Research Article

Green synthesis and characterization of copper nanoparticles using *Bauhinia variegata* extracts: Antibacterial efficacy against Diabetic wound pathogens

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Abstract: The green synthesis of copper nanoparticles (CuNPs) using *Bauhinia variegata* leaf extract presents a cost-effective and environmentally sustainable approach to developing alternative antimicrobial agents. Bauhinia variegata is a medicinal plant rich in bioactive compounds, including anthraquinones, flavonoids, phenolic glycosides, alkaloids, saponins, reducing sugars, and tannins, which facilitate nanoparticle formation and stabilization. The synthesis of CuNPs was indicated by a color change from light to dark green and confirmed through UV-Vis spectroscopy and FTIR analysis, which identified key functional groups responsible for nanoparticle stabilization. The antibacterial potential of the synthesized CuNPs was evaluated against *Pseudomonas spp*., *Klebsiella spp*., and *Staphylococcus spp*., revealing significant inhibitory effects. Among the tested species, *Pseudomonas* spp. exhibited the highest susceptibility, while *Staphylococcus* spp. showed the lowest inhibition. Ethanol extracts demonstrated superior antimicrobial activity compared to other solvent extracts. These findings highlight the potential of *Bauhinia variegata*-mediated CuNPs as promising antimicrobial agents, particularly against multidrug-resistant bacterial strains. Further research should explore their mechanism of action and potential applications in pharmaceutical and biomedical fields.

Keywords: green synthesis, copper nanoparticles, *bauhinia variegata*, antibacterial activity, multidrug-resistant pathogens.

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Introduction

Diabetic wounds, a common and serious complication of diabetes mellitus, often result in chronic infections that are difficult to treat due to impaired healing processes [1]. As the prevalence of diabetes continues to rise globally, there is an increasing need for novel strategies to combat infections and enhance wound healing [2]. One promising approach is the use of nanoparticles, particularly copper nanoparticles (CuNPs), which have gained significant attention for their antimicrobial and wound-healing properties [3]. CuNPs are valued for their unique optical, catalytic, mechanical, and electrical characteristics, along with their affordability and potential to match the effectiveness of more expensive metals like gold and silver[4]. The green synthesis of CuNPs has emerged as a particularly attractive method, as it allows for the controlled production of nanoparticles with specific sizes and shapes under mild reaction conditions, utilizing biological materials as reducing and stabilizing agents [5]. This eco-friendly approach avoids hazardous chemicals, reduces energy consumption, and is more cost-effective compared to traditional synthesis methods [6]. Plants rich in bioactive compounds, such as flavonoids and terpenoids, are ideal for nanoparticle synthesis because they can serve as capping, stabilizing, and reducing agents, producing nanoparticles with consistent quality and minimal environmental impact [7].

Green-synthesized CuNPs have shown significant antimicrobial activity and promising potential in wound care applications. By incorporating biological principles, such as enzymatic reduction, with eco-friendly synthesis methods, these nanoparticles offer a sustainable alternative to conventional wound-healing treatments [8]. Studies have demonstrated that plant-derived CuNPs can effectively disrupt microbial cell membranes, inhibit protein synthesis, and damage nucleic acids, enhancing their therapeutic efficacy [9-12]. Their incorporation into advanced wound dressings is a promising strategy to improve treatment outcomes, particularly for diabetes-related wounds, which are often complicated by poor circulation and slow healing [13]. *Bauhinia variegata*, a medicinal plant known for its rich array of bioactive compounds, has shown great potential in the green synthesis of CuNPs [14]. The plant's phytochemicals, such as flavonoids and terpenoids, possess antioxidant, anti-inflammatory, and antimicrobial properties, which can be harnessed to synthesize CuNPs under mild conditions, providing a sustainable and effective alternative to traditional nanoparticle production [15].

This research aims to address two critical aspects of diabetic wound infections: (1) the identification and characterization of microbial pathogens responsible for these infections, and (2) the synthesis and evaluation of CuNPs derived from *Bauhinia variegata* for their antimicrobial activity against these isolated pathogens. The goal of this study is to assess the potential of these green-synthesized CuNPs as a novel therapeutic agent to manage diabetic wound infections and accelerate wound healing.

