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Evaluation of Antimicrobial Activities of *Salvia verbenaca*

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Abstract

The antimicrobial activity of several extracts and fractions of *Salvia verbenaca* L. (Lamiaceae) was investigated by disc diffusion and broth microdilution methods against *Escherichia coli* ATCC 10536, *Staphylococcus aureus* ATCC 6538P, *Klebsiella pneumoniae* UC57, *Micrococcus luteus* La 2971, *Micrococcus flavus* ATCC 14452, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 19115, *Mycobacterium smegmatis* CCM 2067, *Bacillus cereus* ATCC 7064, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Cryptococcus neoformans* ATCC 90112, *Kluyveromyces fragilis* NRRL 2415 and *Rhodotorula rubra* DSM 70403. The methanol extract, butanol and chloroform fractions have shown potential antimicrobial effects against some bacteria and the yeast cultures tested, with grown inhibition area diameters in the range 10.8 – 22.4 mm, and MIC values between 0.03 and 0.34 µL/mL. The results of the study support the use of the plant in traditional medicine.

Keywords:

Salvia verbenaca, antimicrobial activity, plant extract

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Introduction

The genus *Salvia* L. is one of the most species-rich genera of flowering plants, with about 1000 species currently accepted (Gonzales-Gallegos et al., 2020). Besides, *Salvia* L. is widely distributed

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in subtropical and temperate regions. Turkey has the second highest number of *Salvia* species in the world, after Mexico, with about 100 species, 53 of which are endemic (Celep et al., 2020).

Salvia species are commonly used in Anatolia for colds, stomach aches, and sore throats (Tabanca et al., 2017), to treat inflammatory skin diseases, to stop bleeding, or as an antiseptic for wounds (Suntar et al., 2011).

The secondary metabolites, namely essential oils and plant extract of some *Salvia* species are known to possess antioxidant, antimicrobial, antifungal, and aromatic properties (Gali-Muhtasib et al., 2000; Doğan et al., 2007). Therefore, they are intensely screened and applied in the fields of pharmacology, medicine, and food preservation (Cowan, 1999).

Salvia verbenaca L. (Lamiaceae) is a perennial herbaceous plant, endemic to the Mediterranean region and the Canary Islands and has expanded into Europe and Asia (Codd Lesli and Leistner, 1985). The species is cultivated in several countries mainly to produce dried leaves as raw material in herbal medicine (Baser, 2000). *S. verbenaca* is used as a bactericide against respiratory diseases, as eyedrops (Canzoneri et al., 2011), and in healing wounds and ulcers.

As a result of the interviews, it was determined that *S. verbenaca* was used by the local people for wound treatment and eye disinfection during routine trips. Hence, here the purpose was to determine the antimicrobial effects of the ethanolic leaf extract of this plant collected from Turkey, which is used by the local people to cure some illnesses.

Materials and Methods

The Plant Material

The plant samples were collected during to flowering stage in June 2020 at an altitude of 765 m, Samandere village around, Düzce, Turkey. A voucher specimen of the plant was deposited in the Department of Medical Biology of Duzce University in the author's collection (Voucher number; GD.109-8).

Preparation of Extracts

Plant samples were dried in an oven at 40 °C and powdered. Methanol extracts were obtained by maceration of the plant material with methanol for 3 days at room temperature, the procedure was done three times. The extracts were filtered and dried under reduced pressure at a temperature below 45°C. Then the methanol extracts were separated between chloroform and water ($CHCl_3$, H_2O). Finally, the aqueous fraction was again subjected to separation between n-butanol and water (n-BuOH, H_2O). The yields obtained for each extract and fraction as percentages of the initial dry material were *S. verbenaca* 24.20% for MeOH ($CHCl_3$ 9.82%, BuOH 22.10%, aqueous 31.46%).

Infusions were prepared with 100 g crude powder and 1000 mL water, and the volume was adjusted to a concentration of 1 g/mL under reduced pressure at 40 °C.

Preparation of Samples

The different extracts and fractions were diluted in dimethylsulfoxide (DMSO). The corresponding concentrations are expressed as mg extract or fraction per mL solvent, except for infusions, whose concentrations are expressed as mg initial dry material per mL. For each experiment, a disc containing only (DMSO) was used as a control.

Bioassays

The antimicrobial tests employed the disc diffusion method (Bauer et al., 1966), and the minimum inhibitory concentration (MIC) was determined by microdilution according to the microplate method (Rabanal et al., 2002; Jones et al., 1987).

