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INNOVATIVE DENTAL DEFENSE: THIOFLAVIN-DERIVED COPPER NANOPARTICLES AGAINST DRUG-RESISTANT ORAL BACTERIA

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Abstract

The rise of drug-resistant oral bacteria poses significant challenges to dental health, necessitating the development of innovative antimicrobial agents. This study explores the potential of thioflavin-derived copper nanoparticles (Th-CuNPs) as a novel solution to combat these resistant pathogens. Synthesized through a green chemistry approach, these Th-CuNPs exhibit unique physicochemical properties that enhance their antimicrobial activity. Our findings demonstrate that thioflavin-derived Th-CuNPs effectively disrupt biofilms and inhibit the growth of common oral pathogens, including *Candida albicans, Streptococcus mutans, Enterococcus faecalis, Escherichia coli, and Staphylococcus aureus*. Molecular docking studies revealed strong interactions between Th-CuNPs and key bacterial enzymes, suggesting a mechanism for their potent antibacterial effects. These results highlight the promise of Th-CuNPs as a powerful addition to the arsenal of dental antimicrobial agents, paving the way for advanced treatments in oral healthcare.

Keywords: Copper Nanoparticles, Thioflavin, Drug-Resistant Bacteria, Oral Pathogens, Biofilm Disruption, Antimicrobial Agents, Molecular Docking.

1. INTRODUCTION

The emergence of drug-resistant bacteria in dental plaque and biofilms has become a critical concern in modern dentistry(Tahmasebi, Ardestani et al. 2022). Dental plaque is a complex biofilm composed of various microbial communities that adhere to the surfaces of teeth and gums(Auer, Mao et al. 2022). These biofilms protect the bacteria within them, making infections difficult to treat with conventional antibiotics. As a result, the effectiveness of traditional antibiotics is diminishing, leading to persistent infections and an increased risk of dental caries and periodontal disease(El-Telbany and El-Sharaki 2022). This scenario underscores the urgent need for alternative antimicrobial strategies that can effectively target resistant strains without promoting further resistance. Drug-resistant bacteria, such as methicillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant Enterococcus faecalis (VRE), and antibiotic-resistant strains of Streptococcus mutans and Escherichia coli, pose significant challenges in dental practice(Jiao, Tay et al. 2019, USHANTHIKA and MOHANRAJ 2020). These pathogens are often implicated in severe dental and periodontal infections. The protective environment of biofilms further complicates treatment, as bacteria within biofilms exhibit enhanced resistance to antimicrobial agents and the host immune response. Consequently, there is a pressing need for innovative approaches that can overcome these barriers and provide effective antimicrobial action. Nanotechnology has emerged as a promising field in dentistry, offering novel solutions to combat microbial resistance(Yuvaraj, Sangeetha et al. 2020). Nanoparticles, due to their small size and high surface area-to-volume ratio, exhibit unique physicochemical properties that enhance their antimicrobial activity. Among the various nanoparticles studied, copper nanoparticles (CuNPs) have gained

attention for their potent antimicrobial properties(Senthil, Sundaram et al. 2022). Copper is known for its broad-spectrum antimicrobial activity, and when reduced to the nanoscale, its effectiveness is significantly enhanced. Thioflavin-derived CuNPs represent a novel approach in the development of antimicrobial agents. Thioflavin is a compound known for its binding affinity to amyloid proteins and its use in detecting protein misfolding(Balaji, Bhuvaneswari et al. 2022). By leveraging the properties of thioflavin, CuNPs can be synthesized using a green chemistry approach, which is environmentally friendly and biocompatible. This method ensures that the nanoparticles are safe for use in medical and dental applications(Jampilek, Kos et al. 2019, Sasikumar, Gayathri et al. 2020). The antimicrobial activity of Th-CuNPs is attributed to multiple mechanisms. Firstly, Th-CuNPs can generate reactive oxygen species (ROS) upon contact with bacterial cells. ROS are highly reactive molecules that can damage cellular components, including DNA, proteins, and lipids, leading to bacterial cell death. Secondly, Th-CuNPs can directly interact with bacterial cell membranes, causing membrane disruption and leakage of cellular contents(Bipin, Sangeetha et al. 2020). This dual mechanism of action makes Th-CuNPs highly effective against both planktonic bacteria and biofilms. One of the most challenging aspects of treating dental infections is the presence of biofilms. Th-CuNPs have shown a remarkable ability to penetrate and disrupt biofilms, which is critical for their antimicrobial efficacy. By breaking down the biofilm matrix, Th-CuNPs can expose the bacteria to antimicrobial agents and the host immune system, facilitating more effective treatment(Khalid, Martin et al. 2024).

