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Antimicrobial Potential of the Leaves of *Stachys pseudopinardii* on Microorganisms Isolated from Urinary Tract Infections

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Authors' contributions

This work was developed in collaboration by the both authors. Author GD designed the study, did the field work (samples collection), laboratory work. Author BD participated in the literature search, was also involved in some laboratory work. Both authors read and approved the final manuscript.

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ABSTRACT

Purpose: Urinary tract infections are a serious health problem affecting millions of people each year. Therefore, studies for new alternative remedies are necessary. The aim of this study was to investigate the antimicrobial potential of the leaves of *Stachys pseudopinardii* R. Bhattarcharjee & Hub.-Mor. (Lamiaceae) against the pathogens causing complicated urine tract infections.
Methods: The ethanolic extracts obtained from the leaves of *S. pseudopinardii* were investigated for their antimicrobial activities against the pathogens causing complicated urine tract infections (*Enterococcus feacalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans*) by disc diffusion method and microdilution method. Some antibacterial and antifungal antibiotics were used as a positive reference standard to determine the sensitivity of the strains.
Results: The extracts showed antimicrobial activity against *Enterococcus faecalis*, *Proteus*

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mirabilis and *Candida albicans* with inhibition zones of 18.2, 17.4 and 16.0 mm, with MIC's and MBC's or MFC's of 32.0 (64.0), 32.0 (64.0) and 64.0 (128.0) µg/mL, respectively. Also the extracts exhibited moderate activity against the other test microorganisms.

Conclusion: Our findings support the use of *S. pseudopinardii* in traditional medicine for the treatment against the urine tract pathogens. Hence, it is suggested to isolate and identify the active compounds of the plant for novel antimicrobial agents in future.

Keywords: Urinary tract infection; antimicrobial activity; Stachys pseudopinardii.

1. INTRODUCTION

Diseases caused by microorganisms remain one of the major threats to human health. Although a number of natural synthetic antimicrobial agents have been isolated and developed to kill pathogenic micro organisms effectively, global antimicrobial resistance is an increasing public health problem. Various specific plants have continued to be an important therapeutic aid for alleviating the ailments of humankind. Therefore, novel antimicrobial agents from different biological sources are continuously sought [1].

Stachys Vahl. is a genus about 300 species of annual and perennial herbaceous plants and shrubs in the family Lamiaceae. The distribution of the genus covers Europe, Asia, Africa, and Australia [2]. This plant has 90 species (115 taxa) belonging to 13 subsections, 15 sections and 2 subgenera in Turkey. Of the 115 taxa, 54 (47%) are endemic to Turkey [3].

Phytochemical analyses of *Stachys* species have confirmed the occurrence of diterpenes, phenyl ethanoid glycosides, flavonoids and saponines [4]. *Stachys* species have been reported in folk medicine to treat genital tumors, sclerosis of the spleen, inflammatory tumors and cancerous ulcers [5]. Whole plant or leaves of *Stachys* species are used in phytotherapy and said to possess sedative, antispasmodic, diuretic and emmenagogue activities when used as a tea. Some *Stachys* species are used as a tonic and for stomach ailments in Anatolia [6].

Stachys pseudopinardii R. Bhattarcharjee & Hub.-Mor. (Lamiaceae) is endemic to Turkey [7]. During our field excursions, it was determined that this plant has been used as tea for stomach. So, the aim of the present was to screen this endemic plant extracts has been earlier to have antimicrobial activity against the pathogens causing complicated urine tract infections.

2. MATERIALS AND METHODS

2.1 Plant Material

The plant material was collected from Icel, Turkey in August, 2014 and identified by Dr. Gorkem Dulger. A voucher specimen (voucher number GD84) of the plant was deposited in Department of Medical Biology of Duzce University in the author's personal collection.

2.2 Preparation of Extract

The leaves of the plant were dried in an oven at 40°C for 12 h and powdered. Each dry powdered plant material (20 g) was extracted with 150 mL of 95% ethanol (Merck, Darmstadt, Germany) for 24 h using a soxhlet extractor. The extract was filtered with Whatman filter paper no.1, and the filtrate was evaporated under vacuum in a rotary evaporator at 55°C. The extract yield obtained was 12.4%. The dry extract, which was sticky and black, was stored in labeled sterile screw-capped bottles at -20°C pending use. Prior to testing, 2 g was dissolved in 0.4 L of dimethyl sulfoxide (DMSO) (5 mg/mL).

2.3 Microorganisms

Urinary tract pathogens (*Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Candida albicans*) were isolated from the urine of patients diagnosed with urinary infections in Medical Faculty of Canakkale Onsekiz Mart Universirty, Canakkale, Turkey and from Trakya University, Edirne, Turkey. VITEC 2 (bioMerieux, France) system was used for identification.

2.4 Disc Diffusion Method

The paper disc diffusion method was employed [8]. Sterile 6 mm disc filter paper disc (Schleicher & Schul, No. 2668, Dassel, Germany) were impregnated with 50 µL of the plant extract.

