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Preliminary Screening of Conjunctivitis-Associated Beta Hemolytic Strains of *Staphylococcus aureus* and Their Resistance Against Different Antibacterial Agents

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Abstract

Conjunctivitis is one of the most common ocular infections encountered in clinical and hospital settings, particularly among children under seven years of age. This study aimed to evaluate the antimicrobial susceptibility of ophthalmic isolates of *Staphylococcus aureus* to different antibacterial agents and to compare the effectiveness of antibiotics, plant extracts, and green-synthesized silver nanoparticles (Ag-NPs). A cross-sectional study was conducted from June 2024 to June 2025. Clinical samples were collected from Fatima Memorial Hospital, Lahore, India, and cultured on Mannitol Salt Agar to isolate *S. aureus.* Isolates exhibiting β-hemolysis on blood agar were selected and subjected to antimicrobial susceptibility testing against selected antibiotics, plant extracts, lactic acid bacteria (LAB), and Ag-NPs. Four isolates demonstrated β-hemolysis, confirming pathogenic *S. aureus* association with ocular infections. Among the antibiotics tested, strain 3 showed the highest susceptibility to levofloxacin, with an inhibition zone of 22±0.06 mm. In contrast, *Syzygium aromaticum* extract showed significant antibacterial activities, with inhibition zones of 12.88±0.86 mm and 11.6±0.68 mm for strains 1 and 2, respectively, while strain 4 exhibited lower susceptibility. The LAB also demonstrated antibacterial activities, although the inhibitory effect was gradual. Ophthalmic isolates of *Staphylococcus aureus* exhibit resistance to several commonly used antibiotics. Plant extracts and green-synthesized Ag-NPs demonstrate considerable antibacterial activities, suggesting their potential as alternative therapeutic agents for ocular infections.

Keywords: Antibiotics, corneal infections, hemolysis, mannitol salt agar (MSA), *staphylococcus aureus*

Introduction

Conjunctivitis is one of the most common ocular infections encountered in both community and healthcare settings worldwide.[1] It is characterized by inflammation of the conjunctiva, the transparent membrane covering the sclera and the inner surface of the eyelids.[2,3] Clinical manifestations include redness, irritation, itching, excessive tearing, discharge, and swelling of the conjunctival tissues.[4] Although conjunctivitis is generally considered a self-limiting condition, untreated or severe bacterial infections may lead to complications such as keratitis, corneal damage, visual impairment, and, in rare cases, systemic dissemination of infection. Therefore, prompt identification of causative pathogens and effective antimicrobial treatment remain important for disease management.[5]

Several bacterial species have been implicated in conjunctivitis, including *Staphylococcus aureus*, coagulase-negative staphylococci, *Streptococcus pneumoniae*, and *Haemophilus influenzae.*[6] Among these pathogens, *S. aureus* is one of the leading causes of bacterial conjunctivitis in adults and is frequently associated with recurrent ocular infections.[7] Its ability to produce virulence factors, including hemolysins, toxins, adhesins, and biofilms, contributes substantially to its pathogenicity and persistence in ocular tissues. Furthermore,

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approximately 20–30% of healthy individuals are asymptomatic carriers of *S. aureus*, providing a potential reservoir for recurrent infections and community transmission.[8, 9]

The emergence and rapid spread of antimicrobial resistance among *S. aureus* strains have become a major global public health concern. Excessive and inappropriate use of antibiotics has accelerated the development of resistant bacterial populations, reducing the effectiveness of many commonly prescribed antimicrobial agents.[10] In ophthalmology, antibiotic resistance poses a particular challenge because topical antibiotics are frequently used for the empirical treatment of conjunctivitis and other ocular infections.[11–13] The increasing prevalence of resistant strains may compromise treatment outcomes, prolong recovery, and increase the risk of complications. Consequently, continuous monitoring of antimicrobial susceptibility patterns in ocular isolates is essential for guiding appropriate therapeutic strategies and improving patient care.[14]

