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In vitro evaluation of antimicrobial activity of Poulk (*Stachys schtschegleevii*) hydroethanolic extract against *Nocardia* isolates

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Abstract

Biofilms represent a fundamental mode of microbial existence, enabling bacteria to adhere to surfaces and persist in challenging environments. *Nocardia species* cause opportunistic infections in humans and animals. This study sought to determine the antimicrobial performance of *Stachys schtschegleevii* hydroethanolic extract in inhibiting biofilm-forming *Nocardia* isolates obtained from the water supply systems of industrial layer pullet farms. Data were organized and classified in Excel, then analyzed using SPSS software (SPSS Inc., Version 23, Chicago, USA). Mean comparisons were carried out, and statistical significance was evaluated via one-way ANOVA with Duncan's post hoc test. Biofilm development was measured using the Tissue Culture Plate assay. To determine the extract's antimicrobial properties, serial dilutions were prepared, with the highest concentration tested at 200 mg mL^{-1}. Phytochemical analysis revealed substantial bioactive constituents, including a total phenolic content of 83 mg GAE mL^{-1}, total flavonoid content of 24 mg QE mL^{-1}, and an antioxidant capacity of 65%. The MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values for the *Nocardia* isolates were quantified. For the S1-2 isolate, there was no MIC and MBC up to 200 mg mL^{-1}. While the isolate S1-6B required 100 mg mL^{-1} as MIC and MBC. Also, for the S1-8 isolate, MIC and MBC were 50 mg mL^{-1}. Additionally, the efficacy of sodium hypochlorite (3 mg L^{-1}) was tested via the agar well diffusion assay. Results demonstrated that the S1-8 strain exhibited resistance to sodium hypochlorite, whereas the S1-2 and S1-6B isolates remained susceptible. These results demonstrate the promising potential of *S. schtschegleevii* extract as a novel natural antimicrobial compound against biofilm-forming *Nocardia* isolates, particularly in settings where disinfection resistance poses a challenge.

Introduction

Water serves as a critical physiological component for maintaining avian health and metabolic functions. The quality of drinking water directly impacts poultry welfare and must be carefully monitored to ensure optimal health outcomes for humans by controlling environmental contamination (1). Within the European Union,

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water quality standards for poultry operations are based on regulations established for human drinking water (2). However, existing legislation does not mandate routine microbial assessment of water distribution systems in commercial poultry facilities (3).

Water supply systems in the poultry industry provide a conducive environment for microbial colonization, leading to biofilm formation on internal surfaces (4). These adherent bacterial communities exhibit considerable resilience, remaining largely unaffected by conventional cleaning and disinfection protocols (5). The limited efficacy of such methods underscores the urgent need for enhanced strategies to mitigate biofilm proliferation (6). The bacteria belonging *Nocardia* genus found as environmental contamination in water supply system of poultry houses comprises a diverse set of aerobic, filamentous actinomycetes capable of forming robust biofilms on both biological surfaces and abiotic materials, significantly enhancing their environmental persistence (7). Biofilm formation represents an essential survival strategy for *Nocardial* species, utilizing biofilms as a critical virulence factor (8).

Chlorine-based disinfectants, traditionally employed at concentrations of 3 mg L^{-1}, have long served as a standard treatment for poultry water lines (9). However, prolonged use of chlorine may contribute to the development of bacterial resistance, frequently coinciding with cross-resistance to antibiotics (10). Current approaches include the application of biocides, antibiotics, and ion coatings. Notably, aromatic plants and their extracts have emerged as promising candidates for promoting poultry health and growth, reflecting a shift toward natural and sustainable solutions (11). Previous research has investigated and proven the antibacterial effects of plant extracts and, in some cases, even compared these effects with common antibiotics (12). The genus *Stachys* is recognized for its capability to generate a vast spectrum of bioactive compounds, which demonstrate significant biological activities (13). Extracts derived from *Stachys* species exhibit antimicrobial, anticoccidial, antioxidant, and anti-inflammatory activities, making them of considerable interest for therapeutic applications (14). In Iran, 34 distinct species have been documented. *Stachys schtschegleevii* (*S. schtschegleevii* or poulk) is notable for its historical use in traditional medicine (15). This work was designed to explore the antimicrobial *S. schtschegleevii* extracts' efficacy on biofilm-forming *Nocardia* spp., isolated from water supply systems as environmental contamination in commercial layer pullet farms.