The bacterial cultures were inoculated on Nutrient Broth (Oxoid) and incubated for 24 h at 37±0.1°C, while the yeast cultures were inoculated on Malt Extract Broth (Oxoid) and incubated for 48 h at 28.0±0.1°C. Adequate amounts of Mueller Hilton Agar (Oxoid) were dispensed into sterile plates and allowed to solidify under aseptic conditions. The counts of bacterial and yeast cultures were adjusted to yield 10^7-10^8/mL and 10^5-10^6/mL, respectively, using the standard McFarland counting method. The test microorganisms (0.1 mL) were inoculated with a sterile swab on the surface of appropriate solid medium in plates. The agar plates inoculated with the test microorganisms were incubated for 1 h before placing the extract impregnated paper disc on the plates. The bacterial plates were incubated at 37±0.1°C for 24 h while yeast plates were incubated at 28±0.1°C for 48 h. After incubation, all plates were observed for zones of growth inhibition and the diameter of these zones was measured in millimetres. All tests were performed under sterile conditions in duplicate and repeated three times. Penicillin (10 µg/disc) (Oxoid), tobramycin (10 µg/disc) (Oxoid), ampicillin/sulbactam 1:1 (20 µg/disc) (Oxoid), nystatin (30 µg/disc) (Hi-Media), clotrimazole (30 µg/disc) (Abtek biologicals) and ketoconazole (20 µg/disc) (Liofilchem) discs were used as positive controls.

2.5 Microdilution Method

Determination of the minimum inhibitory concentration (MIC) was carried out according to the method described by Zgoda and Porter [9] with some modifications. A dilution series of the extract, ranging from 10 to 0.5 mg/mL, were prepared and then transferred to the broth in 96–well microtitre plates. The final concentrations were in the range 1000 to 50 µg/mL in the medium. Before inoculation of the test organisms, the bacterial and yeast strains were adjusted to 0.5 McFarland and diluted 1:1000 in Mueller Hinton Broth (Oxoid) and Malt Extract Broth (Oxoid), respectively. The plates were incubated at 35°C for 18-24 h for bacteria and 30°C for 48 h for the yeast cultures. All the tests were performed in broth and repeated twice. While the MIC values of the extracts were defined as the lowest concentration that showed no growth, minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by plating samples from clear wells onto Mueller Hinton Agar and Malt Extract Agar, respectively. MBC and MFC were defined as the lowest concentration yielding negative subculture. Reference antibacterial agents of ampicillin, streptomycin as well as reference antifungal agent of nystatin were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin, pH: 8.0; 0.1 mol/mL), DMSO (nystatin), or in water (streptomycin). The stock solutions of the agents were prepared in medium according to the Clinical and Laboratory Standards Institute [10].

3. RESULTS

The antimicrobial activities of *S. pseudopinardii* extracts against the pathogens causing complicated urinary tract infections examined in this study were qualitatively and quantitatively assessed by the presence of inhibition zones, MIC, MBC and MFC (Table 1 and Table 2).

The ethanolic extracts obtained from the leaves of *S. pseudopinardii* were strong antimicrobial activities against the pathogens with inhibition zones at 10.8-18.2 mm. *E. faecalis* and *P. mirabilis* are susceptible to the extracts of the plant as compared to all standard antibacterial

Table 1. Summary of antimicrobial activity of *S. pseudopinardii* and some standard antibiotics

Microorganisms	Inhibition zones (mm)[a]						
	Plant extract	Standard antibiotics					
		P	AMP	TOB	NYS	KETO	CLT
Enterococcus faecalis	18.2	14.0	16.0	18.0	Nt	Nt	Nt
Escherichia coli	10.8	16.0	14.0	10.0	Nt	Nt	Nt
Klebsiella pneumoniae	12.2	18.0	14.0	15.0	Nt	Nt	Nt
Pseudomonas aeruginosa	11.6	8.0	10.0	12.0	Nt	Nt	Nt
Proteus mirabilis	17.4	13.0	16.0	14.0	Nt	Nt	Nt
Candida albicans	16.0	Nt	Nt	Nt	18.0	22.0	16.0

[a] *includes diameter of disc (6 mm); mean value of three independent experiments; Nt = not tested; P = penicillin (10 µg/disc); TOB = tobramycin discs (10 µg/disc); AMP =ampicillin (20 µg/disc); NYS = nystatin discs (30 µg/disc); KETO = ketoconazole (20 µg/disc); CLT =clotrimazole (30 µg/disc)*

Table 2. Minimum inhibitory concentration (MIC) values of the extracts of *S. pseudopinardii*

Microorganisms	MIC (MBC or MFC)			
	Extract (µg/mL)	Standards		
		ST	AMP	NYS
Enterococcus faecalis	32 (64)	2.0 (4.0)	1.0 (4.0)	Nt
Escherichia coli	1000 (1000)	4.0 (4.0)	32 (64)	Nt
Klebsiella pneumoniae	500 (1000)	8.0 (16.0)	8.0 (8.0)	Nt
Pseudomonas aeruginosa	500 (1000)	1.0 (1.0)	16 (32)	Nt
Proteus mirabilis	32 (64)	4.0 (8.0)	0.5 (1.0)	Nt
Candida albicans	64 (128)	Nt	Nt	8.0 (16)

Nt: not tested; ST: Streptomycin, AMP: Ampicillin, NYS: Nystatin

agents. The extracts showed higher antibacterial activity against *P. aeruginosa* than some of the standard antibiotics. The antifungal effect of the extract against *C. albicans* was equivalent to those of the standard antifungal agent clotrimazole.

