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Phytochemical and Antimicrobial Evaluation of the Essential Oil of Croatian *Salvia brachyodon* Vandas

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Authors' contributions

This work was carried out in collaboration between both authors. Author MS designed the study, performed the analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SL and MS conducted all experiments and analyzed data of the study. Author MS managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Backgraund: This study was designed to evaluate the phytochemical profile and the antimicrobial potential of the essential oil of *Salvia brachyodon* Vandas growing wild in Croatia. Short tooth sage (*S. brachyodon* Vandas),an endemic species that grows in the south east areas of the Adriatic coast in Croatia, Bosnia and Herzegovina, and Montenegro. It is a perennial plant that grows up to 70-80 cm in height and flowers from July to September.
Methodology: The phytochemical components of the essential oil were identified by gas chromatography-mass spectrometry (GC-MS) analysis. The antimicrobial activity was assessed against a panel of representative Gram-positive and Gram-negative bacteria as well as fungi. The antimicrobial activities of the oil against pathogenic microorganisms were determined by using agar disc diffusion and broth microdilution methods.
Results: From the thirty-eight identified constituents representing 95.7% of the oil, 1,8-cineole (16.7%), *β*-pinene (19.7%) and *α*-pinene (7.6%), were the major components. The levels of oxygenated monoterpenes such as camphor (5.6%), borneol (4.2%), myrtenol (2.4 %) and terpinen-4-ol were significant. Other important compounds were sesquiterpenes hydrocarbons *β*-caryophyllene (6.6%), *α*-humulene (4.9%), viridiflorol (3.0%), spathulenol (2.9%) and

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aromadendrene. Preliminary antimicrobial screening revealed that the oil exhibited a very interesting antimicrobial profile. The oil exhibited moderate *in vitro* antibacterial activity after it was tested against twenty pathogenic bacteria and fungal strains, but high antimicrobial activity observed against medically important pathogens such as *E. coli* O157:H7, *Listeria monocytogenes* and *Candida albicans.*

Conclusion: Results presented here may suggest that the essential oil of *S. brachyodon* possess antimicrobial properties, and is, therefore, a potential source of antimicrobial ingredient in food and pharmaceutical industry. The obtained results are preliminary and a further research is needed in order to obtain information regarding the practical effectiveness of essential oil to prevent the growth of foodborne and spoilage microbes under specific application conditions.

Keywords: Salvia brachyodon Vandas; short tooth sage; antimicrobial activity; essential oil composition; 1,8-cineole; α-pinene; β-pinene; GC-MS analysis; minimum inhibitory concentration.

1. INTRODUCTION

Plant essential oils and their components are an enormously variable group of phytochemicals in terms of their number, structural heterogeneity and distribution, which are believed to play a cardinal role in the aetiology and pathogenesis of various chronic diseases. The antimicrobial activities of plant oils and extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. The composition, structure as well as functional groups of the oils play an important role in determining their antimicrobial activities [1-5]. Species of the genus *Salvia* (Lamiaceae) have been extensively used in popular folk medicine and many pharmacognostic researches intended to identify biologically active compounds responsible for their therapeutic effects [5-8].

Salvia, the largest genus of Lamiaceae, includes about 1000 species, widespread throughout the world provide a rich source of variable bioactive compounds [9,10,11]. This genus is represented, by 20 species a narrowly distributed in the coastal and sub-Mediterranean areas of Dalmatia and the continental areas of Croatia. Some members of *Salvia* species are of economic importance since they have been known not only as a culinary herb for flavouring and seasoning, but it has been also of great medicinal importance. The *Salvia officinalis* (Dalmatian sage), originated from Mediterranean area, has been cultivated in many countries, and enjoys the reputation of being a panacea because of its wide range of medicinal effects: it has been used as an antihydrotic, spasmolytic, antiseptic, antitumor and anti-inflammatory and in the treatment of mental and nervous conditions [12].

Recently several authors reported antioxidant properties of sage and some of its constituents, mainly phenolic compounds such as carnosic, rosmarinic, caffeic and salvianolic acids as well as other phenolic structure-based compounds [10,11]. Phytochemical studies so far investigated the composition of the essential oil from *Salvia* species from different sources and chemotypes [13-16], as well as its variation in different seasons [17] and during the plant life cycle [14,18]. Evaluations of the oil composition extracted from different parts of the plant or upon variable environmental, cultivation, and/or storage conditions have also been reported [18,19]. The high diversity of the Salvia genus and phytochemical richness generate great interest for discovering new biological active compounds, including those found in essential oils.