Growing concern regarding antibiotic resistance have stimulated interest in alternative antimicrobial approaches derived from natural and biological sources.[15] Medicinal plants have been used in traditional medicine and are recognized as valuable sources of bioactive compounds with antibacterial, antifungal, anti-inflammatory, and antioxidant properties.[16] Plant extracts obtained from species such as *Syzygium aromaticum* (clove), *Cinnamomum zeylanicum* (cinnamon), *Azadirachta indica* (neem), and *Elettaria cardamomum* (cardamom) have demonstrated antimicrobial activity against a variety of pathogenic microorganisms, including *S. aureus*.[17] These natural products may offer complementary or alternative strategies for combating antibiotic-resistant pathogens. In recent years, green-synthesized silver nanoparticles (AgNPs) have also attracted considerable attention because of their broad-spectrum antimicrobial activity and favorable biocompatibility profile.[18] Similarly, lactic acid bacteria (LAB) produce organic acids, hydrogen peroxide, and bacteriocins that inhibit pathogenic bacteria and have demonstrated antagonistic activity against *S. aureus.*[19] These alternative antimicrobial agents may offer complementary strategies for combating antibiotic-resistant pathogens.

Despite increasing interest in these alternative antimicrobial approaches, comparative studies evaluating conventional antibiotics, plant extracts, green-synthesized silver nanoparticles, and lactic acid bacteria against ocular *S. aureus* isolates remain limited, particularly in Pakistan. Therefore, the present study aimed to isolate and characterize β-hemolytic *S. aureus* strains associated with conjunctivitis and to perform a preliminary comparative evaluation of their susceptibility to conventional antibiotics, aqueous plant extracts, green-synthesized silver nanoparticles, and selected lactic acid bacteria. The findings may provide preliminary evidence regarding potential alternative antimicrobial strategies for managing ocular infections caused by *S. aureus.*

Methods

Clinical ocular swab samples (n=30) were collected from patients presenting with signs and symptoms of conjunctivitis at Fatima Memorial Hospital, Lahore, Pakistan between June 2024 and June 2025. The study was approved by the Institutional Bioethics Committee of Government College University, Lahore (Approval No. 387/ORIC/24). Samples were collected by a qualified ophthalmologist using sterile cotton swabs under aseptic conditions and immediately transported to the Microbiology Laboratory, Government College University, Lahore, for microbiological analysis.

The collected samples were inoculated onto Mannitol Salt Agar (MSA) plates and incubated aerobically at 37°C for 24 h. Colonies exhibiting characteristic yellow pigmentation and mannitol fermentation were selected for further identification. Presumptive isolates were purified by repeated streaking on nutrient agar plates using the quadrant streaking method to obtain single colonies. Identification of *Staphylococcus aureus* was performed using standard microbiological procedures, including Gram staining, catalase testing, and coagulase testing. Catalase positivity was confirmed by visible bubble formation following exposure to 3% hydrogen peroxide. For coagulase testing, 0.5 mL of bacterial suspension was mixed with 0.5 mL of human plasma and incubated at 37°C for 4 h. Clot formation was interpreted as a positive coagulase reaction (Figure 1).

The pathogenic potential of the isolates was assessed using blood agar supplemented with 5% sterile human blood. Following incubation at 37°C for 24 h, hemolytic activity was evaluated by observing the lysis of red blood cells surrounding bacterial colonies. Only β-hemolytic isolates demonstrating complete hemolysis were selected

