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Composition and antimicrobial activity of essential oils of *Salvia fruticosa* and *Salvia ringens* (Lamiaceae)

Sastav i antimikrobna aktivnost etarskih ulja *Salvia fruticosa* i *Salvia ringens* (Lamiaceae)

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Abstract

Background/Aim. Plant essential oils (EOs) can have a significant antibacterial effect especially through additive or synergistic action as antibiotic adjuvants. We investigated the composition and activity of EOs of two species of genus *Salvia (S)* from Greece with the aim to determine their antimicrobial activity as well as the activity in combination with selected antibiotics. **Methods.** The aerial parts of wild-growing *S. fruticosa* and *S. ringens* were subjected to a steam distillation and the obtained EOs were analyzed by gas chromatography and gas chromatography/mass spectrometry. The broth-microdilution method was used in order to determine the minimum inhibitory concentrations (MICs) of EOs on seven strains of bacteria and one yeast. Antimicrobial activity of the combination of EO and antibiotics was investigated by checkerboard method and estimated by calculating fractional inhibitory concentration (FIC) of each component and fractional inhibitory concentration index (FICI). **Results.** Dominant component of *S. fruticosa* EO was *trans*-thujone (54.2%) and for *S. ringens* EO it was α-pinene (28.1%). The MICs of EOs of both species were in the range from 200 μg/mL to > 500 μg/mL. The strongest antimicrobial effect was achieved against *Bacillus subtilis* and *Candida albicans*. According to FICI values, EO of *S. fruticosa* had additive effect with ciprofloxacin against most of bacterial strains but not with amikacin. **Conclusion**. The essential oils of *S. ringens* and *S. fruticosa* showed modest antimicrobial activity. However, the essential oil of *S. fruticosa* showed a promising additive effect in combination with ciprofloxacin.

Key words:
anti-infective agents; lamiaceae; oils, volatile; plants, medicinal; salvia.

Apstrakt

Uvod/Cilj. Etarska ulja različitih biljaka mogu imati značajna antibakterijska svojstva, posebno kao adjuvanti antibiotika sa kojima ostvaruju aditivno ili sinergistično dejstvo. Ispitivali smo sastav i aktivnost etarskih ulja dve vrste roda *Salvia (S)* iz Grčke sa ciljem da odredimo njihovu antimkrobnu aktivnost, kao i dejstvo u kombinaciji sa odabranim antibioticima. **Metode.** Nadzemni delovi samoniklih *S. fruticosa* i *S. ringens* su destilovani vodenom parom i dobijena etarska ulja su analizirana gasnom hromatografijom i gasnom hromatografijom sa masenom spektrometrijom. Radi određivanja minimalnih inhibitornih koncentracija (MICs) etarskog ulja na sedam sojeva bakterija i na jednoj patogenoj gljivici korišćena je mikrodiluciona metoda. Antimikrobna aktivnost kombinacije etarskog ulja i antibiotika ispitana je *checkerboard* metodom i procenjena je na osnovu frakcione inhibitorne koncentracije (FIC) svake komponente i indeksa frakcione inhibitorne koncentracije (FICI). **Resultati.** Dominantna komponenta etarskog ulja *S. fruticosa* je bio *trans*-tujon (54,2%), a etarskog ulja *S. ringens* α-pinen (28,1%). MICs etarskog ulja obe vrste su bile u opsegu od 200 μg/mL do > 500 μg/mL. Najsnažnija antimikrobna aktivnost ostvarena je protiv *Bacillus subtilis* i *Candida albicans*. Na osnovu FICI vrednosti, etarsko ulje *S. fruticosa* je sa ciprofloksacinom, ali ne i sa amikacinom imalo aditivni efekat protiv većine bakterijskih sojeva. **Zaključak.** Etarska ulja *S. ringens* i *S. fruticosa* su pokazala skromnu antimikrobnu aktivnost, ali je etarsko ulje *S. fruticosa* u kombinaciji sa ciprofloksacinom ispoljilo značajan aditivni efekat.