Materials and Methods

Preparation of S. schtschegleevii Hydroethanolic Extract.

The aerial parts (leaves and flowers) of *S. schtschegleevii* were harvested from the Arasbaran region, Iran. The plant material was dried at ambient temperature under shade (25°C) for 72 hours to preserve thermolabile compounds, followed by manual pulverization into a finely ground powder. We performed the extraction using a maceration technique (cold extraction) to minimize degradation of bioactive constituents. Briefly, the powdered plant material was immersed in 70% hydroethanolic solvent (70% ethanol:30% distilled water, v/v) and maintained at 25°C for 96 hours under continuous agitation to facilitate solute diffusion (16). Following maceration, the crude extract was separated from the residual plant matrix via filtration using sterile filtration fabric. Following filtration, the solution was concentrated via rotary evaporation at 55°C under reduced pressure to remove ethanol, thereby yielding a viscous, solvent-free extract. To ensure stability and prevent photodegradation, the final extract was aliquoted into amber glass vials and refrigerated (4°C) until further phytochemical and antimicrobial analyses (16).

Bioactive Compounds of S. schtschegleevii.

Antioxidant capacity

The antioxidant capacity was determined by assessing its radical scavenging activity against 2,2-

Diphenyl-1-picrylhydrazyl (DPPH) using a JENWAY 6405 UV/Vis spectrophotometer at 517 nm (17). For the assay, 150 µL of each extract was tested, and the antioxidant activity was calculated as a percentage DPPH inhibition, calculated by the equation:

$$\% \text{ inhibition} = (A_C - A_S / A_C) \times 100$$

Where A_C denotes the absorbance of the control (untreated DPPH solution (without sample)) and A_S represents the test sample absorbance (DPPH solution with extract).

Total Phenolic Contents (TPC)

Total phenolic content (TPC) was quantified spectrophotometrically following the Folin-Ciocalteu assay established by Singleton et al. (1999). Briefly, 50 µL aliquots of each extract were analyzed at 765 nm using a JENWAY 6405 UV/Vis spectrophotometer (18). TPC was measured against a gallic acid standard curve and expressed as micrograms of gallic acid equivalents per milliliter of extract (µg GAE mL^{-1} extract).

Total Flavonoid Contents

Total flavonoid content (TFC) was determined using an aluminum chloride colorimetric assay (19). The absorbance was measured at 510 nm on a JENWAY 6405 UV/Vis spectrophotometer using 50 µL extract aliquots. Results were quantified against a quercetin standard curve and denoted as micrograms of quercetin equivalents per milliliter of extract (µg QE mL^{-1} extract).

Microbial Strains and Culture Conditions.

A 20-mL amount of water was aseptically collected into sterile sampling containers from 5 different locations, including the farm's main water tank and 4 different halls of a commercial laying pullet poultry farm's water distribution systems for microbiological analysis. In order to isolate bacteria in water, in the microbiology laboratory, after homogenizing the water sample, one mL of it was added to the laboratory culture medium. Isolates were cultured on Brain Heart Infusion agar (BHI; Merck, Germany) (20) and cultured at 37°C aerobically for 48–72 hours. Pure cultures were obtained through successive streak plating and subjected to comprehensive phenotypic characterization.