Short tooth sage *Salvia brachyodon* Vandas is an endemic and one of the rarest plant species of the Dinaric karst with very restricted distribution on the southern Balkan Peninsula. This is present at a limited area of the Pelješac peninsula (Croatia) and another on Mt. Orjen. (border of Bosnia and Herzegovina and Montenegro) [20-22]. Short tooth sage (*S. brachyodon* Vandas) is perennial plant of the *Salvia officinalis* group. Stems are gay-whitish, erect, 70-80 cm high, in lower part slightly white lanate, and in upper part are glabrous (Fig. 1). Leaves are 6-14 cm long, 1.5-4 cm wide, with long petiole. Leaf blade is entire or pinnate divided, terminal leaflet is biggest, with 1-3 lateral leaflets that are 2-4 cm long and 5-7 mm wide, usually are concrescent with terminal leaflet. Inflorescence is laxly flowered panicula, composed of few flowers that are on long lateral branches, flower bracts are caducous. Calyx is tubular-campanulate, 8-10 mm long, glandular-

glutinous with prominent longitudinal veins. Corolla is 35-40 mm long, bilabiate, pale violet, glandular and sparsely hairy. Flowering period of this species is summer time, from July to September [21].

Fig. 1. Morphological characteristics of short-tooth sage (*Salvia brachyodon* Vandas) on the Pelješac peninsula

Due to its very narrow distribution and economically very interesting essential oil composition the antimicrobial activities of *S. brachyodon* essential oils have never been studied, while there is limited report on chemical analysis of the essential oil [18,19,23]. Given the lack of research information in this field, focused study on the phytochemical screening by GC–MS analysis and antimicrobial efficacy was determined by using agar disc diffusion as well as broth microdilution methods.

2. MATERIALS AND METHODS

2.1 Collection of Plant Samples

The flowering aerial parts of *S. brachyodon* Vandas were collected from a wild population in the Pelješac peninsula (Croatia) in June 2003. The botanical identity of the plant material was confirmed by a D. Vladović local botanist and voucher specimens number (PMFST/2003/31) are deposited at the Department of Biology, Faculty of Science, University of Split.

2.2 Isolation of the Essential Oil

The air-drying of the plant was performed in a shady place at room temperature for 10 days. Plant tops (during flowering) were used for the analysis of essential oil composition. A portion (100 g) of the aerial parts of *S. brachyodon* was put into water distillation, using a Clevenger-type apparatus for 3 hours. The obtained essential oil was dried over anhydrous sodium sulphate and 2µl was used for gas chromatography - mass spectrometry (GC/MS) measurements.

2.3 Gas Chromatography-mass Spectrometry Analysis Conditions

The analyses of the volatile compounds were carried out using a Hewlet Packard GC/MS system (GC 5890 Series II; MSD 5971A). The fused silica HP-20 M polyethylene glycol column (50 m x 0.2 mm, 0.2 µm film thicknesses) was directly coupled to the mass spectrometer. The carrier gas was helium (1 ml/min) and the program used was 4 min., isothermal at 70°C. Ionisation of the sample components were performed in the electron ionisation (EI) mode (70eV). The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing the homologous series of C_8-C_{22} *n*-alkanes [24]. Individual constituents were identified by referring to the compounds, known in the literature data [25], and also by comparing their mass spectra with either known compounds or with the Wiley mass spectral database.

2.4 Antimicrobial Activity

Two different methods were employed for the determination of antimicrobial activities of the *S. brachyodon* essential oil: An agar disc diffusion method and broth microdilution method [26]. The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) of the essential oils against the test microorganisms were determined by the broth microdilution method. The MIC and MBC of levofloxacin were also determined in parallel experiments in order to control the sensitivity of the test microorganisms. All tests were performed in duplicate.

