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RESEARCH ARTICLE

Methicillin-Resistant *Staphylococcus aureus*, A One Health Challenge and *Azadirachta Indica* Silver Nanoparticles, A Possible Solution

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ABSTRACT

Pakistan's dairy industry relies heavily on cows and buffaloes for milk production. The industry faces significant infectious disease challenges, including antibiotic-resistant bacterial infections that pose a serious threat to the health and productivity of dairy animals. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of mastitis in dairy cows. In humans MRSA can cause from skin or soft-tissue infections to potentially fatal bloodstream infections. Twenty milk samples were collected from subclinical mastitic cows. Likewise, 20 skin swabs from milkmen were collected. The isolation attempts yielded 36 (20 of animal and 16 of human -origin) *S. aureus* isolates. Kirby-Bauer disc diffusion assay demonstrated significant antibiotic resistance in the *S. aureus* (80% animal and 75% human -derived) isolates. The isolated *S. aureus* were resistant to at least one of the following: Tylosin, Penicillin G, Trimethoprim, Sulfamethoxazole, Oxytetracycline, Gentamicin, and Norflaxacin. The *mec*A gene was detected in 28 (16 animal and 12 human -derived) isolates, suggesting widespread methicillin resistance. As an alternative to antibiotics, green synthesis of silver nanoparticles (AgNPs) using *Azadirachta indica* leaf extract was carried out. The synthesis of silver nanoparticles was optimized by adjusting reaction conditions and the particles were characterized using UV-Vis spectroscopy, FTIR, zeta potential analysis and TEM, confirming their stability and uniform size. The effectiveness of AgNPs against MRSA was evaluated by Time-kill and well diffusion assays. Time-kill assays revealed a dynamic inhibitory effect of biosynthesized AgNPs demonstrating strong antibacterial activity and the well diffusion assay indicated inhibition zones 20.38±6.19 mm. These findings highlight the potential of silver nanoparticles as an alternative to conventional antibiotics, offering a promising approach to combating MRSA in both dairy cows and humans.

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INTRODUCTION

Millions of farmers in Pakistan rely on livestock for their income, jobs, and food security, making it a vital component of the country's agricultural economy and rural livelihoods. The livestock sector supports over 8 million rural families which generates 63.6% of agricultural value addition and contributed 14.97% to the national GDP in 2024–2025. Pakistan's houses an estimated cattle population of 59.7 millions and buffalo population of 47.7 millions that produces over 72 million tonnes of milk, mostly consumed domestically. Therefore, dairy production makes the mainstay of nutrition, economic sustainability, and food security of low-income rural populations of Pakistan. Women and children play a pivotal role in animal husbandry, milk handling, and processing, highlighting the socio-economic significance of the dairy sector in supporting economically vulnerable strata of the population (Anon, 2025).

Bovine mastitis has significant impact on the health and productivity of dairy animals as well as substantial economic implications for the dairy farmers (Corrêa *et al.*, 2024). Bovine mastitis is characterized by inflammation of the mammary gland, that leads to reduced milk production, increased treatment costs, and the early culling of affected animals. Bovine mastitis is caused by a variety of etiological agents including gram-positive and gram-negative bacterial pathogens. Mastitis-causing bacteria can be classified into two subtypes based on origin: contagious or environmental (Lakew *et al*., 2019). Contagious bacteria spreads from cow-to-cow. These bacteria include *Staphylococcus aureus*, *Streptococcus agalactiae*, and Mycoplasma spp. Environmental bacteria are acquired from the farm housing or bedding and serve as opportunistic pathogens. These opportunistic bacteria include *Escherichia coli*, Enterococcus spp., coagulase-negative Staphylococcus and *Streptococcus uberis* (Cheng and Han, 2020).

Staphylococcus aureus is one of the leading causes of bovine mastitis globally, as well as both in Pakistan and Brazil. In Pakistan, bovine mastitis is very common, with subclinical mastitis rates reported at around 50% in cattle and 46% in buffaloes (Khan *et al*., 2019; Yaqoob *et al.,* 2022). Molecular epidemiology studies from Pakistan suggest that *S. aureus* was isolated from 18–20% of animals, with a proportion of isolates carrying the *mec*A gene, indicating the presence of methicillin-resistant strains (Ahmed *et al.,* 2020; Javed *et al.,* 2022). Several risk factors, including inadequate milking hygiene, udder morphology of local cow breeds, and poor farm management practices, contribute to the high prevalence of mastitis in Pakistani dairy herds (Saeed *et al.,* 2021). In Brazil, *S. aureus* is also a significant cause of mastitis. For example, studies across dairy herds in Sao Paulo and Minas Gerais reported *S. aureus* prevalence ranging from 7–71% in bulk tank milk and individual cow samples, including strains producing enterotoxins and showing multidrug resistance (Busanello *et al.,* 2017; Silva *et al.,* 2019). Another study has revealed multiple circulating strains of *S. aureus* with considerable genetic diversity in Brazilian dairy herds (Oliveira *et al.,* 2018).

Antibiotic treatments for mastitis-associated infections are rapidly losing their efficacy owing to the development of multidrug resistance in several bacterial species, necessitating the investigation of novel alternative agents with distinct modes of action (Soltani *et al*., 2021). Antibiotic resistance not only complicates treatment protocols and prolongs the duration of infection but also poses a considerable public health risk through direct contact with infected animals during milking or veterinary practices or potential transmission via the food chain (Ferraz, 2024). Among mastitis-causing pathogens, *Staphylococcus aureus* is of particular concern due to the emergence of methicillin resistance. Methicillin-resistant. *S. aureus* (MRSA) in dairy herds exacerbates mastitis management challenges owing to its heightened resistance to conventional antibiotic therapies (Schnitt and Tenhagen, 2020). Notably, MRSA has been isolated from cows with subclinical mastitis as well as from humans in close contact with infected animals, with bovine and human strains found to be phenotypically and genotypically indistinguishable, indicating cross-species transmission and underscoring the need for a One Health approach to solve this problem (Juhász-Kaszanyitzky *et al*., 2007). Therefore, the pathogenic significance of MRSA in the context of bovine mastitis extends beyond immediate economic losses to dairy farmers as it directly affects the health security (Oliveira *et al*., 2015; Cheng and Han, 2020). Given the high prevalence of bovine mastitis in Pakistan and the increasing threat posed by MRSA, there is an urgent need to explore alternatives to conventional antibiotics (Yaqoob *et al*., 2022). MRSA has been recognized by the World Health Organization and the Food and Agriculture Organization as a pathogen of major importance affecting both human and animal health (Khan *et al*., 2014).

