RESEARCH PAPER

Biogenic Synthesis of Papain Conjugated Copper Metallic Nanoparticles and its Antibacterial and Antifungal Activity

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Abstract: *Biogenic synthesis of papain-conjugated copper metallic Nanoparticles and their antibacterial and antifungal activities Papain metallic conjugated nanoparticles (Papain-CuNPs) were synthesised using Papain and $CuSO_4.5H_2O$. Papain-CuNPs were characterized using UV-visible spectroscopy, FT-IR (Fourier-Transform Infrared), HR-TEM (High-Resolution Transmission Electron Microscopy), XRD (X-ray diffraction), FE-SEM (Field-emission scanning electron microscopy), zeta potential, and a zeta sizer. The antibacterial activity of papain-CuNPs against human infectious microorganisms (Citrobacter spp, Pseudomonas aeruginosa and Candida albicans) was investigated. The mechanism of action of papain-CuNPs was evaluated using FE-SEM and HRTM. UV spectroscopy confirmed the plasma resonance (SPR) at 679 nm, which indicated the formation of papain-CuNPs. The FT-IR spectrum absorbance peaks at 3927, 3865, 3842, 3363, 2978, and 2900 cm^{-1} indicate the presence of O-H and N-H of the secondary amine, and peaks at 1643 and 1572 cm^{-1} represent C=O functional groups in Papain-CuNPs. EDAX analysis confirmed the presence of copper in the papain-CuNPs. The zeta potential (-42.6 mV) and zeta size (99.66 d. nm) confirmed the stability and size of the nanoparticles. XRD confirmed the crystalline nature of the papain-CuNPs. FE-SEM and HRTM showed an oval structure and the nano particles' 16.71244–34.84793 nm. The synthesized papain-NPs showed significant antibacterial activity against clinical P. aeruginosa (15 mm). MIC 125 µg/ml) showed bactericidal activity against P. aeruginosa and the mechanism of action of Papain-NPs was confirmed using an electron microscope by observing cell damage and cell shrinking. Papain-CuNPs have significant antibacterial activity and are thus used in the treatment of P. aeruginosa infections.*

Keywords: *Papain, Copper sulphate, Nanoparticles, SE-SEM, HRTEM, Antibacterial, Antifungal.*

1. INTRODUCTION

The development of antimicrobial resistance in microorganisms which is dangerous to antibiotic efficacy leads to an emergency in global public health [1, 2]. Antimicrobial resistance is considered the top ten diseases for human health risk, which causes serious economic issues. Even

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though modern medicine relies on effective antimicrobial medicines, high rates of resistant infections caused by a wide range of microorganisms have been documented in all World Health Organization (WHO) regions. According to the WHO, 4.95 million deaths were associated with bacterial antibiotic resistance in 2019, with 127 million deaths due to antimicrobial resistance [3]. Although antibiotic overuse and misuse are the primary causes of AMR, other interconnected factors also contribute to its prevalence and spread [4]. Several low- and middle-income countries have higher AMR rates than high-income countries, although lower per-person antibiotic consumption occurs in the former. Infections caused by microbial agents have caused massive deaths worldwide. According to WHO reports, ten million deaths are predicted by 2050. Antimicrobial-resistant microbial infections will increase during hospital stays, mortality, lengthy treatment and raise healthcare expenses [5]. Advanced formulations containing biologically active molecules are required [6].

Converting bioactive antibiotics into nanoparticles will overcome antimicrobial resistance owing to increased binding site accumulation, bioavailability, and reduction in the biodegradation of active molecules [7]. The size of nanoparticles ranged from 1 to 100 nm. A larger surface area enhances bioavailability and rapid antimicrobial activity [8]. Copper is a good choice for synthesizing bioactive molecule-conjugated nanoparticles owing to its characteristic features, which are equal to those of noble metals. Copper-conjugated nanoparticles have gained increasing attention in nanomedicine because of their broad range of biological, chemical, and physical activities [9]. Copper also has good antimicrobial activity, per the research reports [10]. Papain, an essential peptidase enzyme found in papaya, can hydrolyze complex protein molecules into tiny peptides and amino acids due to its proteolytic properties. Papain demonstrates selectivity in its antibacterial activity, exerting minimal harm to mammalian cells. This selectivity is advantageous for therapeutic applications, as it minimizes potential side effects and cytotoxicity associated with many conventional antibiotics. They can be used as antimicrobial agents [11]. In this study, copper sulfate- and papain-conjugated nanoparticles were synthesized and evaluated for their antimicrobial activity.