2.5 Microbial Strains

The antimicrobial activity of *S. brachyodon* essential oil was evaluated using a panel which included laboratory control strains, all of them belonging to the American Type Culture Collection (Rockville, Md., USA): Gram-positive bacteria: *Bacillus cereus* (ATCC 11778), *B. subtilis* (ATCC 26633), *Enterococcus faecium* (ATCC 29212), *E. faecalis* (ATCC 15753),

Listeria monocytogenes (ATCC 700302), *Staphylococcus aureus* (ATCC 25923), Gram-negative bacteria: *Aeromonas hydrophila* (ATCC 7965), *Esherichia coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 43895), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 25933), *Salmonella thyphimurium* (ATCC 19430), and fungal microorganism, *Aspergillus niger* (ATCC 6275), *Aspergillus fumigatus* (ATCC 9142), *Aspergillus flavus* (ATCC 9643), *Candida albicans* (ATCC 10231), *C. rugosa* (ATCC 10571) *Cladosporium cladosporioides* (ATCC 13276), and *Saccharomyces cerevisiae* (ATCC 561). Stock cultures were maintained at 4°C on slopes of Tryptic soy broth (BBL, Cockeysville, MD) amended with 5g/l Yeast extract (Oxoid, Nepean, ON) and 15 g/l Agar agar (BDH, Toronto, ON). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to flasks of Mueller-Hinton broth (MHB) (Oxoid) for bacteria and Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24 h at 25°C. The cultures were diluted with fresh Mueller-Hinton and Sabouraud dextrose broth to achieve optical densities corresponding to 2.0 x 10^6 colony forming units (cfu) /ml for bacteria and 2.0 x 10^5 spore /ml for fungal strains.

2.6 Antibacterial Activity by the Disc Diffusion Method

The essential oil was tested for antibacterial activity by the disc diffusion method according to the National Committee for Clinical Laboratory Standards [26] using 100 µl of suspension of the tested microorganisms, containing 2.0 x 10^6 cfu/ ml for bacteria and 2.0 x10^5 spore/ ml for fungal strains. Mueller-Hinton agar (MHA) (Oxoid) and Sabouraud dextrose agar (SDA) sterilised in a flask and cooled to 45–50 °C were distributed to sterilised Petri dishes with a diameter of 9 cm (15 ml). The filter paper discs (6 mm in diameter, Difco) were individually impregnated with 15 µl of the oil was dissolved in 0.1% (v/v) dimethyl sulfoxide (DMSO), and placed onto the agar plates which had previously been inoculated with the test microorganisms. The Petri dishes were kept at 4°C for 2 h. After aerobic incubation at 37°C or 30°C overnight, the inhibition zones were measured. All tests were performed in duplicate and the antimicrobial activity was expressed in millimeters. Tests with dimethyl sulfoxide (0.1%) used as a negative control, and vancomycin (30 µg), tetracycline (30 µg) for bacteria or nystatin (30 µg) for fungi served as positive controls.

2.7 Determinations of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal or Fungicidal Concentration (MBC/ MFC)

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/ MFC) according to the National Committee for Clinical Laboratory Standards [26]. All tests were performed in Mueller Hinton broth (MHB), with the exception of the yeasts and fungal strains (Sabouraud dextrose broth; SDB). The investigated oil was dissolved in 0.1% dimethyl sulfoxide (DMSO) and then diluted to the highest concentration. A serial doubling dilution of the oil was prepared in a 96-well microtiter plate over the range 0.02-50.00 µl/ml. Overnight broth cultures of each strain were prepared and the final concentration in each well was adjusted to 2.0 x 10^6 cfu/mL for bacteria and 2.0x10^5 spore /ml for fungal strains. Plates were incubated at 37°C for 24 h for bacteria and at 30°C for 48 h for the yeasts and fungal strains. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth after 24 h of incubation at 35°C and the minimum bactericidal or fungicidal concentration (MBC/ MFC) was the point at which 0.1% or less of the initial inoculums survived. The microbial growth was determined by absorbance at 600 nm using the universal microplate reader (Biotek Instrument Inc, Highland Park, Vermont, USA). To determine MBC, the broth was taken from each well and inoculated in Mueller Hinton agar (MHA) and Sabouraud dextrose agar (SDA). The plates were incubated overnight at 37°C for bacteria and at 30°C for fungi. All determinations were performed in duplicate and two growth controls consisting of MH and SDA medium. Tests with dimethylsulfoxide (0.1%) used as a negative control. Levofloxacin for bacteria and amphotericin for fungi was served as a positive control.