Plants are being used to fulfill number of human's everyday needs. For example plants act as ornaments, medicine, cosmetics, and provide flavor to foods. Because of effectiveness against disease causing agents and several health benefits, medicinal plants especially those containing polyphenols have become a keystone in traditional medicine (Mueed *et al.,* 2023). Besides, people living in remote geographical areas with limited supply of modern drugs rely heavily on plants for the treatment of their's or their livestock's aliments. *Azadirachta indica* belonging to the genus Azadirachta (Meliaceae) or neem has been recognized for its therapeutic uses since ancient times. *Azadirachta indica* is the richest source of skeletally diverse triterpenoids and flavanones, well-known for their reducing properties. The silver ions can be reduced by the active compounds present in neem extract: nimbin, nimbinin, and nimbidin. Silver nanoparticles can be effective in inhibiting bacterial growth (Rai *et al.*, 2009; Pazli *et al*., 2023). Currently, the utilization of physical and chemical methods in the synthesis of nanoparticles is facing challenges owing to high costs, labor-intensiveness and potential environmental hazards. Biosynthesis of nanoparticles is a rapidly growing research area that has gained a lot of attention because it facilitates the eco-friendly integration of nanotechnology-based products (Naeem and Mueez, 2023).

The biosynthesis of nanoparticles has gained considerable attention as an eco-friendly approach for developing nanotechnology-based products. In particular, the use of medicinal plants as natural reducing and stabilizing agents for the synthesis of silver nanoparticles (AgNPs) offers a promising alternative to conventional chemical and microbial methods for combating MRSA. This green synthesis method does not require external bio-agents and yields stable nanoparticles with notable antimicrobial properties (Velusamy *et al.,* 2015). The application of AgNPs as antimicrobial agents holds great potential for combating *Staphylococcus* infections, including drug-resistant strains such as MRSA. Silver nanoparticles inhibit growth of *Staphylococcus aureus* and have the potential to be used as a therapeutic agent for bovine mastitis. Ongoing research in this area could contribute to the development of novel strategies to mitigate antibiotic resistance (Haag *et al*., 2019). Neem and its active phytochemical have proven to be effective against a variety of microorganisms such as bacteria, viruses, and pathogenic fungi as well as ectoparasites (Azeem *et al*., 2022). Recent advances in medical plant research and nanotechnology have drawn many researchers into the biosynthesis of metal nanoparticles because of several advantages over orthodox synthesis methods. This has facilitated the accessibility of nanoparticles, making them simpler and cost-effective (Adamu *et al.,* 2024). Synthesis of AgNPs using plant extract has stability, and potential applications as antimicrobial against animal and human pathogens (Velusamy *et al.,* 2015). Silver nanoparticles (AgNPs) contain antibacterial properties with minimum toxicity. To combat staphylococcus infections, neem leaf extract can be used to produce AgNPs (Cunha *et al.,* 2023).

The present study explores the green synthesis of AgNPs using *Azadirachta indica* leaf extract and evaluates their antibacterial efficacy against MRSA isolates. The main purpose of this study was the treatment of mastitis with the help of eco-friendly synthesized silver nanoparticles derived from *Azadirachta indica* leaf extract. Besides being eco-friendly approach of making AgNPs the present study's findings have implications for the development of sustainable solutions for managing MRSA-mediated mastitis in dairy animals and skin infections in milkmen.

MATERIALS AND METHODS

Sample collection: Twenty milk samples were collected aseptically from cows suspected of mastitis from various dairy farms located in Lahore and neighboring districts: Sheikhupura, Kasur, and Sialkot in the Punjab Province of Pakistan. These dairy farms represented typical dairy enterprises of the region's and were located in both rural and peri-urban areas. Sub-clinical mastitis was screened using a Surf Field Mastitis Test described elsewhere (Muhammad *et al.,* 2009; Karabasanavar *et al.,* 2021). Additionally, twenty human skin swab samples of milkmen were collected using sterile swabs.

Isolation and identification of *Staphylococcus aureus:* Primary bacterial isolation was carried out on Nutrient Agar (RPI, Research Products International, Mt. Prospect, IL, USA), followed by selective culturing on Mannitol Salt Agar (Sigma-Aldrich, St. Louis, MO, USA) for *Staphylococcus aureus* isolation. Colonies were subjected to Gram staining, mannitol fermentation, and catalase tests for phenotypic identification. Antibiotic-resistant *S. aureus* strains were confirmed with Kirby-Bauer disc diffusion method using antibiotic discs following CLSI guidelines (Ibrahim *et al.,* 2022).

Identification and sequencing of *mecA* Gene: Genomic DNA was extracted from MRSA isolates using a standard phenol-chloroform method. DNA concentration and purity were determined using a NanoDrop Spectrophotometer (ND-2000, Thermo Scientific, Wilmington, DE, USA). Before running PCR, the extracted DNA was run on 0.8% agarose gel electrophoresis (Thermo Scientific, Waltham, MA, USA). The DNA was subjected to PCR targeting *mec*A gene of MRSA. Following gene-specific primers were used (5′-AGG CCC GGG AAC GTA TTC AC-3′ and 5′-GAG GAA GGT GGG GAT GAC GT-3′) (Venugopal *et al*., 2019). The PCR was run in a total of 25µL of a reaction mixture containing 2.5µL of 10X buffer (NH_3SO_4), 2.5µL 25mM $MgCl_2$, 2µL dNTPs Mix (2 mM each), 15.2µL Nuclease-free water, 0.75µL forward primer, 0.75µL reverse primer, 1µL template DNA and 0.3µL Taq polymerase. PCR amplification was performed using following cycling conditions: an initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 1 minute, and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes. PCR products were resolved on 1.5% agarose gels (Thermo Scientific, Waltham, MA, USA) and visualized using a gel documentation system (Thermo Scientific, Waltham, MA, USA). Sequencing of amplicons was performed via a commercial vendor using dye-labeled dideoxy terminator chemistry on an automated sequencer.

