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Synergy between *Salvia officinalis* L. and some preservatives

Communication

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Abstract: The aim of the present study is to investigate the antibacterial activity of *Salvia officinalis* L. aqueous extracts and its synergistic action with preservatives sodium nitrite, sodium benzoate and potassium sorbate *in vitro* against selected food spoiling bacteria. Synergy 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. Synergy was detected in relation to: *Agrobacterium tumefaciens, Bacillus subtilis* and *Proteus* sp. Synergy was established at plant extract and preservative concentrations corresponding up to 1/8 MIC values.

Keywords: *Salvia officinalis L.* • *Plant extract* • *Preservatives* • *MIC* • *Synergy*

1. Introduction

Salvia officinalis (L.) (commonly known as sage) belonging to the Lamiaceae family is an aromatic, perennial plant widely distributed in Europe. Historically, it has been used as a flavoring agent in a variety of food preparations and it is an integral part of a healthy Mediterranean diet [1]. 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 [2,3]. It is for this reason that sage has found increasing application in food formulations [4]. Lima *et al.* [5] tested the antioxidant potential of the Salvia tea *in vivo* and showed that 14 days after starting to drink Salvia tea, liver antioxidant status improves.

Currently, the attention of research is shifting away from artificial preservatives and towards alternative that consumers perceive to be natural plant extracts [6,7]. Sodium benzoate has proved to be a controversial additive, as recent studies have highlighted health concerns from its use [8,9]. The commonly used preservative sodium nitrite has also been under the spotlight since 2007 [10]. Use of plant extracts as natural preservative has been highlighted since the European Food Safety Authority said that rosemary extract was safe for use as an antioxidant in food [11]. Antibacterial activity of sage against food spoilage bacteria was investigated [12,13]; however, there are no studies that test synergy between aqueous extract and preservatives.

For all these reasons, aim of this work is to establish the antibacterial activity of specimens of this species originating from Serbia and to estimate the efficiency of combined action of plant extracts and selected preservatives, commonly used in food industry (sodium benzoate, sodium nitrite and potassium sorbate) against selected food spoilers and thereby expand the possibilities for more effective and safe conservation of food.

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2. Experimental Procedures

2.1 Preparation of plant extract

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

Aqueous extracts were obtained by cooking dry ground plant material (50 g) in a water bath for 2-3 h at 80°C. Following filtration, the aqueous extract was evaporated in a water bath. Stock solutions were obtained by dissolving dry material in a defined volume of solvent (5% dimethyl sulfoxide) for extract and in a Mueller-Hinton broth (Torlak, Belgrade) for preservatives. Dimethyl sulfoxide (DMSO) was purchased from Merck, Germany.

2.2 Preparation of preservatives

The preservatives used in the experiment were as follows: sodium benzoate (C Product, Belgrade, 2007); sodium nitrite (Laboratory of Biochemistry, Science Faculty, University of Kragujevac); and potassium sorbate (C Product, Belgrade, 2007). The concentrations of the 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.

2.3 Test microorganisms

The antibacterial activity of plant extract 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 stock cultures of the Laboratory of Microbiology (Faculty of Science, University of Kragujevac).

2.4 Minimal inhibitory concentrations

MIC was determined by tube dilution [14]. The aqueous extract solution was serially diluted two-fold in Mueller-Hinton broth so the final concentration ranged from 40 mg/ml to 1.25 mg/ml. Bacterial suspensions were prepared by direct colony suspension and adjusted to obtain the 0.5 McFarland standard. 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 was inhibited. The tubes were incubated at 27°C or 37°C (depending on the bacteria) for 24 h. Each test included 2 growth controls consisting of the medium with the solvent (5% DMSO) and medium with bacterial suspension.

All tests were performed in duplicate. MIC of preservatives was determined in the same way [14], final concentrations of the preservatives ranged from 10 mg/ml to 0.3 mg/ml.

2.5 Synergy

Synergy between aqueous extract and chosen preservatives was assessed by checkerboard assay [15,16]. The following combinations were tested: aqueous extract /sodium benzoate, aqueous extract/ sodium nitrite, aqueous extract/ potassium sorbate. From the first to sixth horizontal row, aqueous extract of the combination was diluted two-fold in Mueller-Hinton broth (from MIC value up to MIC/32), and each of the tested preservatives combinations were diluted two-fold as well (from MIC value of up to MIC/32) and added in a quantity of 0.1 ml from the first to sixth vertical column. 0.1 ml of prepared inocula was added into each tube (turbidity was approximately 10^4 CFU/ ml). Each test tube contained different concentrations of the following combinations;

	MIC (mg/ml)			
Bacterial species	*Salvia officinalis*	Sodium benzoate	Sodium 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 1. MIC values of aqueous extract and preservatives.

aqueous extract/ sodium benzoate, aqueous extract/ potassium sorbate, aqueous extract/ natrium nitrite. Each test included 2 growth controls consisting of the medium with the solvent (5% DMSO) and medium with bacterial suspension.

