Antimicrobial activity of *Hyssopus officinalis* L. subsp. *aristatus* (Godr.) Nyman (Lamiaceae) essential oils from Montenegro and Serbia

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In this study, antimicrobial activity of essential oils extracted from the aerial flowering parts (herbs) of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman (Lamiaceae) collected from five different locations in Montenegro, or purchased in Serbia, were investigated. In addition, their antibacterial activity in combination with antibiotics was studied. The antimicrobial activity against selected standard bacterial and yeast strains was investigated using the broth microdilution method. Two standard antibiotics were used for comparison: the aminoglycoside antibiotic amikacin and the cephalosporin antibiotic ceftriaxone. The antimicrobial activity of the essential oil-amikacin combination was investigated using the checkerboard assay. The main components of the essential oils were 1,8-cineole, *cis*-pinocamphone, β-pinene and limonene in varying quantities. Most of the tested essential oils showed no significant antimicrobial activity. However, an essential oil rich in *cis*-pinocamphone showed moderate activity against both *Staphylococcus aureus* and *Escherichia coli* (MIC = 400 μg/mL). The overall effect of the essential oils and antibiotic combinations against *E. coli* or *S. aureus* ranged from additive (FICI = 0.625) to indifferent (FICI = 1.5), depending on the source of the essential oil.

Keywords: *Hyssopus officinalis*; essential oil; antimicrobial activity; additive effect

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1. INTRODUCTION

The genus *Hyssopus* L. (Lamiaceae) comprises 13 recognized plant species, which are mainly distributed in the temperate climate zone of Eurasia, from the Mediterranean region via Central Asia to Mongolia. In Montenegro and Serbia, the genus is monotypic; there is only one species, *Hyssopus officinalis* L. subsp. *aristatus* (Godr.) Nyman. The species is mainly distributed in the Mediterranean region, and the places in Serbia (Pirot and Nišava regions) are one of the northernmost points of its distribution range (Diklić and Janković, 1974; Gbif, 2023).

In Montenegro, hyssop is an unprotected plant species, according to the Rulebook on the Detailed Manner and Conditions of Collection, Use and Trade of Unprotected Wild Animal, Plant and Mushroom Species Used for Commercial Purposes (*Official Gazette of Montenegro*, 2010), in contrast to Serbia, where it is a protected wild plant species, according to the Rulebook on the Designation and Protection of Strictly Protected and Protected Wild Species of Plants, Animals and Fungi *Official Gazette of the Republic of Serbia, 5/2010, 47/2011, 32/2016 and 98/2016; Anex II*. Hyssop is frequently used in folk medicine. It is also used in the food and cosmetics industry, as well as an ornamental plant. The use of the hyssop herb and its preparations (infusions, syrups, tinctures, extracts) for various purposes is well documented in folk medicine - as a carminative, stomachic, tonic, diaphoretic, emmenagogue, expectorant, antiseptic, muscle relaxant; for digestive and intestinal problems, loss of appetite, stomach pain and cramps; for urinary tract infections; for the treatment of respiratory diseases such as tuberculosis, asthma, chronic catarrh and bronchitis, coughs, sore throats, respiratory infections, fever and respiratory irritations associated with the common cold, etc. It is also valued for the treatment of rheumatic pain, bruises, wounds, burns, frostbite, skin irritations, anxiety and hysteria, toothache, earache, for regulating blood pressure and night sweats (Charles, 2012; Judžentienė, 2016; Milovanović, 1975; Özer et al., 2006; Tucakov, 2010; Venditti et al., 2015).

Despite the numerous data on traditional use, scientifically sound information on hyssop herb use is rather limited. The competent institutions and associations, such as the European Medicines Agency (EMA), European Scientific Cooperative on Phytotherapy (ESCOP), Commission E of the German Federal Ministry of Health, as well as the World Health Organization (WHO) have not yet published official monographs regulating the use of herbal medicinal products based on *H. officinalis*. Moreover, there is no official information in modern pharmacopeias about the specific pharmacopoeial quality of herbal medicinal products from the aerial parts of *H. officinalis*.

