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RESEARCH ARTICLE

The Antibacterial and Antioxidant Potential of *Prosopis juliflora*-Derived Copper Nanoparticles against *Staphylococcus aureus* in Mastitis

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A B S T R A C T

Mastitis, an ongoing challenge in the dairy sector causing major economic losses and rising antibiotic resistance. The most common cause of mastitis is *Staphylococcus aureus* (*S. aureus*). The major aim of the work is to examine the nature friendly production of copper nanoparticles (CuNPs) using bark extract of *Prosopis juliflora* (*P. juliflora*) as a natural reducing and stabilizing agent. The CuNPs were fabricated from an aqueous decoction bark extract of *P. juliflora*. The characertization of synthsized CuNPs was carried out by UV-Vis spectroscopy, X-ray diffraction (XRD), energy dispersive X-ray analysis (EDX), Fourier Transform Infrared Spectroscopy (FTIR), and scanning electron microscopy (SEM). In addition to the color shift from light blue to embraled green, further characterization methods verified that the CuNPs were synthesized with the average size ranged from 10 to 80nm and wide range of morphology. The antibacterial properties of CuNPs were evaluated against *S. aureus*, demonstrating pronounced zone of inhibition in disc diffusion assay. The maximum zone of inhibition recorded at 600, 400 and 200ppm was 19.5±0.41, 13±0.82 and 9.1±0.70mm, respectively. The antioxidant capabilities of the CuNPs were assessed using the DPPH test, demonstrating significant free radical scavenging activity. The UV-Vis spectra of the CuNPs demonstrated a 69.57% reduction in free radical activity, with the highest absorbance occurring at 517nm. This environmentally sensitive synthesis technique provided a sustainable method for nanoparticle production. CuNPs made from *P. juliflora* can be a good alternative to treat mastitis caused by *S. aureus* due to their antibacterial and antioxidant potential.

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INTRODUCTION

The dairy industry in Pakistan is a major driver of the country's GDP. In Asia, buffalo is contributing 89.2 million tons of milk per annum i.e. 96.78% of the world's total milk production (Sharma *et al.,* 2012; Bari *et al.,* 2022). Likewise in Pakistan, buffalo is producing a major share of Pakistan's total milk production i.e. 68.4%. Bovine mastitis is a major limiting factor in production of good quality and quantity of milk, worldwide (Sharma *et al.,* 2012; Jalil *et al.,* 2022; Javed *et al.,* 2022). It can not only lead to reduced milk production but also increase the treatment cost for resource poor farmers (Hussain *et al.,* 2012; Ali *et al.,* 2021; Khan *et al.,* 2023;). Mastitis is characterized by inflammation of the mammary gland parenchyma, leading to abnormal changes in the glandular tissues and altering the physical, chemical and bacterial composition of milk (Kumari *et al.,* 2018; Ali *et al.,* 2021). The sub clinical mastitis leads to reduced milk quality and quantity but no outward signs of infection, which is 15-40 times more common and spreads quickly within herds. However, clinical mastitis can result in clots and milk flakes visible, as well as swollen quarters and the potential for lacerations and necrosis (Bhatt *et al.*, 2012; Cobirka *et al.,* 2020).

Approximately70% of mastitis cases are caused by bacterial infections, while the remaining 30% result from non-infectious factors such as physical trauma and mechanical injury to the gland (Ali *et al.,* 2021; Yadav *et al.*, 2024). Mastitis is associated with over 137 bacterial species, including approximately 20 primary pathogenic agents. *S. aureus* is recognized as the most common bacterial pathogen associated with mastitis in dairy animals (Ibrahim, 2017; Varela-Ortiz *et al.,* 2018; Shahzad *et al.*, 2024).