2. EXPERIMENTAL PROCEDURES

2.1. Materials

Papain, Copper sulfate, and HMDS were purchased from Sigma Aldrich (Mumbai, India). Ciprofloxacin antibiotic discs, Muller Hinton agar, nutrient agar, and Muller Hinton broth were purchased from HiMedia Mumbai.

2.1.1. Synthesis of Papain metallic nanoparticles

Papain metallic nanoparticles were synthesized using the method described by Mali et al. [12] and Amjed et al. [13], with minor modifications. Erlenmeyer flask 5 mM 50 ml of $CuSo_4.5H_2O$ solution was prepared. Fifty millilitres of 10 mg/ml papain solution were prepared and slowly added to the copper sulfate solution with magnetic stirring for 30 min at 10,000 rpm. The color of the solution changed from blue to light green, which confirmed that papain nanoparticles were formed and identified visually. The nanoparticle solution was centrifuged (30 min and 12000 rpm), and the papain copper nanoparticles (Papain-CuNPs) were collected. The papain-CuNPs were washed with sterile water for further characterization and antimicrobial activity.

2.2. Characterisation

2.2.1. Characterisation of Papain-CuNPs

Papain-CuNPs were characterized using UV-visible (Shimadzu) and ATR-FTIR spectrometry to identify papain and copper in the nanoparticles (Shimadzu IR affinity). X-ray diffraction (XRD) was used to confirm the crystalline nature of the papain-CuNPs. EDAX confirmed the elemental analysis of the papain-CuNPs. FE-SEM (TESCAN) and HRTEM (JEOL Ltd., Japan) were used to study the morphology, size, and shape of the Papain-CuNPs nanoparticles. The papain-CuNP size and zeta potential were analyzed using a Zetasizer Nano ZS (Malvern Instruments) [14].

2.2.2. Antimicrobial activity of the Papain-CuNPs

The antibacterial and antifungal activities of papain-CuNPs (clinical *Citrobacter spp*, *Pseudomonas aeruginosa* and *Candida albicans*) were analyzed following the methods of Kotakonda et al. and Muddukrishnaiah K et al.

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[15, 16], with minor changes. A sterile borer was used to create wells in the agar, and 100 µL of Papain-CuNPs was aseptically applied in duplicate. Ciprofloxacin (10 µg/mL) was added to agar wells as a drug control. Treated plates were incubated at 37°C for 24 h.

2.2.3. Papain-CuNPs-minimal inhibitory concentration (MIC)

The Papain-CuNPs MIC assay was performed as described by Moussa et al. [17] with minor changes. Different concentrations of papain-CuNPs (250, 125, 62.5, 31.25, 15.625, 7.8125, 3.90625, and 1.953125 µg/mL) were added to the 96 well plates. Ciprofloxacin was used as the standard. 100 µL of the test microorganism (*P. aeruginosa*) were inoculated into each well. The experimental 96 well plates were incubated in a microbiological incubator for 24 h. MIC was measured using tetrazolium staining.

2.2.4. Electronic Microscopy (FESEM and HRTEM) examination of antimicrobial effects of Papain-CuNPs

Papain-CuNP-treated bacteria were centrifuged, thoroughly cleaned with sterile water, and incubated with 2.5% glutaraldehyde for two hours at room temperature. The water content of the bacterial cells was removed using different concentrations of alcohol, and finally, the bacterial morphology was fixed with hexamethyldisilazane (HMDS). Morphologically fixed bacterial cells were observed for antimicrobial activity using FE-SEM (TESCAN) and HR-TEM (Oxford) [18].

3. RESULTS AND DISCUSSION

3.1. Preparation of Papain-CuNPs

Papain (50 ml of 10 mg/ml) solution was added slowly to the copper sulfate solution (5 mM 50 ml of $CuSo_4.n4H_2O$) with constant stirring. A reaction occurred between papain and copper sulfate, and the color changed from blue to light green. The color change indicates the formation of papain-CuNPs (Figure 1). During the synthesis of copper nanoparticles, a characteristic blue hue typically exists due to the presence of copper ions in the solution. As the reduction reaction progresses and copper ions are converted into copper nanoparticles, the color of the solution undergoes a noticeable transformation to light green. This color change is attributed to the phenomenon of surface plasmon resonance, wherein the collective oscillation of free electrons in the nanoparticles interacts with incident light, resulting in the absorption and scattering of specific wavelengths. The exact color observed during nanoparticle synthesis can vary depending on factors such as nanoparticle size, shape, and stabilizing agents.

