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IN VITRO SYNERGISTIC ANTIBACTERIAL ACTIVITY OF *SALVIA OFFICINALIS* L. AND SOME PRESERVATIVES

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Abstract – The aim of this work was to investigate the antibacterial activity of aqueous extracts of the species *Salvia officinalis* L. and its synergistic action with the preservatives sodium nitrite, sodium benzoate and potassium sorbate *in vitro* against selected food spoiling bacteria. Synergism was assessed by the checkerboard assay method and quantitatively represented by the FIC index. Synergistic action was established for aqueous extract/sodium benzoate, aqueous extract/potassium sorbate, aqueous extract/sodium nitrite combinations. Synergism was detected in relation to: *Agrobacterium tumefaciens, Bacillus subtilis* and *Proteus* sp. Synergism was established at plant extract and preservative concentrations corresponding up to 1/8 MIC values.

Key words: *Salvia officinalis* L., plant extracts, preservative, MIC, synergism

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INTRODUCTION

Salvia officinalis L (commonly known as sage) of the Lamiaceae family is an aromatic, perennial plant widely distributed in Europe. Since ancient times it has been used as a flavoring agent in a variety of food preparations and it is integral part of the popular, healthy Mediterranean diet (Malamas et al., 1992). In the past few decades sage has been the subject of intensive studies for its diterpenoids, triterpenoids, flavonoids and phenolic glycosides, which have been isolated from the plant (Couladis et al., 2002; Durling et al., 2007; Länger et al., 1998). It is for this reason that sage has found increasing application in food formulations (Shahidi, 2000). Lima et al., (2005) tested the antioxidant potential of the Salvia tea *in vivo* and showed that following 14 days of drinking *Salvia* tea the liver antioxidant status improved. The aqueous extract of *S. officinalis* possesses an antioxidant (Geuenich et al., 2008) and antiviral effect (Lima et al., 2007).

In recent years there has been considerable interest in ways to reduce the incidence of food poisoning. As a result of scientific research and negative consumer perceptions of artificial preservatives, attention is shifting towards alternatives that consumers perceive as natural plant extracts (Fiorentino et al., 2008; Gutierrez et al., 2008a). Sodium benzoate has proved a controversial additive, as recent studies have highlighted health concerns from its use (Haaws et al., 2007; McCann et al., 2007) and the commonly used preservative sodium nitrite has been under the spotlight since 2007 (Jiang et al., 2007). The use of plant extracts as natural preservatives has been especially highlighted since The European Food Safety Authority's Pronouncement (2008) that rosemary extract is safe for use as an antioxidant in food. The antibacterial activity of sage against food spoilage bacteria has been investigated (Di Pasqua et al., 2005; Viuda-Martos et al., 2007), but there have been no studies to test the synergy between an aqueous extract and preservatives.

For all these reasons the aim of this work was to establish the antibacterial activity of the aqueos extract of *Salvia officinalis* L. and to estimate the efficiency of the combined action of plant extracts and selected preservatives, commonly used in the food industry (sodium benzoate, sodium nitrite and potassium sorbate) against selected food spoilers and thereby expand the possibilities for more effective and safer conservation of food.

MATERIAL AND METHODS

Salvia officinalis L. was collected during the summer of 2007 on Mt. Suvobor (Serbia). Identification and classification of the plant material was performed at the Science Faculty of the University of Kragujevac. A voucher specimen of the plant is deposited in the herbarium of the Science Faculty.

Aqueous extracts were obtained by cooking dry ground plant material (leaves) (50 g) in a water bath at 80°C and evaporated in a water bath. Solutions of different concentrations of dried extracts were resuspended in 5% DMSO.

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

Test microorganisms

The antibacterial activity of the plant extract was tested *in vitro* against the following Gram-positive bacteria: *Bacillus mycoides* (PMFKg-B1), *Bacillus subtilis* (PMFKg-B2) and *Staphylococcus aureus* (PMFKg-B30); and the following 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 stock cultures of the Laboratory of Microbiology (Science Faculty, University of Kragujevac).

The minimal inhibitory concentration, MIC, was determined by the tube dilution method (NCCLS, 1997). The solution of aqueous extract was serially diluted twofold in Mueller-Hinton broth so that the final concentrations of the extract in the medium ranged from 40 mg/ml to 1.25 mg/ml. Initial inocula were prepared by suspending growth in a sterile saline. Turbidity corresponding to the 0.5 McFarland standard was adjusted spectrophotometrically and then diluted (1:10). 0.1 ml of prepared inocula was added into each tube to obtain a final turbidity (approximately 10^4 CFU/ml). 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/24h. Each test included two controls consisting of the substrate with and without the solvent. The MIC of the preservatives was determined in the same way (NCCLS, 1997) and final concentrations of the preservatives ranged from 10 mg/ml to 0, 3 mg/ml.