The number of cases of mastitis in Pakistan ranges from 42 to 70%, emphasizing the disease's significant effect on Pakistan's dairy sector (Javed *et al.,* 2022). The increased risk of mastitis emphasizes the need for suitable treatment and prevention methods to mitigate financial losses. Nevertheless, this issue additionally prompts milk suppliers to take more antibiotics, accelerating the development of antibiotics resistance (Murphy *et al.,* 2017; Shahzad *et al.,* 2024).

S. aureus has acquired resistance to commonly used antibiotics, complicating the treatment of mastitis (Badua *et al.,* 2020; Taifa *et al.*, 2022). Though the consumption of antibacterial drugs has led to the development of superbugs, traces of antibiotics in food products, and the development of pathogen resistant to several antibiotics, therefore complicating treatment (Krishnamoorthy *et al.*, 2021).

Researchers have been looking into new therapeutic methods in recent ages, especially non-antibiotic-based ones, as concerns about treatment failures have increased (Algharib *et al.,* 2020). In order to cure bacterial infections, nano based treatments become a viable alternative (Dehkordi *et al.,* 2011; Jamaran and Zarif, 2016). Metal-based nanoparticles (NPs) have drawn attention recently as feasible substitutes for treating bacteriological infections (Sharun *et al.,* 2021; Maliszewska and Czapka, 2022). CuNPs have stood out among other metal NPs like silver, gold, copper, zinc and iron due to their strong antibacterial and antifungal properties (Roy *et al.*, 2022; Taifa *et al.*, 2022).

A technique that is both ecologically safe and affordable is the green synthesis of metallic NPs (Mittal *et al.,* 2013; Moroda *et al.,* 2025). A range of plant parts or entire plant, have been utilized since they have contain bioactive compounds. Murthy *et al.* (2020) stated that these plant-based techniques show that bio synthesis can be used to make NPs in a way that is both sustainable and effective.

A plant in the Fabaceae family, *P. juliflora* is thought to help with gastrointestinal, dermatological and inflammatory problems. Bioactive substances like flavonoids, alkaloids and phenolic, which serve as crucial for the synthesis of CuNPs, are thought to be responsible for their curative qualities (Arya *et al.,* 2018; Rathinavel *et al.,* 2024).

Although CuNPs are known to have antibacterial qualities, there is still little research that concentrates on improving their eco-friendly production using *P. juliflora*-derived CuNPs may address oxidative stress and bacterial infections in mastitis. No studies have specifically evaluated its efficacy against *S. aureus*-induced mastitis in Pakistan's dairy industry. Addressing these short-comings will facilitate the development of a viable, non-antibiotic treatment alternative. This study concentrated on the development of sustainable synthesis approach for CuNPs and investigation of their antibacterial efficacy against *S. aureus.* The research assessed the antioxidant characteristics of the fabricated CuNPs by highlighting CuNPs capability as a dual-function therapeutic agent for mastitis treatment and as a free radical scavenger.

MATERIALS AND METHODS

Green synthesis of copper nanoparticles

preparation of plant extract: *P. juliflora* bark was collected from Rawalpindi region, Pakistan. Firstly, the bark was cleaned with distilled water to remove any impurities and dried under the shade for 15 days. Then, it was grinded to fine powder using a grinder. The 40g of powder was added into a flask containing 400mL of distilled water. Kept over magnetic stirrer for 90 minutes at 50°C. Let the solution cool down at the room temperature for 24 hours. Then, the solution was filtered thrice with the help of Whatman No.1 filter paper. Filtered solution was stored at 4°C (Arya *et al.,* 2018).

Synthesis of CuNPs by plant-based methods: The synthesis of CuNPs was performed following the methodology outlined by Khodaie and Ghasemi (2018), with slight modifications. Copper sulphate solution was prepared by dissolving 4g of salt in 1L of distilled water. The flask was placed on magnetic stirrer for further reaction at 70-80°C. Afterward, bark extract was added dropwise into the salt solution and observed for change in color. The fabrication of CuNPs was confirmed by a change of color solution from light blue to embraled green. Then centrifugation was done at 10,000rpm for 15 minutes. CuNPs pallet forms at the bottom of centrifuge tube. Subsequently, CuNPs were re-suspended in distilled water. The CuNPs centrifuged thrice to ensure the complete removal of impurities. Then, the NPs were dried in an oven (Amer and Awwad, 2021; Amjad *et al.,* 2021).