3. RESULTS AND DISCUSSION

The hydrodistillation of the dried aerial parts of *S. brachyodon* Vandas gave yellowish oil with a yield of 0.53% (v/w) on dry weight basis. Thirty-eight compounds were identified in the oil, which represented about 95.7% of the total detected constituents. The general phytochemical profile of the tested oil, the percentages content of the individual components, retention indices and retention times are summarised in Table 1. The

different components of the oil are listed according to the increasing retention times. The essential oil consisted mainly of monoterpene and sesquiterpene hydrocarbons and their related alcohols. In Table 1 is presented only the compounds that participated in the mixture in percentages higher than 0.1%. The levels of monoterpene hydrocarbons, such β-pinene (19.7%), α-pinene (7.6%) was significant. Eucaliptol (16.7%), camphor (5.6%), borneol (4.2%), and myrtenol (2.4%) were the main oxygenated monoterpenes, whereas viridiflorol (3.0%) and spathulenol (2.9%) were the main oxygenated sesquiterpenes. Among other sesquiterpenic hydrocarbons, considerable amounts of α-humulene (4.9%), β-caryophyllene (6.6%) and aromadendrene (2.9%) were detected. Moreover, some sesquiterpenes such as germacerene-D, caryophyllene oxide, β-cubebene, γ-muurolene, α-eudesmol and δ-cadinene were found in low amounts. Regarding the previously reported content of *S. brachyodon* essential oil [18,19,23], it is interesting to point out that there are some differences suggesting that the collection time and environmental factors influence its chemical composition.

Table 1. Phytohemical composition of *Salvia brachyodon* Vandas essential oil (%)

No.	Phytochemicals	Rt (min)	RI	%	Mode of identification
1	*α*-Thujene		1031	0.2	GC, MS
2	*α* -Pinene	4.70	1038	7.6	GC, MS
3	Camphene	5.16	1059	0.1	GC, MS
4	Sabinene	5.95	1117	0.6	
5	*β*-Pinene	6.10	1092	19.7	GC, MS
6	Myrcene	6.61	1148	1.2	GC, MS
7	*α*- Phellandrene	7.24	-	1.7	GC, MS
8	*α*-Terpinene	7.79	1161	0.3	GC, MS
9	*p*-Cymene	8.22	1247	1.4	GC, MS, RC
10	Limonene	8.42	1215	0.7	GC, MS
11	1,8-Cineole	8.86	1191	16.7	GC, MS
12	*γ*-Terpinene	10.28	1236	0.5	GC, MS
13	Myrtenol	11.20	1733	2.4	GC, MS
14	Terpinolene	12.08	1265	0.2	GC, MS
15	Linalool	12.86	1507	0.2	GC, MS, RC
16	Camphor	13.20	1481	5.6	GC, MS
17	Borneol	16.24	1653	4.2	GC, MS, RC
18	Terpinen-4-ol	16.74	1568	1.6	GC, MS
19	1-α-Terpineol	17.41	1652	1.3	GC, MS
20	Bornyl acetate	21.43	1561	0.9	GC, MS
21	Thymol	21.76	2115	0.2	GC, MS
22	Carvacrol	22.10	2140	0.5	GC, MS
23	α-Copaene	24.61	1466	0.6	GC, MS
24	*β*-Bourbonene	24.91	1496	0.4	GC, MS
25	Sabinene hydrate	25.03	1423	0.3	GC, MS
26	*β* -Cubebene	25.14	1680	0.9	GC, MS
27	Aromadendrene	25.47	1585	2.9	GC, MS
28	*β* -Caryophyllene	26.11	1583	6.6	GC, MS
29	*α* -Humulene	27.01	1660	4.9	GC, MS
30	Germacrene D	28.15	1716	1.4	GC, MS
31	***γ*** -Muurolene	28.37	1735	0.7	GC, MS
32	*γ* -Cadinene	28.80	-	0.2	GC, MS
33	*δ*-Cadinene	29.49	1729	0.7	GC, MS
34	Spathulenol	31.14	2061	2.9	GC, MS
35	Caryophyllene oxide	31.31	1927	1.1	GC, MS
36	Viridiflorol	31.83	2023	3.0	GC, MS
37	*γ*-Eudesmol	33.25	2075	0.6	GC, MS
38	α -Eudesmol	33.33	2175	0.7	GC, MS

RI- retention indices (Kovats index) on HP-20 M column;
GC- identification by comparison of retention indices;
MS- identification on the basis of the mass spectra Wiley (MS) only;
RC- identification by comparison of their mass spectra of reference compounds

For example, bornyl acetate (9.8-10.5) and humulene epoxide II (6.6-7%) were detected as the main component in the previous report, in sample bornyl acetate was present at very low concentrations (0.9%), while humulene epoxide II is totally absent.