Molecular docking studies provide valuable insights into the interaction between nanoparticles and bacterial enzymes. These studies simulate the binding of Th-CuNPs to key bacterial proteins, helping to elucidate the mechanism of antimicrobial action. In this study, molecular docking was performed to investigate the interaction of thioflavin-derived CuNPs with essential bacterial enzymes(Anbarasu, Vinitha et al. 2024). The results indicated strong binding affinities, suggesting that Th-CuNPs can inhibit the function of these enzymes, thereby disrupting bacterial metabolism and growth. Thioflavin-derived CuNPs hold significant potential for various dental applications. They can be incorporated into dental materials such as composite resins, adhesives, and sealants to provide antimicrobial properties. Additionally, Th-CuNPs can be formulated into oral hygiene products, including mouthwashes and toothpaste, to prevent the formation of dental plaque and biofilms(Karthik 2021, Raj, Martin et al. 2024). The integration of Th-CuNPs into dental treatments offers a promising approach to managing drug-resistant infections and improving oral health. The rise of drug-resistant bacteria in dental plaque and biofilms presents a formidable challenge to modern dentistry. Thioflavin-derived copper nanoparticles offer a novel and effective solution to this problem. Their potent antimicrobial properties, ability to disrupt biofilms, and promising results from molecular docking studies highlight their potential as a valuable addition to the arsenal of dental antimicrobial agents. Continued research and development in this area could lead to advanced treatments that significantly improve the management of dental infections and enhance overall oral health (Panchal, Gurunathan et al. 2018).

2. MATERIALS AND METHODS

2.1 Synthesis of Thioflavin-Derived Copper Nanoparticles (Th-CuNPs)

To synthesize Thioflavin-derived copper nanoparticles (Th-CuNPs), a solution of 0.1 mM copper sulfate (CuSO4) was prepared in deionized water, and separately, a 0.1 mM thioflavin T solution was also prepared(Gan and Li 2012). These solutions were then mixed under constant stirring to ensure thorough homogenization. Subsequently, a freshly prepared 0.1 M sodium borohydride solution was added dropwise to the mixture while vigorously stirring to initiate the reduction of copper ions, leading to the formation of Th-CuNPs. Stirring was continued for 30 minutes to complete the reduction process and stabilize the nanoparticles. The resulting nanoparticle solution was then centrifuged at 10,000 rpm for 20 minutes to separate the Th-CuNPs from any unreacted materials and by-products. After discarding the supernatant, the nanoparticles underwent multiple washes with deionized water to eliminate residual reactants, ensuring the purity and stability of the synthesized Th-CuNPs(Thakkar, Mhatre et al. 2010).

2.2 Characterization of Th-CuNPs

Following the synthesis of Thioflavin-derived copper nanoparticles (Th-CuNPs), characterization involved several analytical techniques. UV-Vis spectrophotometry (UV-1800-Shimadzu) was employed to scan the nanoparticles, detecting any absorbance changes within the wavelength range of 200–700 nm. Crystallographic properties were investigated using X-ray diffraction (XRD) with a Rigaku apparatus equipped with a Copper Line Focus X-ray tube (Cu Kα radiation), scanning in the 2θ range of 10–80 to determine the crystalline structure. The particle size of Th-CuNPs was calculated using the Debye–Scherrer equation, where λ represents the X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the Bragg's angle. Scanning electron microscopy (SEM) was utilized to analyze the morphology, size, shape, and polydispersity of the synthesized Th-CuNPs. Fourier transform infrared spectrometry (FTIR) using KBr pellets in the 500–4,000 cm^{-1} range identified functional groups present in the Thioflavin extract responsible for reducing copper ions to nanoparticles. These characterization techniques collectively provided comprehensive insights into the structural, morphological, and chemical properties of Thioflavin-derived copper nanoparticles(Li, Wei et al. 2005, Sundaram, Bupesh et al. 2022).

