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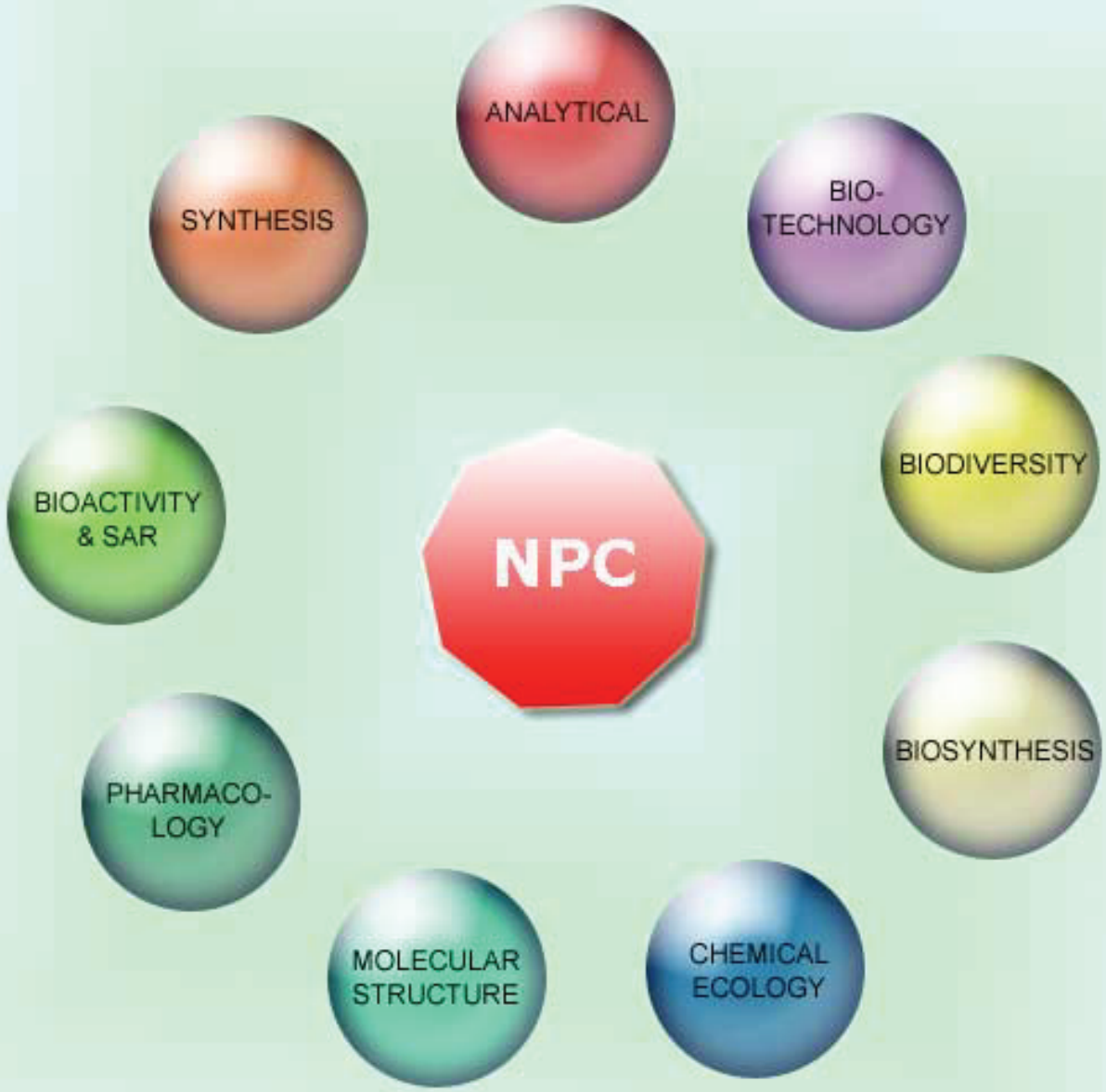

