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Evaluation of the antibacterial effect of *Stachys schtschegleevii* extracts on some foodborne pathogenic bacteria

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Abstract

In recent decades, demand for alternative treatments, such as medicinal plants, has increased due to antibiotic resistance in bacteria. *Stachys schtschegleevii* (*S. schtschegleevii*) is a botanical remedy utilized in traditional medical practices to address various ailments. This study aimed to assess the antimicrobial activity of *S. schtschegleevii* aqueous, ethanolic, and methanolic extracts against important foodborne pathogen bacteria. The antimicrobial effect of these extracts was investigated against *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Salmonella typhimurium* using the disk diffusion method. Furthermore, the investigation encompassed the determination of the plant extracts' minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against the bacterial strains. The findings indicated that the methanolic extract derived from *S. schtschegleevii* exhibited inhibitory properties against *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes*. The MICs were determined to be 50±0, 100±0, and 200±0 mg/mL, respectively, and the MBCs were 100±0, 100±0, and 400±0 mg/mL, respectively. The aqueous extract was efficacious against *Staphylococcus aureus*; growth inhibition and bactericidal activity showed at 50 and 100 mg/mL concentrations, respectively. The findings revealed that the ethanolic extract demonstrated no significant impact on bacterial growth in all tested concentrations. The disk diffusion method showed that all of the extracts did not affect bacterial growth at any evaluated concentrations, and an inhibitory zone was not formed. According to the findings of this investigation, it has been observed that the selection of solvent employed for the extraction of plants plays a crucial role in determining antibacterial efficacy.

Introduction

Foodborne diseases threaten public health in developed and developing areas, leading to considerable health hazards and economic burdens (1). Foodborne illnesses encompass a spectrum of states, ranging from minor, self-resolving ailments to severe cases of food poisoning (2). Consumption of contaminated foods or beverages primarily attributable to foodborne bacteria is a prominent factor contributing to such illnesses (3).

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In 2010, the World Health Organization (WHO) conducted an extensive assessment of foodborne illnesses on a global scale. The findings highlighted a significant incidence, documenting approximately 600 million cases and 420,000 deaths attributed to these illnesses. The primary contributors to this burden were infectious agents known to cause diarrheal diseases, accounting for the majority, with around 550 million cases. Notably, norovirus emerged as a primary culprit, responsible for approximately 120 million cases, followed by *Campylobacter* spp., which accounted for 96 million cases. Other considerable hazards contributing to foodborne illness included hepatitis A virus, *Ascaris* spp., and *Salmonella typhi* causing 14, 12, and 7.6 million cases, respectively (4). Managing these infectious agents is crucial for ensuring food safety and preventing further outbreaks. In recent decades, bacterial resistance towards conventional chemical antibiotics has increased significantly. To manage this challenge, the exploration and utilization of natural antimicrobials sourced from medicinal plants have emerged as a viable strategy for combating bacterial diseases (5).

Stachys species have been employed in traditional practices due to their medicinal properties. Stachys, a genus of approximately 300 species, is widely distributed across temperate and tropical regions, excluding Australia and New Zealand. There are 34 known species in Iran, 13 of them are endemic (6). In traditional medicine, the leaves and branches of this plant are commonly utilized. The species is primarily found in the Arasbaran region, northwest of Iran. Due to the significant resemblance between *Stachys inflata* (*S. infanta*) and *S. schtschegleevii*, inexperienced practitioners occasionally utilize wrongfully and mistakenly in local markets as *S. schtschegleevii* (7). This botanical specimen, commonly called poulk, has gained widespread usage for its traditional applications in urinary tract disinfection, anti-inflammatory effects, and respiratory tract sanitation (8, 9). Moreover, it possesses additional medicinal attributes such as antibacterial, antimicrobial, anti-asthmatic, anti-sinusitis, anti-cold, and anti-rheumatic properties. It has been named natural penicillin (10-12).

The effect of climate, Seasonal Changes, and geographical location of plant culture on phytochemical properties is significant. Also, the choice of solvent in preparing extracts has a notable impact on its antibacterial effectiveness, because different metabolites may be obtained from different solvents. So the aim of this study was to evaluate the antimicrobial effectiveness of three extracts (aqueous, ethanolic, and methanolic) derived from the *S. schtschegleevii* plant, which were collected from the Northwest region of Iran (East Azarbaijan province), against foodborne pathogenic bacteria., employing broth microdilution and disk diffusion techniques.