Initial identification involved microscopic examination following Gram staining, revealing Gram-positive, but often appear beaded or irregularly stained, filamentous, branching rods. Colonial morphology assessment demonstrated 0.5–1.2 µm wide, 5–20 µm long bacilli. The mentioned isolates were further characterized through standardized biochemical assays. Oxidase activity, was tested using tetramethyl-p-phenylenediamine reagent (Merck, Germany), with negative results indicated by absence of purple coloration within 30 seconds. Catalase production, was evaluated by mixing colonies with 3% hydrogen peroxide (Merck, Germany), with positive reactions showing bubble formation. Carbohydrate metabolism, was assessed in Oxidative-Fermentative medium (Merck, Germany) supplemented with 1% glucose. The ability to form biofilms was subsequently evaluated using a standardized microtiter plate assay (8).

Assessment of Biofilm Production Using the Tissue Culture Plate (TCP) Method.

Bacterial colonies isolated on BHI Agar medium were inoculated into Tryptic Soy Broth (TSB; Merck, Germany) and incubated aerobically at 37°C for 24 h. The resulting cultures were standardized to an optical density of 0.5 McFarland (~1.5×10^8 CFU/mL) at 600 nm. Aliquots (20 µL) of the standardized suspension were dispensed into 96-well polystyrene microplates containing 180 µL fresh TSB per well (final volume: 200 µL/well). The plates were incubated statically at 37°C for 24 h to facilitate biofilm formation. Six wells contained only 180 µL sterile TSB and served as negative controls. All experimental conditions were performed in triplicate. Following incubation, biofilm quantification was performed (21). After 24 hours of static incubation, 96-well microplates were emptied by gently aspirating the culture medium from each well. Adhered biofilms were rinsed twice with 200 µL of sterile PBS for the removal of non-adherent cells. Biofilm fixation was achieved by

adding 200 µL of absolute methanol (99%) to each well for 15 minutes at ambient temperature (25 ± 2°C). After methanol removal, plates were air-dried for 60 minutes under laminar flow conditions. Total biofilm biomass was assessed by staining with crystal violet and measuring the solubilized dye spectrophotometrically. Each well received 200 µL of 1% (w/v) crystal violet solution (Neutron Pharmachemical Co., C.I.) for 15 minutes at room temperature. Residual stain was removed by triple-washing with distilled water (200 µL per wash). Bound stain was solubilized with 200 µL of 30% glacial acetic acid (Merck, Germany) for 30 minutes at room temperature. Optical density measurements were performed at 570 nm using a microplate reader (Hyperion MPR4+ ELISA reader) (21). Biofilm formation was categorized based on the absorbance ratio as average optical density (AOD) of test wells to control (TSB-only wells): Non-biofilm producer ($AOD \leq 1.0$), Weak producer ($1.0 < AOD \leq 2.0$), Moderate producer ($2.0 < AOD \leq 4.0$), Strong producer ($AOD > 4.0$). All assays were performed in triplicate, with six negative control wells included per plate (21).

Assessment of Minimum Inhibitory Concentration (MIC) and Bactericidal Activity of Stachys schtschegleevii Extract Against Nocardia Isolates.

To quantify the minimum inhibitory concentration, 100 µL of Mueller-Hinton Broth (MHB; Merck, Germany) was dispensed into all wells of the microplate except the final well. Serial two-fold dilutions of *S. schtschegleevii* extract were prepared as the antimicrobial agent. The terminal well contained exclusively 100 µL of undiluted *S. schtschegleevii* extract, whereas the initial well comprised 100 µL of culture medium combined with 100 µL of *S. schtschegleevii* extract, establishing a dilution series spanning wells 1 through 9 (concentration range: 200 mg mL^{-1} to 0.78 mg mL^{-1}). Following completion of the dilution series, 100 µL was carefully removed from well 9 to standardize the volume across all experimental wells to 100 µL.

Well 10 functioned as the positive control, containing both culture medium and bacterial inoculum. Well 11 served as the medium control, comprising culture medium alone without extract or bacteria. Well 12 (the terminal well) contained solely *S. schtschegleevii* extract without medium or bacterial suspension, thereby serving as the extract control.