Characterization of copper nanoparticles: Ultraviolet-visible (UV-Vis) spectrophotometry was used to characterize the green-mediated CuNPs. To confirm NPs formation, CuNPs were dispersed in distilled water and sonicated for 10-15 minutes. Their UV-Vis spectrum was recorded between 200 and 750nm. The morphology of CuNPs, their shape and size were analyzed with the help of SEM. The elemental composition of the fabricated CuNPs was determined by EDX at energy varies from 0-16keV, confirming the presence of copper and other elements. CuNPs stabilization and functional groups were identified through FTIR analysis. The CuNPs Crystalline structure was discovered through XRD analysis (Hassan *et al.,* 2022; Jaha *et al.,* 2024).

Bacterial sample collection and cultivation: The bacterial strain, *S. aureus*, used in this study was provided by the Department of Microbiology and Parasitology at Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan. Following the protocol by Missiakas and Schneewind (2013), the *S. aureus* strain was grown in nutrient broth and then cultured to Mannitol salt agar (MSA) for selective enrichment.

Antibacterial susceptibility testing of CuNPs: The *in vitro* antibacterial efficacy of CuNPs was assessed using the agar disc diffusion technique, following a modified procedure by Manandhar *et al.* (2019). The bacterial culture of *S. aureus* was adjusted to a concentration of 1.5×10^8CFU/mL using a spectrophotometer, equivalent to the 0.5 McFarland standard. Using a sterilized swab, the bacterial suspension was evenly inoculated across the Muller-Hinton agar (MHA) plate to confirm uniform growth. Discs of sterilized Whatman filter paper, measuring 6mm in diameter were loaded with 25-30µL of CuNPs at 200, 400 and 600ppm doses, respectively. The discs were subsequently placed on agar plate surfaces (Table 1). Enrofloxacin discs were used as positive control, while discs containing only distilled water were included as the negative control. The plates were incubated at 35-37°C for 16-18 hours. After incubation, the antibacterial activity was evaluated by measuring the zones of inhibition around each disc, using a ruler to record the diameter in millimeters. The experiments were conducted in triplicate and the mean zone of inhibition was calculated for further analysis.

Table 1: Different concentration of copper nanoparticles and controls for Antibacterial Activity

Treatments	CuNPs Concentrations (ppm)
T1	200
T2	400
T3	600
Positive Control	Enrofloxacin (5µg)
Negative Control	Distilled water

Antioxidant activity of CuNPs: Free Radical Method was implemented to gauge the antioxidant activity of the synthesized CuNPs by using the DPPH (2,2-diphenyl-1-picrylhydrazyl). In this regard, four test tubes were prepared; one for control and the remaining 3 for 200, 400 and 600ppm, respectively. In the control tube, 3mL of DPPH solution (in ethanol) was added. For the sample tubes, 50µL of the CuNPs solution was added to each tube, followed by 2.96mL of DPPH solution. The DPPH radical, which forms a violet-colored solution in ethanol, is reduced in the presence of an antioxidant, leading to a color change to colorless. After preparing the test tubes, the absorbance was measured using a UV-Vis spectrophotometer at 517nm (Rajasekar & Rajeshkumar, 2021). The antioxidant activity was calculated using the following formula to determine the percentage of inhibition:

$$\text{Percentage of Inhibition} = \frac{(\text{Absorbance of blank - Absorbance of tested sample})}{\text{Absorbance of control}} \times 100$$

This was performed in duplicate and the mean percentage inhibition was calculated to evaluate the antioxidant potential of the CuNPs compared to the control.

