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COMPARATIVE STUDY ON THE ANTIBACTERIAL ACTIVITY OF VOLATILES FROM SAGE *(SALVIA OFFICINALIS* L.*)*

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Abstract - Antibacterial activity of volatiles from sage against Gram-positive and Gram-negative bacteria from the ATCC collection was screened with the disk diffusion test. The essential oil and its fractions showed a significant antibacterial effect against *S. aureus* and *B. subtilis*. The minimum inhibitory concentrations were 1.25-2.5 μL/mL for *S. aureus* and 0.15-2.5 μL/mL for *B. subtilis*. The effect on *S. aureus* was bactericidal, while initial bactericidal effect on *B. subtilis* was impaired by the presence of a resistant fraction of the population, probably endospores. The results obtained with wild type and permeable strains of *E. coli* and *S. typhimurium* indicate that transport through the cell wall limits the antibacterial effect of sage volatiles.

Key words: Sage, volatiles, antibacterial activity

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INTRODUCTION

Plant extracts and essential oils, as well as their constituents, are used in the food, cosmetics, and pharmaceutical industries (Stammati *et al.* 1999). Many essential oils and their ingredients have been shown to possess diverse biological activities, including antibacterial, antifungal, and antiviral effects (Oplachenova and Obreshkova, 2003; Marinković *et al.* 2002; Sattar *et al.* 1995). Nowadays, with the alarming incidence of antibiotic resistance in bacteria, there is a need for effective alternatives. It is therefore of interest to reexamine the way in which essential oils delay or inhibit the growth of pathogenic or food contaminating bacteria and apply them to actual practice.

A number of Lamiaceae species are aromatic plants used in traditional medicine and as culinary herbs worldwide. In traditional medicine sage was used for many ailments, including inflammation of the mouth and throat (Baričević *et al.* 2001). Traditional use of sage has been justified in a number of studies (Baričević *et al.* 2000; Capasso *et al.* 2004; Ren *et al.* 2004; Đarmati *et al.* 1993, 1994). Moreover, antimutagenic and cancer preventive activities of sage have been reported (Craig, 1999; Simić *et al.* 2000; Knežević-Vukčević *et al.* 2001).

In the present work, we compared antimicrobial properties of the essential oil of sage (*Salvia officinalis* L.), its fractions, and major monoterpenes against *S. aureus, S. epidermidis, P. aeruginosa, E. coli, B. subtilis,* and *S. faecalis* strains from the ATCC collection, as well as against laboratory strains of *E. coli* and *S. typhimurium* with increased permeability of their cell walls. We used the disc-diffusion test for pre-screening of the antibacterial potential of agents, the broth macrodilution method to determine the minimum inhibitory concentration (MIC), and the time-kill assay to examine the kinetics of antimicrobial activity.

MATERIAL AND METHODS

Essential oil of sage, its fractions and monoterpenes

Salvia officinalis L. plants were cultivated in Pančevo by the «Dr. Josif Pančić» Institute for Medicinal Plant Research. Essential oil (EO) was prepared by steam distillation of plants cut at ground level in a 2 m^3 steam distiller (Hromil) for 2 hours at the pressure 3-4 bars and temperature of 135-145^oC according to Ph. Jug. IV.

The fractions F1-F5 were prepared by vacuum rectification and analyzed by GC-FID and GC-MS (Brkić *et al.,* 1999). The results of chemical analysis show that EO contains 44 terpenoids. Fractions F1-F4 contain mainly