Fig. 1. Copper and Papain-CuNPs

3.2. Characterisation of Papain-CuNPs

3.2.1. UV Visible spectroscopic analysis of Papain-CuNPs

The surface plasmon resonance (SPR) of papain-CuNPs was investigated utilizing a UV-visible spectrophotometer across the absorbance range of 100–800 nm. Analysis revealed a distinct absorption peak at 679 nm, as depicted in Figure 2, confirming the formation of nanoparticles.

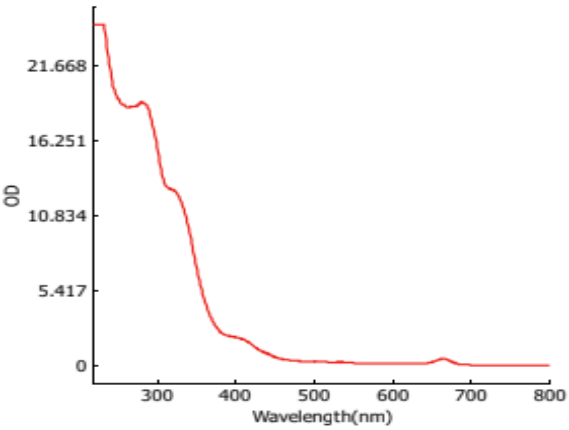

Fig. 2. UV spectrum of Papain-CuNPs

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This characteristic SPR peak indicates the collective oscillation of free electrons on the surface of the nanoparticles upon interaction with incident light. The precise measurement of the SPR peak at 679 nm provides valuable quantitative information about the size, shape, and composition of the papain-CuNPs, enabling further understanding of their optical properties and potential applications in fields such as sensing, imaging, and catalysis.

3.2.2. FT-IR analysis of Papain-CuNPs

The formation of papain-CuNPs was investigated through Fourier-transform infrared (FT-IR) spectroscopy, revealing distinctive absorbance peaks indicative of the reduction process by $CuSO_4 \cdot 5H_2O$, as illustrated in Figure 3. The spectrum exhibited prominent peaks at 3927, 3865, 3842, 3363, 2978, and 2900 cm^{-1}, corresponding to O-H and N-H bonds characteristic of secondary amine groups present in papain. Additionally, peaks at 1643 and 1572 cm^{-1} were observed, representing C=O functional groups within the papain structure. These precise spectral measurements provide quantitative insight into the molecular interactions and chemical changes occurring during the formation of papain-CuNPs, elucidating the mechanisms underlying nanoparticle synthesis and facilitating the optimization of synthesis protocols for tailored nanoparticle properties in various applications.

3.2.3. Papain-CuNPs Zeta Size and Potential Analysis

Papain-CuNPs average zeta size 99.66 d. nm (Figure 4A) and -42.6 mV zeta potential (Figure 4B) indicate that the synthesised Papain-CuNPs were in nano size.