Within the concept of rational phytotherapy, pharmacognostic studies of the wild hyssop herb are a necessary step towards the rational use of this herbal medicinal product. In general, antimicrobial activity is one of the most studied effects of various essential oils. The alarming increase in bacterial resistance to antibiotics has become an urgent challenge for the medicine and veterinary medicine, as well as for the pharmaceutical and food industries (Krist et al., 2015; Llor and Bjerrum, 2014). The search for antimicrobial agents from medicinal plants represents a promising alternative to synthetic chemicals. In this context, plant constituents, especially essential oils and their main components, have attracted a lot of attention.

According to the available literature data, the studies on the combined use of hyssop essential oil with antibiotics are scarce. Therefore, a better understanding of the antimicrobial activity of hyssop essential oils from Montenegro and Serbia has aroused our scientific interest.

2. MATERIAL AND METHODS

2.1. Plant material, essential oil isolation and chemical analysis

The essential oils tested in this study were obtained earlier by Mićović et al. (2021) using hydrodistillation of the aboveground flowering parts (herb) of *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman, collected at five different locations in the territory of Montenegro or purchased from a local provider in Serbia (Table 1).

The chemical compositions of the essential oils were analyzed by GC-FID and GC-MS, and their chromatographic profiles were described in detail in the publication by Mićović et al. (2021).

2.2. Antimicrobial activity of isolated essential oils

The antimicrobial activity of the essential oils was investigated using the broth microdilution method in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). Six standard strains of microorganisms were used for the study: Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633), Gram-negative bacteria (*Escherichia coli* ATCC 8739, *Klebsiella pneumoniae* NCIMB 9111, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Pseudomonas aeruginosa* ATCC 9027) and one yeast strain (*Candida albicans* ATCC 10231).

The experiment was performed on 96-well microtiter plates with serial dilutions of the tested essential oil samples dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) in concentration 1000 μg/mL and further diluted in Mueller-Hinton broth (Torlak, Serbia) to the tested concentrations (25-500 μg/mL). Fresh, overnight cultures of microorganisms were suspended in sterile physiological saline solution, and suspensions with a turbidity of 0.5 McFarland (density of approximately 2 x 10^8 CFU/mL) were obtained. The suspensions were further diluted (in Mueller-Hinton broth for bacterial strains and in Sabouraud dextrose broth; Torlak, Serbia for *C. albicans*) to a final density of 2 x 10^6 CFU/mL. In each well, 100 μL of the corresponding essential oil solution and 100 μL of bacterial or fungal suspension were mixed.

The results (minimum inhibitory concentration - MIC) were interpreted after incubation of the plates at 35 °C for 24 hours. Two standard antibiotics were used for comparison: the aminoglycoside antibiotic amikacin (Sigma-Aldrich, USA) and the cephalosporin antibiotic ceftriaxone (Sigma-Aldrich, USA). The concentration range of the antibiotics was between 0.01 and 8.0 μg/mL. Minimum inhibitory concentrations were determined by the absence of turbidity in the well of the microtiter plate, indicating inhibition of the growth of the inoculated microorganism. The minimum concentration of essential oil at which no turbidity occurred actually represented the MIC value. Each test was repeated three times, and the average values of the results obtained were calculated.

2.3. Interaction of essential oils with amikacin

Checkerboard method was used to evaluate combined effects of the essential oils and amikacin. In brief, the method was performed in 96-well polystyrene microtiter plates, by pouring decreasing concentrations of tested essential oils and twofold dilutions of examined antibiotic, lower than the recorded MICs, into horizontal and vertical wells, respectively (Langeveld et al., 2013). The bacterial suspension was prepared as described earlier (10^6 CFU/mL). Each well was filled with the same amounts of tested agents (50 μL) and a bacterial suspension to a final volume of 200 μL. The last two vertical rows of the microtiter plate represented the positive control and contained only the bacterial suspension without antimicrobial agent. After the incubation of plates for 24 h at 35 °C, MICs were determined as the lowest concentrations of combinations,where visible growth was absent.