Ključne reči:
antiinfektivni agensi; lamiaceae; ulja, etarska; biljke, lekovite; salvia.

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Introduction

Excessive and inappropriate use of antibiotics in treating various infectious diseases has led to the resistance of many pathogens. This is a global problem in healthcare practice today, which is also recorded in Serbia [1]. In order to find new antimicrobial agents that would help to decrease the use of antibiotics, research into traditionally used medicinal plants and herbal products, as well as related species of known medicinal plants, has been intensified.

Earlier studies have shown that essential oils as mixtures of different plant secondary metabolites can have a significant antimicrobial effect, which is based on a different mechanism of action than the one of antibiotics [2]. One possible approach to the use of essential oils in therapy is their use as an antibiotic adjuvant, with the aim of achieving a multitarget effect on pathogens through additive or synergistic actions [3].

Many studies on the anti-multidrug-resistant bacteria activity of aromatic members of Lamiaceae family have been performed, primarily on the essential oils of lavender, mint and thyme [2]. Recently, it has been proved that the essential oil isolated from young leaves of Dalmatian sage (*S. officinalis* L.) also has an antimicrobial effect on certain human pathogens, and that the use of the essential oil potentiated the inhibitory effect of antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) [4].

Within genus *Salvia*, comprised of about 1,000 worldwide distributed species, beside *S. officinalis*, only several species with medicinal properties are aromatic plants with a significant amount of essential oils [5]. The essential oil of *S. tomentosa*, with β-pinene, α-pinene and camphor as the main components, showed antimicrobial activity against a panel of microorganisms [6]. A study of composition and antimicrobial effects of essential oil and extracts of *S. ringens* from North Macedonia, rich in 1,8-cineole, camphene, and borneol has shown that Gram-positive strains were more sensitive to the essential oil [7]. The essential oil of *S. amplexicaulis* characterized by a high amount of sesquiterpenes, with germacrene D, viridiflorol, caryophyllene oxide and β-caryophyllene being the main components, showed inhibitory properties against Gram-positive bacteria and a yeast *Candida (C) albicans* [8]. The Greek sage, *S. fruticosa*, as the most widespread sage species in the Mediterranean, has been used for its healing properties since ancient times [5]. The oil of this species with high contents of 1,8-cineole, α- and β-thujone, and camphor, was tested for antimicrobial activity against eight common bacterial strains [9], as well as against human pathogenic yeast [10]. The combined effect of tetracycline and essential oils of *S. officinalis*, *S. sclarea* and *S. fruticosa* against clinical isolates of methicillin-resistant *Staphylococcus epidermidis* showed synergistic or additive effects [11, 12]. However, there are no consistent results of the antimicrobial activity of the combination of the essential oil of *S. fruticosa* and antibiotics with a different mechanism of action and different standard bacterial strains.

Knowing that the composition of essential oil of *Salvia* species differs throughout different developmental stages [13] and that it is highly influenced by climatic conditions, we investigated the composition and activity of the essential oil of two species of genus *Salvia* from Greece (*S. fruticosa* and *S. ringens*), collected during the late flowering stage with the aim to evaluate their antimicrobial activity as well as the activity of essential oil of *S. fruticosa* in combination with antibiotics [amikacin (AMI) and ciprofloxacin (CIP)].

S. fruticosa Miller (Syn.: *S. triloba* L.) is a shrub up to 120 cm high, widespread in Central and Eastern Mediterranean region. Its leaves are valued and utilized in the region similar to *S. officinalis* L. The plant is recognized for its white tomentose stem with simple or pinnate leaves with a pair of small lobes at the base and a large oblong elliptical rugose terminal segment. Verticillasters contain 2 to 6 flowers, with pink or lilac two-lipped flowers [14, 15].

S. ringens Sibth. & Sm. is a perennial herb, woody at the base, up to 60 cm high, endemic to Southern and Eastern parts of the Balkan Peninsula. This melliferous drought-tolerant plant grows in scrub habitat and open coniferous woodland. It is recognized on rosette of pinnate or lobed rugose leaves with 1–3 pairs of small lobes at the base. Flowering stems are with verticillasters consisting of attractive violet-blue two-lipped flowers [14, 15].