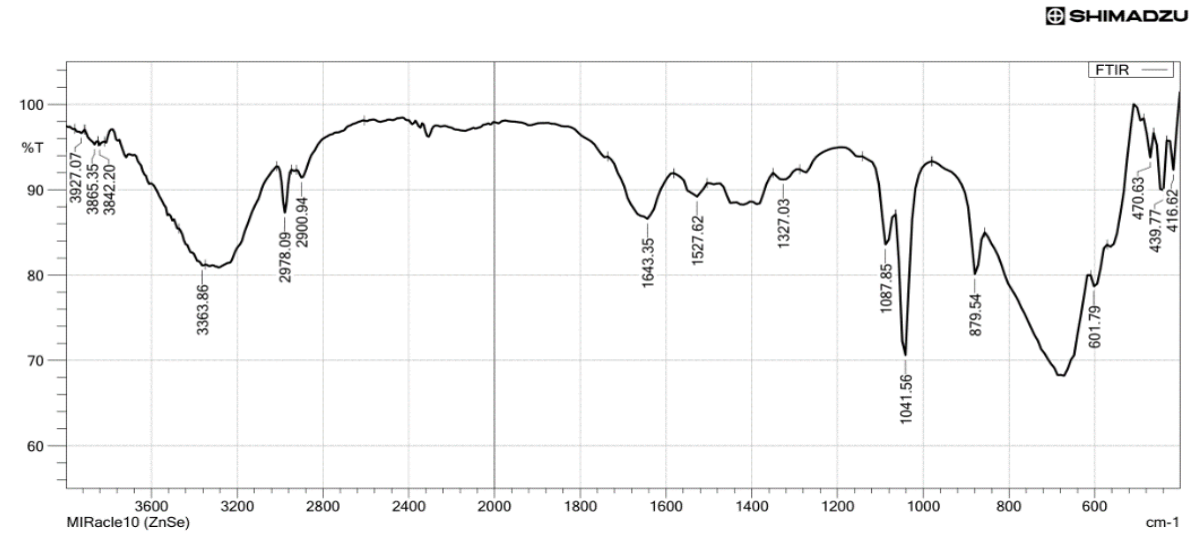

Fig. 3. Papain-CuNPs FT-IR spectrum

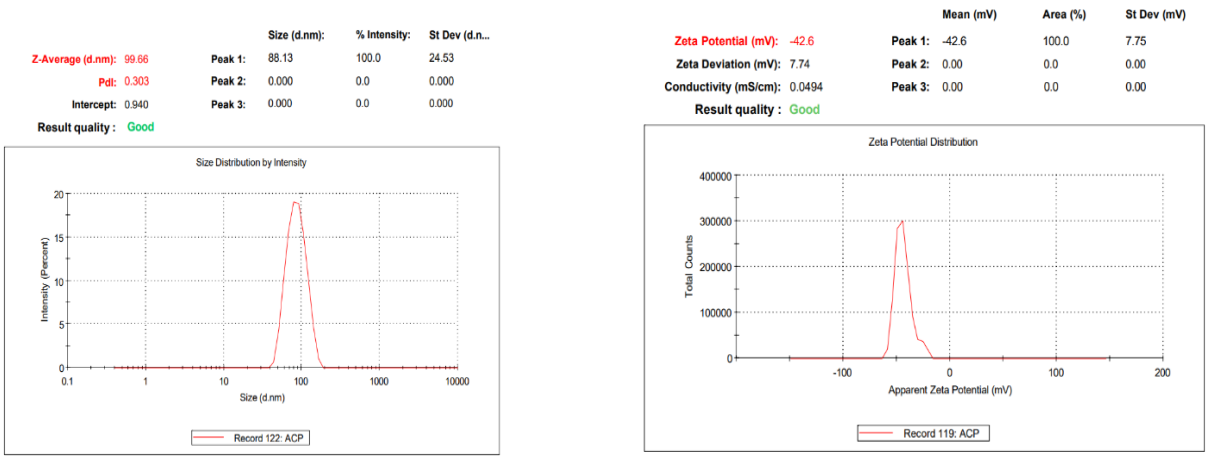

Fig. 4. A: Papain-CuNPs average zeta size 99.66 d. nm, B: Papain-CuNPs zeta potential -42.6 mV.

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The surface charge potential was measured using zeta potential, a major parameter determining the stability of Papain-CuNPs in aqueous solutions.

3.2.4. *XRD analysis of Papain-CuNPs*

X-ray diffraction analysis of papain-conjugated nanoparticles (Papain-CuNPs) revealed a distinct peak at 2θ= 26.63 degrees, indicating the crystalline nature of the nanoparticles. The sharpness and intensity of this peak signify the well-defined crystalline structure of Papain-CuNPs. This observation underscores the ordered arrangement of atoms within the nanoparticles, essential for their functionality and stability in various applications. The confirmation of crystallinity through XRD analysis provides valuable insight into the structural properties of Papain-CuNPs, facilitating further investigation into their potential roles in biomedicine, catalysis, and nanotechnology with confidence in their structural integrity and performance.

3.2.5. *EDAX analysis of the Papain-CuNPs*

The composition of papain-CuNPs was effectively confirmed through EDAX spectroscopy, as depicted in Figure 6, revealing the elemental constituents present within the nanoparticles. Analysis indicated the presence of copper metal (Cu) alongside carbon (C), hydrogen (H), and oxygen (O) derived from the Papain enzyme. The quantitative assessment of these elements through EDAX spectroscopy provides valuable insight into the stoichiometry and composition of the nanoparticles, affirming the successful synthesis and incorporation of copper into the Papain-based nanomaterial.

3.2.6. *Papain-CuNPs observation by electron microscope (FE-SEM and HRTEM)*

Electron microscopy (FE-SEM and HRTEM) was used to confirm that the Papain-CuNPs were nano-sized, and the particles were oval and spherical. Figure 7B shows the papain-CuNP size from 16.71244 nm to 34.84793 nm.