The microorganisms to be tested were inoculated into Brain Heart Infusion Agar (Oxoid) for the bacteria, and Saboraud Dextrose Agar (Oxoid) for the yeasts. After 24h incubation at 35°C and 28°C, respectively, three or four colonies isolated from the media were incubated into 4 mL of Brain Heart Infusion Broth (Oxoid) for the bacteria, and Saboraud Dextrose Broth (Oxoid) for the yeasts, and incubated for 2h at 35°C and 28°C, respectively. The cultures were adjusted with sterile saline solution to turbidity comparable to that of McFarland (0.5) standard. Petri dishes containing Mueller Hinton Agar (Oxoid) or Bacto Yeast Morphology Agar (Difco) were impregnated with these microbial suspensions for the bacteria and yeasts, respectively (Bauer et al., 1966; Rabanal et al., 2002).

MIC was estimated by the broth microdilution method in M24A microplates against the most sensitive microorganisms, using liquid media containing decreasing amounts of the test materials. From the initial solution extract of 375 mg/mL, double dilutions in the culture medium (Mueller Hinton Broth for bacteria, Sabouraud Dextrose Broth for yeasts) were prepared and tested at concentrations ranging from 37.5 to 0.03 mg/mL. After mixing, 5 µL cell suspension (105 cells per µL) was added and mixed vigorously again. The temperature is for 24h in a humid atmosphere. Afterward, the microplates were centrifuged and examined for growth. All data represented at least three replicated experiments per microorganism.

Microorganisms

Escherichia coli ATCC 10536, *Staphylococcus aureus* ATCC 6538P, *Klebsiella pneumoniae* UC57, *Micrococcus luteus* La 2971, *M. flavus* ATCC 14452, *Proteus vulgaris* ATCC 8427, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 19115, *Mycobacterium smegmatis* CCM 2067, *Bacillus cereus* ATCC 7064, *B. subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Cryptococcus neoformans* ATCC 90112, *Kluyveromyces fragilis* NRRL 2415 and

Rhodotorula rubra DSM 70403 were collections maintained in the Laboratory of Medical Biology, Faculty of Medicine at Düzce University.

Results

The antimicrobial activity of the methanol extracts of *S. verbenaca* and standard comparison antibiotics assessed by the disc diffusion method are given in Table 1. No significant effects were found against *Micrococcus luteus*, *M. flavus*, *Listeria monocytogenes*, and the acid-fast bacterium *Mycobacterium smegmatis*. The methanolic extracts of the plant showed potential antimicrobial activity against the test microorganisms, with inhibition zones at 10.8 to 22.4 mm.

Table 1. Antimicrobial activity of *S. verbenaca* methanol extracts as found by the disc diffusion method

Tested microorganisms	*S. verbenaca* MeOH extract (Diameter of inhibition zones (mm))			Standard antibiotics	
	1.25 mg/mL	2.50 mg/mL	3.75 mg/mL	1 (0.03 mg/mL)	2 (0.10 mg/mL)
Escherichia coli	14.2	15.6	15.8	24.6	NT
Staphylococcus aureus	18.2	20.4	22.4	30.2	NT
Klebsiella pneumoniae	12.6	13.8	14.2	22.8	NT
Micrococcus luteus	-	-	-	34.2	NT
Micrococcus flavus	-	-	-	30.6	NT
Proteus vulgaris	14.6	17.8	20.4	24.4	NT
Pseudomonas aeruginosa	12.2	12.6	13.0	14.6	NT
Listeria monocytogenes	-	-	-	22.2	NT
Mycobacterium smegmatis	-	-	-	20.6	NT
Bacillus cereus	14.2	14.2	15.6	23.4	NT
Bacillus subtilis	13.2	14.4	16.0	26.8	NT
Candida albicans	14.0	16.2	18.2	NT	14.4
Cryptococcus neoformans	11.2	12.8	14.6	NT	17.8
Kluyveromyces fragilis	10.8	11.2	12.4	NT	16.2
Rhodotorula rubra	14.2	15.6	16.2	NT	14.6

(-): no inhibition zones; NT: Not tested; 1: Chloramphenicol; 2: Amphotericin B

The antimicrobial activity of the aqueous, butanol, and chloroform fractions obtained from the methanol extracts of the plant are presented in Table 2. Antimicrobial activity was not observed in the aqueous fraction against all the tested microorganisms. The diameter of the growth inhibition

area ranged from 10.2 to 17.2 for the BuOH fraction and 10.2 to 15.4 for the $CHCl_3$ fraction. The extracts of the plant showed the highest activity against *Staphylococcus aureus* and *Proteus vulgaris* (inhibition values close to that of Chloramphenicol) and *Candida albicans* and *Rhodotorula rubra* (inhibition values close to that of Amphotericin B). The inhibition zone diameters around the control disc (containing only DMSO) were 0-0.5 mm.