Green synthesis of Silver Nanoparticles (AgNPs): A 1 mM silver nitrate ($AgNO_3$) solution was prepared by dissolving 0.018 g of $AgNO_3$ (Sigma-Aldrich, St. Louis, MO, USA) in 500mL of double-distilled water. *Azadirachta indica* leaves were collected, thoroughly washed with tap water followed by washing with the distilled water and shade-dried to preserve bioactive compounds (Awwad *et al.,* 2013; Banerjee *et al.,* 2014). Fifty grams of dried leaves were homogenized using a blender and an extract was prepared by boiling the homogenate in distilled water at 80–90°C for 20 minutes. The resulting mixture was filtered through Whatman No. 1 filter paper to remove particulate matter. The filtrate was collected and double-distilled water was added to the final volume of 500mL. Optimization of synthesis was performed at ambient temperature by mixing varying concentrations of $AgNO_3$ and neem extract.

Effect of substrate concentration: To optimize the substrate ratio for AgNPs synthesis, four different combinations were prepared using 1:1 (10mL $AgNO_3$: 10mL neem extract), 1:2, 1:3, and 1:4 ratios. Each combination was monitored for color change as a preliminary indicator of nanoparticle formation and further assessed for stability, particle size, and synthesis efficiency using spectroscopic and microscopic methods.

Effect of pH on nanoparticle synthesis: The effect of pH on nanoparticle formation and stability was assessed by adjusting the pH of the reaction mixture using 0.1M NaOH to obtain pH values of 7, 9, 10, and 12. Samples were incubated at room temperature, and nanoparticle formation was analyzed using UV-Vis spectrophotometry (Ahmed *et al.,* 2016).

Effect of reaction time: Reaction kinetics were evaluated by incubating the synthesis mixture at various time intervals: 5, 30, 60, and 120 minutes. Samples were collected at each interval and analyzed spectrophotometrically to assess the yield and stability of AgNPs (Ghosh *et al.,* 2012).

Purification of synthesized AgNPs: The synthesized nanoparticles were purified by centrifugation (Hermle Z326 K; Hermle Labortechnik GmbH, Weh, Germany) at 10,000 rpm for 15 minutes at 4°C. The pellet was washed thrice with deionized water to remove residual phytochemicals and unreacted silver ions, followed by drying at 60°C in a vacuum oven (Thermo Scientific, Waltham, MA, USA).

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Characterization of silver nanoparticles: Preliminary characterization of AgNPs was performed using UV–Vis spectrophotometry (Cecil 7200, Cecil Instruments Ltd., Cambridge, United Kingdom) within the 250–550 nm wavelength range. Fourier Transform Infrared Spectroscopy (IRAffinity-1, Shimadzu, Japan) was used to identify functional groups involved in nanoparticle capping. Surface charge was measured via zeta potential analysis. Morphological and size characterization was performed using transmission electron microscopy (JEOL Ltd., Tokyo, Japan).

Assessment of antimicrobial activity of AgNPs: The antimicrobial potential of biosynthesized AgNPs was evaluated using the agar well diffusion method against MRSA isolates. Bacterial cultures were adjusted to a concentration of 10^5 CFU/mL, and varying concentrations of AgNPs were applied. Zones of inhibition were measured after incubation at 37°C for 24 hours. Bacterial viability post-treatment was quantified using colony-forming unit counts (Rajeshkumar and Malarkodi 2014). Moreover, Time-kill curve assay was also used to evaluate the effect of AgNPs on the reduction of colony-forming unit per milliliter of MRSA isolates.

Statistical analysis: One-way analysis of variance (ANOVA) was used to determine the significance of differences among various concentrations of AgNPs and antibiotic treatments. The alpha level was set at 0.05. Data analysis was performed using SPSS software (IBM, Armonk, NY, USA) version 26.

RESULTS

Bacterial identification and antibiotic sensitivity: Out of 40 samples analyzed, 36 isolates showed positive mannitol fermentation. All mannitol fermenting Gram positive bacterial isolates were positive for catalase test. Among these, 20 isolates were of animal and 16 were of human -origin, respectively.

On Kirby-Bauer disc diffusion assay the isolated *Staphylococcus aureus* demonstrated significant antibiotic resistance to Tylosin, Penicillin G, Trimethoprim, Sulfamethoxazole, Oxytetracycline, Gentamicin, and Norflaxacin (Fig. 1). Overall, 16 (80%) animal-origin and 12 (75%) human origin isolates exhibited resistance to at least one tested antibiotic.

PCR amplification of *mec*A gene: PCR amplification targeting *mec*A gene, a key marker of methicillin resistance, yielded a 292 bp amplicon on 2 % agarose gel (Fig. 2) in 28 *S. aureus* isolates. The *mec*A gene was detected in 16 animal-origin and 12 human-origin isolates. In the remaining human and animal isolates, the *mec*A gene was not found.

Synthesized AgNPs: The synthesized neem (*Azadirachta indica*) leaves extract derived silver nanoparticles were marked by a distinct color change, from pale yellow to dark brown, in the reaction mixture. A schematic diagram of the silver nanoparticle synthesis is provided (Fig. 3).