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Synergy between Essential Oils of *Calamintha* Species (Lamiaceae) and Antibiotics

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The subject of the study was the investigation of the chemical composition and antimicrobial activity of the essential oils (EOs) isolated from *Calamintha sylvatica*, *C. vardarensis*, *C. nepeta* and *C. glandulosa*, as well as their antibacterial activity in combination with antibiotics. The quantitative and qualitative analysis of EOs was performed using the GC/FID and GC/MS methods. The antimicrobial activity of EOs against six standard bacterial strains and one strain of yeast was tested using the broth microdilution method, while the antimicrobial activity of a combination of essential oils and gentamicin/ciprofloxacin was tested by the checkerboard method. The dominant components (> 10%) of the essential oils were: *cis*-piperitone epoxide and menthone (*C. sylvatica*), pulegone and menthone (*C. vardarensis*), pulegone and piperitenone (*C. nepeta*), pulegone, piperitenone, menthone and piperitone (*C. glandulosa*). EOs did not exhibit significant antimicrobial activity except the essential oil of *C. vardarensis* which was selectively active against *Staphylococcus aureus* (MIC - 21.25 µg/mL). The overall effect of essential oil-antibiotic combinations varied from synergistic (FICI ≤ 0.5) to antagonistic (FICI ≥ 2) depending on the bacterial strain tested.

Keywords: *Calamintha* species, Essential oil, Antimicrobial activity, Synergistic effect.

Antibiotic resistance is a major global public health problem and despite the huge diversity of antibacterial compounds, bacterial resistance to first-choice antibiotics has been drastically increasing [1]. The necessity for finding new antimicrobial agents and approaches in curing infections caused by resistant bacterial strains is increasing every day [2]. One of the approaches to this problem is the use of essential oils as antibiotic adjuvants, with the aim of achieving synergistic interactions [3].

Species of genus *Calamintha* are known as aromatic plants. The essential oil content in these species ranges from 0.2-2%. Their essential oils are rich in menthane class monoterpene compounds such as: pulegone, *cis*-piperitone epoxide, piperitenone and piperitenone oxide [4].

The results of the research done so far on the antimicrobial activity of the essential oils of the species of genus *Calamintha* have confirmed this activity [4g-4h,5]. However, since the effect of the combination of conventional antibiotics and *Calamintha* species' essential oils on bacterial growth has yet to be examined, the aim of this research was to examine the antimicrobial activity of the essential oils isolated from *Calamintha* species widespread in the central part of the Balkan Peninsula as well as their antibacterial activity in combination with antibiotics. For our research we selected four species: *Calamintha sylvatica* Bromf., *C. vardarensis* Šilić, *C. nepeta* (L.) Savi and *C. glandulosa* (Req.) Benth. *C. vardarensis* is a Balkan endemite which grows in FYR Macedonia, Albania, Bulgaria and Greece, while the other investigated species, besides the Balkan Peninsula, extend eastwards to western Asia [6].

All the investigated species were characterized by a high quantity of essential oils (0.6%-1.8%) dominated by oxygenated monoterpene compounds (higher than 89%) (Table 1). The dominant components of the investigated essential oils were: *C. sylvatica* - *cis*-piperitone epoxide (63.3%) and menthone (10.8%), *C. vardarensis* - pulegone (51.6%) and menthone (19.9%), *C. nepeta* - pulegone (58.0%) and piperitenone (27.4%), and *C. glandulosa* - pulegone (35.1%), piperitenone (23.4%), menthone (15.7%) and piperitone (11.5%). The biggest difference in the proportionality of dominant oil components is seen in the lack of pulegone, piperitone and piperitenone in species *C. sylvatica* and the dominance of *cis*-piperitone epoxide. In the oil of the rest of the species, pulegone dominates. *cis*-Piperitone epoxide appearing in *C. vardarensis* (7.1%), was not detected in the oil of *C. nepeta* and *C. glandulosa.* Piperitenone, one of the main components of the oil of *C. nepeta* and *C. glandulosa*, appears in the oil of *C. vardarensis* in the amount of 5.9% and was not detected in the oil of *C. sylvatica*. Piperitone is one of the main components only in the oil of *C. glandulosa* (Figure 1).