In the microdilution test, the lowest MICs and MBCs of the extracts were 32 and 64 µg/mL, respectively, against *E. faecalis* and *P. mirabilis*, followed by *C. albicans*, with MIC/MFC of 64/128 µg/mL, respectively. The extracts showed weak antimicrobial activity against the other test microorganisms with MIC/MBC ranging from 1000/1000 to 500/1000 µg/mL. These values were well below those of the standards.

4. DISCUSSION

To the best of our knowledge, the information on antimicrobial activity of *S. pseudopinardii* is inadequate. Furthermore, investigations on antimicrobial activity of the other *Stachys* species are few. In previous study, the antimicrobial activity of the leaves of *S. pseudopinardii* were investigated against some bacteria and the yeast cultures [11]. The extracts showed strong antibacterial activity *B. cereus* ATCC 7064, with an inhibition zone of 25.0 mm, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 16 and 32 µg/mL, respectively. *D. hansenii* DSM 70238 was among the most susceptible of the yeast cultures, with an inhibition zone of 17.0 mm and MIC and minimum fungicidal concentration (MFC) of 32 and 32 µg/mL, respectively. The extracts exhibited moderate activity against the other test microorganisms.

Antimicrobial activity of some endemic *Stachys* species (*S. sivasica, S. anamurensis, S. cydnia, S. aleurites* and *S. pinardii*) was reported before. The methanol extracts of *Stachys* species had antimicrobial activity only against bacteria [12,13]. It was reported that the ethanol extracts of *S. byzantina* was shown not to be effective against *C. albicans* strains [14]. In another study, the methanol extracts of *Stachys byzantina, S. inflata, S. lavandufolia* and *S. laxa* were more active against Gram-positive bacteria but did not show any antifungal effect [15]. In contrast, the essential oil of *S. plumosa* exhibited antimicrobial activity bacteria and *C. albicans* strains [16]. Besides, the essential oil of eight *Stachys* species (*S. alopecuros, S. scardia, S. cretica* subsp. *cretica, S. germenica* subsp. *heidrichii*, *S. recta, S. spinulosa S. euboica* and *S. menthifolia*) were investigated for their antimicrobial activity. The essential oil of *S. scardia* was shown a strong antimicrobial activity against both bacteria and fungi. According to the findings, the essential oil of *Stachys* species have antifungal activity against the yeast cultures, especially *C. albicans*, but the antifungal activity was not observed for the leaf extracts [17]. Notably, the extracts of *S. pseudopinardii* demonstrated antimicrobial activity against the pathogens causing complicated urine tract infections in this study. The differences between our results and those of the other investigations may be due to several factors, for example, the intra-specific variability in the production of secondary metabolites. In addition, there may be differences in the extraction protocols used to recover the active metabolites as well as differences in the assay methods.

Phytochemical analyses of *Stachys* species have confirmed the occurrence of diterpenes, phenyl ethanoid glycosides, flavonoids and saponines [4]. Terpenes or terpenoids are active against bacteria, fungi, viruses and protozoa [18]. Flavonoids have been found in vitro to be effective antimicrobial substances against a wide array of microorganisms. Flavonoids are known to be synthesized by plants in response to microbial infection. Their activity is probably due

to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall. Lipophilic flavonoids may also disrupt microbial membranes [18,19]. Several reports are available in support to antimicrobial activity of plant extracts was due to the presence of saponins. Several reports have shown that the plant saponins posses significant antibiotic, antifungal, antiviral, hepatoprotective, anti-inflammatory and anti-ulcer activities [20].

5. CONCLUSION

Urinary tract infection (UTI) is among the most common infections ranking next to upper respiratory infection with an increasing resistance to antimicrobial agents. These ailments affect patients in all age of groups and sexes. Majority of UTIs are not life threatening and do not cause any irreversible damage. Some of the major contributing factor for high level of urinary tract infection (UTIs) in predominantly areas is poor sanitary conditions and lack of proper hygiene. So, the medicinal plants are the best alternate for treating UTI. This study provides data about the antimicrobial properties of the extracts obtained from *S. pseudopinardii* against the pathogens causing complicated urine tract infections. These extracts may be applied clinically for pathogens. So, this plant extracts should be analyzed further, as it might provide a new compound effective against UTI pathogens. In addition, the findings explain the use of *S. pseudopinardii* in folk medicine for the treatment of various diseases, some related to microbial infections.

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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