Table 1. Composition of essential oil of sage and its fractions

Constituent	Percentage (m/m) in the sample					
	EO	F-1	F-2	F-3	F-4	F-5
cis-salven	0.518	0.134				
triciklen	0.123	0.146				
α-thujene	0.178	0.100				
α-pinene	5.059	5.194	0.620			
camphene	3.683	6.017	1.361			
sabinene	0.124	0.134				
β-pinene	2.717	3.429	0.962			
myrcene	0.874	0.295	0.042			
α-felandren	0.062					
α-terpinene	0.225					
p-cymene	0.460	1.423	1.342	0.611	0.102	
limonene	1.224	1.235	0.667	0.325		
1,8-cineole	14.425	31.661	21.864	4.853	0.475	
β-ocimene	0.032	0.023	0.058	0.039		
γ-terpinene	0.391	0.101	0.144		0.236	
cis-sabinene-hydrate	0.114			0.202	0.144	
cis-linalol-oxide	0.069			0.123	0.135	
terpinolen	0.262	0.095	0.135	0.125	0.924	
trans-sabinenehydrate	0.501	0.824	0.484	0.489		1.112
α-thujone	37.516	29.656	48.233	61.512	57.335	11.267
β-thujone	4.665	3.002	4.781	7.439	7.895	2.150
camphor	13.777	8.293	14.364	21.614	27.623	12.075
trans-pinocamphon	0.461			0.364	0.545	
borneol	0.753	0.903		0.509	1.200	4.227
cis-pinocamphon	0.033			0.111	0.160	
terpin-4-ol	0.351			0.155	0.337	0.997
p-cimene-8-ol	0.025					
α-terpinol	0.117			0.201	0.084	1.116
mirtenal	0.208				0.236	
bornil-acetate	0.391	0.508		0.197	0.425	1.777
trans-sabinilacetate	0.099				0.070	
α-kubeben	0.029				0.048	
β-burbonen	0.058				0.136	
caryophilene	1.824			0.185	0.454	
α-humulene	4.994			0.239	0.586	29.852
allo-aromadendren	0.085					
γ-murolen	0.053					
viridiflorene	0.109				0.054	
γ-kadinen	0.031					
δ-kadinen	0.066					
caryophillene-oxide	0.089					
viridiflorol	1.371					8.745
humulene-epoksid	0.340					2.683
manool	0.277					1.892
Σ	98.762	93.172	95.058	99.293	99.205	88.315

monoterpenes, while fraction F5 is with high content of sesquiterpenes (Table 1). The α+β thujone (94.48/3.50) used was from Extrasynthese, D,L-camphor was from ICN, and 1,8-cineole was kindly provided by the «Dr. Josif Pančić" Institute for Medicinal Plant Research.

Bacteria and media

The following bacterial strains were used: *Staphylococcus aureus* ATCC25923, *Staphylococcus epidermidis* ATCC12228, *Pseudomonas aeruginosa* ATCC27853, *Escherichia coli* ATCC25922, *Bacillus subtilis* ATCC10707, *Streptococcus faecalis* ATCC29212, and *Escherichia coli* K12 strains SY252 and IB112 from our laboratory collection; and *Salmonella typhimurium* TA100 and TA102 (Maron and Ames, 1983). The IB112 and *Salmonella* strains are with increased permeability, due to *lpc*A and *rfa* mutations, respectively.

Bacteria were cultivated at 37°C in Luria broth (LB) (yeast extract 5 g, bacto-tryptone 10 g, NaCl 5 g, distilled water 1 L) or Mueller Hinton broth (MHB) from Oxoid. Luria agar (LA, LB plus 15 g agar) and Mueller Hinton agar (MHA) from Oxoid were used as solid media. The essential oils, its fractions, and monoterpenes were dissolved in ethanol (1/10) and applied in different concentrations. Ethanol was used as a negative control and antibiotics (chloramphenicol, streptomycin, and gentamycin) as positive controls.

Antibacterial activity

The disk-diffusion assay was applied to determine the growth inhibition of bacteria by sage extracts (H i n d l e r , 1995). Overnight bacterial cultures (100 μL) were spread onto MHA. Sage extracts were applied to 10 mm disks (W h a t m a n paper No.1). After 24 h of incubation at 37°C, the diameter of growth inhibition zones was measured.

MIC determination

The broth dilution test was performed in test tubes. In two-fold serial dilutions of EO or its fractions, a standardized suspension (M c F a r l a n d turbidity standard) of test bacteria (100 μL) was added to obtain a final concentration of $5x10^5$ CFU/mL. A growth control tube and sterility control tube were used in each test. After overnight incubation at 37°C, the MIC was determined visually as the lowest concentration that inhibits growth, evidenced by the absence of turbidity (H i n d l e r , 1995). Differences of more than two steps of dilutions were considered significant (O p l a c h e n o v a and O b r e s h k o v a , 2003).

