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SYNERGISTIC EFFECTS OF *SALVIA OFFICINALIS* L. ESSENTIAL OILS AND ANTIBIOTICS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

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Abstract: Infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) due to the acquisition of resistance to current antimicrobials pose a serious challenge for therapy, and new measures to treat and prevent this infectious pathogen are of crucial importance. Plant essential oils (EOs) and their constituents are promising agents with antimicrobial properties. The aim of this study was to evaluate the antistaphylococcal effect of essential oils from *Salvia officinalis* using the broth-microdilution method. Essential oils of *S. officinalis* were isolated from the same individual, but at different life stages – young and old leaves. The effects of combinations of sub-inhibitory concentrations of oil and different antibiotics were evaluated by the checkerboard method. The results, expressed as the fractional inhibitory concentration (FIC) and index (FICI), indicate that the essential oil isolated from young leaves potentiated the inhibitory effect of antibiotics against tested MRSA strains.

Key words: *Salvia officinalis*; essential oil; antibiotics; MRSA

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INTRODUCTION

Salvia officinalis L. (Lamiaceae), known as Dalmatian sage, is a perennial subshrub native to the northern coastal region of the Mediterranean and grows wild in central Spain, southern France and the western part of the Balkan Peninsula (Hedge, 1972). Dalmatian sage is an official drug in most pharmacopoeia, and chemical variations of its essential oils were investigated depending on the origin, ecological conditions and different stages of plant development (Perry et al., 1999; Santos-Gomes and Fernandes-Ferreira, 2001; Marić et al., 2006; Bernotienė et al., 2007; Maksimović et al., 2007; Ben Farhat et al., 2009; Jug-Dujakovic et al., 2012; Lakušić et al., 2013).

The wide spread of multidrug-resistant (MDR) bacteria is documented as a serious problem that affects the choice of appropriate antibiotic therapy and increases the probability of unfavorable infection outcome. Resistance to all classes of antibiotics has emerged, leading to a continuous need to produce new drugs. One promising method in coping with bacterial resistance is the use of alternative classes of antimicrobial agents and the application of the synergistic activity between antibiotics, and between antibiotics and non-antibiotics (Kalan and Wright, 2011).

In recent years, methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a major public health concern worldwide (Kennedy et al., 2008; DeLeo et al., 2010). This important nosocomial and community-acquired pathogen has developed resistance to various antibiotics (β-lactams, quinolones and aminoglycosides). Since infections caused by MRSA

Table 1. Chemical composition of *Salvia officinalis* essential oils (%)

	Samples	KIE	A- Sal-Off-102 young leaves	B - Sal-Off-109 old leaves
No.	**Constituent (%)**			
1	*cis*-Salvene	855	0.3	0.3
2	*trans*-Salvene	861	0.0	0.1
3	Tricyclene	921	0.0	0.1
4	α-Thujene	927	0.3	1.1
5	α -Pinene	933	1.9	0.7
6	Camphene	947	1.2	3.6
7	Sabinene	969	0.4	0.9
8	β-Pinene	975	6.5	0.3
9	Myrcene	994	0.8	1.2
10	α-Phellandrene	1005	0.0	0.2
11	α -Terpinene	1018	0.1	1.5
12	p-Cymene	1026	0.3	2.0
13	Limonene	1029	1.0	2.6
14	1,8-Cineole	1031	19.4	2.5
15	*cis*-β-Ocimene	1035	0.3	0.0
16	*trans*- β-Ocimene	1045	0.1	0.0
17	γ-Terpinene	1060	0.4	2.9
18	*cis*-Sabinene hydrate	1070	0.4	1.0
19	Terpinolene	1089	0.2	0.9
20	*trans*-Sabinene hydrate	1101	0.8	1.8
21	*cis*-Thujone	1107	16.5	25.9
22	*trans*-Thujone	1118	2.4	4.3
23	α-Campholenal	1124	0.0	0.2
24	*trans*-Sabinol	1137	0.1	0.1
25	Camphor	1145	10.7	30.4
26	*trans*-Pinocamphone	1163	0.2	0.2
27	Borneol	1167	1.9	0.6
28	*cis*-Pinocamphone	1172	0.0	1.8
29	Terpinen-4-ol	1180	0.3	0.1
30	α-Terpineol	1195	0.2	0.1
31	Bornyl acetate	1288	0.4	0.4
32	*trans*-Sabinyl acetate	1289	0.0	0.1
33	β-Caryophyllene	1420	1.2	0.4
34	α-Humulene	1455	16.2	4.5
35	γ-Muurolene	1478	0.0	0.1
36	Viridiflorene	1496	0.1	0.0
37	Caryophyllene oxide	1583	0.2	0.1
38	Viridiflorol	1592	2.9	3.3
39	Humulene epoxide I	1593	0.4	0.2
40	Humulene epoxide II	1609	2.4	1.1
41	n.i.	n/a	0.1	0.3
42	n.i.	n/a	0.0	0.2
43	Manool	2056	9.0	2.0
44	n.i.	n/a	0.4	0.1
	Total		100	100