Figure 1 Pure Colonies of Staphylococcus aureus on Nutrient Agar

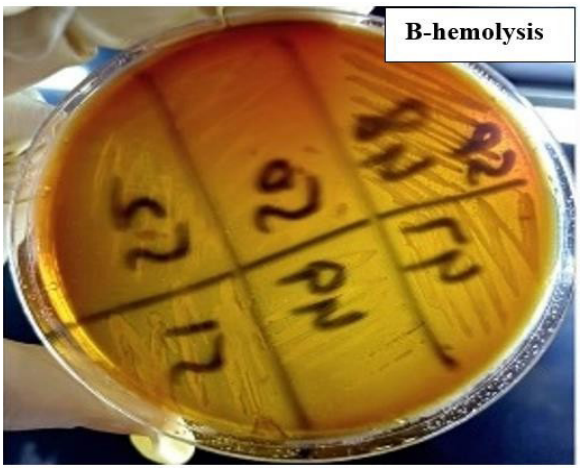

Figure 2 Pathogenicity Test of *Staphylococcus aureus*

for subsequent antimicrobial susceptibility testing. This selection criterion was adopted because β-hemolysin production is considered an important virulence factor associated with increased pathogenicity and tissue invasion in *S. aureus*. Among the 30 ocular samples examined, four isolates fulfilled the criteria of β-hemolysis together with positive catalase and coagulase reactions and were therefore retained for further analysis (Figure 2).

For antimicrobial susceptibility testing, a single colony from each confirmed isolate was inoculated into nutrient broth and incubated overnight at 37°C. Prior to testing, bacterial suspensions were adjusted to a turbidity equivalent to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) to ensure uniform inoculum density. Mueller–Hinton agar plates were inoculated with standardized bacterial suspensions using sterile cotton swabs to obtain confluent bacterial growth. Wells of 6 mm diameter were prepared aseptically, and 50 µL of antibiotic solution was added to each well. The antibiotics evaluated included levofloxacin (5 µg), ciprofloxacin (5 µg), gentamicin (10 µg), and droncef (10 µg). Sterile distilled water served as the negative control. Following incubation at 37°C for 18–24 h, inhibition zones were measured in millimeters using a digital Vernier caliper.

The antibacterial activity of aqueous plant extracts was also evaluated. Dried plant materials of *Syzygium aromaticum* (clove), *Cinnamomum zeylanicum* (cinnamon), *Azadirachta indica* (neem), and *Elettaria cardamomum* (cardamom) were finely powdered. Forty grams of each powdered sample were suspended in 200 mL of sterile distilled water and heated at 50°C for 20 min. The extracts were filtered through Whatman No. 1 filter paper and stored at 4°C until use. The resulting crude extract concentration corresponded to approximately 200 mg/mL. Antibacterial activity was assessed using the agar well diffusion method, in which 50 µL of each extract was introduced into agar wells prepared on inoculated plates. Sterile distilled water served as the negative control.

Silver nanoparticles (Ag-NPs) were synthesized using a green synthesis approach. A 1 mM silver nitrate solution was prepared by dissolving 0.018 g of $AgNO_3$ in 100 mL of deionized water. Plant extracts were mixed with the silver nitrate solution at a ratio of 1:9 and exposed to sunlight until a visible color change was observed, indicating nanoparticle formation. The reaction mixtures were subsequently incubated under dark conditions for 24 h. For antimicrobial testing, 50 µL of the AgNP suspension was added to each agar well, and inhibition zones were measured following incubation at 37°C for 24 h.

Lactic acid bacteria (LAB) were isolated from milk, curd, and pickle samples obtained from local markets. Samples were serially diluted and cultured on de Man–Rogosa–Sharpe (MRS) agar plates, followed by incubation at 37°C for 48h. Distinct colonies were purified and identified by 16S rRNA gene sequencing. The antimicrobial activity of the LAB isolates against pathogenic *S. aureus* was determined using the agar well diffusion method. Genomic DNA from both LAB and pathogenic *S. aureus* isolates was extracted

using the phenol–chloroform extraction method. Amplification of the 16S rRNA gene was performed using universal primers, and PCR products were verified by agarose gel electrophoresis before sequencing.

All antimicrobial susceptibility assays were performed using three independent biological replicates, and inhibition zones were measured in triplicate for each treatment. Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were conducted using GraphPad Prism software. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was applied to compare mean inhibition zone diameters among treatment groups. Because only four pathogenic β-hemolytic isolates were available for analysis, the statistical evaluation was considered exploratory and was not intended for population-level inference. Therefore, statistical findings should be interpreted with caution. A p-value <0.05 was considered statistically significant.