Table 2. Antimicrobial activity of *S. verbenaca* fractions (aqueous, BuOH, and $CHCl_3$) as found by the disc diffusion method

Tested microorganisms/ The plant fractions	**Aqueous fraction**			**BuOH fraction**			**$CHCl_3$ fraction**		
	1.25 mg/mL	**2.50 mg/mL**	**3.75 mg/mL**	**1.25 mg/mL**	**2.50 mg/mL**	**3.75 mg/mL**	**1.25 mg/mL**	**2.50 mg/mL**	**3.75 mg/mL**
Escherichia coli	-	-	-	12.2	13.4	14.6	11.2	12.0	12.0
Staphylococcus aureus	-	-	-	14.6	15.4	17.2	13.2	14.6	15.0
Klebsiella pneumoniae	-	-	-	11.4	12.2	12.6	14.0	13.2	14.2
Proteus vulgaris	-	-	-	13.2	14.6	15.2	13.2	15.2	14.6
Pseudomonas aeruginosa	-	-	-	11.4	12.0	12.4	10.6	11.0	11.2
Bacillus cereus	-	-	-	12.6	14.2	15.0	11.2	12.0	13.2
Bacillus subtilis	-	-	-	12.8	14.6	16.2	12.2	13.0	12.4
Candida albicans	-	-	-	14.0	15.2	17.2	14.2	15.0	15.4
Cryptococcus neoformans	-	-	-	10.2	11.0	11.0	10.2	11.0	11.6
Kluyveromyces fragilis	-	-	-	10.4	10.6	11.0	10.6	11.2	12.6
Rhodotorula rubra	-	-	-	13.6	14.2	15.4	12.4	13.2	14.0

(-): no inhibition zones

Table 3 summarized the MIC values of the active extracts and fractions. These values ranged from 0.03 to 0.34 mg/mL. The highest antibacterial activity was observed in the $CHCl_3$ fraction with particularly low MIC values against *Bacillus subtilis* and *Staphylococcus aureus* (0.03 mg/mL) following against *Bacillus subtilis* (0.05 mg/mL). *Rhodotorula rubra* is susceptible to $CHCl_3$ fraction among the yeasts at MIC values of 0.05 mg/mL. In general, the extracts and fractions obtained from *S. verbenaca* had a potential antimicrobial effect on bacteria especially *Staphylococcus aureus*, *Bacillus subtilis*, *B. cereus* as well as the yeast culture *R. rubra*.

Table 3. Minimum inhibitory concentration as found by the microdilution method

Tested microorganisms	MIC (mg/mL)				
	Extract/Fractions			Standard antibiotics	
	MeOH extract	BuOH fraction	$CHCl_3$ fraction	1	2
Escherichia coli	0.22	0.22	0.18	0.002	NT
Staphylococcus aureus	0.09	0.09	0.03	0.002	NT
Klebsiella pneumoniae	0.29	0.34	0.22	0.002	NT
Proteus vulgaris	0.11	0.09	0.05	0.006	NT
Pseudomonas aeruginosa	0.29	0.34	0.22	0.008	NT
Bacillus cereus	0.09	0.09	0.05	0.0005	NT
Bacillus subtilis	0.09	0.09	0.03	0.0005	NT
Candida albicans	0.09	0.09	0.05	NT	0.008
Cryptococcus neoformans	0.29	0.29	0.29	NT	0.002
Kluyveromyces fragilis	0.29	0.29	0.22	NT	0.006
Rhodotorula rubra	0.11	0.09	0.05	NT	0.006

NT: Not tested; 1: Chloramphenicol; 2: Amphotericin B

Discussion

In this study, the methanol extract, butanol and chloroform fractions obtained from *Salvia verbenaca* L. (Lamiaceae) was investigated by disc diffusion and broth microdilution methods. The extracts have shown potential antimicrobial effects against some bacteria and the yeast cultures tested, with grown inhibition area diameters in the range 10.8 – 22.4 mm, and MIC values between 0.03 and 0.34 µL/mL. When similar previous studies on the antibacterial activity of *S. verbenaca* were examined, Al-Howiriny (2002) investigated the MIC values of the oil obtained from *S. verbenaca* were 2.0 mg/mL against *B. subtilis* and *S. aureus*, and 3.0 mg/mL against *M. smegmatis* but *E. coli* and *P. aeruginosa* were resistant to the oil. Salah et al. (2006) studied the antibacterial activity in the methanolic extract of *S. verbenaca* leaves against *P. aeruginosa* and reported a MIC value greater than 1000 µg/mL. Kamatou et al. (2007) determined the antibacterial effects of methanolic extract of aerial parts of *S. verbenaca* against *E. coli*, *K. pneumoniae*, *B. cereus*, and *S. aureus*, with MIC values of 8, 2, 2, and 3 mg/mL, respectively. Sarac & Ugur (2007) reported the growth inhibitory effect of ethanol extract of *S. verbenaca* against various pathogenic bacteria with