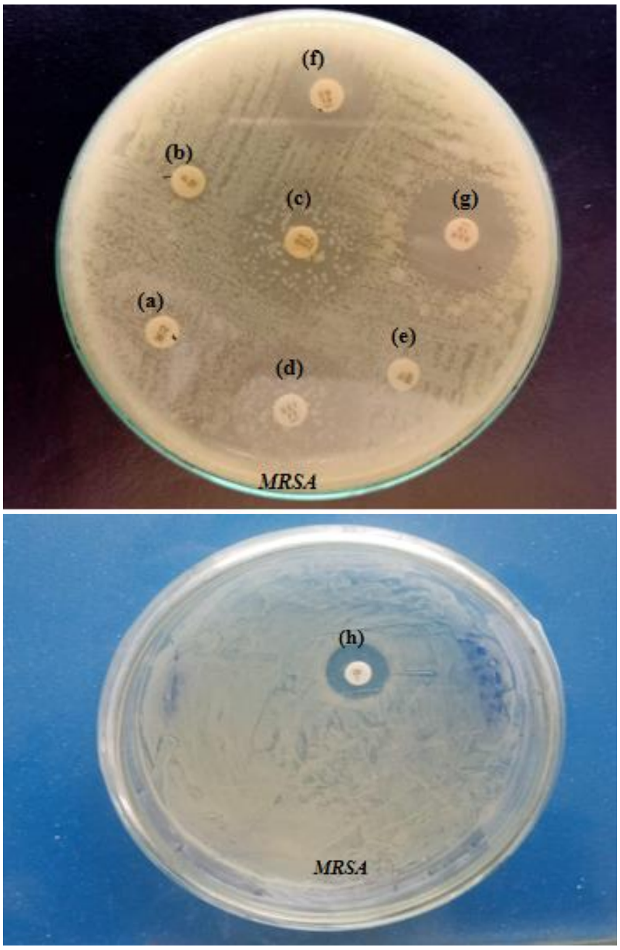


Fig. 1: Kirby Bauer Disc Diffusion Method: *Staphylococcus aureus* strains were resistant to (a) Tylosin (0 mm); (b) Penicillin G (0 mm); (c) Trimethoprim (6 mm); (d) Sulfamethoxazole (7 mm); (e) Oxytetracycline (0 mm); (f) Gentamicin (7 mm); and (h) Oxacillin (9 mm, Resistant) but an Intermediate zone was noted for (g) Norfloxacin (12 mm).

Fig. 2: Gel image of the PCR targeting *mec*A gene of Methicillin-resistant *S. aureus*, Lane M: 1000 bp ladder, Lanes 1-10: 292 bp amplified using *mecA*- specific primers.

Silver nanoparticles optimization results: The influence of distinct physico–chemical factors was analyzed as below.

Effect of varying substrate concentration: The 1:1 ratio out of 500mL of 1mM $AgNO_3$ and 500mL of neem extract were prepared, and the two were then combined in equal volumes ($AgNO_3$ solution and neem leaf extract), which showed the highest absorbance at 411 nm, demonstrating its superior synthesis efficiency (Fig. 4).

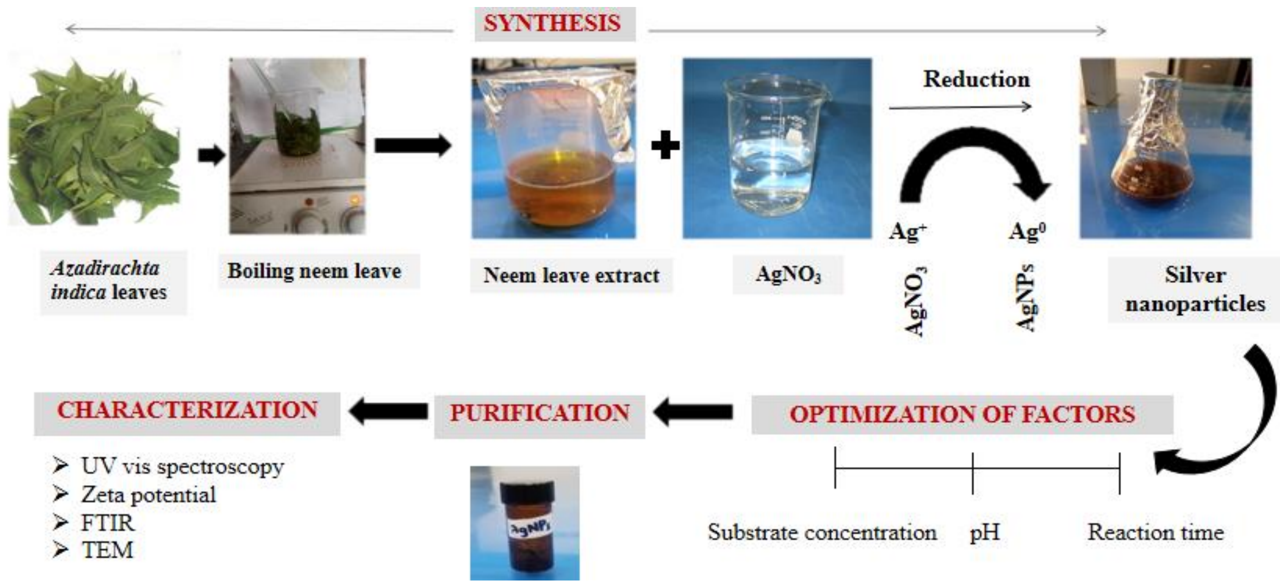


Fig. 3: Schematic representation of *Azadirachta indica* leaves extract silver nanoparticles (AgNPs) synthesis.