Materials and methods

Preparation of extracts

The plant was collected during late spring and identified by the Herbarium of Faculty of Pharmacy, University of Tabriz, Tabriz, Iran, then dehydrated, and aerial parts crushed to obtain a fine powder. Water, ethanol, and methanol were used as solvents to prepare the plant extracts. The ratio of solvents to plant material used for extraction was 10:1. The extract was obtained by using a shaker within 24 h. Shaking and mixing were done repeatedly to speed up the extraction process. The mixture was then filtered by passing through Whatman filter paper. The extract was concentrated using a rotary device at about 50°C and then dried in an oven at the same temperature**.**

Bacterial culture

Five bacterial strains were obtained from the Scientific and Industrial Research Organization of Iran, comprising three gram-positive bacteria (*Listeria monocytogenes* ATCC 19115, *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* PTCC 1015) and two gram-negative bacteria (*Escherichia coli O157:H7* ATCC 10536, *Salmonella typhimurium* ATCC 14028). The bacteria were cultivated in BHI broth at 37°C for 18

h. Dilutions of the bacterial cultures were prepared utilizing the 0.5 McFarland turbidity standard.

Antimicrobial activity of extracts

Disk diffusion method

In order to assess the antimicrobial properties of the extracts by using the disk diffusion method, a bacterial suspension containing 1.5×10^8 cfu/mL was inoculated on Brain Heart Infusion plates. The inoculated plates were dried for 5-10 minutes. Sterile paper disks with a diameter of 6 mm were aseptically placed on the surface of the agar plates using sterile forceps. Subsequently, 20 µl of serially diluted extracts ranging from 1.56 to 400 mg/mL were poured on the sterile paper disks in the agar medium. The inoculated plates were then incubated at 37°C for 24 h. The antimicrobial activity was assessed by measuring the zones of inhibition. Each experimental assay was repeated three times to ensure accuracy (13).

Determination of minimum inhibitory concentration (MIC) of the extracts by broth microdilution method

The MIC of extracts was determined using a broth microdilution assay. Briefly, 96-well microplates with a total volume of 300 µl were utilized, containing BHI broth (95 µL), extract dilution (100 µL), and bacterial samples. Stock solutions of the aqueous, ethanolic, and methanolic extracts were prepared at 400 mg/mL. Subsequently, different concentrations of the extracts were prepared using a serial two-fold dilution method and added to each well. Bacterial inoculum was transferred to each microwell, resulting in a final of 1×10^5 cfu/mL concentration. As a positive control, the same volume of bacterial culture was added to the BHI broth without any extract. The microplate contents were mixed for 2 minutes using a microplate shaker. Following an incubation period of 24 h at 37 °C, visual monitoring of the wells was conducted for turbidity. The MIC was determined as the minimum concentration of the extract that inhibited bacterial growth. The experiments to determine the MIC value were performed in triplicate to ensure reliability and accuracy (13, 14).

Determination of minimum bactericidal concentration (MBC)

To ascertain the MBC, the wells with bacterial growth inhibition were utilized. A sterile swab was saturated with the substance in each well and subsequently transferred onto the surface of BHI agar plates. Following incubation at 37°C for 24 h, the concentration of extract with a growth inhibition rate of 99.9% for bacteria was identified as the MBC. The experiments were conducted in triplicate (14).

Statistical analysis

For statistical data analysis, version 26 of the SPSS software (IBM Corporation, Armonk, NY, USA) was utilized. Significance levels were determined by a p-value of less than 0.05. To examine the relationship between the variables, the Chi-square (X^2) test was employed.

Table 1. MIC and MBC concentrations of aqueous, ethanolic, and methanolic extracts of *S. schtschegleevii* against foodborne bacteria

Bacteria	**MIC and MBC**	**extracts Concentration (mg/mL)**		
		aqueous	**ethanolic**	**methanolic**
Staphylococcus aureus	MIC	50±0	>400	100±0
	MBC	100±0	>400	100±0
Bacillus cereus	MIC	>400	>400	50±0
	MBC	>400	>400	100±0
Listeria monocytogenes	MIC	>400	>400	200±0
	MBC	>400	>400	400±0
Escherichia coli	MIC	>400	>400	>400
	MBC	>400	>400	>400
Salmonella typhimurium	MIC	>400	>400	>400
	MBC	>400	>400	>400

Results

The MIC of *S. schtschegleevii* aqueous, ethanolic, and methanolic extracts was investigated against significant foodborne bacteria using the broth microdilution method and results are shown in Table 1.

MIC and MBC of extracts by broth microdilution method

The methanolic extract of the plant demonstrated the greatest efficacy against *B. cereus*, exhibiting a MIC of 50 mg/mL in the determination of MIC and MBC of extracts by broth microdilution method. Remarkably, the methanolic extract exhibited the highest lethal impact on *S. aureus* and *B. cereus* at a 100 mg/mL concentration. Analysis of results demonstrated that there is a significant relationship between the type of extracts and the inhibition of bacterial growth or bactericidal activity in the MIC and MBC tests, and methanolic extract had a more substantial inhibitory impact on bacterial growth and bactericidal activity in comparison to other extracts. Notably, methanolic extract demonstrated efficacy against all gram-positive bacteria included in this study, as outlined in Table 1.

S. aureus was recognized as the most sensitive bacteria in the relationship between the type of bacteria and inhibition of bacterial growth in the MIC test and bactericidal activity in the MBC determination tests. The growth of *S. aureus* was exclusively hindered by the aqueous extract of this plant, displaying a MIC of 50 mg/mL. This MIC value was impressively lower than the methanolic extract's MIC (100 mg/mL) against these bacteria. The antimicrobial potential of the ethanolic extract was assessed, and it was observed that there was no activity against the tested microorganisms, indicating a lack of antimicrobial effects (Table 1).