Subsequently, 10 µL of standardized bacterial suspension (0.5 McFarland; 1.5×10^8 CFU mL^{-1}) of *Nocardia* isolates was inoculated into all experimental wells, excluding the control wells (10 and 11). This was followed by the addition of 20 µL resazurin indicator solution (SIGMA-ALDRICH) to each well, producing an initial blue coloration. The microplate was then subjected to incubation at 37°C for 24 hours, after which the chromatic transition in each well was evaluated. Bacterial metabolic activity induced a pH-dependent reaction, manifested by a distinct colorimetric shift from blue to red, indicative of microbial viability.

The agar well diffusion and agar spot inoculation methods were used to assess the growth inhibition of pathogenic microorganisms. Wells identified during MIC determination, along with adjacent wells, were selected for this analysis. To enhance experimental reliability and facilitate observation of bacterial growth inhibition and destruction, an extended series of wells was examined and spot-inoculated on a single agar plate. Specifically, 2 µL of bacterial culture suspension mixed with *S. schtschegleevii* extract was inoculated onto Brain Heart Infusion agar (BHI; Merck, Germany) plates by agar spot inoculation method. Following cooling to room temperature, the plates were cultured aerobically at 37°C for 24 hours. Subsequent evaluation of bacterial growth presence or absence was conducted to determine antimicrobial effectiveness (22).

Evaluation of the Effect of Sodium Hypochlorite on Nocardia Species under in Vitro Conditions

A bacterial suspension equivalent to 0.5 McFarland standard was prepared from overnight cultures (24 h) of *Nocardia* isolates. The standardized

suspension was aseptically swabbed onto the entire surface of Mueller-Hinton agar plates (5 mm thickness) that had been pre-prepared with a 6 mm central well. Thirty microliters (30 µL) of sodium hypochlorite solution (3 mg L^{-1}) was dispensed into the central well of each inoculated plate. All plates were then incubated aerobically at 37°C for 24 hours. Following incubation, the formation of inhibition zones around the central wells was visually assessed, and their diameters were measured using calibrated calipers for each test isolate (23).

Statistical Analysis.

A completely randomized design was used to investigate the effects of various concentrations of *S. schtschegleevii* hydroethanolic extract on *Nocardia* species. Data were organized and classified in Excel, then analyzed using SPSS software (SPSS Inc., Version 23, Chicago, USA). Mean comparisons were carried out, and statistical significance was evaluated via one-way ANOVA with Duncan's post hoc test. Means and standard errors were calculated for all treatments, and all experiments were performed in triplicate (n=3).

Results

Bioactive Compounds of S. schtschegleevii *Hydroethanolic Extract*

The results of the bioactive compounds of the *S. schtschegleevii* hydroethanolic extract are presented in Table 1. These include the antioxidant capacity, total flavonoid content, and total phenolic content of the hydroethanolic extract.

Confirmation of Biofilm Formation by Nocardia Species Using TCP assay

The biofilm production capacity of *Nocardia* isolates was quantitatively assessed by measuring optical density (OD) values in the tissue culture plate assay. Isolate S1-2 demonstrated moderate biofilm-forming ability with a mean ELISA titer of 0.448, which demonstrated significantly elevated levels relative to the control well (*p=0.041*). In contrast, isolates S1-6B and S1-8 showed weak biofilm production with an average optical density of 0.116 and 0.115, respectively. These quantitative results (summarized in Table 1) indicate significant variation in biofilm production capacity among the tested *Nocardia* isolates.

Table 1. (A) Determination of Total Phenolic Compounds, Flavonoid Content, and Antioxidant Potential in *S. schtschegleevii* Extract. (B) Assessment of Biofilm Formation in *Nocardia* Isolates Using the TCP assay

(A) Scientific name	Total phenolics (mg GAE mL^{-1})	Flavonoids (mg QE mL^{-1})	Antioxidant capacity (%)
Stachys schtschegleevii	83 ± 2.6	24 ± 3.5	65 ± 2.5

(B) Samples	S1-2			S1-6B			S1-8		
Optical Density Titer*	0.297	0.402	0.647	0.098	0.142	0.108	0.086	0.084	0.177
Average titer	0.448			0.116			0.115		
Control	0.086			0.086			0.086		
Difference with control	0.362			0.030			0.029		
Interpretation	Strong			Weak			Weak		

*ELISA absorbance measurements were recorded at 570 nm wavelength

Assessment of Minimum Inhibitory Concentration (MIC) and Bactericidal Activity of Stachys schtschegleevii Extract against Nocardia Isolates.