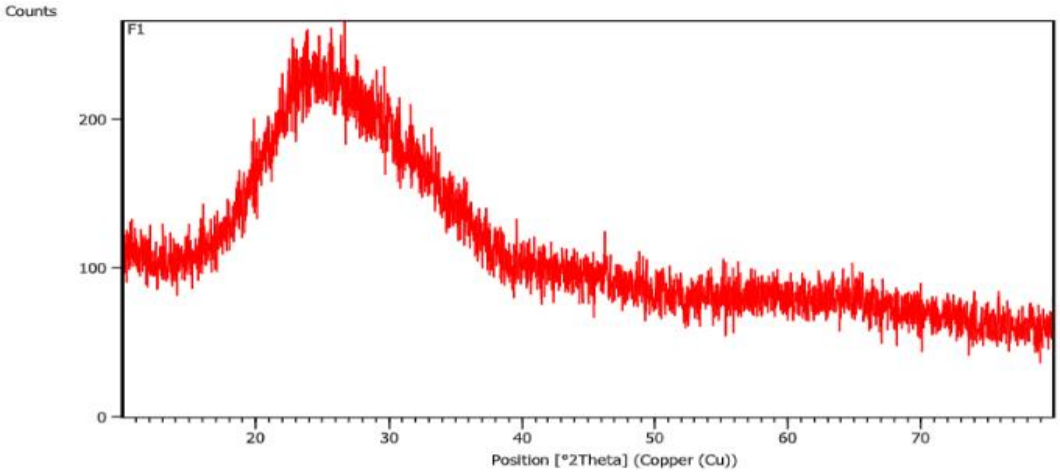

Fig. 5. Papain-CuNPs X-ray diffraction analysis

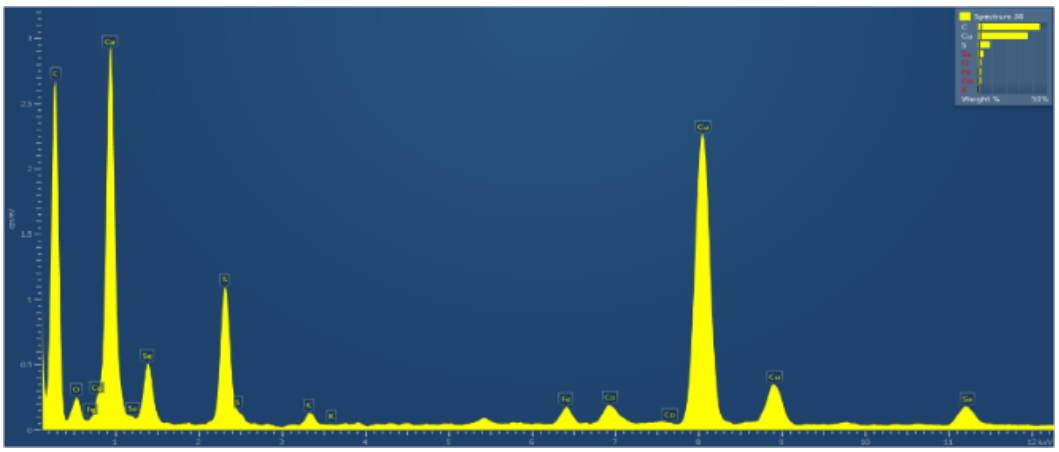

Fig. 6. Papain-CuNPs elements composition

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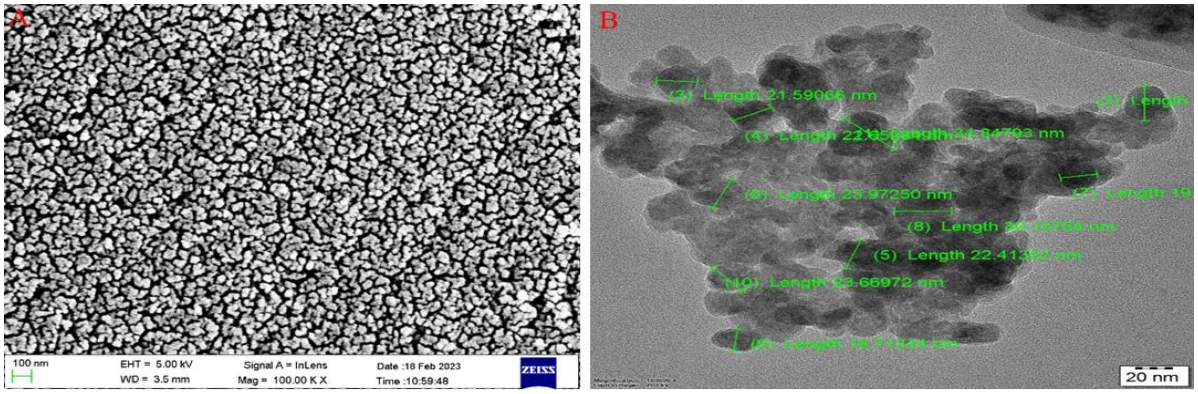