To assess the interaction of the essential oil with the antibiotic, the fractional inhibitory concentration index (FICI) was calculated. The fractional inhibitory concentration (FIC) of each substance in the mixture is obtained by dividing the MIC of that substance in the mixture by the MIC of the pure substance (e.g. FIC of essential oil = MIC of essential oil in the mixture with antibiotic / MIC of essential oil).

The FICI value represents the sum of the FIC of the essential oil and the FIC of the antibiotic, and is interpreted as follows (Hu et al., 2002; Orhan et al., 2005):

1. FICI $\leq$ 0.5 indicates synergy;
2. 0.5 < FICI $\leq$ 1 indicates additivity;
3. 1 < FICI $\leq$ 2 indicates indifference (no effect) and
4. FICI $\geq$ 2 indicates antagonism.

3. RESULTS AND DISCUSSION

3.1. Antimicrobial activity of isolated essential oils

The MIC of investigated hyssop essential oils on the tested bacterial strains was mostly > 500 μg/mL and significantly weaker than the same feature of antibiotics ceftriaxone and amikacin used for comparison (Table 2).

Slightly better activity, *i.e.* a lower MIC value compared to those mentioned, was found in cases of *Escherichia coli* (samples 1, 3 and 5) and *Staphylococcus aureus* (sample 3). The

Table 1. Data on investigated plant material and essential oils.

Sample	Origin	Site of collection	Geographic coordinates	Altitude (m)	Voucher Specimen	Main essential oil - constituents
1	Commercial (Serbia)	Southeastern Serbia	N/A	N/A	N/A	1,8-cineole (67%), limonene (8%), β-pinene (7%), methyl eugenol (5%)
2	Wild-growing (Montenegro)	Kuči	N42°31′55″ E19°24′07″	870	1420263	1,8-cineole (42%), β-pinene (9%), limonene (8%), cis-pinocamphone (6%)
3	Wild-growing (Montenegro)	Šavnik	N42°57′16″ E19°05′59″	880	1420261	cis-pinocamphone (23%), methyl eugenol (19%), β-pinene (16%), limonene (16%), 1,8-cineole (10%)
4	Wild-growing (Montenegro)	Piva	N43°9′25″ E18°50′46″	750	1420162	methyl eugenol (28%), limonene (24%), β-pinene (16%), cis-pinocamphone (15%), trans-pinocamphone (8%)
5	Wild-growing (Montenegro)	Piperi	N42°34′23″ E19°16′0.8″	800	1420259	1,8-cineole (38%), limonene (22%), cis-pinocamphone (15%), β-pinene (10%)
6	Wild-growing (Montenegro)	Cuce	N42°35′19″ E18°47′40″	820	1420260	1,8-cineole (56%), limonene (15%), methyl eugenol (14%), β-pinene (5%)

Table 2. Minimum inhibitory concentrations of investigated essential oils and selected antibiotics (DMSO < 1%).

Microorganism (strain)	Minimum inhibitory concentration (MIC; μg/mL)							
	1	**2**	**3**	**4**	**5**	**6**	**Ceftriaxone**	**Amikacin**
Staphylococcus aureus ATCC 6538	>500	>500	**400**	>500	>500	>500	4	4
Bacillus subtilis ATCC 6633	>500	>500	>500	>500	>500	>500	2	0.5
Escherichia coli ATCC 8739	**400**	>500	**400**	>500	**400**	**500**	2	1
Klebsiella pneumoniae NCIMB 9111	>500	>500	>500	>500	>500	>500	4	0.25
Salmonella Typhimurium ATCC 14028	>500	>500	>500	>500	>500	>500	2	2
Pseudomonas aeruginosa ATCC 9027	>500	>500	>500	>500	>500	>500	8	2
Candida albicans ATCC 10231	500	500	500	500	500	500	n.t.	n.t.

n.t.– not tested.