Statistical analysis: All experiments were performed in triplicates, and the data obtained from the disc diffusion assay were statistically analyzed using Statistical Package of Social Sciences (SPPSS). The results are presented as mean ± standard deviation (SD) of triplicate experiments. $P<0.05$ was considered statistically significant.

RESULTS

Nanoparticles synthesis and analysis: The production of CuNPs using *P. juliflora* as the principal reducing and stabilizing agent was extremely successful. The effective production of CuNPs in the reaction mixture consisting of *P. juliflora* extract and $CuSO_4.5H_2O$ solution as verified by UV-visible spectrum analysis is depicted in Fig. 1. Significantly, a clear absorption peak at 550nm showed a color shift from the light blue green to emerald green solution, which may be explained by surface plasmon resonance. This unique signal at 550nm provides unambiguous proof that CuNPs are produced. Visual depictions of both the dimension and shape of the synthesized CuNPs through SEM are provided in Fig. 2. The EDX analysis unveiled discernible peaks associated with elemental Cu and O as shown in Fig. 3. Remarkably, no more peaks were seen, confirming the purity of the produced CuNPs and in line with XRD investigations. The XRD pattern of copper sulphide (Cu_2S) showed face-centered cubic (FCC) lattice and a cubic crystal system. It is the crystal lattice's (hkl) planes that are responsible for the diffraction peaks (Fig. 4). In FTIR spectrum of CuNPs, many absorption bands were seen in the resultant spectra, including a strong peak at 3376.65cm^{-1} that was suggestive of hydroxy groups displaying typical "polymeric" OH stretching and hydrogen bonding (Fig. 5; Table 2).

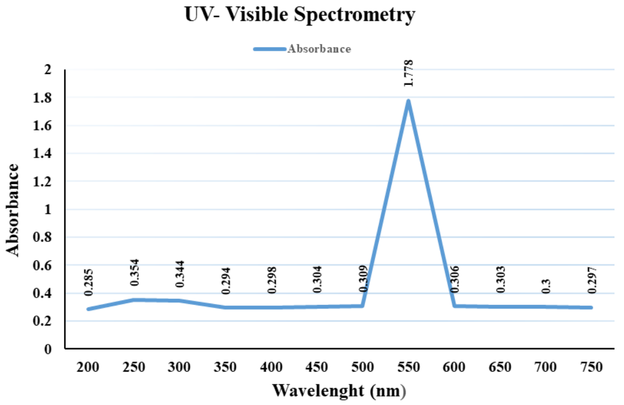

Fig. 1: UV-Vis spectrophotometric analysis of CuNPs, displaying the distinctive absorption peak, indicating nanoparticle formation and stability

***Staphylococcus aureus* enrichment, identification and MSA colonies:** On MSA, the colonies were smooth-textured, round, convex, and small to medium size (Fig. 6). As a result of mannitol fermentation, they looked light to golden yellow. The colonies' distinct borders and ability to withstand the high salt content of the MSA medium confirmed the presence of *S. aureus*. The gram-staining procedure revealed the distinctive characteristics of *S. aureus* as gramme-positive cocci under a microscope. They still had crystal violet stains on them and were a purple color. Typically, the cells were clustered together to form structures similar to grapes.

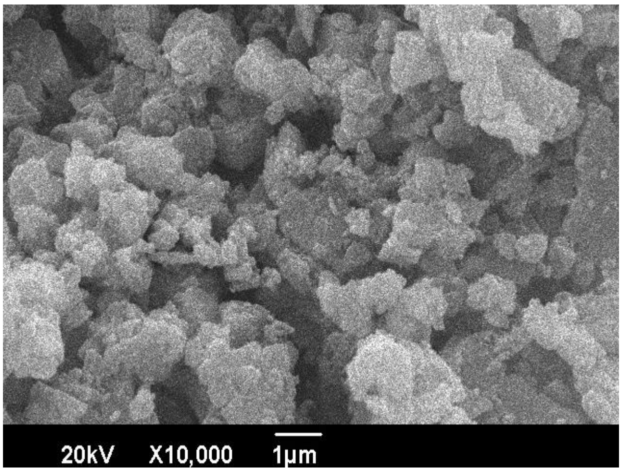

Fig. 2: SEM results of CuNPs showing morphology of syntesized nanoparticles.