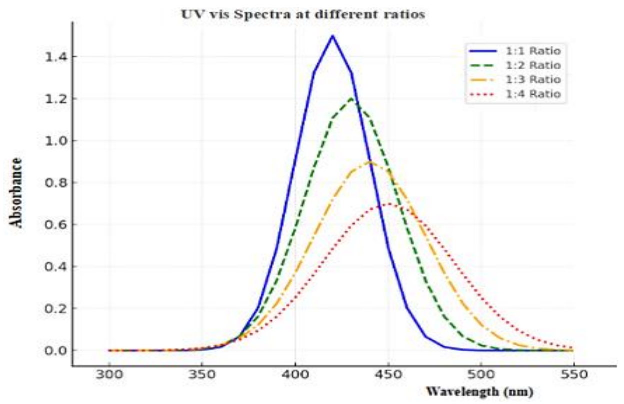


Fig. 4: UV-Vis spectroscopy graph comparing the absorbance of *Azadirachta indica* leaves silver nanoparticles synthesized with different ratios (1:1, 1:2, 1:3, 1:4).

Effect of the pH: The influence of pH on nanoparticle formation was noted by adjusting the pH. The reaction mixture showed sharp and intense peak at 400 nm with a uniform size at pH 10. A narrow surface plasmon resonance band reflected clear and well-defined peak, indicating the formation of stable silver nanoparticles (Fig. 5).

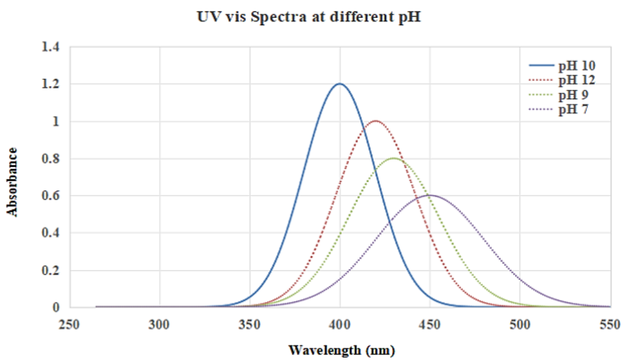


Fig. 5: UV-Vis spectroscopy graph comparing the absorbance of *Azadirachta indica* leaves silver nanoparticles synthesized with different pH (7, 9, 10, 12).

Effect of reaction time: UV-Vis spectrum at 120 minutes showed the highest absorbance at 433 nm. Prolonged incubation improved the reduction of silver ions and the stability of synthesized AgNPs, as indicated by steady increase in absorbance intensity over time (Fig. 6).

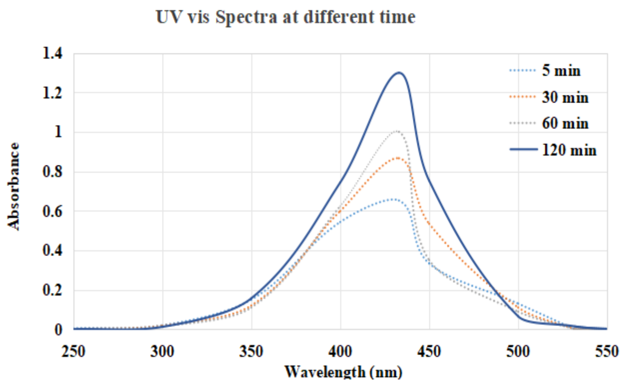


Fig. 6: UV-Vis spectroscopy graph comparing the absorbance of *Azadirachta indica* leaves silver nanoparticles synthesized with different duration (5, 30, 60, and 120 min).

Characterization of silver nanoparticles

UV-Visible Spectroscopy analysis: UV with a wavelength range of 200-1100 nm was used to evaluate neem leave extracted nanoparticles. Absorbance was measured between 250-550 nm, confirming the presence of AgNPs at 439 nm of optimized nanoparticles (Fig. 7).

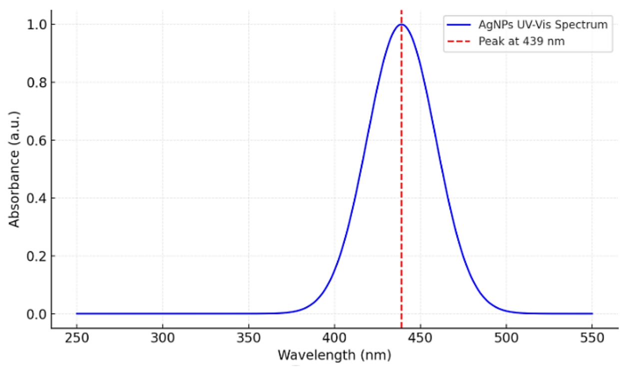


Fig. 7: UV-Visible Spectrum of optimized *Azadirachta indica* leaves silver nanoparticles.

Fourier-transform infrared (FTIR) spectroscopy analysis: The samples' FTIR spectra demonstrated a number of distinctive absorption peaks, a wide peak at roughly 3329.15 cm^{-1} was observed of O-H stretching vibrations (Fig. 6). Other peaks were found at ~2895.34 cm^{-1}, ~1605.28 cm^{-1}, ~1217.16 cm^{-1}, and ~1046.12 cm^{-1}, corresponding to C-H stretching, C=N stretching, N-H bending, and C-O stretching modes, respectively. When compared to the O-H stretching band, a noticeable peak linked to AgNPs was also seen, indicating that the AgNPs had a high degree of crystallinity (Fig. 8).

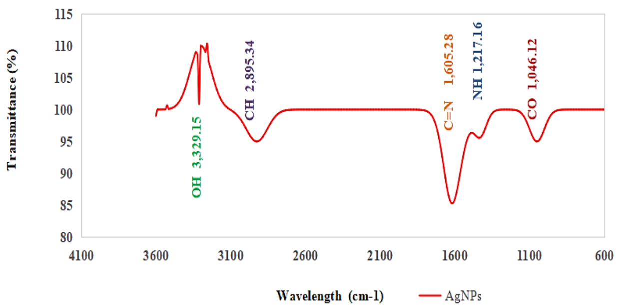


Fig. 8: Fourier transformed infrared spectroscopy analysis of *Azadiracta indica* silver nanoparticles.

Zeta potential analysis: The ZP values for ideal formulation of encapsulated neem leaves extract was -20 mV (Fig. 9).