Fig. 7. A: Papain-CuNPs FE-SEM observation, B: Papain-CuNPs HRTEM observation

3.2.7. Antimicrobial activity of the Papain-CuNPs

The antimicrobial activity of papain-CuNPs against clinical Citrobacter spp, P. aeruginosa and C. albicans was evaluated using the well plate method. Papain-CuNPs showed good antibacterial activity against P. aeruginosa (15 mm) and no activity against Citrobacter spp or C. albicans. Figure 8 shows the antimicrobial activity of Citrobacter spp, P. aeruginosa and C. albicans. The papain-CuNP zone of inhibition indicated the degree of antimicrobial susceptibility. Figure 9 shows the MIC of papain-CuNPs for P. aeruginosa (125 µ/ml). MIC was determined by using tetrazolium staining. Live bacterial cells converted yellow tetrazolium to red color with the help of an active reductase enzyme. Dead bacterial cells could not alter the tetrazolium yellow color to red owing to the absence of an active reductase enzyme. The tetrazolium staining results confirmed that papain-CuNPs inhibited the growth of P. aeruginosa at a concentration of 125 µg/ml.

Papain, a proteolytic enzyme derived from the latex of the papaya fruit (Carica papaya), has gained significant attention for its diverse biological activities, including its remarkable antibacterial properties [19]. Extensive research has revealed that papain exerts its antibacterial effects through multiple mechanisms, making it a promising candidate for various therapeutic applications. One of the primary mechanisms by which papain demonstrates antibacterial activity is through the disruption of bacterial cell membranes. Papain possesses the ability to break down proteins, a characteristic attributed to its proteolytic nature. This enzymatic activity enables papain to target and degrade proteins present in the bacterial cell membrane, leading to structural damage and permeability changes. As a result, the integrity of the bacterial cell membrane is compromised, causing leakage of cellular contents and eventual cell death. This membrane-disrupting action is particularly effective against a broad spectrum of bacteria, making papain a potential agent for combating various bacterial infections. Furthermore, papain exhibits antibacterial activity by interfering with bacterial cell adhesion and biofilm formation.

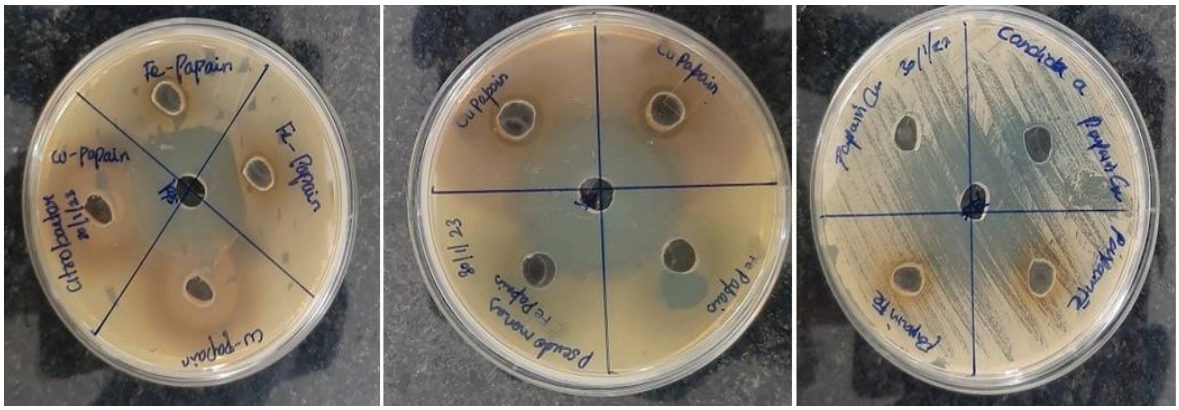

Fig. 8. Antimicrobial activity of Papain-CuNPs against Citrobacter spp, P. aeruginosa and C. albicans.