Earlier research [4a-4b, 4d-4f, 4h] has determined a similar content and composition of the essential oil isolated during the species' flowering stage which indicates that these are stable characters of the plants. These characteristics should be taken into consideration when gathering material for possible further pharmacological research i.e. the material should be gathered in the same developmental stage of the plants so that the results obtained are compatible for comparison with those already published.

Values of minimum inhibitory concentrations of essential oils and antibiotics (gentamicin/ciprofloxacin) are presented in Table 2 and Table 3, respectively. The essential oils of the investigated species have shown similarities in antimicrobial activity, which is probably the result of their similar essential oil composition. We did not observe strong antimicrobial activity, except for the essential oil of *C. vardarensis* which was selectively active against *Staphylococcus aureus* (MIC-21.25 µg/mL). Since pulegone is the dominant component in the essential oil of *C. vardarensis*, we might assume that its antimicrobial activity comes from this compound. But, it is interesting to note that the essential oil of *C. nepeta* and

Table 1: The content and composition (%) of essential oils of the studied *Calamintha* species.

				C. sylvatica	*C. vardarensis*	*C. nepeta*	*C. glandulosa*
Oil content (%, v/w)				0.6	0.9	1.8	1.6
	RT	R I	Compounds				
1.	7.008	935	α-Pinene	0.1	0.7	0.3	0.8
2.	8.194	945	Sabinene	0.1	0.4	0.2	-
3.	8.320	979	β-Pinene	0.2	0.8	0.6	1.3
4.	8.698	992	β-Myrcene	0.2	0.5	0.3	0.7
5.	8.834	996	3-Octanol	0.4	0.2	-	1.6
6.	9.925	1026	ρ-Cymene	0.7	0.4	-	-
7.	10.077	1030	Limonene	0.8	2.8	1.8	3.2
8.	10.357	1034	(Z)-β-Ocimene	0.2	0.3	-	0.1
9.	11.199	1059	γ-Terpinene	0.2	0.2	-	-
10.	11.557	1069	*cis*-Sabinene hydrate	1.7	0.6	-	1.1
11.	12.782	1101	*trans*-Sabinene hydrate	0.5	-	-	-
12.	15.630	1161	**Menthone**	**10.8**	**19.9**	3.9	**15.7**
13.	16.035	1167	Isomenthone	-	2.0	0.8	0.6
14.	16.060	1177	Isopulegone	-	1.4	1.1	-
15.	16.130	1181	Terpinen-4-ol	3.1	-	-	-
16.	16.384	1189	Isomenthol	3.2	-	-	-
17.	16.583	1192	Neoisomenthol	0.3	-	-	-
18.	16.665	1194	α-Terpineol	0.4	-	-	-
19.	16.957	1253	**Pulegone**	-	**51.6**	**58.0**	**35.1**
20.	19.480	1257	**Piperitone**	-	-	2.4	**11.5**
21.	19.703	1265	***cis*-Piperitone epoxide**	**63.3**	7.1	-	-
22.	20.876	1289	Dihydroedulan II	0.3	-	-	-
23.	21.026	1295	Thymol	1.8	0.2	-	-
24.	21.419	1305	Carvacrol	0.2	0.1	-	-
25.	21.717	1312	Menthyl acetate	1.1	-	-	-
26.	21.896	1316	Dihydrocarveol acetate	0.6	-	-	0.7
27.	23.135	1346	**Piperitenone**	-	5.9	**27.4**	**23.4**
28.	24.188	1371	Piperitenone oxide	2.4	0.3	1.5	1.3
29.	24.918	1388	β-Bourbonene	0.2	-	-	-
30.	26.371	1423	(E)-Caryophyllene	3.1	0.9	-	-
31.	27.724	1457	α-Humulene	1.2	0.3	-	0.5
32.	28.837	1485	Germacrene D	1.6	1.6	0.6	0.7
33.	29.399	1500	Bicyclogermacrene	-	0.4	0.1	-
34.	32.558	1581	Spathulenol	-	0.2	-	-
35.	32.801	1587	Caryophyllene oxide	0.6	0.2	-	0.2
Monoterpene hydrocarbons				2.5	6.1	3.2	6.1
Oxygenated monoterpenes				**89.7**	**89.1**	**95.1**	**89.4**
Monoterpenoids				92.2	95.2	98.3	95.5
Sesquiterpene hydrocarbons				6.1	3.2	0.7	1.2
Oxygenated sesquiterpenes				0.6	0.4	-	0.2
Sesquiterpenoids				6.7	3.6	0.7	1.4
Other compounds				0.4	0.2		1.6
Total				99.3	99.0	99.0	98.5