Antimicrobial activity of extracts by disk diffusion method

According to the disk diffusion method, none of the tested concentrations of the extracts resulted in the formation of an inhibition zone.

Discussion

The current research aimed to explore the in vitro antimicrobial properties of *S. schtschegleevii*, which is grown in Northwest Iran against various foodborne pathogenic bacteria, both Gram-positive and Gram-negative. Three different solvents were utilized to extract *S. schtschegleevii*, because different metabolites may be obtained from different solvents. The potential of these plant extracts to combat bacteria is significantly influenced by their chemical composition. While some species within the Stachys genus have been recognized for their strong antibacterial effects, others exhibit limited efficacy. These variations can be attributed to factors such as the geographic regions where the plants are grown, differing climatic conditions, and genetic diversity within and among species (15).

Methanol, a polar solvent, plays a crucial role in efficiently extracting a broad spectrum of phytochemicals, including phenolic compounds, flavonoids, and other secondary metabolites known for their antibacterial activities. The enhanced solubility of these bioactive compounds in methanol compared to water is a key factor contributing to the increased antibacterial effectiveness observed in this study (16).

In *S. schtschegleevii* plant, specific phenolic compounds and terpenoids may be pivotal, with the antibacterial action primarily linked to the medium's polarity or the polar compounds like phenolics found in the methanolic extract (7). These compounds are recognized for their ability to disrupt bacterial cell membranes and inhibit vital metabolic processes, particularly effective against Gram-positive bacteria due to their thicker peptidoglycan layers that are more vulnerable to such compounds.

According to a similar study, researchers examined the antimicrobial effects of various extracts derived from *S. schtschegleevii*. The results indicated that the methanolic extract demonstrated the highest

effectiveness compared to the other extracts (n-hexane, dichloromethane) (7).

The methanolic extract exhibited bacteriostatic rather than bactericidal properties. This finding aligns with other studies regarding the bacteriostatic activity of *Stachys glutinosa* (*S. Glutinosa*) and the antibacterial effects of *S. schtschegleevii* (7, 8,18). In contrast, Gram-negative bacteria showed no susceptibility, a finding consistent with several studies; one reason for this lack of effectiveness is the double membrane structure surrounding each bacterial cell (19). The ethanolic extract derived from *S. schtschegleevii* did not exhibit any inhibitory effect even at higher concentrations in our study; this suggests that the ethanolic extract of this species might not possess significant efficacy against bacterial infection, contradicts a study that explored the antibacterial properties of different species within the Stachys genus (6).

Similar to our results, a study reported that the aqueous extract of *S. schtschegleevii* inhibits the growth of *S. aureus* with a MIC of 100 mg/mL. These results indicate the potential of the extract as a promising antibacterial agent specifically targeting this strain of bacteria (20). Furthermore, it was observed that gram-positive bacteria were more susceptible to the herbal extracts than gram-negative bacteria (21).

The bacterium most susceptible to these extracts was *Staphylococcus aureus*, known for causing severe infections in humans and animals, including skin lesions, abscesses, and foodborne illnesses (7, 8, 17). The extracts may lead to cell lysis or impede cell wall synthesis, resulting in bacterial death; this mechanism is typically more pronounced in Gram-positive bacteria due to their structural weaknesses. Sonboli et al. conducted a study investigating compounds of *S. schtschegleevii* essential oil (EO) and the antibacterial effects of this plant. The findings revealed that the EO exhibited moderate activity against the tested Gram-positive bacteria, with *Staphylococcus aureus* being the most sensitive. However, no significant activity was observed against the examined Gram-negative bacteria, except for *Escherichia coli* (7).

In a study the efficacy of dried flowering aerial parts of *Stachys byzantine* (*S. byzantine*), *S. inflata*, *S. lavandulifolia*, and *S. laxa* was assessed for their antimicrobial properties using the disc diffusion method and determination of the MIC values. Mainly, the methanol extracts exhibited higher effectiveness. Similar to our findings, the disc diffusion method did not produce any inhibition zones. In addition, phytochemical analysis of the plant in this study revealed the presence of flavonoids in the aerial parts of the Stachys genus, which could contribute to their antibacterial activity (22). This outcome suggests that the extracts may lack effective antimicrobial compounds or that the concentrations used were insufficient to inhibit these specific bacterial strains.

Conclusions

The choice of solvent in preparing *S. schtschegleevii* extract has a notable impact on its antibacterial effectiveness. Additional investigations are required to identify the most suitable solvent for extracting the active antibacterial compounds from *S. schtschegleevii*. Nevertheless, the analysis of findings indicates that the methanolic extract of *S. schtschegleevii* demonstrates potential antibacterial Activity which deserves further examination.

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Ethical approval

Not applicable.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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