Results

A total of 30 ocular swab samples were analyzed. Four isolates (13.3%) were identified as β-hemolytic, coagulase-positive *Staphylococcus aureus* and were selected for subsequent antimicrobial susceptibility testing. The antibacterial activity of four antibiotics against the isolated *S. aureus* strains is presented in Table 1. All isolates were resistant to gentamicin, with no measurable inhibition zones observed. Levofloxacin demonstrated the greatest antibacterial activity, with inhibition zones ranging from 10.4±0.02 mm to 22.0±0.06 mm. Ciprofloxacin produced inhibition zones ranging from 4.6±0.25 mm to 20.2±0.06 mm. Droncef exhibited limited antibacterial activity, with measurable inhibition observed only in strains 1 and 4 (8.0±0.10 mm and 7.0±0.12 mm, respectively).

Data are presented as mean zone of inhibition (ZOI) ± standard error (SE), expressed in millimeters (mm). R = resistant (no measurable inhibition zone). Susceptibility was interpreted according to CLSI guidelines: susceptible (≥21 mm), intermediate (16–20 mm), and resistant (≤15 mm). Please verify that these breakpoint criteria are applicable to all antibiotics tested. The antibacterial activity of aqueous plant extracts against the isolated *S. aureus* strains is summarized in Table 2.. No antibacterial activity was observed for*Azadirachta indica* against any of the isolates. The inhibition zones for *Elettaria cardamomum* against strains 1, 2 and 3 were recorded as 10.66±0.87 mm, 12.06±0.32 mm and 9.6±0.62 mm respectively while strains 4 was resistant and strain 3 showed the least zone of inhibition. *Cinnamomum zeylanicum* exhibited the highest antibacterial activity against strain 2 with an inhibition zone of 12.34±0.62 mm. For *Syzygium cumini*, the inhibition zones recorded for strains 1–4 were 10.3±1.44 mm, 14.6±0.87 mm, 12.17±0.61 mm, and 12.84±0.7 mm respectively. The aqueous extract of *Syzygium aromaticum* showed inhibition zones of 12.88±0.86 mm, 11.6±0.68 mm, 8.6±0.53 mm, and 8.42±0.22 mm for strains 1–4 respectively, strain 1 exhibited the highest antibacterial activity for *Syzygium aromaticum* with value of 12.88±0.86 mm (Table 2).

The antibacterial activity of green-synthesized silver nanoparticles (AgNPs) against the isolated *Staphylococcus aureus* strains is presented in Table 3. AgNPs synthesized from *Cinnamomum zeylanicum* produced inhibition zones ranging from 8.22±1.68 mm to 12.44±0.32 mm across the four isolates. AgNPs synthesized from *Elettaria cardamomum* exhibited inhibition zones of 9.58±0.64 mm, 11.24±0.44 mm, 8.62±0.24 mm, and 9.24±0.66 mm against strains 1–4, respectively. The largest inhibition zone was observed for AgNPs synthesized from *Syzygium aromaticum*, which produced an inhibition zone of 14.42±0.86 mm against strain 3. In contrast, no

Table 1 Antibacterial Activity of Antibiotics Against Isolated *Staphylococcus aureus* Strains

Strain	Levofloxacin (mm)	Droncef (mm)	Ciprofloxacin (mm)	Gentamycin (mm)
Strain 1	10.4±0.02	8±0.10	20.2±0.06	R
Strain 2	12±0.10	4±0.12	4.6±0.25	R
Strain 3	22±0.06	2±0.11	9.6±0.02	R
Strain 4	18±0.22	7±0.12	5.4±0.25	R

Table 2 Antibacterial Activity of Aqueous Plant Extracts Against Isolated *Staphylococcus aureus* Strains

Bacterial Strains	Plant extracts ZOI (mm)±S. E			
	Cinnamomum Zeylanicum (mm)	Syzygium Aromaticum (mm)	Azadirachta Indica (mm)	Elettaria Cardamomum (mm)
Strain 1	10.58±0.89	12.88±0.86	R	10.66±0.87
Strain 2	12.34±0.62	11.6±0.68	R	12.06±0.32
Strain 3	9.42±0.63	8.6±0.53	R	9.6±0.62
Strain 4	9.68±0.59	8.42±0.22	R	R

S.E=standard error deviation; R=resistance

measurable antibacterial activity was observed for AgNPs synthesized from *Azadirachta indica* against any of the tested isolates.