As shown in Table 2, the hydroethanolic extract used in this study showed minimum inhibitory concentrations (MIC) ranging from 50 to 100 mg mL^{-1} toward two *Nocardia* isolates and no MIC against one of the isolates.

Table 2. Assessment of inhibitory and bactericidal concentrations of *S. schtschegleevii* extract toward *Nocardia* isolates and morphological and enzymatic properties of *Nocardia* spp. isolation and evaluation of sodium hypochlorite (3 mg L^{-1}) antimicrobial efficacy.

		Concentration gradient of hydroethanolic *Stachys schtschegleevii* extract (mg mL^{-1})									Control wells			Morphological and enzymatic properties of *Nocardia* spp. Isolates and evaluation of sodium hypochlorite (3 mg L^{-1}) antimicrobial efficacy						
Sample location	Test type	200	100	50	25	12.5	6.25	3.125	1.56	0.78	Bacterial control	Culture medium	Extract control	Catalase	Oxidase	OF*	Morphology	Genus	NaClO (3 mg L^{-1}) Inhibitory zone (mm)	Biofilm**
		1	2	3	4	5	6	7	8	9	10	11	12							
S1-2	MIC	+	+	+	+	+	+	+	+	+	+	-	-	+	-	OX	Coccobacilli	*Nocardia*	11	+
	MBC	+	+	+	+	+	+	+	+	+										
S1-6B	MIC	-	-	+	+	+	+	+	+	+	+	-	-	+	-	OX	Coccobacilli	*Nocardia*	10	+(W)
	MBC	-	-	+	+	+	+	+	+	+										
S1-8	MIC	-	-	-	+	+	+	+	+	+	+	-	-	+	-	OX	Coccobacilli/Co	*Nocardia*	0	+(W)
	MBC	-	-	-	+	+	+	+	+	+										

*Oxidative-Fermentative Test

** The ability to form biofilm (W: Weak)

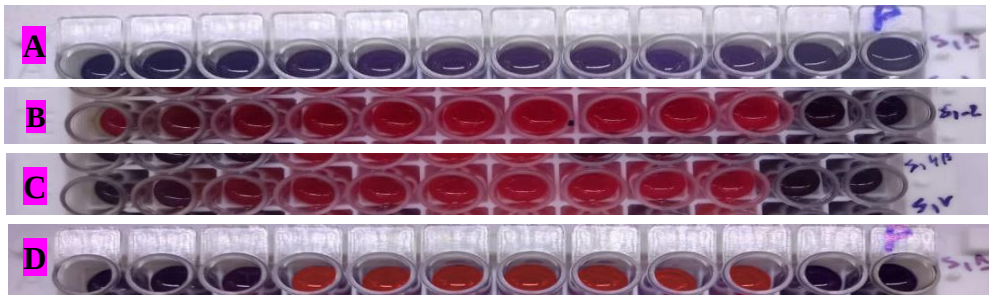

Fig. 1. Determination of Minimum Inhibitory Concentration (MIC) Using Resazurin Indicator (A) Uninoculated control wells (baseline measurement); (B-D) Post-incubation MIC determination results for clinical isolates: (B) S1-2 (C) S1-6B (D) S1-8.