The studied of essential oils displayed different phytochemical profile from those *Salvia* species observed of other geographical origins [15,16, 27-30]. The oils of *S. officinalis*, *S. lavandulifolia*, *S. fruticosa* and *S. ringens*, members of the section *Salvia* to which *S. brachyodon* also belongs, are characterised by the presence of 1,8 cineole, *α*-pinene and *β*-pinene, camphor, thujone, borneol, bornyl acetate, camphene, as principal components. Bicyclic terpenes (*α*-pinene and *β*-pinene) give a different profile in the sage essential oils. Both enantiomers have been found as major compounds in the oils from those *Salvia* species such as *S. bracteata, S. cryptantha, S. multicaulis*, *S. tentillifolia, S. tomentosa* and *S. aucheri* [27-29].

More recently, Ozcan et al. [16] reported that the main components of *Salvia aucheri* var. *canescens* 1,8-cineole (32.3%, 28.6%), camphor (18.9%, 22.8%), borneol (8.2%, 8.9%), *α*-pinene (6.3%, 9.0%) and *β*-pinene (5.3%, 6.2%). The chemical composition of *Salvia* species essential oils of shows a large interspecies variability and, within the same species, it seems to depend on the genetic characteristics of the plant and on the conditions under which it has grown.

In a preliminary screening assay the antimicrobial efficacy of *S. brachyodon* essential oil against medically important pathogens employed and its antimicrobial potential were qualitatively assessed by the disc diffusion method. The results are presented in the Table 2 showed that the oil displayed a variable degree of antimicrobial activity on different strains, in the conditions studied. The data obtained from disc diffusion method indicated that *E. coli* O157:H7 and *Candida albicans* were the most sensitive

Table 2. Antimicrobial activity of the *Salvia brachyodon* Vandas essential oil by the disc diffusion method

Mcroorganisms	Source no.	Essential oil zone inhibition (mm)	Antimicrobial agents* zone inhibition (mm)		
			Vancomicin	Tetracycline	Nystatin
Gram-positive					
Bacillus cereus	ATCC 11778	11	12	28	nd
Bacillus subtilis	ATCC 26633	14	15	24	nd
Enterococcus faecium	ATCC 12755	6	24	-	nd
Enterococcus faecalis	ATCC 29212	7	28	-	nd
Listeria monocytogenes	ATCC 15313	22	24	28	nd
Staphylococcus aureus	ATCC 25923	14	12	26	nd
Gram-negative					nd
Aeromonas hydrophila	ATCC 7965	14	18	31	nd
Esherichia coli	ATCC 25922	9	22	28	nd
E. coli O157:H7	ATCC 43895	24	18	26	nd
Klebsiella pneumoniae	ATCC 13883	11	21	26	nd
Pseudomonas aeruginosa	ATCC 27853	6	8	32	nd
Proteus mirabilis	ATCC 25933	8	20	24	nd
Salmonella thyphimurium	ATCC 19430	10	19	22	nd
Fungi					
Aspergillus niger	ATCC 6275	19	nd	nd	13
Aspergillus fumigatus	ATCC 9142	15	nd	nd	10
Aspergillus flavus	ATCC 9643	19	nd	nd	12
Candida albicans	ATCC 10231	19	nd	nd	25
Candida rugosa	ATCC 10571	21	nd	nd	18
Cladosporium cladosporioides	ATCC 13276	19	nd	nd	16
Saccharomyces cerevisiae	ATCC 561	11	nd	nd	18

** Vancomycin 30 µg/disc; tetracycline 30 µg/disc; nystatin 30 µg/disc.*
nd - Not detected
- Not inhibit the growth

microorganisms, with the stronger inhibition zone 24 mm, followed by *Listeria monocytogenes* (22 mm). The *Bacillus subtilis, Staphylococcus aureus* and *Aeromonas hydrophila* were displayed moderate inhibition zones (14 mm). The *Enterococcus* group D was found to be more resistant among the Gram-positive bacteria. Gram-negative bacteria, *E. coli* exhibited weak inhibition zone (9 mm). Similar, other Gram-negative bacteria *P. aeruginosa* also exhibited weak inhibition zones (6 mm), since it is known to have high level of intrinsic resistance to virtually all known antibiotics and more essential oils and due to a very restrictive outer membrane barrier.