Statistical analysis demonstrated significant differences in antibacterial activity among the tested lactic acid bacteria (LAB) isolates (one-way ANOVA: $F=23.33$, $R^2=0.9488$, $df=15$, $p<0.0001$). Tukey's post-hoc analysis identified a significant difference between *Limosilactobacillus fermentum* and *Enterococcus faecium* ($p<0.05$). For antibiotic susceptibility testing, one-way ANOVA comparing ZOI values across the four strains showed significant variation for levofloxacin ($p<0.05$) and ciprofloxacin ($p<0.05$), but not for droncef ($p>0.05$). Similarly, significant differences in ZOI were observed among strains for *Syzygium aromaticum* and *Cinnamomum zeylanicum* extracts ($p<0.05$), and for *Syzygium aromaticum*-derived Ag-NPs ($p<0.05$). Results are expressed as mean±standard error of the mean (SEM). Tukey's post-hoc test was used to check statistically significant differences ($p<0.05$) among the groups. The results were stated as mean±standard error of the mean (SEM) in graphical form. The analysis showed a very significant difference with $p<0.0001$, F value of 23.33, R^2 value of 0.9488, and total degrees of freedom (df) equal to 15.

The amplified 16S rRNA gene products of the four *S. aureus* isolates were sequenced, and the resulting sequences were deposited in the National Center for Biotechnology Information (NCBI) database. The accession numbers assigned to the isolates were PX579129, PX588603, PX622975, and PQ565662.

Discussion

The present study evaluated the antimicrobial susceptibility of conjunctivitis-associated β-hemolytic *Staphylococcus aureus* isolates

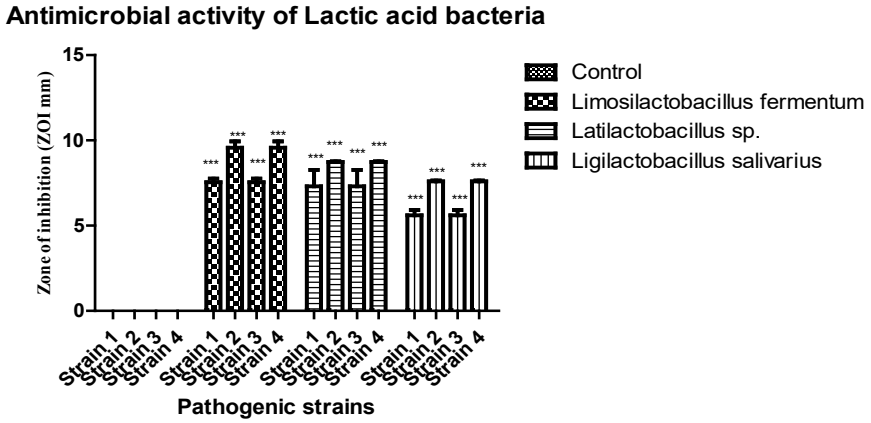

Figure 3 Comparative Antimicrobial Activity of Different Lactic Acid Bacteria Against *Staphylococcus aureus*

against conventional antibiotics, aqueous plant extracts, green-synthesized silver nanoparticles (Ag-NPs), and lactic acid bacteria (LAB). Among the antibiotics tested, levofloxacin demonstrated the greatest antibacterial activity, whereas no measurable inhibition was observed for gentamicin against any of the tested isolates. These findings are generally consistent with previous reports indicating that fluoroquinolones remain among the most active agents against ocular *S. aureus* isolates, while resistance to commonly used antibiotics continues to emerge. [20,21] 1 The absence of gentamicin activity in the present study may suggest reduced susceptibility among the tested isolates; however, the underlying mechanisms could not be determined because molecular resistance analyses were not performed. Given the limited number of isolates examined, these findings should be interpreted as preliminary observations rather than representative estimates of regional antimicrobial resistance patterns.s.