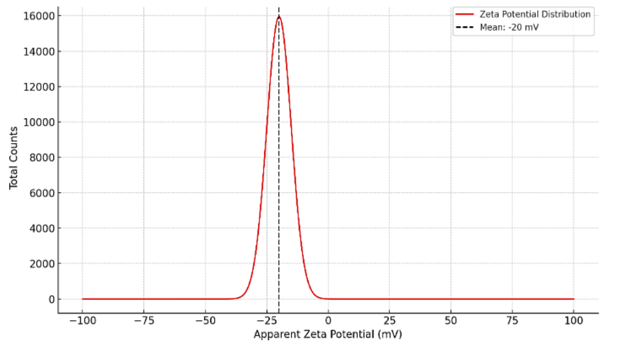


Fig. 9: Zeta potential results of silver nanoparticles.

Transmission electron microscopy analysis: Transmission Electron Microscopy revealed that the synthesized nanoparticles were 50 nm in size (Fig. 10).

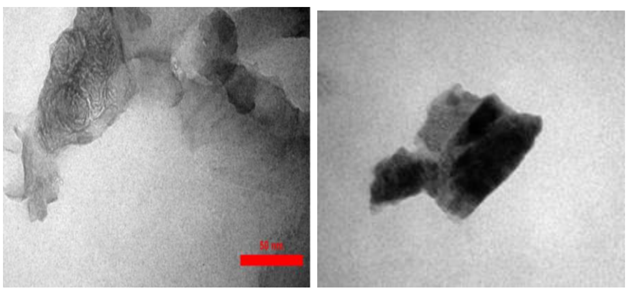
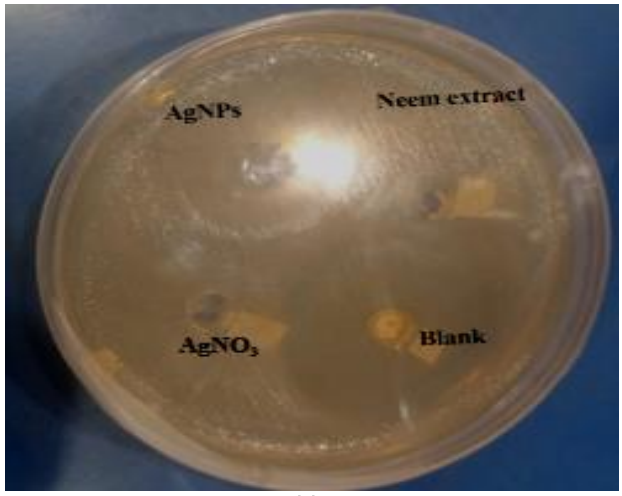
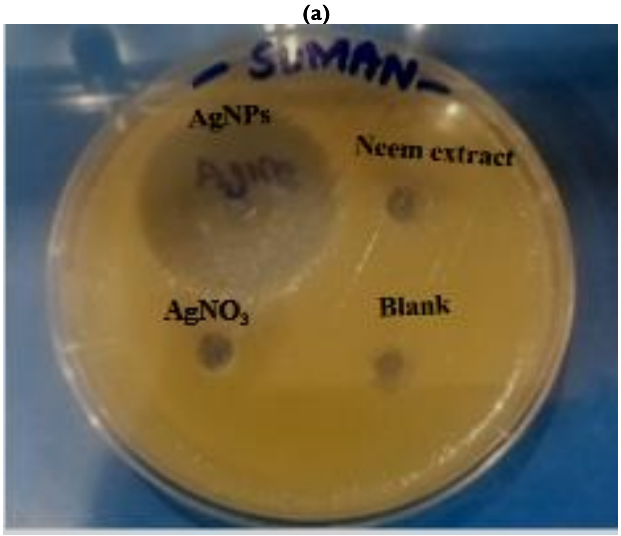


Fig. 10: Transmission electron microscopy of silver nanoparticles.

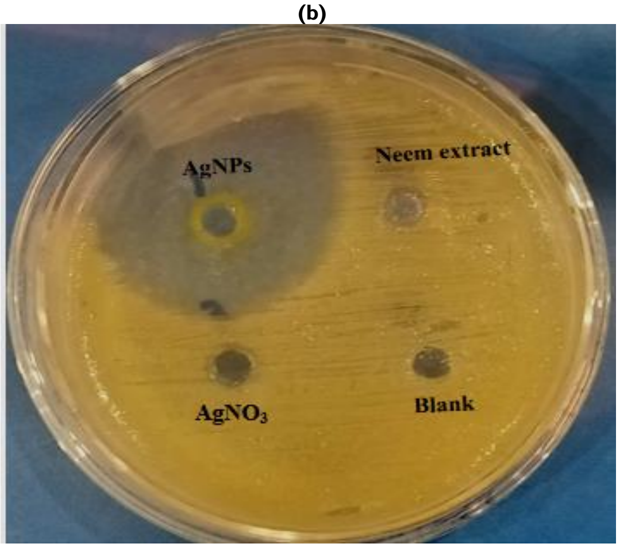
Well diffusion assay: The biosynthesized AgNP's nanoparticles encapsulated in an aqueous leaf extract indicated significant antibacterial activity against Methicillin-resistant *S. aureus* (MRSA) with a zone of inhibition of 20.38±6.19 mm (Fig. 11). In contrast, $AgNO_3$ showed a notably lower inhibition zone of 0.46 ± 0.27 mm. With a zone of 0.00 mm, the aqueous leaf extract by itself showed no inhibitory effect. As a standard for comparison, oxacillin, was employed as a positive control, that showed an inhibition zone of 16.24 ± 4.8 mm (Fig. 12 and Tables 1 and Table 2).



(a)



(b)



(c)

Fig. 11: Muller-Hinton Agar plate showing the antibacterial activity after 24 h incubation at 37°C. AgNPs (a) 12.5mg/mL (b) 25mg/mL and (c) 50mg/mL. Each plate contained $AgNO_3$, Neem leaf extract and Blank wells as controls.