RT - Retention times; RI – Linear retention indices determined in relation to a homologous series of *n*-alkanes (C_9-C_{24}).

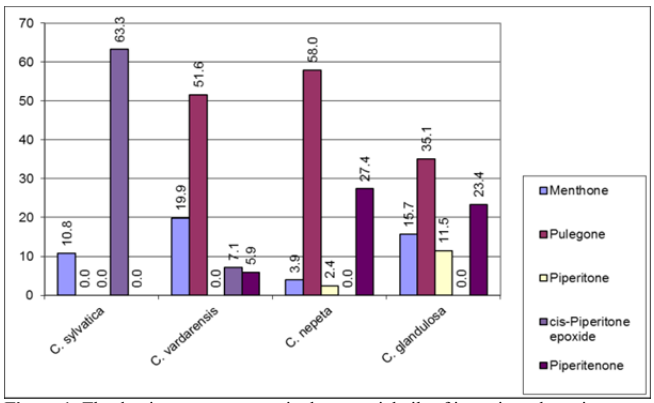

Figure 1: The dominant components in the essential oils of investigated species.

C. glandulosa, also dominated by pulegone did not have significant antimicrobial effect.

Gormez *et al*. [5f] reported the antibacterial activity of *C. nepeta* against phytopathogenic bacteria. The authors explained the high antimicrobial effect by the presence of pulegone, *cis*-piperitone epoxide and piperitenone oxide. Furthermore, the synergistic and antagonistic effects of these chemicals and minor components can

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

Microorganisms	MIC (μg/mL)			
	C. sylvatica	*C. vardarensis*	*C. nepeta*	*C. glandulosa*
Staphylococcus aureus ATCC 6538	>182	21.25	>191	>186
Staphylococcus epidermidis ATCC 12228	>182	>170	>191	>186
Bacillus subtilis ATCC 6633	>182	>170	>191	>186
Escherichia coli ATCC 10536	>182	>170	>191	>186
Pseudomonas aeruginosa ATCC 9027	>182	>170	>191	>186
Salmonella abony ATCC 6017	>182	>170	>191	>186
Candida albicans ATCC 10231	>182	>170	>191	>186

Table 3: Minimum inhibitory concentrations (MIC) of antibiotics.

Bacteria	MIC (μg/mL)	
	Gentamicin	Ciprofloxacin
Staphylococcus aureus ATCC 6538	0.62	0.16
Staphylococcus epidermidis ATCC 12228	1.25	0.62
Bacillus subtilis ATCC 6633	0.62	0.62
Escherichia coli ATCC 10536	0.31	0.16
Pseudomonas aeruginosa ATCC 9027	2.50	0.62
Salmonella abony ATCC 6017	1.25	0.62

also affect the antibacterial activity of the essential oils. Božović *et al*. [4h] reported the anti-candida activity of *C. glandulosa* (Syn. *C. nepeta* (L.) Savi subsp. *glandulosa* (Req.) Ball) with the MIC values ranging from 6.24 mg/mL to 12.48 mg/mL. It should be emphasized that MIC values in this study were expressed in miligrams/mL, whereas in our study we presented MIC values in micrograms.