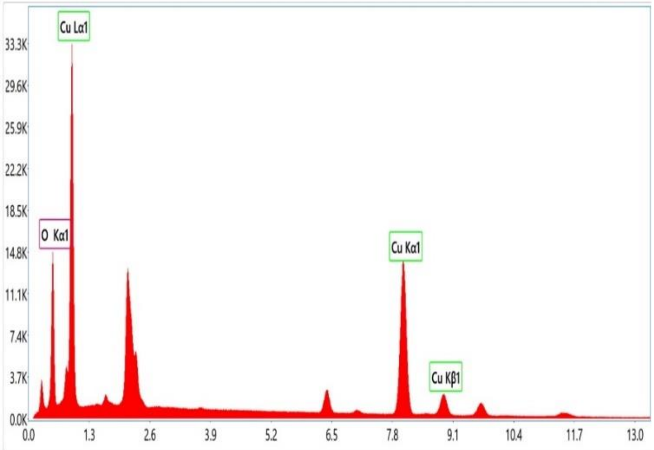

Fig. 3: Energy dispersive x-ray Spectroscopy (EDX) results confirming the elemental composition of the synthesized material, highlighting the presence of copper (Cu) and other associated elements.

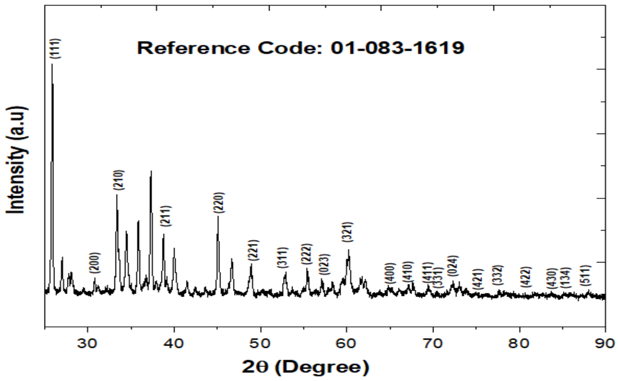

Fig. 4: X-ray Diffraction (XRD) pattern illustrating the crystalline structure of copper nanoparticles, with distinct peaks corresponding to characteristic Cu phases

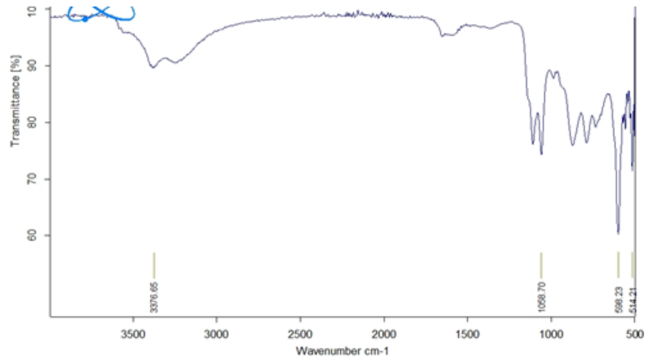

Fig. 5: Fourier Transform Infrared Spectroscopy (FTIR) spectrum of copper nanoparticles, showing characteristic absorption peaks corresponding to functional groups involved in nanoparticle stabilization.