The MIC was defined as the lowest concentration of the plant extract at which visible growth is inhibited. The synergy between plant extracts and preservatives was determined by calculating the fractional inhibitory index according to the formula:

FIC index= 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. The effects were classified as follows: FIC≤0.5, synergy; FIC 0.5-1, additive effect; FIC 1-4, indifferent effect; and FIC > 4, antagonism [17].

3. Results

Our findings suggest that the sage aqueous extract exerts significant antibacterial activity. MIC varies depending on taxonomic characteristics of the species of microorganism tested and concentration of the extract. Results are represented in Table 1. The negative control (5% DMSO) does not inhibit growth in the tested bacteria.

Aqueous extract is most effective against *B. mycoides, B. subtilis, E. cloaceae* and *Proteus* sp (MIC was 10 mg/ml), and the most resistant is *E. coli* (40 mg/ml).

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

By the checkerboard method, the aqueous extract showed synergy with all tested preservatives. Synergy was detected in relation to *A. radiobacter* var. *tumefaciens*, *B. subtilis* and *Proteus* sp. FIC index fluctuated from 0.25 to 0.50. We also demonstrated that synergy between plant extracts and preservatives, as deduced from calculation of the FIC index, were additive and indifferent effect (Table 2). Aqueous extract/ sodium benzoate combination showed synergy against 3 (33, 3%) species: *A. radiobacter* var. *tumefaciens*, *B. subtilis* and *Proteus* sp.; indifference against 3 (33, 3%) species; additive against 3 (33, 3%) species. MIC of aqueous extract and sodium benzoate in combination were reduced up to 1/8 MIC values. Combining aqueous extract/potassium sorbate showed synergy against 1 (11.1%) species *B. subtilis*; and an additive effect against 6 (66.6%) bacterial species; indifferent effect against 2 (22.2%) bacterial species. MIC values of aqueous extract and potassium sorbate were reduced up to 1/8 MIC values. Aqueous extract/ sodium nitrite exhibited synergy in relation to 1 (11.1%) bacteria species *A. radiobacter* var. *tumefaciens*; and an additive effect against 5 (55.5%) bacterial species; which was also indifferent against 3 (33.3%) species. MIC values of aqueous extract were reduced up to 1/4 MIC and MIC of sodium nitrite up to 1/8 MIC values. FIC index results for the most effective combinations (most active concentrations of tested agents) by checkerboard method are presented in Table 2.

4. Discussion

S. officinalis is a plant which has been used in a variety of food preparations. In this work we show significant antibacterial activity of the aqueous extract. Couldais *et al.* reported that the main compounds of essential oil were oxygenated monoterpenes: α-thujone, β-thujone,

	Aqueous extract + Sodium benzoate		Aqueous extract + Potassium sorbate		Aqueous extract + Sodium nitrite	
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	1.2	I
Pseudomonas fluorescens	1.25	I	1.00	A	1.25	I
Staphylococcus aureus	0.75	A	0.75	A	1.0	A

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

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

1.8-cineol, camphor, borneol and bornyl acetate [18]. Among the dominant sesquiterpenes were: α-humulene, viridiflorol and manool. These isolated bioactive compounds are responsible for its antimicrobial activity. Some earlier studies have demonstrated sage antibacterial activity against foodborne bacteria [19,20].

Preservative effect of *S. officinalis* L. was exerted in the test against *Salmonella* sp. [21]. In the present study we showed preservative effect of sage aqueous extract on some other bacterial species. It is known that aqueous extract of *S. officinalis* possess antioxidant [22] and antiviral effect [23].

Arslan *et al.* [24] in their work showed that sage along with different plant extracts were comparable to the synthetic preservatives like sorbic acid and butylated hydroxyanisole. Karpińska-Tymoszczyk [25] study showed that sage extract alone or in combination with sodium isoascorbate could be used for preservation of certain food products. In accordance with these studies, our results show the preservative effect of sage extract. The most effective combination observed was sage with the commonly used preservative sodium benzoate. MIC values of sodium benzoate in combination with sage extract were reduced to one third of the original value; in relation to *A. radiobacter* var. *tumefaciens* and *Proteus* sp.; one fourth in relation to *B. subtilis*. MIC values of potassium sorbate were reduced to one fourth of the original value in relation to *B. subtilis*, while MIC value of natrium nitrite was reduced to one third in relation to *A. radiobacter* var. *tumefaciens*.

In the present study *E. coli* was the most resistant strain to the plant extract, applied alone or in combination. MIC and FIC values were highest comparing to other bacterial species tested. *B. subtilis* and *A. radiobacter* var. *tumefaciens* were the most sensitive bacterial species. In relation to these strains, mostly synergy was established in the combinations of aqueous extract and preservatives.

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

Results obtained herein, confirmed that the aqueous extract of *S. officinalis* possess antimicrobial activity and according to exhibited synergy with sodium benzoate, potassium sorbate and sodium nitrite, suggest that it may be used in biotechnological fields as natural preservative ingredient in food.

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