Methods

The plant material was sampled in Greece in June 2017. The aerial parts of wild-growing *Salvia fruticosa* Miller were collected on Mount Dhirfis on Evia island, and the aerial parts of *S. ringens* Sibth. & Sm. were collected on Mount Kyllini near Trikala, both during the late flowering stage. Voucher specimens are kept in the Herbarium of Belgrade University, Serbia. Sample details are given in Table 1.

For the isolation and analysis of the essential oil, air-dried plant material was subjected to a 2-hour steam distillation in a Clevenger-type apparatus according to Ph. Eur. 8.0 [16].

A qualitative analysis of the essential oils was performed using analytical gas chromatography (GC/FID) and gas chromatography/mass spectrometry (GC/MS). GC/FID and GC/MS analyses were carried out using an Agilent 6890N GC system equipped with FID and an Agilent 5975 MSD. The capillary column used was a HP-5 MS (30 m ×

Table 1

Plant material sample details

Species	Latitude	Longitude	Altitude	Voucher
S. fruticosa	N 38.632981°	E 23.786606°	520 m	BEOU 46566
S. ringens	N 37.984287°	E 22.458935°	1,250 m	BEOU 46586

BEOU – Herbarium of University of Belgrade.

0.25 mm i.d., film thickness 0.25 μm). The thermal program was 60ºC to 280ºC at a 3ºC/min rate. Injector temperature: 200ºC. FID temperature: 300ºC. Transfer-line temperature: 250ºC. Carrier gas, He (1.0 mL/min); injection volume, 1 μL; split ratio, 10:1. EI Mass spectra (70 eV) were acquired over the *m/z* range of 35–550. Identification of the compounds was based on the comparison of their retention times (RT), retention indices (RI), and mass spectra with those obtained from authentic samples and/or the NIST, Wiley libraries and literature [17]. The linear retention indices (RI) were determined in relation to a homologous series of *n*-alkanes (C_9–C_{24}) under the same operating conditions. Relative area percentages obtained by FID were used for quantification.

The antimicrobial activity of the essential oils was determined by broth-microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. This method was used in order to determine minimum inhibitory concentrations (MICs) of the essential oil that inhibit the growth of microorganisms. For the experiment, we used seven standard strains of bacteria (Gram-positive: *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633 and Gram-negative: *Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* NCIMB-9111, *Salmonella* Abony NCTC 6017, *Pseudomonas aeruginosa* ATCC 9027 as well as *Acinetobacter baumannii* ATCC 19606) and one standard strain of yeast – *Candida albicans* ATCC 10231.

Each strain was inoculated into Müller-Hinton broth (MHB) for 24h incubation at 35°C prior to the experiment. The cultures were then diluted with MHB to the final concentration in each plate well, adjusted to 2×10^6 CFU/mL. For *C. albicans* yeast, Sabouraud dextrose broth was used. The essential oils were dissolved in 1% dimethylsulfoxide for the stock and diluted to the desired concentrations with MHB. The essential oils were tested in the concentration range 31.25–500 μg/mL. Two standard antibiotics were used for comparison: an aminoglycoside antibiotic amikacin and a fluoroquinolone antibiotic ciprofloxacin and the range of antibiotic concentrations was 0.01–8.0 μg/mL.

After the incubation for 24h at 35°C in aerobic conditions, MICs were determined in the following way: lack of broth turbidity in a well of a microtiter plate indicates an inhibition of growth or death of an inoculated microorganism. The well with the lack of turbidity at the minimum concentration of an essential oil represents MIC. Each broth-microdilution test was repeated three times and the mean values are presented.

Antimicrobial activity of the combination of essential oil and antibiotics was investigated by checkerboard method according to Langeveld et al. [3]. The determination was performed with the aerial parts' essential oil of *S. fruticosa* because there were small quantities of essential oil in aerial parts of *S. ringens*. This method is based on the application of decreasing concentrations of essential oil horizontally and decreasing concentrations of antibiotics vertically to the 96-well microtiter plate (AMI or CIP). Beside the microorganism strains used in broth-microdilution method, standard strain of *Acinetobacter baumannii* ATCC 19606 was added to the experiment.