The oil was effective against all fungal strains tested, with inhibition zones 11-24 mm. Maximum activity was observed against *Candida albicans*. Other fungal strains such as *Aspergillus niger*, *A. flavus* and *Cladosporium cladosporioides* were exhibited equal susceptibility to the investigated oil, with inhibition zones 19 mm.

Antimicrobial activity of the *S. brachyodon* Vandas essential oil was expressed as minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/ MFC) by the broth microdilution method. The results are presented in Table 3 showed that the oil exhibited moderate *in vitro* activity against *Bacillus subtilis* and *Salmonella thyphimurium,* in a range between 12.25 µl/ml and 25.00 µl/ ml. Furthermore, the data indicate that the essential oil against both *Escherichia* species expressed very different activity. The results showed that non-pathogenic *Echerichia coli,* belonging to the normal microflora of humans, displayed moderate susceptibility, with MIC/MBC values ranging from 6.25 µl/ml to 12.50 µl/ml. Interestingly, high antimicrobial effect of the oil observed against hemorrhagic strain *E. coli* O157:H7 in a range from MIC 1.56 µl/ml to MBC 3.12 µl/ml. The difference may be explained by the biological activity of oil and differences in genome structure between the two types of *E. coli.*

The bacterial strains *Enterococcus faecium* and *Proteus mirabilis* were resistant than that other strains. The Gram-negative *Pseudomonas aeruginosa* seemed to be also resistant, exhibiting high MIC/MBC values from 12.50 to 50.00 µl/ml. Present data confirmed the findings of the previous studies reported that

Table 3. Antimicrobial activity of the *Salvia brachyodon* Vandas essential oil, expressed as minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/ MFC) by the broth microdilution method

Mcroorganisms	Source no.	Essential oil (µl/ml)		Antimicrobial agents (µg/ml)	
		MIC	MBC/MFC	MIC	MBC/MFC
Gram-positive				**Levofloxacin**	
Bacillus cereus	ATCC 11778	6.25	25.00	1.00	2.00
Bacillus subtilis	ATCC 26633	12.50	25.00	0.50	2.00
Enterococcus faecium	ATCC 12755	50.00	na	0.50	8.00
Enterococcus faecalis	ATCC 29212	25.00	50.00	1.00	8.00
Listeria monocytogenes	ATCC 15313	3.12	6.25	0.50	2.00
Staphylococcus aureus	ATCC 25923	3.12	12.50	0.12	4.00
Gram-negative					
Aeromonas hydrophila	ATCC 7965	6.25	12.50	0.050	1.00
Esherichia coli	ATCC 25922	6.25	12.50	0.004	2.00
E. coli O157: H7	ATCC 43895	1.56	3.12	0.016	2.00
Klebsiella pneumoniae	ATCC 13883	6.25	12.50	0.062	1.00
Pseudomonas aeruginosa	ATCC 27853	12.50	50.00	0.250	2.00
Proteus mirabilis	ATCC 25933	50.00	na	0.062	4.00
Salmonella thyphimurium	ATCC 19430	12.25	25.00	0.050	0.50
Fungi				**Amphotericin**	
Aspergillus niger	ATCC 6275	6.25	12.50	0.25	1.00
Aspergillus fumigatus	ATCC 9142	6.25	12.50	0.25	2.00
Aspergillus flavus	ATCC 9643	3.12	12.50	0.50	2.00
Candida albicans	ATCC 10231	1.56	3.12	0.25	2.00
Candida rugosa	ATCC 10571	3.12	6.25	0.50	2.00
Cladosporium cladosporioides	ATCC 13276	6.25	12.50	0.12	8.00
Saccharomyces cerevisiae	ATCC 561	12.50	25.00	0.25	4.00

high contents 1,8-cineole has been show inhibitory effects on bacteria and fungi, with the exception of *P. aeruginosa* [31-33]. The oil also exhibited the highest inhibitory effect against Gram-positive bacteria *Listeria monocytogenes* in a range between 3.12 µl/ml and 6.25 µl/ml. Many studies have shown that essential oils have greater activity against Gram-positive bacteria than against Gram-negative bacteria [34,35], because the cell wall of Gram-negative bacteria is composed of LPS, lipoproteins, and phospholipids, which form a barrier that blocks the penetration of hydrophobic oil and avoids the accumulation of essential oils in target cell. In contrast, the cell wall of Gram-positive bacteria includes a thin layer of peptidoglycan as well as teichoic acid and abundant pores that allow foreign molecules to penetrate, resulting in cell membrane damage and cell death.