Methodology

Sample Collection and Bacterial Isolation

Diabetic wound samples were collected from patients at Salem's Government Hospital using sterile cotton swabs. These samples were transported to the Microbiology laboratory and stored at 4°C until further processing. Upon arrival, the samples were inoculated onto Blood agar, Mannitol Salt Agar (MSA), and MacConkey agar plates using a sterile wire loop. The plates were incubated at 37°C for 24 hours to allow bacterial growth. After incubation, bacterial colonies were examined for their morphological characteristics. Pure colonies were selected for further identification. To ensure proper bacterial concentration, bacterial cultures were isolated using the 0.5 McFarland technique, which facilitated subsequent testing and analysis [16].

Identification of Bacterial Pathogens

To proceed with the identification of the isolated colonies, Gram staining was performed to differentiate between Gram-positive and Gram-negative bacteria, based on the structural differences in their cell walls. This initial step was crucial for classifying the bacterial pathogens. Subsequently, the isolated bacteria were further characterized using a series of biochemical tests, including the Oxidase test, Indole test, Catalase test, Urease test, Citrate test, Coagulase test, Triple Sugar Iron (TSI) test, and Triple Sugar Iron with Slant & Butt (TSI) test [17]. These tests allowed for the comprehensive identification of the bacterial pathogens present in the diabetic wound samples.

Collection and Processing of Medicinal Herbs

Bauhinia variegata plant samples were collected from the Kolli Hills, Tamil Nadu, India. The collected plant material was thoroughly washed to remove any impurities [18]. All media and chemicals required for the antibacterial investigation, including nutrient agar, Muller-Hinton agar, and copper sulfate ($CuSO4$) for nanoparticle synthesis, were procured from HiMedia Laboratories (Mumbai, India). Nanopure filtered water was utilized throughout the experiment to ensure purity and reproducibility**.**

Preparation of Plant Extract

Bauhinia variegata leaves were initially washed under running tap water and then shade-dried to preserve their bioactive constituents. The dried leaves were finely ground into a powder. For extract preparation, 5 g of the powdered sample was mixed with 50 mL of nanopure filtered water. The mixture was heated in a water bath at 60°C for 15 minutes. Following heating, the solution was continuously stirred using a magnetic stirrer for 30 minutes after reaching the boiling point [19]. The extract was subsequently filtered using Whatman No. 1 filter paper, and the obtained filtrate was stored for further experimental analysis.

Phytochemical Analysis

The preliminary phytochemical analysis of *Bauhinia variegata* leaf extracts was conducted using standard qualitative assays to detect the presence of various secondary metabolites. Alkaloids were identified using Mayer's and Wagner's reagents, with the formation of a precipitate indicating a positive result. Flavonoids were detected using the aluminum chloride test, where a yellow coloration confirmed their presence. The presence of phenolic compounds was established through the ferric chloride test, characterized by the development of a blue-black coloration. Saponins were confirmed using the froth test, in which the formation of stable and persistent foam indicated a positive result. Tannins were detected via the lead acetate test, as evidenced by the formation of a brown precipitate. Terpenoids were identified through the Salkowski test, where a reddish-brown coloration confirmed their presence. The presence of sterols was validated using the Liebermann-Burchard test, indicated by a green or blue color change. Finally, glycosides were detected using the Keller-Killiani test, with the formation of a brown ring at the interface confirming their presence [20]. These qualitative assessments provide valuable insight into the phytochemical composition of *Bauhinia variegata*, reinforcing its pharmacological potential in antimicrobial, antioxidant, and therapeutic applications.

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Synthesis of Copper Nanoparticles (CuNPs)

To synthesize copper nanoparticles, 20 mL of *Bauhinia variegata* extract was added to 80 mL of 2 mM $CuSO_4$ solution. The reaction mixture was maintained at room temperature for four days to facilitate the formation of CuNPs. After the incubation period, the mixture was centrifuged, and the resulting pellet was collected and dried for further characterization [21]. The bioreduction of Cu^{2+} ions to CuNPs was monitored by recording absorbance using a UV-Vis spectrophotometer at regular intervals within the wavelength range of 300-900 nm [22].