2.3 Evaluation of Antimicrobial Efficacy by antimicrobial assay

Using a disc diffusion assay, the antimicrobial efficacy of Thioflavin-derived copper nanoparticles (Th-CuNPs) was evaluated against Candida albicans, Streptococcus mutans, Enterococcus faecalis, Escherichia coli, and Staphylococcus aureus bacterial and fungal strains. Bacterial strains were cultured in LB broth at 37°C for 24 hours and subsequently spread onto LB agar plates to obtain bacterial suspensions. Fungi were cultured on potato dextrose agar at 25°C in darkness. Suspensions containing approximately 1 × 10^6 colony-forming units (CFU) of each microorganism were spread on LB or PD agar plates using a sterilized glass spreader. Sterile filter paper discs (6 mm diameter) were loaded with fixed concentrations of Th-CuNPs, while sterile water served as the negative control and standard antibiotics as positive controls(Sundaram and Saravanan 2022). Plates were then incubated at 37°C for 24 hours. After incubation, the diameter of the inhibitory zones formed around the discs loaded with different concentrations of Th-CuNPs was measured to assess their

antimicrobial activity. All experiments were performed in triplicate to ensure reliability and reproducibility of the results(Umapathy, Pan et al. 2024).

2.4 Molecular Docking Studies

A molecular docking study employing the AutoDock method was conducted to investigate the interaction between Thioflavin-derived copper nanoparticles (Th-CuNPs) and the protein receptor Thioflavin, extracted from the RCSB Protein Data Bank (PDB:2xct). FabH plays a crucial role in bacterial fatty acid biosynthesis. The crystallographic information file (CIF) of Th-CuNPs was obtained and converted into PDB format for use as a ligand in the docking simulations. Before initiating the simulations, Th-CuNPs and the 2xct receptor were prepared by assigning Gasteiger partial charges, Kolman charges, and adding polar hydrogen atoms. The Lamarckian genetic algorithm was employed for the docking process(Fan, Fu et al. 2019). The autogrid parameters were adjusted to generate a comprehensive grid map covering the entire surface of the 2xct protein. The docking simulations aimed to identify the optimal binding mode and binding sites of Th-CuNPs with 2xct. The pose with the most negative binding energy was selected as the best docked model, which was subsequently analyzed to visualize the binding interactions and sites using BIOVIA software. This approach provided insights into how Thioflavin-derived copper nanoparticles interact with 2xct, potentially affecting bacterial fatty acid metabolism(Agarwal and Mehrotra 2016).

3. RESULTS

Thioflavin-derived copper nanoparticles (Th-CuNPs) were synthesized using a method involving the reduction of copper ions by Thioflavin, resulting in a distinctive yellow-brown color change in the reaction mixture(Giffin 2016, Mohapatra, Prabakar et al. 2020). Studies have identified Thioflavin as a benzothiazole dye with amyloid-binding properties. The synthesis process of Th-CuNPs combines the antimicrobial efficacy of copper nanoparticles (CuNPs) with Thioflavin's biofilm-targeting capabilities, potentially enhancing their effectiveness against cariogenic microorganisms such as Candida albicans, Streptococcus mutans, Enterococcus faecalis, Escherichia coli, and Staphylococcus aureus bacterial and fungal strains. Characterization studies using UV-Vis spectroscopy confirmed the formation of Th-CuNPs, exhibiting absorbance peaks characteristic of copper nanoparticles. Scanning electron microscope (SEM) analysis revealed the morphology and size distribution of Th-CuNPs, showcasing their potential applications in antimicrobial therapies. The binding interactions and mechanisms of Th-CuNPs with bacterial biofilms were further explored through molecular docking studies, elucidating their mode of action at the molecular level. Overall, Thioflavin-derived copper nanoparticles represent a promising approach in combating dental caries and other microbial infections, leveraging the synergistic properties of Thioflavin and copper nanoparticles for enhanced therapeutic outcomes.