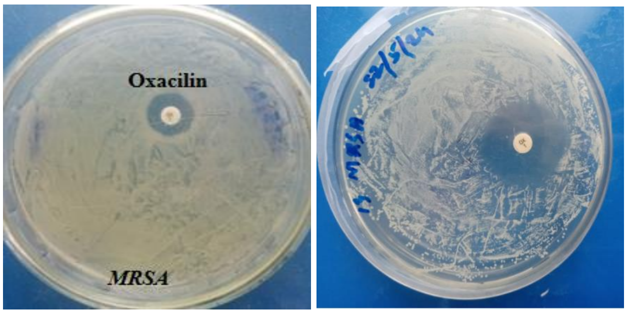


Fig. 12: Muller-Hinton Agar plate showing the antibacterial activity of Oxacillin as a positive control against MRSA.

Table 1: Antibacterial activity of nanoparticles encapsulated aqueous neem leave extract against Methicillin-resistant *S. aureus*

Treatment	Zone of inhibition
AgNPs	20.38 ±6.19
$AgNO_3$	0.46± 0.27
Aqueous leave extract	0
Negative control blank	0
Positive control Oxacillin	16.24±4.8

The data is represented as mean±Standard deviation (n=6)

Table 2: Zones of inhibition of AgNPs, $AgNO_3$, and neem leave extract

Sr. No	Concentration (mg/ml)	Zones of inhibition (mm) Oxacillin	AgNPs	$AgNO_3$	Neem leave extract
1.	50	22	32	1	0
2.	25	21	24	0.5	0
3.	12.5	18	19	0.4	0
4.	6.25	14.02	18.45	0.3	0
5.	3.12	12.43	16.05	0.1	0
6.	1.56	10	12.8	0.5	0

Time-kill curve assay: Quantification of colony-forming unit per milliliter (CFU/mL) was documented with varying proportion of AgNPs. Various concentrations of AgNPs led to reduction in the growth of *S. aureus* in a dose-dependent manner. Higher concentrations of AgNPs caused faster and more significant reductions in CFU/mL, demonstrating their stronger inhibitory or toxic effects (Fig. 13).

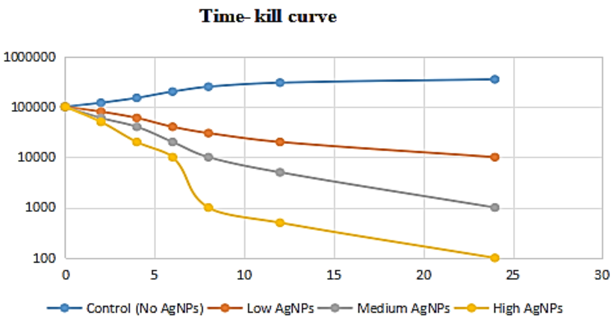


Fig. 13: Time-kill curve illustrating the effect of varying concentrations of silver nanoparticles. Yellow and gray lines indicate faster and more significant reductions in CFU/mL.

DISCUSSION

The present study highlights the growing importance of green-synthesized silver nanoparticles (AgNPs) as antimicrobials, particularly for combating multidrug-resistant pathogens. Antimicrobial resistance is a global concern, and infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) remain a major challenge in both human and veterinary medicine. Investigating alternative antimicrobial strategies is crucial in addressing the growing menace of antibiotic resistance. The present study aimed to synthesize AgNPs using aqueous neem leaf extract, characterize their physicochemical properties, and evaluate their antibacterial activity against MRSA isolated from mastitis affected cow's milk and human skin samples. The biosynthesized silver nanoparticles exhibited strong antibacterial activity against MRSA, with a mean inhibition zone of 20.38 ± 6.19 mm, Time-kill kinetic assay further confirmed their potency, revealing a clear, concentration-dependent reduction in *S. aureus* growth. Green synthesis offers an eco-friendly approach to developing novel antimicrobial agents. Taken together, this laboratory-based study suggests that neem leaf extract AgNPs exhibited substantial zone of inhibition against MRSA compared to oxacillin or $AgNO_3$ alone, suggesting silver nanoparticles are effective against MRSA. The present study highlights the potential of neem-derived AgNPs against MRSA isolated from mastitis affected cows's milk samples and human skin swabs. The study also highlights potential impact of plant-origin nanoparticles in managing multidrug-resistant infections.

Mastitis continues to be a significant challenge to the dairy farmers, especially in developing countries such as Pakistan where dairy farming is a major economic activity especially of rural communities. Of myriad bacterial etiologies of bovine mastitis, the present study focused on MRSA. The MRSA is one of the major causes of bovine mastitis which can lead to reduced milk production and poor quality milk production. The financial losses to dairy farmers are not only limited to milk production and quality loss but also include the cost of treating mastitic animals. Irrational use of antibiotics to treat mastitic animals can contribute to the development of antibiotic resistance (Javed *et al.*, 2022). The development of multidrug resistance in MRSA, its continued evolution, and the presence of virulence genes leads to a significant therapeutic challenge (Shahid *et al.,* 2023; Li *et al.,* 2025; Thacharodi *et al.,* 2025). Boiling and pasteurization can effectively eliminate viable MRSA in milk but consuming inadequately boiled or unpasteurized milk constitutes a public health risk (Kannan *et al.,* 2023; Wörmann *et al.,* 2023). In Pakistan some people prefer to consume raw milk as they consider raw milk healthier than boiled or pasteurized one. A recent study from Pakistan have highlighted the role of *S. aureus* in bovine mastitis, emphasizing the need for effective control measures (Javed *et al.,* 2022). The emergence of MRSA strains has further complicated intervention strategies. Isolation of MRSA from human skin samples in the study suggests the possibility of cross-contamination and spread of antimicrobial-resistant strains between animals by humans when milking, as well as the zoonotic spread of MRSA to humans. A study conducted in the Pothohar Region of Pakistan reported that 21% of *S. aureus* isolates from subclinical mastitis were methicillin-resistant and contained *mec*A gene (Khan *et al.,* 2019). Another recent study from the Punjab Province of Pakistan has demonstrated even higher percentage of 96.8% (61/63) of MRSA in human clinical human samples (Idrees *et al.,* 2023). This underscores the urgent need for alternative therapeutic approaches to combat MRSA. In this context, silver nanoparticles synthesized using plant extracts have gained attention due to their potent antimicrobial properties.

