatives. The synergistic effect, by checkerboard method of *M. officinalis*, was tested in an earlier study (Gutiérrez *et al*., 2008) but with other plant extracts. To our knowledge, there are no studies that tested synergism *M. officinalis* with preservatives.

We established a synergism for the combination of all three extracts of *M. officinalis* with all the tested preservatives. When tested alone or in combination with preservatives, the ethanol extract was most effective. It showed synergism in relation to seven bacterial species and with all the tested preservatives. The MIC values of the three preservatives in combination with ethanol extract were reduced up to four times. Comparing to ethanol, ethyl acetate and aqueous extracts were less effective. They exerted synergy in relation to less bacterial species then the ethanol extract and the MIC value of one preservative in combination was reduced four times (sodium benzoate in case of ethyl acetate and potassium sorbate in case of aqueous extract).

The most sensitive on combinations of tested agents were *B. subtilis* and *B. mycoides,* common food spoilage bacterial species. In relation to these species the synergism was established in the combination of all the three plants extract with almost every tested preservative.

Escherichia coli showed some resistance to plant extracts when they were tested separately, but in combination: ethanol extract/sodium benzoate, ethanol extract/sodium nitrite and ethyl acetate/sodium benzoate manifested sensitivity. For these combinations synergism was detected.

According to the FIC index *Proteus* sp. and *S. aureus* exerted the greatest resistance to all combinations plant extract/preservative. No synergism was detected.

The combination of ethanol, ethyl acetate and aqueous extracts respectively with sodium nitrite, sodium benzoate, potassium sorbate exhibited synergism by inhibiting the growth of significant number of bacterial species at a lower concentration than when each agent was assayed separately.

The combination of ethanol, ethyl acetate and aqueous extracts with sodium nitrite, sodium benzoate and potassium sorbate inhibited the growth of significant number of bacteria species at a lower concentration

Table 5. Types of interactions (ΣFIC, most effective combination) between plant extracts and preservatives

Bacterial species	Aqueous extract+			Ethanol extract+			Ethyl acetate extract+		
	Sn	Sb	Ps	Sn	Sb	Ps	Sn	Sb	Ps
Agrobacterium tumefaciens	1.00 A	1.00 A	0.75 A	0.375 S	0.75 A	0.75 A	1.00 A	1.00 A	1.00 A
Bacillus mycoides	0.50 S	0.75 A	1.00 A	0.75 A	0.375 S	0.375 S	0.50 S	0.75 A	0.50 S
Bacillus subtilis	1.00 A	0.75 A	0.375 S	0.25 S	0.375 S	1.00 A	0.75 A	0.75 A	0.50 S
Enterobacter cloaceae	2.00 I	2.00 I	1.50 I	0.50 S	1.50 I	1.00 A	2.00 I	2.00 A	0.75 A
Erwinia carotovora	0.75 A	1.50 I	0.75 A	0.50 S	1.00 A	0.375 S	0.50 S	0.75 A	0.75 A
Escherichia coli	0.75 A	2.00 I	2.00 I	0.375 S	0.50 S	1.50 I	0.75 A	0.375 S	0.75 A
Proteus sp.	2.00 I	1.00 A	1.00 A	2.00 I	2.00 I	2.00 I	1.50 I	1.50 I	1.25 I
Pseudomonas fluorescens	1.00 A	0.75 A	0.375 S	1.00 A	0.75 A	0.375 S	1.00 A	1.00 A	1.25 I
Staphylococcus aureus	2.00 I	1.50 I	1.25 I	0.75 A	1.50 I	0.75 A	0.75 A	1.25 I	1.00 A

Sn: sodium nitrite. Sb: sodium benzoate. Ps: potassium sorbate. S: synergistic effect. A: additive effect. I: indifferent effect.

than when the single agents were assayed separately. The synergism was recorded at 1/4 and 1/8 MIC values of preservatives which indicated the possibility of avoiding the use of higher concentrations of tested preservatives that could lead to accumulation of toxic products in conserved food. The compounds of ethanol, ethyl acetate and aqueous extract did not decrease the activity of preservatives because antagonism was not indicated.

The results obtained herein, confirmed that *Melissa officinalis* L. possess antimicrobial activity and according to exhibited synergism with sodium benzoate, potassium sorbate and sodium nitrite, suggest that it may be used in biotechnological fields as natural preservative ingredients in food.

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