Table 2: FTIR spectral analysis of CuNPs, showing characteristic absorption bands at different wavelengths and their corresponding functional groups, indicating possible interactions and stabilization of nanoparticles

Sr No.	Wavelength cm^{-1}	Functional Group
1	3376.65 cm^{-1}	Hydroxy group, H-bonded OH stretch, Normal "polymeric" OH stretch,
2	1058.70 cm^{-1}	Skeletal C-C vibrations, Aromatic C-H in-plane bend, Aliphatic fluoro compounds, C-F stretch, Alkyl-substituted ether, C-O stretch, Alkyl-substituted ether, C-O stretch, Primary amine, CN stretch, Phosphate ion, Silicate ion
3	598.23 cm^{-1}	Aliphatic iodo compounds, C-I stretch, Alcohol, OH out-of-plane bend, Disulfides (C-S stretch),
4	514.21 cm^{-1}	Aliphatic iodo compounds, C-I stretch,

Antibacterial activity of CuNPs: The results showed an increase in the zone of inhibition as the concentration of CuNPs increased. This proposes that higher concentrations of CuNPs have a more potent antibacterial effect against *S. aureus*. When the concentration was at 200ppm, the zone of inhibition measured 9.1±0.70mm. As the concentration increased to 400ppm, the zone of inhibition expanded to 13±0.82mm. Finally, at 600ppm, the zone of inhibition reached a size of 19.5±0.41mm. Enrofloxacin (positive control) exhibited a zone of inhibition measuring 24mm, whereas the negative control (distilled water) displayed no inhibition at all, measuring 0mm (Fig. 7). It appears that CuNPs have demonstrated strong antibacterial properties against *S. aureus*.

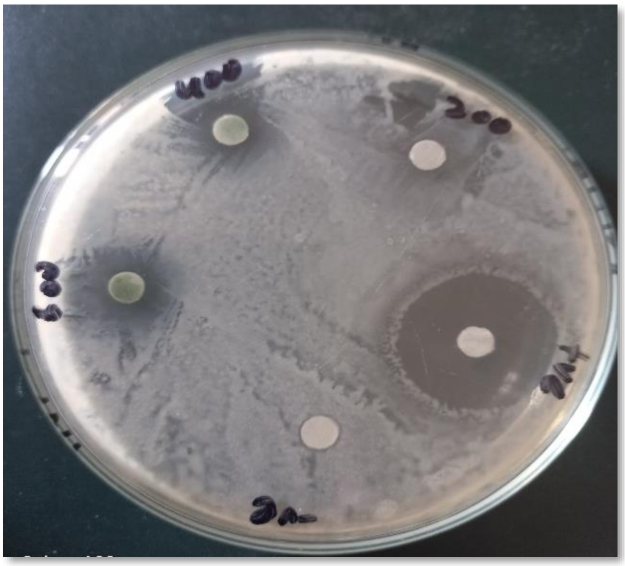

Fig. 7: Antibacterial activity assay displaying inhibition zones for the positive control, negative control, and CuNPs, indicating comparative effectiveness against the tested bacterial strain.

Antioxidant activity of CuNPs: During recording of UV-Vis Spectra for the CuNPs derived from *P. juliflora* highest absorbance (69.57%) of CuNPs at 517nm was observed at a concentration of 600ppm. The results presented in Table 3 indicated that CuNPs exhibit significant antioxidant properties, confirming their efficacy in antioxidant activity and supporting their potential as effective free radical scavengers.

Table 3: Antibacterial and antioxidant activity of CuNPs at different concentrations (200, 400 and 600ppm)

Concentration of CuNPs (ppm)	Zone of Inhibition (mm)	Absorbance (%)
200	9.1 ± 0.70^{a}	58.96
400*	13 ± 0.82^{b}	64.89
600*#	19.5 ± 0.41^{c}	69.57
Enrofloxacin	24	-
Distilled water	0	-

The different subscript letters (a,b,c) within a column show statistical difference among treatment levels. *p-values less than 0.05 indicated a statistically significant difference among the effect of treatment levels i.e. between Concentration of CNPs_200 and Concentration of CNPs_400; between Concentration of CNPs_200 and Concentration of CNPs_600; between Concentration of CNPs_400 and Concentration of CNPs_600. *$P<0.05$ *vs.* 200, #$P<0.05$ *vs.* 400.