The *in vitro* activity of essential oils in combination with antibiotics and FICI values are presented in Table 4. The overall effect of essential oil-antibiotic combinations varied from synergistic (FICI ≤ 0.5) to antagonistic (FICI ≥ 2). The combination of all the essential oils and both antibiotics exhibited a synergistic and additive effect against *Salmonella abony*. It is interesting that when combined with gentamicin all the tested essential oils exhibited an antagonistic interaction against *Escherichia coli,* while with ciprofloxacin they exhibited synergistic and additive effect.

Table 4: The *in vitro* activity of essential oils in combination with antibiotics

Bacteria	FICI							
	Gentamicin				Ciprofloxacin			
	C. sylvatica	*C. vardarensis*	*C. nepeta*	*C. glandulosa*	*C. sylvatica*	*C. vardarensis*	*C. nepeta*	*C. glandulosa*
Staphylococcus aureus	1.23 I	0.99 A	0.25 S	0.25 S	2.17 AN	2.12 AN	0.75 A	0.75 A
Escherichia coli	2.12 AN	2.12 AN	2.12 AN	2.12 AN	0.25 S	0.25 S	1.12 I	0.62 A
Pseudomonas aeruginosa	0.62 A	0.62 A	0.75 A	0.62 A	0.62 A	1.12 I	0.5 S	0.62 A
Salmonella abony	0.50 S	0.50 S	0.5 S	0.62 A	0.25 S	0.25 S	0.25 S	0.62 A

I – indifferent; AN – antagonism; A – addition; S – synergism

We previously reported different interactions between *Salvia officinalis* essential oil and antibiotics (ceftriaxone, ciprofloxacin, and gentamicin). The same combination showed different effects, from synergistic to antagonistic, on different clinical isolates of MRSA [7]. In a study conducted by Rosato *et al*. [8] the effect of the combinations of gentamicin and four essential oils (*Aniba rosaeodora* Ducke, *Melaleuca alternifolia* (Maiden &Betche) Cheel, *Origanum vulgare* L. and *Pelargonium graveolens* L 'Hér) was evaluated. A synergistic effect against all the tested strains was observed in gentamicin/*A. rosaeodora* and gentamicin/*P. graveolens* combinations, while in the other two combinations the effect ranged from synergistic to indifferent. In the present study we demonstrated that the essential oils of *C. sylvatica, C. vardarensis* and *C. nepeta* in combination with antibiotics (gentamicin or ciprofloxacin) exert a synergistic activity, while *C. glandulosa* essential oil had an additive effect against *Salmonella abony*. Conversely, antagonism was observed against *Escherichia coli* when essential oils were combined with gentamicin. Antagonistic interactions between essential oils and antibiotics should not be ignored [9]. These findings demonstrate the necessity for systematic *in vitro* studies of the interactions between essential oils and antibiotics to reveal any undesirable combinations.

According to the results of this study we can conclude that all the investigated species were characterized by a high quantity of essential oils dominated by oxygenated monoterpene compounds of the menthane class, as well as: *cis*-piperitone epoxide and menthone (*C. sylvatica*), pulegone and menthone (*C. vardarensis*), pulegone and piperitenone (*C. nepeta*), pulegone, piperitenone, menthone and piperitone (*C. glandulosa*).

The essential oils of *C. sylvatica, C. nepeta and C. glandulosa*, did not show significant antimicrobial activity except the essential oil of *C. vardarensis* which demonstrated antimicrobial activity against *Staphylococcus aureus*.