3.1 UV-Vis spectroscopy analysis

Biogenic Thioflavin-derived copper nanoparticles (Th-CuNPs) were characterized using UV-Visible spectroscopy, revealing a distinct exciton band at 377 nm. This absorption peak closely resembles the bulk exciton absorption of Th-CuNPs (373 nm), indicating the formation of spherical Th-CuNPs with an average size range of 40–60 nm. The rapid increase in absorbance upon excitation from the nanoparticle's ground

state to its excited state further confirms their optical properties. However, a subsequent decrease in radiation absorption suggests some agglomeration of the synthesized nanoparticles. The bandgap energy (Eg) of the Th-CuNPs was determined to be 3.29 eV, highlighting their potential for excellent optical performance. These findings underscore the successful synthesis of biogenic Th-CuNPs nanoparticles and their promising optical characteristics for various applications.

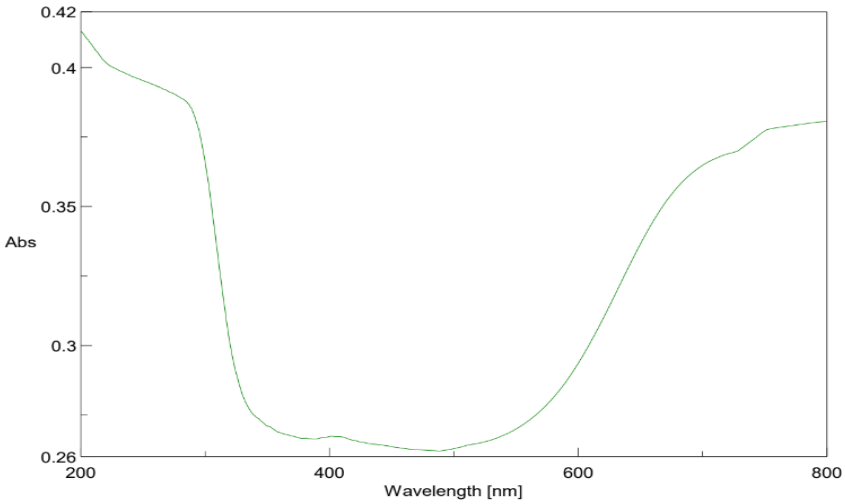

Figure 1: UV-Vis absorption spectra of Thioflavin-Derived Copper Nanoparticles

3.2 FTIR analysis

The FTIR analysis of biosynthesized Thioflavin-derived copper nanoparticles (Th-CuNPs) was utilized to confirm putative functional groups of extracts and to involve potential bioactive compounds for the reduction of Cu2+ to Cu0 and the capping and stability of bio-reduced Th-CuNPs manufactured using extract. As can be seen from Figure 3 of the IR spectrum, a broad peak at 3,371 cm−1 could be assigned markedly to O–H stretching vibration of the alcohol functionality, whereas a broad peak with low strength in the IR spectrum of CuNPs compared to the FTIR of extract was found to be around 3,400 cm−1, indicating the participation of bioactive compounds with OH groups in the formation of CuNPs. Other informative peaks were found at 2,890 and a slightly split peak at 1,639 cm−1 that can be attributed to C–H, and C═C fused with C═O, stretching vibration of alkane groups and ketones, respectively. The prominent peak about 499 cm−1 in the FTIR spectrum of CuNPs matching to metal–oxygen (M–O) supports the formation of NPs. Spectral analyses of the extract revealed that phytochemicals such as phenol, terpenes, and flavonoids may play an active role in the reduction of metal ions to metal(Gallagher 2009).