KIE = Kovats (retention) index experimentally determined (AMDIS); n.i.=not identified

are increasing, as are rates of antibiotic therapy failure, alternative strategies need to be found to treat and prevent these infections. A possible solution may be to combine existing antibiotics with phytochemicals to enhance the efficacy of antibiotics. A group of phytochemicals that is said to have such effects, according to *in vitro* studies, is essential oils (EOs) and their components (Kon and Rai, 2012).

The aims of our study were to examine the antimicrobial effect of the essential oil of Dalmatian sage (*Salviae aetheroleum*) from different stages of leaf development and combined application of the essential oil with the best antimicrobial effect and various antibiotics on the growth of methicillin-resistant *Staphylococcus aureus* strains (MRSA).

MATERIALS AND METHODS

Plant material

Salvia officinalis from Učka in Croatia was successfully grown for ten years in a private garden in Belgrade (Serbia). Two samples of essential oils were analyzed: essential oil from young leaves (sample A – SalOff-102) and from old leaves (sample B - Sal-Off-109). In the research, essential oil of sage produced by Kirka producer (sample C) was used.

Oil isolation

Oils were isolated from fresh material, always using the same distillation apparatus under the same conditions. The essential oils were isolated by hydrodistillation, according to the standard procedure reported

in the Sixth European Pharmacopoeia (2007) using a Clevenger-type apparatus. Duration of distillation was 2 h. Oil samples were dissolved in ethanol and analyzed by gas chromatography/flame ionization detector (GC/FID) and gas chromatography/mass spectrometry (GC/MS).

Analytical gas chromatography (GC/FID)

GC/FID analysis of the oils was carried out on a HP-5890 Series II GC (Hewlett-Packard, Waldbronn, Germany), equipped with split-splitless injector and automatic liquid sampler (ALS), attached to an HP-5 column (25 m; 0.32 mm; 0.52 μm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H_2) was 1 ml/min, split ratio 1:30, with injector temperature 250°C, detector temperature 300°C, while the column temperature was linearly programmed from 40-260°C (at 4°/min). Solutions of essential oil samples in ethanol (~1%) were consecutively injected by ALS (1 μl, split mode 1:30). Area percent reports, obtained from the standard processing of chromatograms, were used as a basis for quantification.

Gas chromatography/mass spectrometry (GC/MS)

The same analytical conditions as those mentioned for GC/FID were used for GC/MS analysis, along with an HP-5MS column (30 m; 0.25 mm; 0.25 μm film thickness), using an HP G 1800C Series II GCD system (Hewlett-Packard, Palo Alto, CA, USA). Instead of hydrogen, helium was used as carrier gas. The transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in the range of 40-450 m/z. Sample solutions in ethanol (~1 %) were injected by ALS (200 nl, split mode 1:30). The components of the oil were identified by comparison of their mass spectra with those from Wiley275 and NIST/NBS libraries, using different search engines. The experimental values for retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1.), compared with those from available literature (Adams, 2007) and used as an additional tool to confirm the MS findings.