DISCUSSION

Among the several bacterial and fungal diseases that affect dairy cattle, mastitis is the most common and economically devasting, caused by *S. aureus.* Bradley (2002), highlights the impact of mastitis on milk production, quality of milk and treatment expenses in the dairy sector. One major obstacle to managing mastitis effectively is the potential for the pathogen to become resistant to frequently used antibiotics (Wernicki *et al.,* 2014; Wierzbicki *et al.,* 2024). The ability to build biofilms, modify target sites and synthesis of beta-lactamase enzyme are some of the mechanisms that produce resistance in *S. aureus* that shield it from antibacterial drugs and the immune system of its host (Hemaiswarya *et al.,* 2008; Kim *et al.,* 2022). Due to the limitations and negative effects associated with traditional antibiotic drugs, there is increasing interest in alternative approaches, including nanotechnology and plant-based antimicrobial compounds (Hemaiswarya *et al.,* 2008; Anand *et al.,* 2019). Owing to their nano size and large surface area, NPs interact with microbial cells, causing damage to cell membrane and disruption of crucial biological process. Despite interesting such advances are still in early stages of development and require further research to validate their efficacy and safety in *in vivo* models. It is essential to consider the environmental and health impacts associated with the widespread use of nanoparticles (Lahiri *et al.*, 2021).

Recent study conducted by Sundar and Madhvan (2024) explained the potential of CuNPs to effectively scavenge free radicals. They found that at concentration of 125µg/mL of CuNPs, 83% of DPPH free radicals scavenged, compared to 60, 64 and 70% at concentration of 50, 75 and 100µg/mL, respectively. In line with the earlier study, similarly at high concentration, the green-mediated CuNPs in our study showed 69.57% inhibition of free radicals. The results explained that the antioxidant characteristics of CuNPs persist, even when produced through green-mediated environmentally friendly approach. The results supports the effectiveness of CuNPs in scavenging free radicals, emphasizing its importance in antioxidant application (Rajasekar and Rajeshkumar, 2021).

CuNPs were successfully synthesized using *P. juliflora* extract, demonstrating the effectiveness of plant-based NPs manufacturing and emphasizing its sustainable and environmentally beneficial characteristics. The existence of flavonoids, alkaloids, and phenolic compounds played a pivotal role in reducing Cu (II) ions and in stabilizing the resultant NPs. This twofold functionality aids in reducing the requirement of hazardous chemicals or external stabilizing agents and making the process eco-friendly. In contrast to microbial or chemical methods, NPs production is more simplified through plant-mediated fabrication as it usually does not require complex culture maintenance or toxic reagents. The efficacy of this green technique has expanded its potential for large-scale nanotechnology applications, especially in the health and environmental domains (Amer and Awwad, 2021; Amjad *et al.*, 2021). Surface plasmon resonance caused a notable absorption peak at 550nm in the UV-visible spectrum analysis, which verified the successful production of CuNPs. This outcome is consistent with other research, including those conducted by Chung *et al.* (2017) and Amjad *et al.* (2021), which reported similar peaks for CuNPs synthesized using different plant extracts. The presence of this peak substantiates the formation of CuNPs in our study, providing clear evidence of the reduction process facilitated by *P. juliflora* extract. SEM images revealed the shape and size distribution of the CuNPs, showing a range of shapes including spherical, irregular, truncated hexagonal, cylindrical, triangular and prismatic forms, with particle sizes between 10 and 80nm. This diversity in morphology is consistent with findings from Arya *et al.* (2018) and Murthy *et al.* (2020), who reported similar variations in CuNPs synthesized by different methods. The observed agglomeration of particles is likely due to their high surface area.