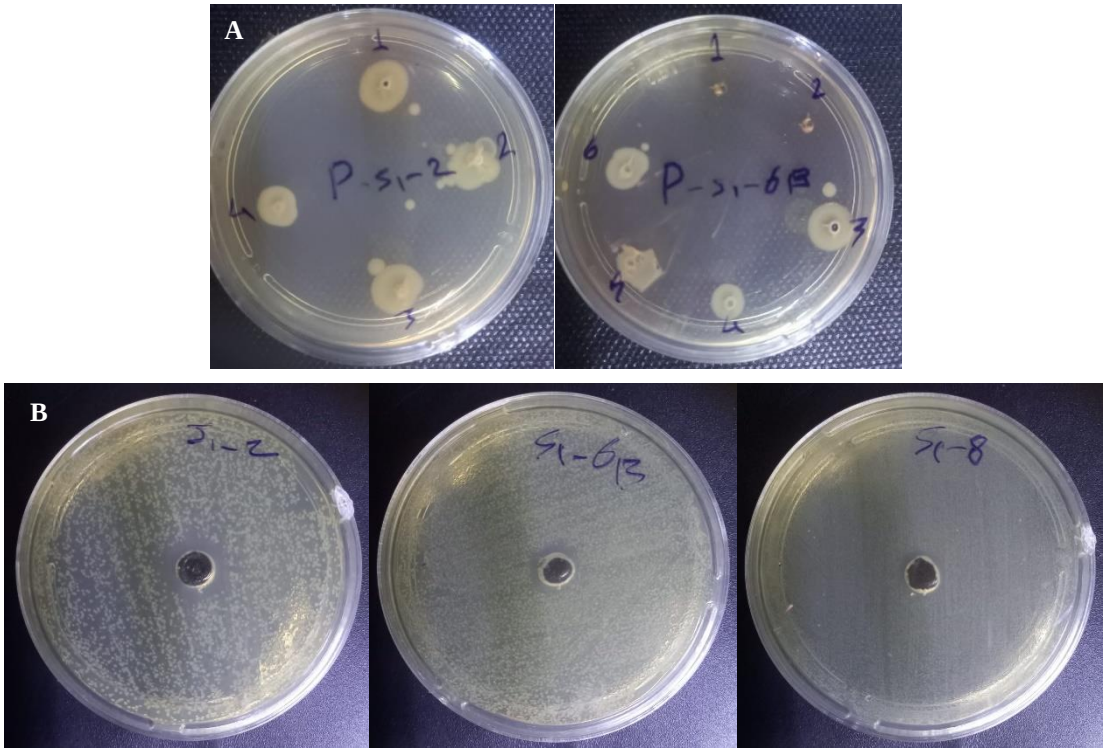

Fig. 2. (A) To improve the accuracy of assessing bacterial growth inhibition and bactericidal effects, aliquots were aseptically transferred from selected wells and subcultured onto fresh microplates using sterile inoculation needles. The minimum bactericidal concentrations (MBC) were determined for the following isolates: S1-2 & S1-6B. (B) Antimicrobial susceptibility testing by agar well diffusion method: Sodium hypochlorite activity against *Nocardia* spp. (isolates S1-2, S1-6B and S1-8).

Analysis of Figures 1 and 2(A) and Table 2 reveals that the *S. schtschegleevii* extract exhibited the following Minimum Bactericidal Concentration (MBC) against *Nocardia* strains: 100 mg mL^{-1} for S1-6B, and 50 mg mL^{-1} for S1-8, and no MBC activity against strain S1-2. These results demonstrate strain-dependent variability in susceptibility to the extract.

In vitro Evaluation of Sodium Hypochlorite (3 mg L^{-1}) Activity against Nocardia Isolates

The in vitro assessment of sodium hypochlorite (NaClO) susceptibility revealed significant strain-dependent variation among the *Nocardia* isolates. Isolates S1-2 and S1-6B demonstrated the highest sensitivity to 3 mg L^{-1} NaClO, exhibiting distinct growth inhibition zones of 11 and 10 mm diameter, respectively. In contrast, isolate S1-8 displayed complete resistance to the same concentration of sodium hypochlorite, showing no measurable growth inhibition (Table 2). This dichotomous response pattern suggests fundamental differences in oxidative stress defense mechanisms among these clinically relevant strains, potentially reflecting variations in their membrane composition or antioxidant capacity. The observed resistance in one isolate raises important clinical considerations regarding disinfection protocols for *Nocardia* contamination.