The essential oil and antibiotic mixture was prepared by adding 50 μL of each to the same well of microtiter plate, and inoculated with 100 μL of previously prepared bacterial suspensions (10^6 CFU/mL). The last two vertical rows of microtiter plate were positive control wells of bacterial growth suspensions. After the incubation for 24h at 35°C in aerobic conditions, MIC of the combination was determined. The interaction between the essential oil and antibiotic was estimated by calculating fractional inhibitory concentration (FIC) of each component and fractional inhibitory concentration index (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 $FIC_{essential\ oil}$ and $FIC_{antibiotic}$. They were interpreted as following: $FICI \leq 0.5$ synergy; $0.5 < FICI \leq 1$ additivity; $1 < FICI \leq 2$ indifference (no effect) and $FICI \geq 2$ antagonism [19, 20].

Results

The aerial parts of *S. fruticosa* were characterized by the high content of essential oil (0.9%) with dominant oxygenated monoterpenes (56.6%) followed by sesquiterpene hydrocarbones (25.3%). The main constituents were *trans*-thujone (54.2%), γ-cadinene (9.2%), (*E*)-caryophyllene (5.1%), β-pinene (4.7%) and myrcene (4.3%). *S. ringens* arial parts yielded 0.4% essential oil characterized by monoterpene hydrocarbons (57.4%), and oxygenated monoterpenes (33.5%) with α-pinene (28.1%), 1,8-cineole (13%) and β-pinene (12.2%) as its main constituents. The detailed chemical composition of the analyzed essential oils is shown in Table 2.

The MICs of *S. fruticosa* and *S. ringens* essential oils were in the range from 200 μg/mL to > 500 μg/mL (Table 3). Antimicrobial activity of the investigated oils was modest against most of the selected bacteria when compared to antibiotics. The greatest antibacterial activity was noted against *B. subtilis*, with MICs of 200 and 250 μg/mL of *S. ringens* oil and *S. fruticosa* oil respectively. Also, *C. albicans* growth was inhibited by 300 μg/mL and 200 μg/mL of essential oils of the listed plant species.

Due to the small quantities of *S. ringens* aerial parts essential oil, further investigation of combined oil/antibiotics antimicrobial activity was performed with more abundant essential oil of *S. fruticosa*.

Antimicrobial activity of the combination of the essential oil and antibiotics is presented in Table 4. According to FICI values, there was a significant difference in contribution of *S. fruticosa* essential oil to antimicrobial activity of antibiotics with different mechanisms of action (AMI and CIP). With CIP, the additive effect was observed for all bacterial strains except for standard strain of *Acinetobacter baumannii*, against which the combination was found to be indifferent. The combination of essential oil and AMI yield

Table 2

The content (in percentages) and chemical composition of essential oils of studied *Salvia* species