Bacterial strains and susceptibility testing

Antibacterial activity of essential oils was tested against four clinical isolates of MRSA and one laboratory control strain of methicillin-resistant *S. aureus* ATCC 43300 (KWIK-STIK™, Microbiologics, USA) as a positive control. MRSA strains were isolated from wound (Strain Nº 5), blood (Strains Nº 6 and 7) and endotracheal tube (Strain Nº 8). Identification of the isolates and methicillin resistance were firstly determined by VITEK 2 automated system (VITEK 2 test cards GP and AST-P580; bioMérieux, France). All tested strains were identified as *Staphylococcus aureus* (with 98.57-99.00% level of confidence) and expressed resistance to methicillin. Furthermore, identification and methicillin resistance were confirmed by PCR for the *nuc* gene that encodes the thermostable nuclease of *Staphylococcus aureus* (Brakstad et al., 1992) and *mec*A gene that encodes the penicillin-binding protein PBP2' specific for MRSA strains (Bignardi et al., 1996). All tested strains were *nuc*- and *mec*A-positive.

Table 2. Minimum inhibitory concentrations (MIC) of essential oils and antibiotics

	MIC (μg/ml)					
	Essential oil			Antibiotics		
MRSA strain Nº	A	B	C	CTX	CIP	GEN
5	25	>400	>400	32	1.0	2.0
6	25	>400	400	32	0.5	2.0
7	>400	>400	>400	64	8.0	16.0
8	25	>400	400	64	0.2	0.5
ATCC 43300	25	>400	>400	32	16.0	256.0

* CTX-ceftriaxone, CIP-ciprofloxacin and GEN-gentamicin

The antimicrobial activity of essential oils was determined by broth-microdilution test according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2007). Briefly, essential oils were diluted to the desired concentrations (ranging from 3.12-400 μg/ml) with fresh Mueller-Hinton broth (MHB) with the addition of 0.05% triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich) as a growth indicator, set in triplicate and inoculated with $5x10^5$ CFU/ml of bacteria. After incubation for 24 h at 35°C in aerobic

conditions, minimum inhibitory concentrations (MIC) were determined. TTC is a redox indicator used for differentiation between metabolically active and non-active cells. The colorless compound is enzymatically reduced to red 1,3,5-triphenylformazan by bacterial dehydrogenases, indicating bacterial metabolic activity (i.e. red color of the microtiter plate well). MIC values were determined as the lowest concentration of essential oil or antibiotic that inhibited bacterial growth (i.e., wells with unchanged-yellow color of medium). Each broth-microdilution test was repeated three times.

Synergy testing

The synergism between essential oils and commercial drugs was investigated by the checkerboard method according to White et al. (1996). Three antibiotics representing different groups of antimicrobial agents were used: β-lactam ceftriaxone (CTX) and non β-lactam antibiotics, ciprofloxacin (CIP) and gentamicin (GEN) (Sigma-Aldrich). The synergistic effect of combinations was investigated in one concentration above and several concentrations below the MIC of each compound (both antibiotic and tested essential oil). The test was performed in 96-well microtiter plates as a modified broth-microdilution test. Essential oils and antibiotics were diluted to the desired concentrations with MHB with the addition of 0.05% TTC. The combination of essential oils and antibiotics was prepared by adding 50 µl of each to the same well of microtiter plate, and inoculating with 100 µl of previously prepared bacteria ($5x10^5$ CFU/ml). After incubation for 24 h at 35°C in aerobic conditions, the MIC of the combination was determined. The interaction between the two antimicrobial agents was estimated by calculating the fractional inhibitory concentration (FIC) indices (FICI). The FIC of each compound was calculated by dividing the concentration of the compound in effective MIC of the combination with the MIC of the drug alone (e.g. $FIC_{essential\ oil} = MIC_{essential\ oil\text{-}antibiotic\ combination}/MIC_{essential\ oil}$). FICI values were calculated as the sum of the $FIC_{essential\ oil}$ and $FIC_{antibiotic}$ and interpreted as following: FICI ≤ 0.5 synergy; 0.5 < FICI ≤ 1 additivity; 1 < FICI ≤ 2 indifference (no effect) and FICI ≥ 2 antagonism (Hu et al., 2002; Orhan et al., 2005). Each test was repeated three times.

RESULTS

Leaves of the same plant of *S. officinalis* at different stages of development form different types of oil (Lakušić et al., 2013). In the essential oil of young leaves (sample A – SalOff-102) 1,8 cineole (19.4%), *cis*-thujone (16.5%), α-humulene (16.2%), camphor (10.7%) and manool (9.0%) were dominant, whereas in the oil of the old leaves (sample B - SalOff-109) *cis*-thujone (25.9%) and camphor (30.4%) were dominant (Table 1).