Discussion

Based on previous studies demonstrating the toxic effects of methanolic extracts, the hydroethanolic extract was selected as a safer alternative. This product shows potential for various industrial applications, including poultry farming and food processing industries, due to its favorable safety profile. The antimicrobial properties of the hydroethanolic extract of *S. schtschegleevii* were assessed against various isolates of *Nocardia* using the MIC and MBC assays. Research on various species of the *Stachys* genus has revealed potent antibacterial properties effective in targeting a diverse array of Gram-positive and Gram-negative bacteria. Data from this study corroborate previous work demonstrating the antibacterial effects of *Stachys* extracts on Gram-positive bacterium, caused by the plant's phenolic and flavonoid constituents, specifically, the hydroethanolic extract of *S. schtschegleevii* has been identified as a rich source of bioactive compounds with significant antimicrobial activity. This extract contains diverse phytochemicals, including flavonoids, terpenoids, and other secondary metabolites that exert antibacterial effects through multiple mechanisms. Its antibacterial activity is revealed in Gram-positive organisms like *Corynebacterium* (24). Additionally, the extract interferes with critical metabolic pathways by inhibiting key enzymes such as catalase and peroxidase, thereby disrupting bacterial energy production (25). The flavonoid components further contribute to antibacterial activity by inducing oxidative stress through the production of reactive oxygen species, leading to structural and functional damage to bacterial cells (26). These collective mechanisms - membrane disruption, metabolic interference, and oxidative stress induction - make *S. schtschegleevii* extract particularly effective against pathogenic bacteria, including *Nocardia* species.

Various studies have reported the detection of *Nocardia* species from environmental and clinical sources. Recent research by Kim et al. (2023) highlights that pathogenic species are frequently associated with both opportunistic infections in immunocompromised humans and zoonotic transmissions from animals. (27). In this research, a total of 24 isolates from various genera including *Nocardia*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, etc. were obtained, with the current study focusing specifically on *Nocardia* isolates.

The MIC results are consistent with those of Sarker et al. (2007), with observed variations likely attributable to differences in the bacterial strains examined. Notably, the hydroethanolic extract demonstrated a superior safety profile compared to methanolic extracts, making it suitable for applications in human, veterinary, and poultry products (28). In a related study, the MIC of *S. schtschegleevii* methanolic extract, assessed via resazurin microtiter assay, ranged from 1.56 to 6.25 mg mL^{-1} (28). Another investigation evaluating methanolic extracts of various *Stachys* species reported MIC values of 1.25–10 mg mL^{-1} (29), consistent with those reported by Sarker et al. (2007). Discrepancies between these studies and our results may stem from differences in plant

species or extraction methods. However, as noted by Jassbi et al. (2013), some extracts showed no antibacterial activity against certain bacterial strains, failing to inhibit growth altogether (29). No other studies were found that specifically examined the effect of *S. schtschegleevii* extract on *Nocardia* invitro. Comparative analysis with previous studies shows that methanolic extracts from various *Stachys* species had MIC values ranging from 1.25 to 10 mg mL^{-1} (29), consistent with findings by Sarker et al. (2007). Also, other researches have revealed 0.071 to 0.637 mg mL^{-1} MIC values for ethanolic extracts (30). The observed differences between these studies and our current results may be attributed to: (1) interspecies variation among the studied *Stachys* plants, and (2) differential antimicrobial activity between methanolic and hydroethanolic extraction methods. Notably, Jassbi et al. (2013) reported a complete absence of antibacterial activity in some extracts against certain bacterial strains (29), highlighting the importance of extract composition and target organism specificity in antimicrobial studies.