Number	RI	Constituent	*S. fruticosa*	*S. ringens*
			0.9 (oil content, % v/w)	0.4 (oil content, % v/w)
1	851	3-(*Z*)-Hexenol	0.2	–
2	926	α-Thujene	0.4	0.4
3	934	α-Pinene	0.8	28.1
4	949	Camphene	0.4	6.9
5	973	Sabinene	0.7	t
6	978	β-Pinene	4.7	12.2
7	991	Myrcene	4.3	1.0
8	1024	ρ-Cymene	t	1.1
9	1029	Limonene	0.6	3.7
10	1029	β-Phellandrene	0.5	3.7
11	1032	1,8-Cineole	0.2	13.0
12	1036	(*Z*)-β-Ocimene	0.3	–
13	1059	γ-Terpinene	0.2	t
14	1100	Linalool	t	0.9
15	1109	*cis*-Thujone	0.7	t
16	1118	*trans*-Thujone	54.2	0.7
17	1123	*cis*-*p*-Menth-2-en-1-ol	–	2.7
18	1140	*trans*-*p*-Menth-2-en-1-ol	t	2.0
19	1147	Camphor	0.3	3.7
20	1168	Borneol	0.6	6.6
21	1179	Terpinen-4-ol	0.3	1.0
22	1209	*trans*-Piperitol	–	1.0
23	1287	Bornyl acetate	0.3	1.8
24	1352	α-Cubebene	3.2	t
25	1378	α-Copaene	1.3	t
26	1391	β-Cubebene	0.4	–
27	1423	(*E*)-Caryophyllene	5.1	1.6
28	1456	α-Humulene	1.1	2.9
29	1479	γ-Muurolene	0.3	–
30	1482	α-Amorphene	0.5	–
31	1495	*trans*-Muurola-4(14)-5-diene	0.2	–
32	1496	γ-Amorphene	2.2	–
33	1521	γ-Cadinene	9.2	t
34	1526	δ-Cadinene	1.8	t
35	1544	γ-Cuprenene	-	1.2
36	1587	Caryophyllene oxide	1.7	0.8
37	1591	$C_{15}H_{26}O$	0.3	–
38	1612	Humulene epoxide II	0.3	1.8
39	1624	1,10,-di-*epi*-Cubenol	1.2	–
40	1631	1-*epi*-Cubenol	0.8	–
41	1645	Cubenol	0.4	–
42	1672	*epi*-β-Bisabolol	–	0.1
43	1815	(*Z*)-α-*trans*-Beragmotol acetate	–	0.7
Monoterpene hydrocarbones			13.2	57.4
Oxygenated monoterpenes			56.6	33.5
Sesquiterpene hydrocarbones			25.3	5.7
Oxygenated sesquiterpenes			4.4	3.4
Other			0.2	0
Total identified			99.7	100

RI – linear retention indices determined in relation to a homologous series of *n*-alkanes (C_9-C_{24}); t – trace.

Table 3

Minimum inhibitory concentrations (MICs) of essential oils and antibiotics

Microorganisms	MIC (μg/mL)			
	S. fruticosa	*S. ringens*	Amikacin	Ciprofloxacin
Staphylococcus aureus ATCC 6538	> 500	> 500	2	0.12
Bacillus subtilis ATCC 6633	250	200	0.5	0.06
Escherichia coli ATCC 8739	> 500	> 500	2	0.06
Klebsiella pneumoniae NCIMB 9111	> 500	> 500	0.5	0.06
Salmonella enterica subsp. *enterica* serovar S. abony NCTC 6017	> 500	> 500	2	0.06
Pseudomonas aeruginosa ATCC 9027	> 500	> 500	1	0.25
Acinetobacter baumannii ATCC 19606	> 500	> 500	8	0.25
Candida albicans ATCC 10231	200	300	–	–

Table 4

The activity of *Salvia fruticosa* essential oil in combination with antibiotics

Bacterial strain	MIC (FIC)		FICI	Effect	MIC (FIC)		FICI	Effect
	Amikacin	*S. fruticosa*			Ciprofloxacin	*S. fruticosa*		
Staphylococcus aureus ATCC 6538	2 (1)	62.5 (0.0625)	1.0625	IN	0.125 (0.25)	500 (0.5)	0.75	AD
Bacillus subtilis ATCC 6633	0.125 (0.25)	250 (1)	1.25	IN	0.031 (0.5)	125 (0.5)	1	AD
Escherichia coli ATCC 8739	2 (1)	125 (0.125)	1.125	IN	0.031 (0.5)	125 (0.125)	0.625	AD
Klebsiella pneumoniae NCIMB 9111	1 (2)	250 (0.25)	2.25	ANT	0.031 (0.5)	62.5 (0.0625)	0.5625	AD
Salmonella enterica subsp. *enterica* serovar *S. abony* NCTC 6017	2 (1)	62.5 (0.0625)	1.0625	IN	0.031 (0.5)	125 (0.125)	0.625	AD
Pseudomonas aeruginosa ATCC 9027	1 (1)	125 (0.125)	1.125	IN	0.125 (0.5)	62.5 (0.0625)	0.5625	AD
Acinetobacter baumannii ATCC 19606	8 (1)	62.5 (0.0625)	1.0625	IN	0.25 (1)	62.5 (0.0625)	1.0625	IN