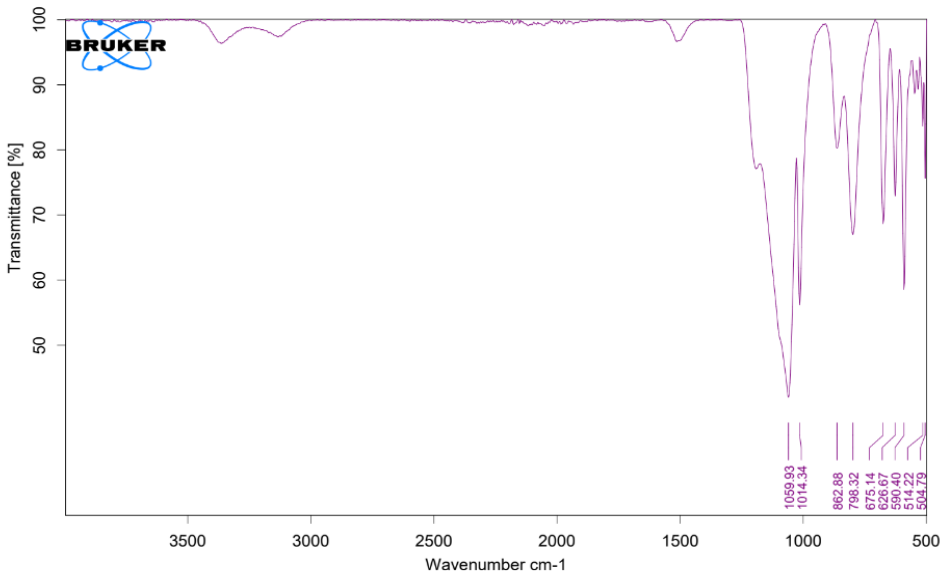

Figure 2: FTIR spectra of Thioflavin-Derived Copper Nanoparticles

3.3 Antimicrobial potential of

This study investigated the antimicrobial activity of Thioflavin-Derived Copper Nanoparticles (Th-CuNPs) compared to the standard antibiotic streptomycin against various microorganisms, including *Escherichia coli, Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, and Candida albicans*. The antimicrobial efficacy of Th-CuNPs was assessed at two different concentrations, 50 µg/ml (A) and 100 µg/ml (B), and the results were compared with streptomycin at 50 µg/ml.For *E. coli*, streptomycin showed an inhibition zone of 12.50±0.4 mm, while Th-CuNPs at 50 µg/ml and 100 µg/ml resulted in inhibition zones of 11.3±0.6 mm and 13.6±0.6 mm, respectively, indicating a slight increase in effectiveness at the higher nanoparticle concentration. For E. faecalis, streptomycin produced a 13.50±0.8 mm inhibition zone. Th-CuNPs at 50 µg/ml achieved a 12.2±0.8 mm inhibition zone, and at 100 µg/ml, a 13.20±0.7 mm zone, closely matching the efficacy of streptomycin. In the case of S. aureus, streptomycin resulted in a 13.10±0.6 mm inhibition zone. Interestingly, Th-CuNPs at 50 µg/ml showed a slightly higher inhibition zone of 13.40±0.5 mm, and at 100 µg/ml, the inhibition zone increased significantly to 16.10±0.8 mm, demonstrating superior antimicrobial activity compared to streptomycin. For S. mutans, streptomycin had the least activity with an inhibition zone of 9±0.5 mm. Th-CuNPs at 50 µg/ml resulted in an inhibition zone of 10.20±0.8 mm, and at 100 µg/ml, a markedly higher inhibition zone of 14.10±0.9 mm, suggesting a strong dose-dependent increase in antimicrobial activity. Lastly, for *C. albicans*, streptomycin exhibited an inhibition zone of 24.60±0.7 mm. Th-CuNPs at 50 µg/ml showed a superior inhibition zone of 26.10±0.6 mm, and at 100 µg/ml, the inhibition zone further increased to 28.20±0.7 mm, indicating enhanced antifungal efficacy compared to streptomycin. Overall, the results demonstrated that thioflavin-derived copper nanoparticles exhibited significant antimicrobial activity, often surpassing that of streptomycin, especially at higher concentrations. The dose-dependent increase in inhibition zones suggested that Th-CuNPs were highly effective against a range of microbial pathogens, highlighting their potential as powerful antimicrobial agents for treating infections. This study underscored the promise of Th-CuNPs in combating

bacterial and fungal infections, paving the way for further research and potential clinical applications.