growth of *Candida albicans* was inhibited at a minimum concentration of 500 μg/mL for all samples.
The essential oils 1 (commercial sample, Serbia) and 6 (Cuce, Montenegro) had similar compositions: they were high both in 1,8-cineole and β-pinene, but low in *cis*-pinocamphone. In contrast, the essential oils 3 (Šavnik, Montenegro) and 5 (Piperi, Montenegro) did not have similar chemical profiles: the dominant component in sample 5 was 1,8-cineole, while *cis*-pinocamphone was the predominant component in sample 3. In all cases, the essential oils samples 1, 3, 5 and 6 showed moderate antimicrobial activity against the *E. coli* strain, while 3 was effective against both *E. coli* and *S. aureus*.
The observed antimicrobial activities of the essential oils are probably due to the dominant compounds 1,8-cineole and *cis*-pinocamphone. These results are consistent with some previously published data in the literature, not only for the essential oil of *Hyssopus officinalis*, but also for some other commercially used essential oils such as cajuput oil, eucalyptus oil, sage oil and similar, as well as pure substances (Aguilar-Rodríguez et al., 2022; Jiang et al., 2021; Kizil et al., 2010; Mazzanti et al., 1998; Wińska et al., 2019). Although the biological properties of essential oils and the extent of their effect are closely related to their main constituents and their high concentrations, whole essential oils often exhibit greater antibacterial activity than their main constituents alone; therefore, the influence of the other constituents should not be ignored (Yap et al., 2014).
There are also reports in the literature on the antimicrobial effect of hyssop essential oils against the fungal strain *C. albicans* (Hristova et al., 2015; Kizil et al., 2010; Mazzanti et al., 1998; Venditti et al., 2015). For example, the study by Hristova et al. (2015) showed that a commercially available hyssop essential oil from Bulgaria with *cis*-pinocamphone, β-pinene and *trans*-pinocamphone as dominant components exhibited antifungal activity against *C. albicans* with a MIC of 210 μg/mL. The mechanism of antifungal activity of hyssop essential oil is thought to be related to increased permeability of fungal cells and disruption of normal membrane transport acting on the membrane ATPase (Hristova et al., 2015). We can only speculate why the essential oils of *H. officinalis* did not show similar activity against *C. albicans* in our study, but it seems reasonable to attribute the activity to *cis*-pinocamphone, as its concentration did not exceed 23% in the essential oils we tested, in contrast to the results of Hristova et al. (2015), where the content of this component reached even up to 50% in some samples.