Synergism between water extract with chosen preservatives was assessed by the checkerboard assay method (Bayani and Azanza, 2005; Rochon-Edouard et al., 2000). The following combinations were tested: aqueous extract/sodium benzoate, aqueous extract/sodium nitrite, aqueous extract/potassium sorbate. From the first to the sixth horizontal column, the aqueous extract of the combination was doubly diluted in Mueller-Hinton broth (from MIC value of up to MIC/32), while each of the tested preservatives of the combination was doubly diluted (from MIC value of up to MIC/32) and added in a quantity of 0.1 ml from the first to sixth vertical row. 0.1 ml of prepared inocula was added into each tube (turbidity was approximately 10^4 CFU/ml). Each test tube contained a different concentration of the combinations; aqueous extract/sodium benzoate, aqueous extract/potassium sorbate, aqueous extract/natrium nitrite. Each test

included two controls consisting of the substrate with and without the solvent. 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 according to the formula: ∑FIC = FICA + FIC B = [A]/MIC_A + [B]/MIC_B. 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. The 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 (Bajaksouzian et al., 1997).

RESULTS

The sage aqueous extract exerted significant antibacterial activity. The MIC varied depending on the taxonomic characteristics of the species of microorganism tested and the concentration of the extract. Results are represented in Table 1. The aqueous extract was most effective against *Bacillus mycoides, Bacillus subtilis, Enterobacter cloaceae* and *Proteus* sp. (MIC was 10mg/ml), while most resistant was *Escherichia coli* (40 mg/ml).

Among the preservatives the most effective was sodium nitrite; the MIC fluctuated from 0.5-2 mg/ml. The most sensitive species was *Pseudomonas fluorescens.* The MIC for sodium benzoate and potassium sorbate was from 5-10 mg/ml. The most sensitive species in relation to these preservatives were *Pseudomonas fluorescens, Escherichia coli* and *Erwinia carotovora.* Results are represented in Table 1.

Using the checkerboard method, the aqueous extract in this test showed synergy with all tested preservatives. Synergism was detected in relation to *Agrobacterium radiobacter* var. *tumefaciens, Bacillus subtilis* and *Proteus* sp. The FIC index fluctuated from 0.25-0.50. Along with the synergism, types of interactions between plant extracts and preservatives noticed in the experiment as deduced from the calculation of the FIC index were additive and of indifferent effect (Table 2). The aqueous extract/sodium benzoate combination showed synergy against 3 (33.3%) species: *Agrobacterium radiobacter* var. *tumefaciens, Bacillus subtilis* and *Proteus* sp.; indifference against 3 (33.3%) species; an additive effect against 3 (33.3%) species. The MIC of the aqueous extract and sodium benzoate in combination were reduced up to 1/8 MIC values (Table 3.). The combination aqueous extract/potassium sorbate showed synergy against 1 (11.1%) species *Bacillus subtilis;* an additive effect against 6 (66.6%) bacterial species; an indifferent effect against 2 (22.2%) bacterial species. The MIC values of the aqueous extract and potassium sorbate were reduced up to 1/8 MIC values (Table 3). The aqueous extract/sodium nitrite combination exhibited synergy in relation to 1 (11.1%) bacteria species *Agrobacterium radiobacter* var. *tumefaciens;* an additive effect against 5 (55.5%) bacterial species;

Table 1. MIC values of aqueous extract and preservatives

Bacterial species	MIC(mg/ml)			
	Aque. ext. Salvia off.	Sodium benzoate	Natrium nitrite	Potassium sorbate
Agrobacterium tumefaciens	20	10	1	10
Bacillus mycoides	10	10	2	10
Bacillus subtilis	10	10	1	10
Enterobacter cloaceae	10	10	2	10
Erwinia carotovora	20	10	2	5
Escherichia coli	40	5	2	5
Proteus sp.	10	10	2	10
Pseudomonas fluorescens	20	5	0.5	10
Staphylococcus aureus	20	10	1	10

Table 2. FIC values of tested bacterial species and types of interaction between plant extract and preservatives

	Aque.ext+ S.benzoate		Aque.ext+ P.sorb.		Aque.ext+ S.nitr.	
Bacterial species	**ΣFIC***	**activity**	**ΣFIC***	**activity**	**ΣFIC***	**activity**
Agrobacterium tumefaciens	0.50	S	1.5	I	0.375	S
Bacillus mycoides	1.00	A	0.75	A	0.75	A
Bacillus subtilis	0.25	S	0.25	S	0.75	A
Enterobacter cloaceae	0.75	A	0.75	A	0.75	A
Erwinia carotovora	0.75	A	1.00	A	1.00	A
Escherichia coli	1.5	I	2.00	I	2.00	I
Proteus sp.	0.50	S	0.75	A	1.25	I
Pseudomonas fluorescens	1.25	I	1.00	A	1.25	I
Staphylococcus aureus	0.75	A	0.75	A	1.00	A

ΣFIC*-most effective combination; S-synergism; A-additive effect; I-indifferent effect

Table 3. FIC values showing synergism for combination aqueous extract/ preservatives.