The study presents optimized AgNPs synthesized using aqueous leaf extract of neem (*Azadirachta indica*). The synthesized AgNPs exhibited a characteristic absorption peak at 439 nm. The synthesis involved reducing $AgNO_3$ with bioactive compounds from neem extract as evidenced by a characteristic surface plasmon resonance peak between 250 nm and 550nM. These findings are consistent with those published by Barik *et al.* (2024), who reported peaks between 430 and 450nM. The FTIR analysis revealed functional groups corresponding to N–H, O–H, and C=O stretching, indicating the presence of bioactive compounds responsible for stabilization of AgNPs. The present study's results corroborate Kumari *et al.* (2022) results, who performed FTIR analysis and observed distinct absorption bands various chemical groups associated with plant-derived bioactive molecules. The zeta potential of the synthesized AgNPs was found to be –20 mV, suggesting moderate colloidal stability. Zeta potentials between –15 and –25mV were associated with stable, phytochemical-capped nanoparticles in previous plant-mediated AgNPs studies (Singh *et al*., 2021; Kumari *et al*., 2022). Transmission electron microscopy analysis revealed that the nanoparticles were accumulated with an average size of approximately 50 nm consistent with a previous study on AgNPs derived from *A. indica* extracts (Barik *et al.,* 2024).

The synthesized AgNPs showed strong antibacterial action against MRSA. This antibacterial activity of AgNPs was likely due to their large surface area, nanoscale size, and ability to interface with bacterial cell membranes. AgNPs demonstrated potent antibacterial activity against MRSA's common resistance mechanisms because of their stronger inhibitory effect when compared to the positive control antibiotic (oxacillin). This enhanced activity is supported by other studies showing that AgNPs cause damage to the bacterial membrane, generate reactive oxygen species, and impede essential cellular functions such as DNA replication and protein synthesis. Consistent with previous studies, neem's aqueous leaf extract alone exhibited no inhibitory effect against MRSA (0 mm zone) suggesting that polar solvents such as water extract and fewer bioactive compounds from neem leaves, resulted in negligible antibacterial activity. The aqueous neem extract did not result in any inhibition, consistent with previous findings that water extracts do not contain adequate amounts of active phytochemicals (Biswas *et al*., 2002; Sultana *et al*., 2015; Basha and Rekha, 2017).

The low activity of $AgNO_3$ alone, on the other hand, suggests that silver ions without nanoparticle stabilization have limited effectiveness, either because of quick ion precipitation or decreased contact with bacterial surfaces. These results collectively show that metallic silver and neem-derived reducing agents work in concert to produce nanoparticles with strong antibacterial capabilities against MRSA. Together, these results suggest that AgNPs exhibit potent antibacterial activity against MRSA, surpassing both the leaf extract and $AgNO_3$ alone in effectiveness.

The present study findings are also consistent with a previous study by Ahmed *et al.* (2020) who reported significant antibacterial activity of green synthesis AgNPs against MRSA. These results also corroborate Khan and Javed's (2021) findings, who demonstrated that biologically synthesized AgNPs exhibited antimicrobial activity compared to conventional antibiotics. The tiny particle size, larger surface area, and synergistic interactions between silver ions and bioactive chemicals from neem extract may contribute to the effectiveness of neem-origin AgNPs in the present study. The eco-friendly synthesis process, coupled with the potent antimicrobial properties of AgNPs, makes them suitable candidates for developing new therapeutic agents to combat bovine mastitis caused by MRSA strains. The present study suggested that silver nanoparticles are effective against field isolates of MRSA of bovine and human origin in laboratory settings. The AgNPs may have the potential to treat antibiotic-resistant bacteria as an alternative to antibiotics.

This study has a number of limitations despite its valuable contribution to the already existing body of knowledge on the topic. The controlled laboratory setting in which the tests were carried out may not translate in clinical or farm setting, where a variety of external variables affect the infection dynamics, even though the isolates were collected from the field samples. Because of the small sample size, the results' generalizability is limited. Furthermore, the study did not test the cytotoxicity of AgNPs. *In-vivo* studies of neem-derived AgNPs for evaluating the efficacy and safety for treating staphylococcal infections may shed light on their potential therapeutic effects. Likewise, the toxicity of AgNPs needs to be evaluated in both animals and humans. To determine the safety, therapeutic dose, pharmacokinetics, and long-term effects of neem-mediated AgNPs assessments in animal models are crucial. Deeper understanding of how AgNPs affect bacterial physiology may be possible through mechanistic research using cutting-edge methods including proteomics, transcriptomics, and membrane-interaction tests. Additionally, further studies should explore the environmental effects of usage and optimize AgNP synthesis techniques for large-scale veterinary or human use. Together, the current work adds to the growing body of knowledge demonstrating the potential of green-synthesized AgNPs as sustainable antibacterial agents in both veterinary and human medicine. The present laboratory-based study's results present neem leaf-derived AgNPs as a promising alternatives to antibiotics. The study results hold significant as the race for developing novel antimicrobials is on because of the growing threat of antimicrobial resistance.

Conclusions: Neem-derived AgNPs exhibit concentration-dependent antibacterial activity against MRSA highlighting their potential as an antibacterial agent.

Authors Contribution: SN: Experimental work, data collection, methodology, data analysis and manuscript writing; SF: Supervision, Validation, and resource provision; MW: Data visualization and analysis; SA: Manuscript's critical review, editing, proofreading and finalization; ARA: Sample collection and Data analysis; MT: Lab work execution; SS: Resource provision.

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