In combination with antibiotics the investigated essential oils exhibited different effects (from synergistic to antagonistic) depending on the bacterial strain tested. The essential oils of *C. sylvatica, C. vardarensis* and *C. nepeta* in combination with antibiotics (gentamicin or ciprofloxacin) exerted a synergistic activity, while *C. glandulosa* essential oil had an additive effect against *Salmonella abony.* Synergistic and additive effects suggest that the essential oils of the investigated species could be used as potential adjuvants to antibiotics. However, a large number of different microorganisms should be included in further research, with an emphasis on clinical isolates.

Experimental

***Plant material*:** The plant material was collected in the flowering stage from the following localities: *C. sylvatica* on the edge of a beech and hornbeam forest, under Mt. Rudnik (Serbia), *C. vardarensis* in the underbrush in Radika canyon (FYR Macedonia), *C. nepeta*, by Vratarnica near Zaječar (Serbia) and *C.glandulosa* on Luštica in Stari Krašići (Montenegro). Voucher specimens were deposited in the Herbarium of the Department of Botany, Faculty of Pharmacy, University of Belgrade (HFF) under numbers: 3251 (*C. sylvatica*), 3037 (*C. vardarensis*), 3714 (*C. nepeta*) and 1223 (*C. glandulosa*).

***Isolation and analysis of the essential oil**:* Essential oils were obtained from dried aboveground parts of plants by hydrodistillation in a Clevenger-type apparatus according to Ph. Eur. 8 [10]. Quantitative and 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 x 0.25 mm i.d., film thickness 0.25 μm). The thermal programme 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 [11]. The linear retention indices (RI) were determined in relation to a homologous series of n-alkanes (C9-C24) under the same operating conditions. Relative area percentages obtained by FID were used for quantification.

***Antimicrobial activity*:** The antimicrobial activity of the essential oils was determined by broth-microdilution method according to Clinical and Laboratory Standards Institute guidelines [12]. In the study we used six standard strains of microorganisms (Gram positive bacteria: *Staphylococcus aureus* ATCC 6538*, S. epidermidis* ATCC 12228*, Bacillus subtilis* ATCC 6633, Gram negative: *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 9027*, Salmonella abony* ATCC 6017*,* and one strain of yeast - *Candida albicans* ATCC 10231). Overnight broth cultures were prepared for each strain, and the final concentration in each well was adjusted to 2×10^6 CFU/mL. The essential oils were dissolved in 1% dimethylsulfoxide and diluted to the desired concentrations with Mueller-Hinton broth with the addition of 0.05% triphenyl tetrazolium chloride (TTC) (Sigma-Aldrich). After incubation for 24h at 35°C in aerobic conditions, minimum inhibitory concentrations (MIC) were determined. Each broth-microdilution test was repeated three times and the mean values are presented in this paper.

The synergism between essential oils and standard antibiotics ciprofloxacin and gentamicin (Sigma-Aldrich) was investigated by the checkerboard method according Langeveld *et al*. [3e] and Hu *et al*. [13]. The synergistic effect of the combinations was investigated in one concentration above and several concentrations below the MIC of each compound (antibiotic and tested essential oil). The test was performed in 96-well microtiter plates as a modified broth-microdilution test. The combination of essential oils and antibiotic was prepared by adding 50 μL of each to the same well of microtiter plate, and inoculating with 100 μL of previously prepared suspension of microorganisms (10^6 cfu/mL). After incubation for 24h at 35°C in aerobic conditions, the MIC of the combination was determined. The interaction between the essential oil and antibiotic was estimated by calculating the fractional inhibitory concentration 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 $\leq$ 0.5 synergy; 0.5 < FICI $\leq$ 1 additivity; 1 < FICI $\leq$ 2 indifference (no effect) and FICI $\geq$ 2 antagonism [13,14].

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