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Fig. 9. MIC of Papain-CuNPs against P. aeruginosa (125 µ/ml)

Biofilms, complex communities of microorganisms embedded in a self-produced extracellular matrix, contribute to the persistence of bacterial infections and resistance to antibiotics [20]. Papain disrupts biofilm formation by preventing the adhesion of bacteria to surfaces and inhibiting the production of the extracellular matrix. This dual action not only hinders the initial stages of biofilm development but also destabilizes established biofilms, enhancing the susceptibility of bacteria to antimicrobial agents.

The ability of papain to target biofilms is of significant therapeutic relevance, as biofilm-associated infections are notoriously challenging to treat. Additionally, papain's antibacterial properties extend to its ability to modulate the host immune response. Research suggests that papain can stimulate the production of various immune mediators, including cytokines and chemokines, which play crucial roles in orchestrating the immune defence against bacterial infections [21]. By promoting the immune response, papain enhances the body's ability to recognize and eliminate invading bacteria. This immunomodulatory effect complements its direct antibacterial actions, contributing to a comprehensive and effective defence against bacterial pathogens [22]. The specificity of papain towards bacterial cells is attributed to differences in the composition of bacterial and mammalian cell membranes, making papain a promising candidate for the development of antibacterial agents with high efficacy and low toxicity.

3.2.8. Electronic microscopic examination of Papain-CuNPs antimicrobial activity

The morphology of papain-CuNP (125 µg/ml)-treated bacterial cells was observed using an electron microscope. From the electronic microscopic observation, bacterial cell lysis and shrinking confirmed that papain-CuNPs (125 µg/ml) inhibited the growth of the clinical bacteria P. aeruginosa. Papain proteolytic (hydrolyzes complex protein molecules into tiny peptides and amino acids) and copper conjugation properties, Papain-CuNPs showed cell lysis of P. aeruginosa (Figure 10).

The combination of papain, a proteolytic enzyme derived from papaya, with copper nanoparticles presents a synergistic approach to antibacterial activity. Papain's intrinsic ability to disrupt bacterial cell membranes and interfere with biofilm formation is complemented by the unique properties of copper nanoparticles. Copper nanoparticles exhibit potent antibacterial effects by inducing oxidative stress and disrupting bacterial cell walls. When combined, papain and copper nanoparticles create a formidable antimicrobial alliance, effectively targeting a broad spectrum of bacteria [23]. This synergistic interaction enhances the overall antibacterial activity, potentially leading to the development of novel therapeutic strategies with increased efficacy against bacterial infections (Figure 11). The combination leverages the specific strengths of each component, showcasing promise for applications in antibacterial formulations and medical interventions.

4. CONCLUSIONS

In this study, papain-CuNPs were successfully synthesized and characterized using advanced microscopy. In this study, it was observed that the Papain-CuNPs showed significant antibacterial activity, which can be used to treat clinical infections of *P. aeruginosa.* Furthermore, this formulation must be explored for *In-vivo* and pre-clinical studies.

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CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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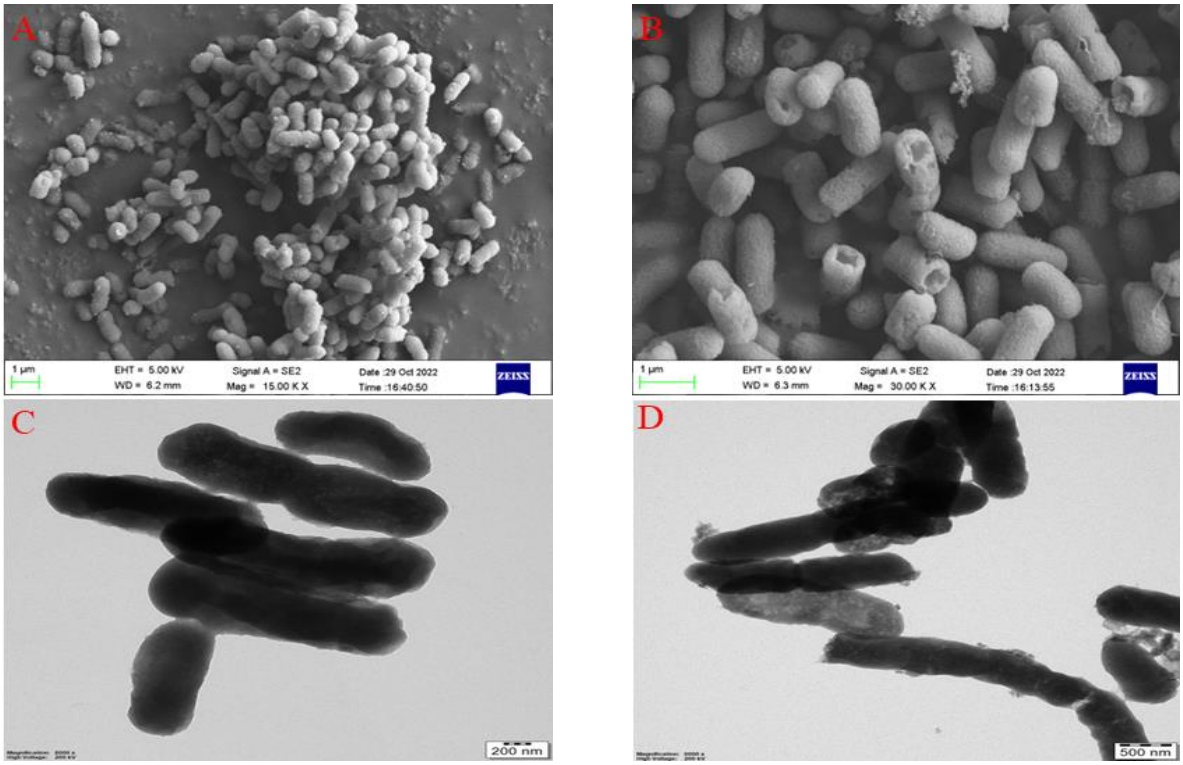