Antibacterial Activity

Freshly seeded test organisms were plated to evaluate the antibacterial properties of the synthesized copper nanoparticles. The nanoparticles interacted with the organisms by diffusing into the medium. The diameters of the inhibitory zones formed were measured to determine antibacterial efficacy [23]. Twenty milliliters of Muller-Hinton Agar medium were poured onto Petri plates containing bacterial cultures and allowed to solidify overnight. Subsequently, the 100µL inoculum was dried under laminar airflow for five minutes. Wells of 6 mm diameter were created using a well cutter in the seeded agar plate. Different volumes of the green solution containing synthesized copper nanoparticles were introduced into these wells using a micropipette. The plates were then incubated at 37°C for 24 hours. The diameter of the inhibition zones formed around the wells served as an indicator of antibacterial activity. The ability of the nanoparticles to diffuse into the agar and inhibit microbial growth demonstrated their antibacterial potential.

Results and Discussion

Identification of Isolated Organisms

The isolation and identification of pathogenic microorganisms from diabetic wound samples revealed a diverse microbial profile, including *Staphylococcus* spp., *Pseudomonas* spp., and *Klebsiella* spp (Table 1). These organisms are well-known contributors to chronic wound infections, particularly in diabetic patients, where impaired immune responses and poor circulation create an environment conducive to bacterial growth and persistence. The ability of these pathogens to form biofilms, evade immune responses, and develop antimicrobial resistance further complicates wound healing and infection management.

Among the isolated species, *Staphylococcus* spp. and *Pseudomonas* spp. are of particular concern due to their frequent association with multidrug resistance. *Staphylococcus aureus*, for example, has developed resistance to a broad range of antibiotics, including methicillin, creating significant challenges in treating infections in diabetic wounds [24]. Similarly, *Pseudomonas aeruginosa*, a highly opportunistic pathogen, exhibits intrinsic resistance to several antibiotics and can rapidly develop resistance to others during treatment, making it a key player in chronic wound infections [25]. The multidrug-resistant nature of these organisms emphasizes the need for alternative treatment strategies, such as the use of novel antimicrobial agents or combination therapies, which can effectively target these pathogens while minimizing the risk of resistance development [26].

Table 1. Biochemical test results for *Staphylococcus spp*., *Pseudomonas spp*., and *Klebsiella spp.*

Biochemical	***Staphylococcus spp***	***Pseudomonas spp***	***Klebsiella spp***
Gram Staining	+	-	-
Motility	Motile	Motile	Non Motile
Indole	-	+	-
Methyl Red	+	-	-
VP test	-	-	+
Citrate	+	+	+
Urease	+	-	+
TSI Test	A/A	K/K	A/A
Oxidase	-	+	-
Catalase	+	+	+
Coagulase	+	-	-

Klebsiella spp. though often less resistant than *Staphylococcus* and *Pseudomonas*, still contribute to wound infections, especially in the setting of hospital-acquired infections or immunocompromised patients [27]. In light of these findings, the need for continuous surveillance of wound infections in diabetic patients is critical to identify prevalent pathogens and their resistance profiles. This can inform the development of more targeted, effective treatment strategies that address both bacterial and fungal components of wound infections. Additionally, the identification of novel antimicrobial agents, as well as therapies targeting biofilm formation and resistance mechanisms, could provide valuable tools in managing these challenging infections [28].

Preparation of Plant Extract

The successful preparation of *Bauhinia variegata* leaf extract was confirmed through its appearance and solubility. Extracts were prepared using methanol, ethanol, and water to compare their efficiency in extracting bioactive compounds. The methanolic extract exhibited a deep greenish-brown color, indicating a high concentration of polyphenols and flavonoids. The ethanolic extract appeared slightly lighter but still retained a significant presence of bioactive constituents. The aqueous extract showed a brownish coloration, suggesting the presence of water-soluble phytochemicals such as tannins and saponins [29-31]. These variations in extract color and solubility highlight the differential solubility of various bioactive compounds in different solvents. The extraction method, which involved heating at 60°C and continuous stirring, likely enhanced the solubility and release of these bioactive constituents into the respective solvents. The final filtrates were clear, suggesting the efficient removal of plant debris and insoluble particles, making them suitable for further applications in nanoparticle synthesis.

Phytochemical Analysis

Phytochemical screening of *Bauhinia variegata* leaf extract revealed the presence of diverse secondary metabolites, including alkaloids, flavonoids, sterols, terpenoids, proteins, saponins, phenols, and glycosides. These bioactive compounds are well-documented for their antimicrobial, antioxidant, and anti-inflammatory properties. The results of the phytochemical analysis are summarized in Table 2. The presence of flavonoids and phenolic compounds indicates strong antioxidant potential, which may contribute to wound healing and microbial inhibition [32]. Furthermore, the detection of alkaloids and saponins suggests a potential synergistic antimicrobial effect, particularly in the context of nanoparticle synthesis [33]. These findings reinforce the therapeutic significance of *Bauhinia variegata* in traditional medicine and highlight its promising application in nanoparticle-based antimicrobial therapies.