EDX analysis identified peaks corresponding to elemental copper and oxygen, confirming the purity of the produced CuNPs. The presence of oxygen is attributed to the bioactive molecules encapsulated within the CuNPs. These results are consistent with previous studies (Khodaie and Ghasemi, 2018; Murthy *et al.*, 2020) which noted the presence of CuO and Cu_2O due to surface oxidation. XRD analysis showed that the CuNPs have FCC lattice structure, with prominent peaks corresponding to (220), (111), (200) and (311) planes. Well-defined crystal structures were indicated by the average crystallite size, which was determined to be around 9.972nm using the Scherrer equation. This finding aligns with the results of Jay *et al.* (2015) and others who have studied the crystalline nature of CuNPs.

FTIR spectra exposed the presence of several functional groups such as hydroxy groups, aromatic C-H, and C-O stretching, indicative of the bioactive compounds from *P. juliflora* involved in stabilizing the CuNPs. Peaks at 3376.65 and 1058.70cm^{-1} were particularly significant, suggesting the presence of polymeric OH stretch and aliphatic fluoro compounds, respectively. These results correspond with the findings of Coates (2000) and Nandiyanto *et al.* (2019), who identified similar functional groups in their studies.

During the antibacterial efficacy of CuNPs against *S. aureus*, the zone of inhibition increased in a concentration-dose manner, according to the data, with more antibacterial activity observed at higher CuNPs concentrations. At 600ppm, the inhibition zone measured 19.5±0.41mm, in contrast to 24mm for the enrofloxacin (positive control). These results align with earlier research by Padma *et al.* (2018), Murthy *et al.* (2020) and Rambau *et al.* (2024), which also documented substantial antibacterial efficacy of CuNPs against diverse pathogens.

The biological approach for the synthesis of CuNPs used in our study aligns with the study conducted by Tyagi *et al.* (2018), as they described it as an eco-friendly and sustainable method for the fabrication of NPs. Such as Rajakumar *et al.* (2012) and Jayaseelan *et al.* (2018), uncovered bioactive compounds in *P. juliflora* are vital for the reduction and stabilization processes. The antioxidant and antibacterial effects reported during current study supports potential of CuNPs in range of applications, as mentioned by Betancourt-Galindo *et al.* (2014) and Crisan *et al.* (2021).

As highlighted by Khan and Ahmad (2013) and Arumugam *et al.* (2015), green fabrication of NPs has several benefits for both environmental and therapeutic purpose. Their ability to damage cell membrane and induce oxidative stress is one of the primary mechanisms responsible for antibacterial properties of NPs (Chaloupka *et al.*, 2010). Environmental cleaning and water purification using CuNPs, as mentioned by Singh and Naraa (2013) and Kalaiselvi *et al.* (2015), show how beneficial and versatile the CuNPs are for bacterial pathogens.

In this regard, our work shows *P. juliflora* can be used for effectively producing CuNPs. They have strong antibacterial and antioxidant properties. These results are a valuable addition to the expanding body of research on the potential application of CuNPs in a variety of disciplines and green synthesis approaches.

Conclusions: This study highlights the antibacterial potential of CuNPs, particularly against *S. aureus*, a major cause of mastitis. The results show that CuNPs effectively hinder bacterial growth in a dose dependent manner, with higher concentration yielding stronger inhibition. Moreover, the CuNPs show remarkable antioxidant qualities that might help reduce inflammation in affected tissues and oxidative stress. Using CuNPs offers a sustainable and effective way to control mastitis, a common and financially challenging problem in the dairy sector. Their dual function- combating bacterial infections while reducing oxidative damage-make them a promising alternative to conventional treatments. These findings pave the way for further research into CuNPs-based therapies offering a more sustainable approach to improving animal health and dairy production.

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Authors contribution: RZ, NA, and AQ contributed equally to the conceptualization and design of the study. RZ and AQ performed experiments and collected the data. NA, AR, SUR, MK, MS, AA, ZUR, and MAS contributed to data analysis and interpretation. ASA and NB provided technical guidance and resources. ASA and NB contributed to statistical analysis. MUH and ZM supervised the project, critically reviewed the manuscript, and were the corresponding authors. All authors reviewed and approved the final manuscript.

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