Fig. 10. A: FE-SEM observation of P. aeruginosa, B: FE-SEM Papain-CuNPs treated P. aeruginosa (Bacterial cell damage). C: HR-TEM observation of P. aeruginosa, D: HR TEM Papain-CuNPs treated P. aeruginosa (Bacterial cell damage).

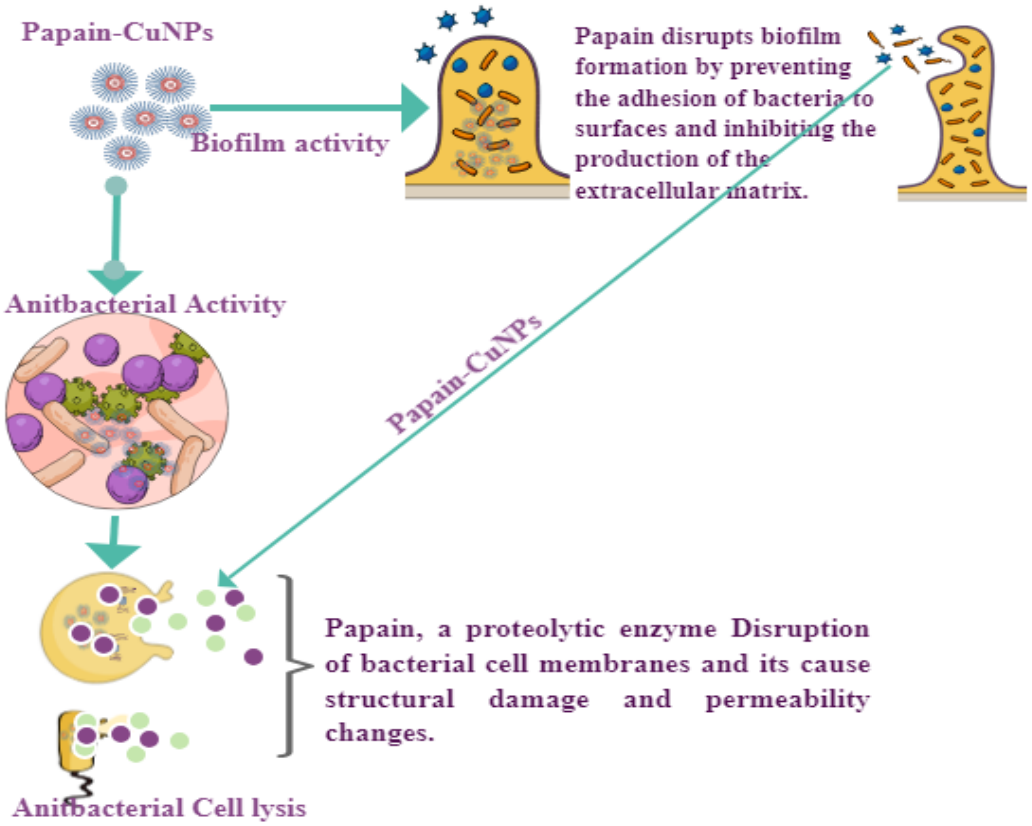

Fig. 11. Possible antibacterial and antibiofilm activity

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FUNDING

Nil

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