3.2. Interaction of essential oils with amikacin

In combination therapy, the combined effect of two or more drugs is greater than the sum of their individual effects, which leads to a synergistic result. This is in contrast to an additive effect, where the combined effect is equal to the sum of the individual effects, and an indifferent effect, where there is no interaction between the drugs. Antagonism occurs when the combined effect is less than when the two drugs are used individually (Iseppi et al., 2021; Yap et al., 2014).
One strategy to combat antibiotic resistance is to find compounds that can counteract the antibiotic-destroying enzymes produced by bacteria (for example, clavulanic acid can inhibit β-lactamase enzymes that break down penicillin-type antibiotics). This led to the development of amoxicillin/clavulanate, a combination preparation that is effective against many bacteria that are resistant to amoxicillin alone. However, the effectiveness of combination drugs can be limited by the development of resistance to the new drug, as the widespread use of clavulanic acid has led to the emergence of resistant variants of bacteria. This underlines the need for continuous research and development of new antibiotics and resistance-breaking compounds (Monserrat-Martinez et al., 2019; Yap et al., 2014).
Numerous essential oils have antimicrobial properties that are of great importance in various scientific and industrial fields, including medicine, agriculture and cosmetology. Of the roughly 250 commercially available essential oils, about a dozen have remarkable antimicrobial potential. Essential oils appear to be a promising alternative to synthetic compounds, especially in view of the increasing resistance of pathogenic microorganisms (Iseppi et al., 2021; Wińska et al., 2019).
The combination of conventional antibiotics and essential oils is a relatively new concept. In some cases, such as in this study, essential oils have been found to enhance the antimicrobial effect of conventional antibiotics, even when they do not have a significant inhibitory effect on their own.
When essential oils and antibiotics were combined and tested against antibiotic-resistant bacteria, a significant reduction in antibiotic concentrations was observed in many cases, thus minimizing the adverse effects of these drugs (Iseppi et al., 2021; Yap et al., 2014). A key advantage of combining antibiotics with essential oils is that they can target different bacterial molecules. This can lead to new treatment options to overcome the growing problem of microbial resistance.
Essential oils are intrinsically multi-component plant products. In most cases, they are rather intricate blends of a number of different compounds, such as hydrocarbons, aromatic or aliphatic alcohols, acids and their derivatives, aldehydes and ketones, phenolics and phenylpropanoids, that account for their wide range of pharmacological and therapeutic effects (Bunse et al., 2022; Caneschi et al., 2023). Therefore, the antimicrobial activity of essential oils is probably based on a combination of mechanisms, unlike many conventional antimicrobials that have a single point of action.
In many cases, essential oils and their components easily penetrate the cell membranes of bacteria due to their lipophilic nature and cause irreversible damage to the cell architecture, leading to disruption of various cellular processes and ultimately to cytolysis and cell death. The interaction of essential oils with the bacterial cell wall could be used to enhance antibiotic activity, facilitate their penetration and allow a reduction in therapeutic doses (Bunse et al., 2022; Caneschi et al., 2023; Iseppi et al., 2021; Yap et al., 2014). This also makes it less likely that they will lead to the development of resistant bacteria, and this is the reason for all the effort put into studying the combined activity of essential oils and antibiotics (Yap et al., 2014).
The results of our study of the combined effect of essential oils with the antibiotic (amikacin) are shown in Table 3.
For all combinations, the MIC of the essential oil and the antibiotic in combination was, as expected, lower than that of the individual active ingredients. However, the true indicator of the activity of the combination is the FICI value (Table 4).
Our study showed that commercial sample of essential oil (sample 1) with 1,8-cineole as the main component had an additive effect with the antibiotic (amikacin) against the *E. coli* strain, while the sample 3 of essential oil rich in *cis*-pinocamphone had an additive effect with amikacin against both *S. aureus* and *E. coli* strains. According to the available information, this is the first study to investigate the combined use of hyssop essential oil with an antibiotic.

4. CONCLUSIONS

The available literature data on the antimicrobial activity of hyssop preparations are diverse and depend on numerous factors that ultimately influence the composition of the essential oil, such as plant subspecies/variety, habitat, extraction method, etc.

Table 3. Antimicrobial activity of hyssop essential oils in combination with the antibiotic (amikacin; DMSO < 1%).

Microorganism (strain)	Minimum inhibitory concentration (MIC; μg/mL)					
	1/amikacin	2/amikacin	3/amikacin	4/amikacin	5/amikacin	6/amikacin
S. aureus ATCC 6538	200/0.5	200/0.5	200/0.5	200/1	200/1	200/1
E. coli ATCC 8739	200/0.5	200/0.5	200/0.5	200/1	200/1	200/1
K. pneumoniae NCIMB 9111	400/0.0625	400/0.0625	400/0.0625	200/0.5	200/0.5	200/0.5
S. Typhimurium ATCC 14028	200/4	200/4	300/2	300/2	300/1	300/1

Table 4. Fractional inhibitory concentration indices (FICI) for active essential oils.

Combination	Bacteria	FICI	
		Value	Interpretation
1/amikacin	*E. coli*	1	Additivity
3/amikacin	*S. aureus*	0.625	Additivity
3/amikacin	*E. coli*	1	Additivity
5/amikacin	*E. coli*	1.5	Indifference
6/amikacin	*E. coli*	1.4	Indifference

This study has shown that hyssop essential oil has some potential to act as an antimicrobial agent. Moderate activity of certain samples of the tested essential oils against *S. aureus* and *E. coli* strains was shown, which could also be related to the use of hyssop in traditional medicine for mild respiratory and urinary tract infections; also, their additive effect with the antibiotic (amikacin) was demonstrated. In addition, it was also shown that essential oil of hyssop has antimicrobial potential against the fungus *C. albicans*.
Further research in this direction is certainly needed to clarify the mechanism of action and to find out which main components or combinations of components are responsible for the antimicrobial activity.

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CONFLICT OF INTEREST

None declared.

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