Time-kill assay

Exponential cultures of test bacteria (100 μL) are inoculated into several tubes of LB containing MIC concentration of sage extract for *S. aureus* and double MIC for *B. subtilis*. A growth control tube was used in each test. Tubes were incubated at 37°C for 24 h. At regular time intervals, samples were taken, diluted to obtain a countable number of colonies, and plated onto LA plates.

Growth curves

Overnight culture of SY252 was diluted 20-fold in

Table 2. Antibacterial effect of sage EO, its fractions and major monoterpenes

Bacterial strain		*S.aureus*	*B.subtilis*	*S. faecalis*	*E.coli* SY252	*E. coli* IB112
Fraction	μL/disc	Diameter of the growth inhibition zone (mm)				
EO	2	13	20	14	0	19
	10	22	25	17	0*	no bacteria
	30	33	34	25	0*	no bacteria
F1	2	25	17	[25]	0	0
	10	30	30	[30]	23	30
	30	48	34	40	35	37
F2	2	23	18	17	0	21
	10	30	27	24	20	24
	30	45	60	40	30	32
F3	2	22	14	0	0	13
	10	28	23	16	18	22
	30	61	38	28	25	30
F4	2	15	14	0	0	14
	10	30	17	14	14	22
	30	31	33	20	21	34
F5	2	20	13	0	0	12
	10	33	20	0	0	14
	30	36	21	0	0	16
Thujone	1	0	0	0	12	14*
	1.5	0	14	14	14	16*
	2	15	16	16	14	19*
Cineole	1	0	0	0	0	0*
	1.5	0	0	0	14	15*
	2	0	0	0	15	17*
Camphor[a]	1	0	0	0	0	0*
	5	0	0	0	0	17*
	10	0	0	0	16	19*
Crystal violet	10[b]	23	20	16	0	24*

[a]μg/disk;

[b]stock concentration 1mg/mL;

0 – no growth inhibition zone; nt – not tested; brackets indicate incomplete inhibition of growth; *- in addition to a growth inhibition zone, there is a thinner bacterial lawn on plates compared to control.

LB with or without sage EO and incubated for 5 h at 37°C with aeration. At regular time intervals, samples were taken and optical density at 600 nm was measured using a Shimadzu spectrophotometer.

RESULTS AND DISCUSSION

In a preliminary experiment, we screened the effect of essential oil (EO) of sage (*S. officinalis* L.) against *S. aureus* ATCC25923, *S. epidermidis* ATCC12228, *P. aeruginosa* ATCC27853, *E. coli* ATCC25922, *B. subtilis* ATCC1070 and *S. faecalis* ATCC29212 in disk-diffusion assay. The results showed antibacterial activity of EO (2-20 μL/disc) against all tested bacteria (data not shown). Moreover, Gram-positive bacteria were more sensitive than Gram-negative bacteria to the killing effect of EO, confirming results already reported (P a l o m b o and S e m p l e , 2001; K u d i *at al.* 1999; M a r i n o *et al.* 2001). The disk-diffusion test was further used to compare antibacterial activity of EO and its fractions (F1-F5) with different content of mono- and sesquiterpenes. The test was performed with the most sensitive bacteria in the pre-screening test: *S. aureus*, *B. subtilis,* and *S. faecalis.* All tested concentrations of EO and fractions showed antibacterial activity (Table 2). The largest zones of growth inhibition appeared with the highest tested concentration (30 μL/disk). *Streptococcus faecalis* displayed lower sensitivity to EO and fractions than *S. aureus* and *B. subtilis*; it was even resistant to F5.

Table 3. MIC values of EO and fractions F1-F5

Bacterial strain	Concentration μL/mL					
	EO	F1	F2	F3	F4	F5
S. aureus	1.25	1.25	2.50	1.25	1.25	1.25
B. subtilis	0.30	0.60	1.25	0.30	2.50	0.15