Table 2. Phytochemical Screening of *Bauhinia variegata* Extracts in Methanol, Ethanol, and Water

	Water	Methanol	Ethanol
Reducing sugar	-	-	+
Anthraquinone	-	-	+
Terpenoids	-	-	+
Phenols	+	+	+
Flavonoids	+	+	+
Saponin	+	-	+
Tannin	+	+	+
Alkaloid	-	-	+
Cardiac glycosides	-	-	-

Synthesis of Copper Nanoparticles (CuNPs)

The formation of copper nanoparticles was visually observed through a gradual change in the color of the reaction mixture from light blue to a dark brownish-red shade over the four-day incubation period. This color transformation suggests the reduction of Cu(II) ions to Cu(0), a process mediated by the phytochemicals present in the plant extract [34]. The bright blue color of the solution confirms the presence of Cu^{2+} ions from copper sulfate ($CuSO_4$), which serves as the precursor for nanoparticle synthesis (Fig.1).

Figure 1. Copper nanoparticle formation, indicated by the color change from light blue to dark brownish-red over a four-day incubation period in the

presence of the plant extract. This color shift reflects the reduction of Cu(II) ions to Cu(0).

This characteristic color arises due to the d-d electronic transitions in aqueous Cu^{2+} ions. The extract appears dark green, suggesting the presence of bioactive phytochemicals such as flavonoids, tannins, and phenolic compounds. These compounds play a crucial role in the reduction of Cu^{2+} to Cu^0 during nanoparticle formation. The extract was covered with aluminum foil, likely to prevent light-induced degradation of its active constituents. Upon mixing $CuSO_4$ with the plant extract, a color change is observed, transitioning from bright blue (Cu^{2+}) to a darker greenish-brown. This change indicates the reduction of Cu^{2+} ions into copper nanoparticles (CuNPs), facilitated by the phytochemicals present in the extract. The final color of the solution suggests successful nanoparticle synthesis, with plant metabolites acting as both reducing and stabilizing agents. The centrifuged CuNPs were collected as a dark-colored precipitate, indicative of successful nanoparticle formation. The drying process resulted in a fine, dark powder, suggesting the presence of nanosized copper particles. The involvement of plant metabolites in the bio-reduction and stabilization of CuNPs was further supported by the formation of a stable colloidal suspension without excessive aggregation.

UV–Visible Spectroscopic Analysis of CuNPs

Metallic nanoparticles exhibit unique optical properties due to their nanoscale dimensions, leading to an exponential reduction in particle size and enhancement of surface plasmon resonance. UV–Visible spectroscopy is widely used to characterize nanoparticles, as the peak position and shape of absorption spectra are highly sensitive to nanoparticle size and distribution [35]. In this study, the synthesized CuNPs were analyzed within the wavelength range of 280–500 nm, and significant absorption peaks were observed, confirming the presence of copper nanoparticles (Fig.2). These peaks are indicative of the bio-reduction process, wherein plant phytochemicals act as reducing and stabilizing agents in nanoparticle formation. Quartz cuvettes with a 2.5 mL capacity and a 1 cm path length were employed for spectroscopic measurements.

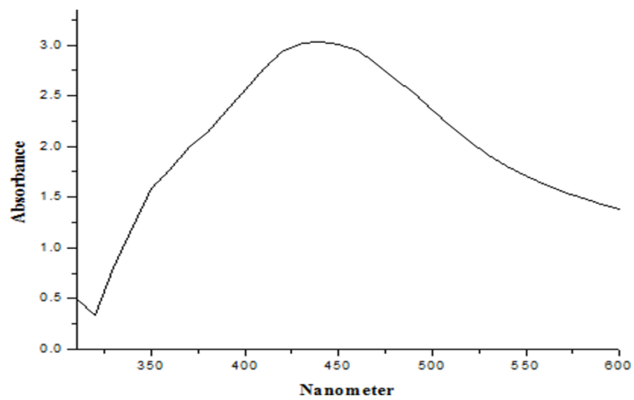

Figure 2. UV-Visible Spectroscopic Confirmation of Copper Nanoparticles

The UV-Vis spectrum of the synthesized copper nanoparticles (CuNPs) exhibits a strong absorbance peak around 450 nm, confirming the formation of CuNPs. This characteristic surface plasmon resonance (SPR) band is indicative of the nanoscale nature of the copper particles. The broad peak suggests polydispersity and possible aggregation, which may be influenced by synthesis parameters such as reaction time and concentration of the plant extract. The high absorbance intensity (above 3.0) indicates efficient nanoparticle formation, likely facilitated by phytochemicals present in the extract acting as reducing and stabilizing agents.