MIC – minimal inhibitory concentration; FIC – fractional inhibitory concentration; FICI – fractional inhibitory concentration index; FICI ≤ 0.5 synergy; 0.5 < FICI ≤ 1 additivity; 1 < FICI ≤ 2 indifference (no effect); FICI ≥ 2 antagonism IN – indifference; ANT – antagonism; AD – additivity.

ed no significant results, as it was indifferent against all strains except *Klebsiella pneumoniae* where the antagonistic effect was manifested.

Discussion

Previous research has shown a highly variable composition of essential oil of *S. fruticosa*, even when it comes to samples from similar habitats. In an early study [9] on composition and antimicrobial activity of *S. fruticosa* oil, the samples had 1,8-cineole (47.48%), thujone (11.93%) and camphor (9.04%) as the main components, while our sample had *trans*-thujone (54.2%) as a dominant compound and a low amount of 1,8-cineole (0.2%). In the same study, investigation of antimicrobial activity of the essential oil and its main compounds by disk diffusion assay showed relatively low levels of antimicrobial activity against the bacteria tested [9]. Taking into account the differences between antimicrobial assessment methods, similar results were presented by Khoury et al. [21] for *S. fruticosa* essential oil from Lebanon. The research was conducted using broth-microdilution method and obtained MICs were greater than 500 μg/mL for *S. aureus*, *E. coli* and *C. albicans*, which is consistent with our findings.

The overall composition of *S. ringens* essential oil is also variable, according to the results given by Alimpić et al. [7] when compared with our results. While the main constituents of *S. ringens* oil from Greece in our study were α-pinene (28.1%), β-pinene (12.2%) and 1,8-cineole (13%), the oil obtained from areal parts of the plant from North Macedonia was rich in 1.8-cineole (32.0%), camphene (17.1%) and borneol (11.9%) [7]. In the same study, moder-

ate antibacterial activity of the essential oil was shown against Gram-positive bacteria, especially *S. aureus* and it was attributed to the high content of 1,8-cineole. Considering the lower content of this monoterpene in our sample, the results of its antibacterial activity in our study were expected.

Given that the combined antibacterial activity of *S. fruticosa* oil with AMI or CIP was not tested before, we used these antibiotics in our study [3]. On the other hand, Chovanová at al. [12] have recently showed that among *Salvia* species tested, the essential oil of *S. fruticosa* was the best in reducing the MIC of tetracycline due to decreasing antibiotic efflux and decreasing the expression of *tet*(K) gene in tetracycline resistant clinical isolates of *Staphylococcus epidermidis* [12]. Although tetracycline and AMI belong to different classes of antibiotics, we expected some similar synergistic effect because their mode of action is based on the inhibition of protein synthesis by targeting 30S ribosomes, but the anticipated results did not occur even though the antagonistic effect was observed with *K. pneumoniae*. However, according to FICI, the additive effect of *S. fruticosa* essential oil with CIP was evident against almost all tested bacterial strains, thus suggesting that the essential oil of the examined species could be used as a potential adjuvant to fluoroquinolone class of antibiotics. Considering that these investigations have not been performed so far, our results provide a good basis for future testing on different bacterial strains.

Conclusion

The essential oils of Greek samples of *S. ringens* characterized by high concentration of α-pinene and *S. fruticosa* characterized by high concentration of *trans*-thujone, showed modest antimicrobial activity according to MIC values obtained by broth-microdilution method on standard bacterial strains and a moderate antifungal activity on *C. albicans*. In combination with antibiotics, according to FICI values, the contribution of *S. fruticosa* essential oil to antimicrobial activity of amikacin was not evident, but with ciprofloxacin, a promising additive effect was achieved.

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