A common feature of plant volatiles is their hydrophobic nature, and the cell membrane has been proposed as the primary target of their antimicrobial action. Plant volatiles appear to accumulate in the cell membrane causing the leakage of ions, enzymes, and metabolites (I n o u e *et al.,* 2004). It has been suggested that high resistance to plant extracts in Gram-negative bacteria is due to the outer membrane of their cell wall, acting as a barrier to many environmental substances including antibiotics (P a l o m b o and S e m p l e , 2001; K u d i *at al.* 1999; M a r i n o *et al.* 2001). To test this hypothesis, we compared the antibacterial effect of EO and fractions in wild type *E. coli* K12 strain SY252 and its permeable counterpart IB112. The IB112 strain was more sensitive to EO and fractions than SY252 in the disk-diffusion test. Similar results were obtained when major sage monoterpenes (thujone, cineole, and camphor) were tested, although the differences in size of the growth inhibition zones of IB112 and SY252 were less pronounced (Table 2). The permeable *S. typhimurium* strains TA100 and TA102 showed sensitivity to EO, its

Table 4. Results of the time kill assay in *S. aureus* and *B. subtilis* * MIC of EO and F1-F5. ** double MIC of EO and F1-F5

S. aureus	Time (h)				
	0	4	6	8	24
Fraction*	viable cells/mL				
EO	$3.5x10^7$	0	0	0	0
F1	$1.8x10^8$	$5.0x10^5$	$6.5x10^5$	$1.0x10^3$	$1.9x10^2$
F2	$5.9x10^7$	0	0	0	0
F3	$1.2x10^8$	0	0	0	0
F4	$8.5x10^7$	$1.9x10^6$	$4.5x10^6$	$5.1x10^5$	$2,0x10^5$
F5	$1.4x10^8$	$5.0x10^5$	$1.5\ x10^5$	$2.5x10^4$	$9.9x10^3$
B. subtilis	Time (h)				
	0	4	6	8	24
Fraction**	viable cells/mL				
EO	$4.0x10^7$	$1.5x10^4$	$1.5x10^4$	$1.1x10^4$	$1.7x10^7$
F1	$1.0x10^7$	$8.5x10^3$	$7.0x10^3$	$6.0x10^3$	$6.8x10^3$
F2	$8.0x10^6$	$9.0x10^3$	$9.0x10^3$	$4.5x10^3$	$4.0x10^3$
F3	$7.1x10^7$	$2.4x10^4$	$1.7x10^4$	$4.3x10^4$	$1.9x10^7$
F4	$1.1x10^7$	$1.2x10^4$	$1.5x10^3$	$3.5x10^3$	$3.0x10^3$
F5	$1.1x10^8$	$1.9x10^4$	$4.0x10^3$	$4.5x10^3$	$1.8x10^3$

fractions, and monoterpenes similar to that of IB112 (data not shown). The obtained results indicate that transport of sage volatiles through the cell wall of Gram-negative bacteria is the major process limiting their antibacterial effect.

As evident from Table 2, the strongest antibacterial effect compared to EO was detected with the F2 and F3 fractions. The chemical structure of F2 and F3 is similar, and they both contain high concentration of α-thujone (Table 1). Toxicity of thujone has been demonstrated in mice, and the concentration with thujone was in correlation to its toxicity (F a r h a t *et al.* 2001). Our results demonstrate that, among major sage monoterpenes, thujone is toxic to all tested bacteria (Table 2). However, the antibacterial activity of EO and fractions is not correlated with their content of thujone, cineole, or camphor, indicating that their antibacterial effect probably involves some type of synergism between many constituents. The F5 fraction showed lower antibacterial effect in all tested bacteria. This fraction F5 contains mainly sesquiterpenoids, which probably enter bacterial cells in distinct quantities.

Taking into account data obtained in the disk-diffusion test, we decided to determine MIC of EO and fractions for *S. aureus* and *B. subtilis*. Both species are important food-borne pathogens (P a l o m b o and S e m p l e , 2001). In addition, multidrug resistant *S. aureus* strains are often isolated from human clinical specimens (O p l a c h e n o v a and O b r e s h k o v a , 2003). The results reported in Table 3 show differential sensitivity of *S. aureus* and *B. subtilis*. The concentration of EO and fractions required to inhibit bacterial growth were higher for *S. aureus* than for *B. subtilis*. Moreover, the MIC values for *S. aureus* were similar for all tested fractions, while MIC for *B. subtilis* varied between 0.15 µl/ml and 2.50 µl/ml, depending on the fraction applied. Lower sensitivity of *S. aureus* compared to *B. subtilis* to sage EO was also reported by other authors (C a r v a l h o *et al.* 1999).