FTIR Analysis

Fourier-transform infrared (FTIR) spectroscopy was employed to identify the functional groups present in the ethanolic extract of *Bauhinia variegata*. The analysis was performed using Attenuated Total Reflectance (ATR) within the spectral range of 4000–650 cm^{-1}. The FTIR spectra indicated the presence of hydroxyl (-OH), carbonyl (-C=O), and amine (-NH) groups, which correspond to various bioactive compounds such as flavonoids, tannins, terpenoids, and glycosides [36]. These functional groups play a significant role in the bioreduction and stabilization of CuNPs, contributing to their potential antibacterial properties.

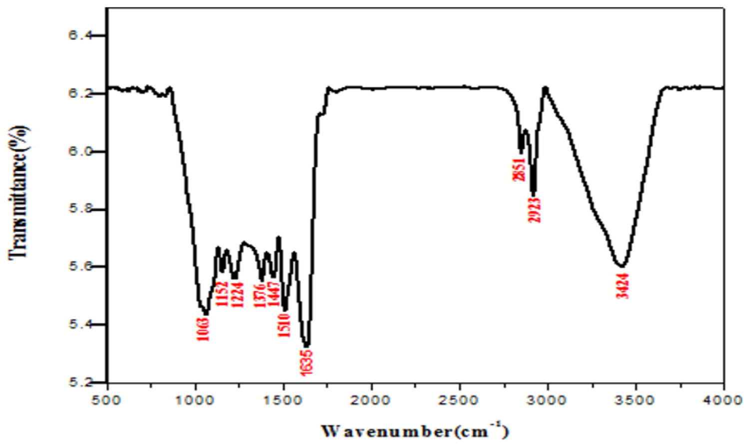

Figure 3. FTIR Spectrum of Copper Nanoparticles. The FTIR spectrum reveals the functional groups present in the plant extract, which are responsible for the reduction and stabilization of copper nanoparticles (CuNPs).

The FTIR spectrum provides valuable insights into the functional groups responsible for the reduction and stabilization of CuNPs (Fig 3). The absorption band at 3424 cm^{-1} corresponds to O–H stretching vibrations, suggesting the presence of phenols and flavonoids, which aid in reducing Cu^{2+} to Cu^{0}. The peaks at 2923 cm^{-1} and 2851 cm^{-1} are attributed to C–H stretching vibrations of aliphatic compounds. Additionally, the bands at 1651 cm^{-1} and 1576 cm^{-1} indicate C=O stretching from amides or ketones, likely originating from proteins and polyphenols involved in nanoparticle capping. The region between 1108 cm^{-1} and 1510 cm^{-1} is associated with C–O and C–N stretching vibrations, further confirming the presence of bioactive molecules from the plant extract.

The UV-Vis and FTIR analyses confirm the successful green synthesis of CuNPs using *Bauhinia variegata* extract. The UV-Vis spectrum validates nanoparticle formation through an SPR peak in the expected range, while the FTIR spectrum identifies biomolecules responsible for reduction and stabilization. The presence of hydroxyl, carbonyl, and amide groups suggests that polyphenols, flavonoids, and proteins play key roles in nanoparticle synthesis.

This green synthesis approach provides an eco-friendly alternative to conventional chemical methods, leveraging plant metabolites for nanoparticle formation [37]. Further characterization using XRD and SEM can provide additional insights into the crystalline structure and morphology of CuNPs. The study suggests that *Bauhinia variegata* extract effectively mediates CuNP synthesis, potentially offering applications in biomedical, antimicrobial, and catalytic fields.