inhibition zones ranging between 9-11 mm. In another study, ethyl acetate extract of *S. verbenaca* from aerial part growth in Algeria was investigated against eight microorganisms. The authors studied the effect of two different concentrations (100 mg/mL and 200 mg/mL) and indicated a proportional effect of *S. verbenaca* ethyl acetate extract concentration. At 200 mg/mL, the inhibitory zone varied between 13 and 16 mm, with a large inhibitory zone was reported against *S. aureus* (16 mm) (Belkhiri et al., 2017). Kabouche and Kabouche (2008) investigated the antibacterial activity of root acetone extract at 128 mg/mL against several strains (Kabouche and Kabouche, 2008). The results indicated that *B. subtilis* (28 mm, MIC=4 µg/mL). *S. aureus* (26 mm, MIC=26 µg/mL) and *Streptococcus haemolyticus* (22 mm, MIC=6 µg/mL) were extremely sensitive to the concentration of 128 mg/mL, while a weak antibacterial effect was reported for *E. coli*, *K. pneumoniae*, *P. mirabilis* and *S. haemolyticus*. In our study, extract and fractions showed predominantly activity against *S. aureus*, *B. cereus* and *B. subtilis* as Gram positive bacteria. Notably, *P. vulgaris* as Gram negative bacterium is susceptible to the extract and fractions. The other Gram-negative bacteria have shown smaller diameters of inhibition zones. The results obtained from this section are similar to the previously the mentioned literature results. Besides, the structure of the cellular wall of Gram-positive bacteria appeared to be more sensitive to the plant extracts compared to the Gram-negative bacteria cellular wall, which is also composed of several layers of peptidoglycan but additionally surrounded by a membrane containing fatty substances and polysaccharides, giving it less permeable characteristics compounds (Kozlowka et al., 2022).

There are limited studies on *S. verbenaca* examining its antifungal activities (Al-Howiriny, 2002; Salah et al., 2006). Al-Howiriny (2002) studied the antifungal activity of essential oil extracted from aerial parts of *S. verbenaca* against Candida albicans and observed a MIC value of 2.0 mg/mL. Salah et al. (2006) investigated the antifungal effect of *S. verbenaca* leaves against *C. albicans* and *Cryptococcus neoformans* and determined MIC values greater than 1000 µg/mL for both strains. In our studies, extract and fractions showed predominantly antifungal effects against *C. albicans* and *R. rubra*. A weaker activity against the other the yeast cultures as *C. neoformans* and *K. fragilis* have determined. Our findings in this section are partially similar to the results of the literature data the mentioned above. According to the findings of Sas-Piotrowska and Piotrowski (2003), the biological activity of plant extracts depends on several factors, and first of all on content of specific chemical compounds and on their ability to diffuse. Besides that, some those compounds may stimulate a pathogen development and increase a degree of contamination and the others can act as inhibition factors differences between action of brew, macerate, decoction and oils probably resulting from possible losses caused by evaporation of the solvent during preparation and the difference in the solubility of the extracted.

Studies on the bioactivity of *S. verbenaca* have shown that it is an important antimicrobial agent depending on different factors such as the extract used, the localization of the plant, the collection time, the part used, the extraction methods, the experiments used and the bioactive

compounds in the plant (Bouyahya et al., 2018). However, the mechanism by which extracts and essential oils are activated is not fully understood. Indeed, different investigations reported that bioactive molecules such as flavonoids belonging to the flavanones subclass showed important antibacterial activity by decreasing biofilm formation and decreasing fatty acid secretion (Song et al., 2020). Moreover, modifying cell morphology and gene expression, increasing cell permeability, and inhibiting the quorum-sensing system are also mechanisms of pathways by which molecules exert their effects on bacteria (Bouyahya et al., 2019). Furthermore, the synergistic effects of the major and the minor phenolic compounds should be taken into consideration.

In conclusion, this study may suggest that various extracts of *S. verbenaca* possess compounds with antimicrobial properties which can be used as antimicrobial agents in new drugs for the therapy of infectious diseases in humans, especially against *S. aureus*, *Bacillus* species, *C. albicans* and *R. rubra*.

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

All authors' contributions are equal for the preparation of research in the manuscript.

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