Bacterial species	MIC(mg/ ml) Aqu. extr.	S. b.	P. s.	S.n.	MIC*(mg/ ml) Aqueous extract+ Sodium benzoate	FIC values	Potassium sorbate	FIC values	Sodium nitrite	FIC values
Agrobacterium radiobacter	20	10	10	1	5+2.5	**0.50**			5+0.125	**0.375**
Bacillus subtilis	10	10	10	1	1.25+1.25	**0.25**	1.25+1.25	**0.25**		
Proteus sp.	10	10	10	2	2.5+2.5	**0.50**				

MIC *- most effective combination of tested agents

an indifferent effect against 3(33.3%) species. The MIC values of the aqueous extract were reduced up to ¼ MIC and the MIC of sodium nitrite up to 1/8 MIC values (Table 3). ΣFIC results for the most effective combinations (most active concentrations of tested agents) by the checkerboard method are presented in Table 2.

DISCUSSION

Salvia officinalis L. is a plant which has been used in a variety of food preparations. In this work we showed the significant antibacterial activity of the aqueous extract of *S. officinalis* L. Some earlier studies have demonstrated sage antibacterial activity against foodborne bacteria (Gutierrez et al., 2008b; Longaray Delamare et al., 2007).

The preservative effect of *S. officinalis* L. was exerted in the test against *Salmonella* sp. (Hayouni et al., 2008). In this study we showed the preservative effect of sage aqueous extract but we expanded the number of bacterial species tested. Arslan et al. (2009) in their work showed that sage along with different plant extracts was comparable to synthetic preservatives such as sorbic acid and butylated hydroxyanisole. The Karpińska- Tymoszczyk (2007) study showed that sage extract alone or in combination with sodium isoascorbate could be used for the preservation of certain food products. In accordance with these studies our results exerted

the preservative effect of sage extract. The most effective was the combination with the commonly used preservative sodium benzoate. The MIC value of sodium benzoate in combination with sage extract was reduced three times; in relation to *Agrobacterium radiobacter* var. *tumefaciens* and *Proteus* sp.; four times in relation to *Bacillus subtilis*. The MIC value of potassium sorbate was reduced four times in relation to *Bacillus subtilis*, while the MIC value of natrium nitrite was reduced three times in relation to *Agrobacterium radiobacter* var. *tumefaciens*.

In this study the most resistant species when the plant extract was tested alone or in combination was *Escherichia coli.* The MIC and FIC values were highest compared to the other bacterial species tested.

Bacillus subtilis and *Agrobacterium radiobacter* var. *tumefaciens* were the most sensitive bacterial species. In relation to these species, synergism was established in the biggest number of aqueous extract/preservative combinations.

The combination of the aqueous extracts with sodium nitrite, sodium benzoate, potassium sorbate inhibited the growth of a significant number of bacteria species at a lower concentration than when the single agents were assayed separately. Synergism was recorded at 1/4 and 1/8 MIC values of the preservatives which indicates the possibility of avoiding the use of higher concentrations of the tested preservative which could lead to an accumulation of toxic products in conserved food. The water extract did not decrease the activity of preservatives because antagonism was not indicated.

CONCLUSION

The results obtained confirmed that the aqueous extract of *Salvia officinalis* L. possesses an antimicrobial activity and according to exhibited synergism with sodium benzoate, potassium sorbate and sodium nitrite, suggests that it may be used in biotechnological fields as a natural preservative ingredient in food.

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IN VITRO АНТИБАКТЕРИЈСКА АКТИВНОСТ *SALVIA OFFICINALIS* L. И ОДАБРАНИХ КОНЗЕРВАНАСА

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У последње време постоји тенденција проналаска алтернативе вештачким конзервансима, због све већег броја научних радова који доказују њихов штетан ефекат. Све чешћа замена се налази у биљним екстрактима. У овом раду смо тестирали постојање синергизма *Salvia officinalis* L. и натријум бензоата, натријум нитрита и калијум сорбата, конзерванаса који се често користе у прехрамбеној индустрији. Синергизам је констатован при концентрацијама које одговарају 1/4 и 1/8 MIC вредности биљног екстракта и конзерванса. У комбинацијама са воденим екстрактом вредности конзерванаса су редуковане и до четири пута. Ови резултати указују на могућност коришћења *Salvia officinalis* L. у процесима конзервирања хране.