The antimicrobial effect of the essential oils against MRSA was observed only with sample A (SalOff-102). Certain MIC values of oil A were almost identical for most strains and amounted to 25 µg/ml, except for hospital strain N°. 7, which proved to be completely resistant. Sample B (SalOff-109) did not show antimicrobial effect. The commercial oil (sample C) showed antimicrobial activity only on MRSA strains N° 6 and 8, at high concentration (Table 2). The MIC values of antibiotics were in the range of 32-64 µg/ml (CTX), 0.2-16 µg/ml (CIP) and 0.5-256 µg/ml (GEN). All tested MRSA strains were resistant to ceftriaxone (MIC > 4 µg/ml), two strains to ciprofloxacin (MIC > 1 µg/ml) and four strains to gentamicin (MIC > 1 µg/ml) (EUCAST 2014). The MICs of the antibiotics are presented in Table 2.

The checkerboard method was performed with three antibiotics that represent different groups of antimicrobial agents: β-lactam antibiotic-cephalosporins (CTX), fluoroquinolone (CIP) and aminoglycoside (GEN). The overall effect of essential oil-antibiotic combinations varied from synergistic (FICI ≤ 0.5) to antagonistic (FICI ≥ 2). The most significant synergistic effect was observed in the combination of essential oil A and ceftriaxone and ciprofloxacin against all tested strains of MRSA. The effects were exhibited in essential oil A/CTX combinations at

concentration of 1/16 MIC (1.56 μg/ml) of essential oil A and 1/8-1/4 MIC (8 μg/ml) of CTX, and in essential oil A/CIP combinations at a concentration of 1/16 MIC (1.56 μg/ml) of essential oil A and 1/128-1/2 MIC (0.125 μg/ml) of CIP. The results of *in vitro* activity of tested oil in combination with conventional antibiotics are given in Table 3 and Fig. 1. The most prevalent synergistic interaction was noted when *Salviae aetheroleum* was combined with ceftriaxone (FICI: 0.312-0.453) and ciprofloxacin (FICI: 0.070-0.562); when combined with gentamicin, an antagonistic interaction was noted (Strain Nº 6, FICI 2.125). It is interesting that the same combination of essential oil and gentamicin exhibited synergistic effect on other MRSA strains.

DISCUSSION

Considering that the essential oil of young leaves (sample A) contains a significant amount of 1,8-cineole, α-humulene, manool, camphor and *cis*-thujone, it may be assumed that these components play a crucial role in the antimicrobial activity of the oil. On the other hand, the essential oil of old leaves (sample B) did not show antimicrobial effect against MRSA, although it contains a significantly higher amount of *cis*-thujone and camphor, which could indicate that camphor and *cis*-thujone do not inhibit the growth of MRSA. However, it is most probable that the different antibacterial activity exhibited by the oils is the result of the synergistic effect of the all components in the oil, even those that are present in minimal concentration.

The emergence of multi-drug resistant microbial strains is becoming a formidable threat in the fight against infective diseases. Thus, alternatives to the standard treatments with single agents are presently being sought. Generally, the combined use of antimicrobial agents can potentially increase overall antimicrobial activity and reduce toxicity of both agents towards human cells (Kon and Rai, 2012; Verma, 2007).

Different combinations of EOs and traditional antibiotics demonstrated an enhancing effect, which is important in restoring the activity of inactive antibiotics and prolonging the activity of presently highly efficient drugs. Combinations of essential oils with conventional antibiotics could be an effective alternative in the treatment of infections caused by MRSA. Essential oils of *Zataria multiflora* demonstrated high activity against clinical isolates of MRSA and methicillin-susceptible *S. aureus* (MSSA), but also enhanced the activity of vancomycin (Mahboubi and Bidgoli, 2010).