The current study provides compelling evidence that the three identified isolates represent distinct bacterial strains, based on significant variations in their phenotypic characteristics. Quantitative analysis revealed substantial differences in antimicrobial susceptibility patterns, with MIC and MBC varying considerably among isolates. Furthermore, the strains exhibited markedly different biofilm-forming capabilities, as quantified through optical density measurements. Perhaps most notably, the isolates demonstrated heterogeneous responses to oxidative stress, showing variable growth inhibition zones when exposed to 3 mg L^{-1} sodium hypochlorite (NaClO). These comprehensive phenotypic profiles strongly suggest that the isolates constitute genetically distinct variants, potentially corresponding to previously characterized strains reported in the literature. The observed spectrum of antimicrobial resistance patterns, biofilm production capacity, and stress response characteristics not only confirms strain-level differentiation but also underscores the importance of such detailed phenotypic characterization in clinical microbiology studies.

The primary objective of comparing the antibacterial properties of *S. schtschegleevii* hydroethanolic extract with sodium hypochlorite (NaClO) was to assess its efficacy as a plant-based substitute for commercial disinfectants. Due to the non-equivalent concentrations of active compounds - a crucial factor for valid comparative assessment of inhibitory effects - the zone of inhibition was not evaluated for the plant extract. Instead, inhibition zone diameters were exclusively measured to determine *Nocardia* isolates resistance to 3 mg L^{-1} NaClO.

Our findings revealed significant strain-dependent susceptibility patterns. Isolates S1-2 and S1-6B showed greater sensitivity to chlorine antimicrobial agent, demonstrating a 11 and 10 mm inhibition zone with NaClO, respectively. Conversely, MIC/MBC values of the *S. schtschegleevii* extract for complete inactivation for tested isolates showed a reverse trend, and the extract had no effect on S1-2, but the MIC was 100 mg mL^{-1} for S1-6B isolate. Conversely, isolate S1-8 exhibited marked resistance to the chlorine antimicrobial agent, showing no inhibition zone (0 mm) against NaClO, but required lower concentrations of the plant extract for inhibition in MIC/MBC assays.

These results demonstrate that the hydroethanolic extract of *S. schtschegleevii* can achieve comparable disinfection efficacy to standard sodium hypochlorite treatment against *Nocardia* species, while offering three distinct advantages: enhanced safety for consumers, oral administration potential, and additional beneficial properties beyond the studied antibacterial effects.

The different resistance patterns observed against both antimicrobial agents suggest possible dissimilar mechanisms of bacterial resistance that warrant further investigation. Importantly, the plant extract's multifunctional nature and safety profile position it as a promising candidate for

development as a natural disinfectant or therapeutic agent, particularly for applications where chemical disinfectants may be contraindicated.

Conclusion

In conclusion, the replacement of hazardous chemical compounds with organic plant-based alternatives has demonstrated significantly enhanced inhibition of biofilm-forming bacteria, proving more effective than conventional disinfection methods. This approach not only protects consumer health from the adverse effects of chemical disinfectants but also eliminates risks associated with residual contamination. Data from this study indicated that the hydroethanolic extract of the medicinal plant *S. schtschegleevii* exhibits potent antibacterial activity against biofilm-forming *Nocardia* species, displaying strong bactericidal properties against these bacteria. Future studies are expected to identify and characterize the specific roles of individual components in the antibacterial effects and more precisely determine the active compounds present in the *S. schtschegleevii* plant extract. Our findings suggest promising potential for developing natural, plant-based alternatives to traditional antimicrobial agents, particularly for applications requiring both efficacy and safety. The demonstrated bactericidal activity against biofilm-forming pathogens positions this botanical extract as a viable candidate for further development into standardized antimicrobial formulations.

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Conflict of Interest Statement

No potential conflicts of interest were declared by the authors. All research procedures were carried out in compliance with ethical and professional guidelines, with measures implemented to mitigate potential biases.

Ethical approval

This study evaluated the antimicrobial activity of *Stachys schtschegleevii* hydroethanolic extract against *Nocardia* isolates in vitro. All experimental procedures and methods conducted in this study were performed according to the institutional ethical guidelines in research.

Artificial Intelligence Statement

During the preparation of this work, the authors used artificial intelligence-based websites to improve readability and language and reduce grammatical errors. After using this tool, the authors reviewed and edited the content thoroughly and take full responsibility for the manuscript's integrity.

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