The oil of *S. brachyodon* was effective against all fungal strains tested in the study with a range from 1.56 µl/ml to 25.00 µl/ml. Maximum activity was observed against the yeast such as *Candida albicans* from 1.65 µl/ml to 3.12 µl/ml. The negative control was DMSO, which did not inhibit the growth of microorganisms in the conditions studied.

The antimicrobial activities of the essential oil of the *S. brachyodon* growing wild in Croatia are comparable to the previously published activities for *Salvia* species growing in other Mediterranean countries [22] and it seems that different oil components contribute to differences in microbial susceptibility. In a plant screening [23] of *Salvia ringens* containing concentrations of 1,8-cineole (46.42–50.74%), comparable to that obtained for *S. brachyodon* no activity was reported for *Staphylococcus aureus* and but very good activity for Gram negative bacteria. These results of *S. brachyodon* show that the oil is moderately active against *Staphylococcus aureus* but low activity observed against Gram-negative bacteria, with the exception of *E. coli* O157:H7. The positive effects of sage oil on human health have been attributed to its various biologically active constituents. Taking into account the phytochemical composition of *S. brachyodon* oil, it becomes evident that there is a relationship between the antimicrobial activities of this oil and the presence of principal components, such as pinene enantiomers (*α*-pinene and *β*-pinene), camphor and their derivatives, eucalyptol (1,8-cineole), borneol, and can be considered as the main antimicrobial constituents of *S. brachyodon* oil. These results suggested that the bicyclic terpene *a*-pinene and *β*-pinene, are the main components of *S. brachyodon*, appeared to have an important role in the antimicrobial activities of oil observed in the present work and that is in accordance with previous studies reports [5,33]. *In vitro* studies indicated that pinenes have significant antimicrobial potential but there is no consensus regarding the differences in both potency and spectrum of antimicrobial activity, potentially because of the lack of enantiomer identification. Previous investigations on the antibacterial efficacy of pinenes have confirmed that only the positive enantiomers of pinenes exhibited an antimicrobial effect with MIC values ranging from 117 µg/ml to 6.25 µg/ml [33]. The solubility of terpenes is also hypothesised as correlating with their antimicrobial activity. However, the pinenes are examples of compounds with very low water solubility, but relatively high antimicrobial activities.

Furthermore, the data indicate that, in general, the antimicrobial activities of the oil studied apparently related to high contents of 1,8 cineole (16.7%), a monoterpene oxide that have been show inhibitory effects on bacteria and fungi with the exception of *Pseudomonas aeruginosa* and that is in accordance with previous report [36]. It is known that the 1,8 cineole inhibit production of inflammatory mediators following an inflammatory stimuli and systemic anti-inflammatory and expressed analgesic, gastro and hepato protective effects [37]. The antimicrobial activity of the *S. brachyodon* oil could, in part, be associated with camphor and borneol, were revealed to inhibit the growth of bacteria and fungi, especially *Candida albicans* [38]. Most studies focused on the antimicrobial activity of the single compounds. The adulteration of essential oils with synthetic components may give rise to a different proportion of enantiomers for a large number of components than in a pure botanical sample: this can greatly influence the bioactivity. In addition, several aspects of the properties of *Salvia sp.* oil components such as synergistic action between two or more components or other beneficial pharmacological or medicinal properties have not yet been explored fully. In fact, it was also possible that the components in lower percentage might be involved in some type of synergism with the other bioactive compounds. For example, synergistic activity of 1,8-cineole and camphor against some bacteria had already been reported [36]. In addition, the components in lower amount such as *β*-caryophyllene,

phellandrene, aromadendrene, terpinen-4-ol, germacrene D and *p*-cymene could also contribute to the antimicrobial activity of investigated oil. To the best of knowledge, the essential oil composition and antimicrobial activity of *S. brachyodon* Vandas, has not been reported before and therefore results can be evaluated as the first report about the antimicrobial properties in respect to the phytohemical profile.

4. CONCLUSION

The results of this study suggested that the possibility of using the essential oil or some of their components as natural food preservatives and as pharmaceuticals and natural therapies of infectious diseases because the oils possess antimicrobial activity. Further research is needed in order to obtain information regarding the practical effectiveness of essential oil to prevent the growth of pathogens and spoiling microbes under the specific application conditions and *in vivo* antimicrobial potential of in animal models.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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