In a study conducted by Rosato et al. (2010), the effect of combinations of gentamicin and four EOs (*Aniba rosaeodora*, *Melaleuca alternifolia*, *Origanum vulgare* and *Pelargonium graveolens*) was evaluated by the checkerboard method against several reference bacterial strains. A synergistic effect against all tested

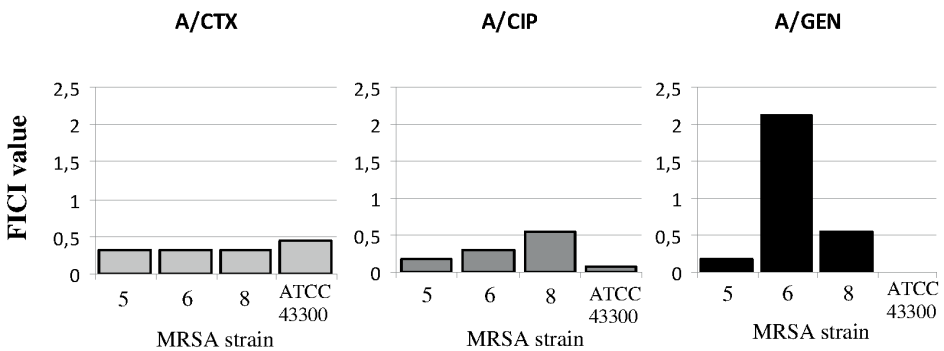

Fig. 1. FICI values of the essential oil A/antibiotic combination. A/CTX (ceftriaxone); A/CIP (ciprofloxacin) and A/GEN (gentamicin). FICI ≤ 0.5 synergy; 0.5 < FICI ≤ 1 additivity and FICI ≥ 2 antagonism.

strains was observed in gentamicin/*A. rosaeodora* and gentamicin/*P. graveolens* combinations, while in the other two combinations the effect was from synergistic to indifferent.

It should be noted that whether an essential oil with known antimicrobial activity will produce beneficial or harmful effects when combined with conventional antimicrobials depends on the ratio of the two components. Van Vuuren et al. (2009) studied the activity of four EOs (*Melaleuca alternifolia*, *Thymus vulgaris*, *Mentha piperita* and *Rosmarinus officinalis*) in combination with ciprofloxacin against *S. aureus* and *Klebsiella pneumoniae* in nine different ratios. The study demonstrated the presence of concentration-dependent interactions between EOs and antibiotics: the same EO showed effects from synergistic to antagonistic when combined with ciprofloxacin depending on the ratios of both agents.

It is worth emphasizing that although many EOs and their components have a high level of antibacterial activity (Lorenzi et al., 2009; Si et al., 2008), precautions should be taken because of the presence of a rather large number of antagonistic interactions (van Vuuren et al., 2009) that should not be ignored (van Vuuren and Viljoen, 2011).

Our preliminary study demonstrated the presence of different interactions between *Salvia officinalis* essential oil and antibiotics: the same combination showed different effects, from synergistic to antagonistic, on different clinical isolates of MRSA. These findings demonstrate the necessity for systematic *in vitro* studies of interactions of EOs and antibiotics to reveal any undesirable combinations. Combinations of EOs with antibiotics should only be chosen for treatment when synergistic or additive interactions are documented. The main limitation of this study was small number of both MRSA strains and antibiotics tested. Having this in mind, in future we will include some additional MRSA strains and antibiotics in our research. In addition, essential oil characteristics need to be monitored throughout the development of plants, because it is evident that the oil isolated at different stages of development has different antimicrobial activity.

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Table 3. The *in vitro* activity of essential oil in combination with conventional antibiotics against MRSA.

	A/CTX		A/CIP		A/GEN	
Strain Nº	**MIC (μg/ml)**	**Eff**	**MIC (μg/ml)**	**Eff**	**MIC (μg/ml)**	**Eff**
5	1.56/8	syn	1.56/0.125	syn	1.56/0.25	syn
6	1.56/8	syn	1.56/0.125	syn	3.12/4	ant
7	>12.5/128	/	>12.5/2	/	>12.5/4	/
8	1.56/8	syn	1.56/0.125	add	1.56/0.25	add
ATCC43300	1.56/8	syn	1.56/0.125	syn	>12.5/4	/

*CTX-ceftriaxone, CIP-ciprofloxacin and GEN-gentamicin. Effect (eff): synergy (syn), additivity (add) and antagonism (ant).

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