The aqueous plant extracts demonstrated variable antibacterial activity against the tested isolates. *Syzygium aromaticum* exhibited the strongest inhibitory activity, whereas *Azadirachta indica* showed no measurable antibacterial effect. These findings are consistent previous studies reporting antibacterial activity of clove-derived extracts against Gram-positive bacteria.[22]. The antimicrobial activity of clove is primarily attributed to eugenol, a phenolic compound known to disrupt bacterial cell membranes and interfere with enzymatic activity. In contrast, the absence of antibacterial activity observed for *A. indica* differs from reports demonstrating inhibitory effects against methicillin-resistant *S. aureus*.[23,24] Differences in extraction procedures may partly explain this discrepancy, as previous studies frequently used organic solvents that are more effective in extracting bioactive phytochemicals than aqueous preparations.

Green-synthesized silver nanoparticles also exhibited measurable antibacterial activity against the ocular isolates. Among the tested formulations, AgNPs synthesized using *S. aromaticum* extract produced the largest inhibition zones. Similar observations have been reported in previous studies demonstrating the antibacterial potential of plant-mediated AgNPs against pathogenic bacteria.[25, 26] The antibacterial effects of Ag-NPs are believed to result from multiple mechanisms, including disruption of bacterial membranes, generation of reactive oxygen speciesand interference with DNA replication and protein synthesis. The enhanced activity observed in clove-mediated nanoparticles may be attributed to synergistic interactions between silver ions and plant-derived bioactive compounds. Nevertheless, the inhibition zones observed in the present study were smaller than those reported in some previous investigations, which may be due to differences in nanoparticle size, concentration, synthesis conditions, and bacterial strain variability.

All tested LAB isolates demonstrated inhibitory activity against S. aureus, although the observed inhibition zones were relatively modest compared with those of antibiotics and Ag-NPs. The greatest activity was observed for Limosilactobacillus fermentum. These findings are consistent with previous studies reporting antagonistic effects of LAB against ocular and multidrug-resistant S. aureus isolates. Similarly, Chaudhry et al. demonstrated significant antibacterial activity of LAB against multidrug-resistant *S. aureus*.[13] The inhibitory activity of LAB is commonly attributed to the production of organic acids, hydrogen peroxide, and bacteriocins that suppress bacterial growth.22 Although the antibacterial effects observed in this study were modest, LAB may represent a potential complementary approach for future antimicrobial applications.

The novelty of this investigation lies not in the individual antimicrobial agents tested, as similar studies involving plant extracts, Ag-NPs, and LAB have previously been reported, but rather in the comparative evaluation of these approaches against conjunctivitis-associated β-hemolytic *S. aureus* isolates obtained from a clinical setting in Pakistan. Nevertheless, several limitations should be acknowledged. Only four pathogenic isolates were available for analysis, limiting statistical power and generalizability. Furthermore, antimicrobial activity was evaluated solely by agar well diffusion assays, while MIC and MBC determinations were not performed. In addition, resistance gene profiling, biofilm characterization, toxicity assessment, nanoparticle characterization, and mechanistic studies were beyond the scope of this investigation. Future studies involving larger numbers of clinical isolates, detailed molecular characterization, quantitative susceptibility testing, biofilm inhibition assays, and in vivo validation are required to confirm and extend these preliminary findings.

In conclusion, selected plant extracts, green-synthesized silver nanoparticles, and lactic

acid bacteria exhibited measurable in vitro antibacterial activity against conjunctivitis-associated β-hemolytic *S. aureus* isolates. However, given the limited number of isolates and the exploratory nature of the susceptibility testing, these findings should be interpreted cautiously. The present results provide preliminary evidence supporting further investigation of alternative antimicrobial approaches but do not constitute sufficient evidence for therapeutic recommendations. Additional well-designed studies are required before clinical application can be considered.

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