Antibacterial Activity of Synthesized CuNPs

The antimicrobial activity of *Bauhinia variegata* leaf extracts was initially assessed using ethanol, methanol, and water extracts at a fixed concentration of 25 µg/mL against *Staphylococcus* spp., *Pseudomonas* spp., and *Klebsiella* spp.. Among these extracts, the ethanol extract exhibited the highest antimicrobial activity, followed by the methanol extract, while the water extract demonstrated the least inhibition. The superior activity of the ethanol extract suggests that ethanol effectively extracts bioactive compounds, such as flavonoids, tannins, and polyphenols, which contribute to microbial inhibition.

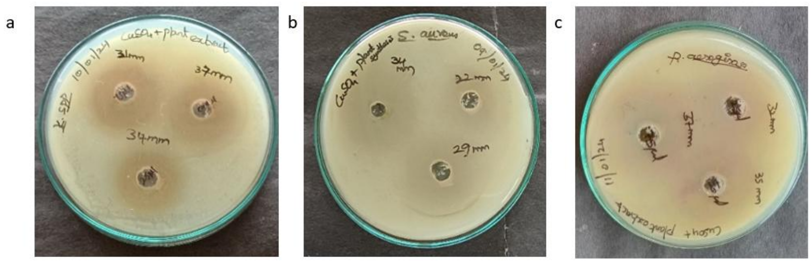

Figure 4. Antimicrobial Activity of *Bauhinia variegata* Leaf Extracts and $CuSO_4$ Nanoparticles. The antimicrobial efficacy of ethanol extract and $CuSO_4$ nanoparticles (NPs) was evaluated at various concentrations (25 µg/mL, 50 µg/mL, and 75 µg/mL) against (a) *Klebsiella* spp.. (b) *Staphylococcus* spp., and (c) *Pseudomonas* spp.,

Following this, $CuSO_4$-derived nanoparticles (CuNPs) synthesized using the ethanol extract were further evaluated for their antimicrobial activity at increasing concentrations (25 µg/mL, 50 µg/mL, and 75 µg/mL) against the same bacterial strains (Table 3). The results indicate that the synthesized CuNPs exhibit enhanced antimicrobial properties [38], with inhibition zones increasing proportionally with concentration. *Klebsiella* spp. showed the highest sensitivity to CuNPs, with an inhibition zone of 31 mm at 25 µg/mL, which increased to 37 mm at 75 µg/mL.

Table 3. Antimicrobial activity of $CuSO_4$-derived Nanoparticles (CuNPs) synthesized using ethanol extract.

	25 µg/mL	50 µg/mL	75 µg/mL
Staphylococcus spp	22	29	34
Pseudomonas spp	32	35	37
Klebsiella spp	31	34	37

Pseudomonas spp. also exhibited strong susceptibility, with an inhibition zone of 32 mm at 25 µg/mL, reaching 37 mm at 75 µg/mL. *Staphylococcus* spp. demonstrated moderate inhibition, with zones increasing from 22 mm at 25 µg/mL to 34 mm at 75 µg/mL. These findings highlight the enhanced antimicrobial activity of CuNPs synthesized using *Bauhinia variegata* ethanol extract. The nanoparticles likely exhibit a synergistic effect due to their small size, increased surface area, and bioactive phytochemical coating, which enhances bacterial interaction and disruption. The mechanism of action may involve the generation of reactive oxygen species (ROS), disruption of bacterial cell membranes, and interference with intracellular processes.

Conclusion

The present study highlights the successful green synthesis of copper nanoparticles (CuNPs) using *Bauhinia variegata* leaf extract, demonstrating a simple, cost-effective, and environmentally sustainable approach. The phytochemical analysis confirmed the presence of key bioactive compounds, including anthraquinones, flavonoids, phenolic glycosides, alkaloids, saponins, reducing sugars, and tannins, which contributed to nanoparticle stabilization. The formation of CuNPs was validated through visual color change, UV-Vis spectroscopy, and FTIR analysis. The synthesized CuNPs exhibited significant antibacterial activity against *Pseudomonas* spp., *Klebsiella* spp., and *Staphylococcus* spp., with *Pseudomonas* spp. showing the highest susceptibility. Ethanol extracts demonstrated the strongest inhibitory effect, further emphasizing their antimicrobial potential. These findings underscore the potential of *Bauhinia variegata*-mediated CuNPs as promising alternative antimicrobial agents, particularly in combating multidrug-resistant bacterial infections. Future studies should focus on exploring their mechanistic action and potential applications in clinical and pharmaceutical settings.

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