To date, there has been no standard method for studying the susceptibility of microorganisms to essential oils (O p l a c h e n o v a and O b r e s h k o v a , 2003). Our results show significant differences between antibacterial activities of some fractions obtained with the disk-diffusion test and the MIC assay, probably caused by differences in water solubility and diffusion rates of the compounds, as well as by physiology of the tested bacteria. Although the MIC test is considered more accurate for quantitative evaluation of antimicrobial activity, it does not represent an absolute value either. The «true» MIC is somewhere between the lowest test concentration which inhibits the bacterial growth and the next lower test concentration. Also, inhibitory concentrations of plant extracts are higher when the incubation time is extended for 5 or 7 days (O p l a c h e n o v a and O b r e s h k o v a , 2003).

Bactericidal activity of antimicrobial agents can also be assessed by performing an *in vitro* time-kill assay. Table 4 presents data on the kinetics of survival of *S. aureus* and *B. subtilis* in the presence of EO and fractions (MIC for *S. aureus* and double MIC for *B. subtilis*). The EO, F2, and F3 exhibited a strong bactericidal effect on S. *aureus*, and within 4 hours the bacterial population was completely inactivated. Fraction F1, F4, and F5 gradually reduced the counts of *S. aureus* during 24 h of incubation, indicating that longer incubation or increased concentrations are needed for complete loss of viability. In contrast, EO and all tested fractions rapidly reduced the counts of *B. subtilis*, but after the initial reduction a constant fraction of the bacterial population survived; with EO and the F3 fraction, it even recovered after 24 h of incubation. The survival of *B. subtilis* is probably due to the presence of endospores, which are resistant to conditions to which vegetative cells are intolerant. Considering our results and the finding that EO of sage at a concentration of 0.35 µL/mL could not prevent the germination of *B. subtilis* INRA L2104 spores, but extended the lag phase of the culture by 60% (V a l e r o and S a l m e r o n , 2003), we can speculate that further incubation with EO and fractions would probably result in complete recovery of the *B. subtilis* population.

We also examined the effect of sage EO on the initial growth parameters of *E. coli* SY252 by monitoring optical density of the culture. There was no increase in the optical density of a culture containing 1 µL/mL of EO during 5 h of incubation, time sufficient for the control to enter the stationary phase (data not shown).

Obtained with different experimental methods, the presented data show a significant antibacterial effect of sage EO and its fractions. The activities demonstrated against *B. subtilis, S. aureus,* and *E. coli*, the traditional use of sage as a culinary herb, and the recently reported antigenotoxic potential of sage extracts in mice (V u j o š e v i ć and B l a g o j e v i ć , 2004) indicate that sage oil or its fractions can be considered for application in controlling food contaminations.

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УПОРЕДНА АНАЛИЗА АНТИБАКТЕРИЈСКЕ АКТИВНОСТИ ИСПАРЉИВИХ КОМПОНЕНТИ ИЗ ЖАЛФИЈЕ (*SALVIA OFFICINALIS* L.)

ДРАГАНА МИТИЋ-ЋУЛАФИЋ, БРАНКА ВУКОВИЋ-ГАЧИЋ, ЈЕЛЕНА КНЕЖЕВИЋ-ВУКЧЕВИЋ, С. СТАНКОВИЋ и ДРАГА СИМИЋ

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Испитан је антибактеријски ефекат испарљивих компоненти из жалфије на бактеријама из ATCC колекције у диск-дифузионом тесту. Етарско уље и његове фракције показују значајан антибактеријски ефекат на *S. aureus* и *B. subtilis*. Минимална инхибиторна концентрација за *S. aureus* је 1,25-2,5 μL/mL, а за *B. subtilis* 0,15-2,5 μL/mL. Ефекат на *S. aureus* је бактерицидан, док се почетни бактерицидни ефекат код *B. subtilis* губи, вероватно због присуства ендоспора. Резултати добијени на дивљим и пропустљивим сојевима *E. coli* и *S. typhimurium* указују да присуство интактног ћелијског зида ограничава антибактеријско деловање испарљивих компоненти из жалфије.