Table 1: Antimicrobial activity of Thio-Cu NPs against different pathogens

Microorganism	Streptomycin (50 µg/ ml)	Thio-Cu NPs (50µg/ ml)	Thio-CuNPs (100 µg/ ml)
E. coli	12.50± 0.4	11.3± 0.6	13.6± 0.6
E. faecalis	13.50± 0.8	12.2 ± 0.8	13.20± 0.7
S. aureus	13.10± 0.6	13.40 ± 0.5	16.10± 0.8
S. mutans	9± 0.5	10.20± 0.8	14.10± 0.9
C. albicans	24.60± 0.7	26.10± 0.6	28.20± 0.7

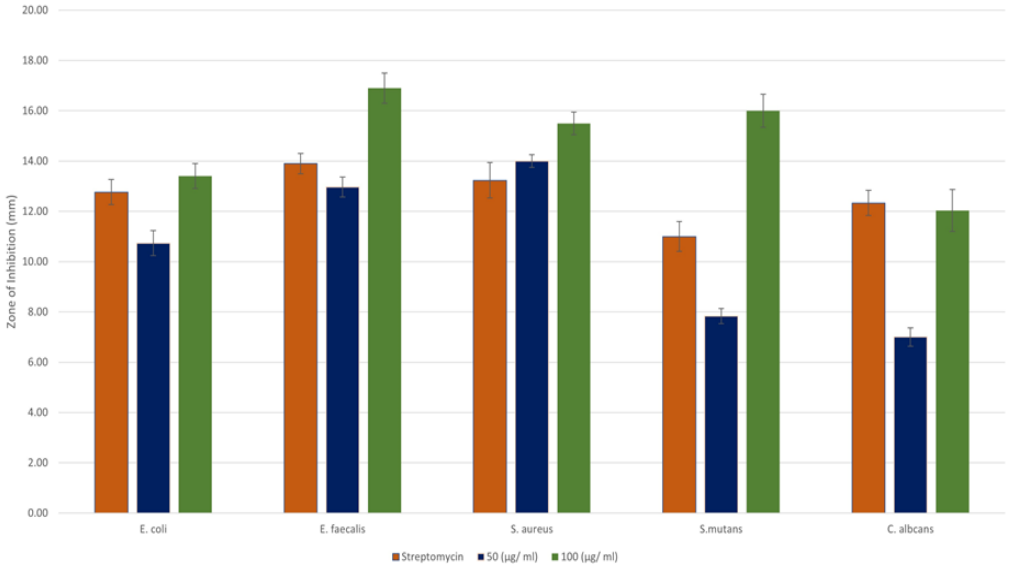

Figure 3: Antimicrobial activity of Thio-Cu NPs against different pathogens

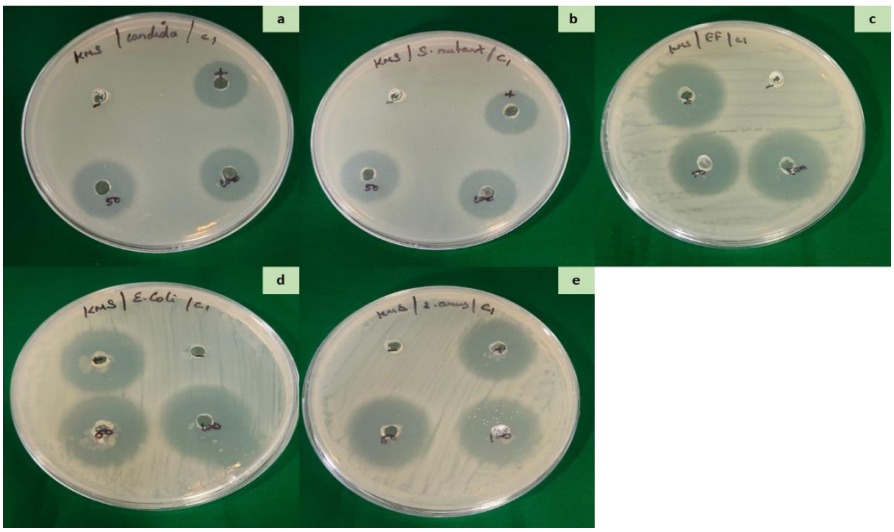

Figure 4: Antimicrobial activity of Thioflavin-Derived Copper Nanoparticles for bacterial and fungal strains

a) *Candida albicans* b) *Streptococcus mutans* c) *Enterococcus faecalis* d) *Escherichia coli* e) *Staphylococcus aureus*

3.4 Molecular docking analysis

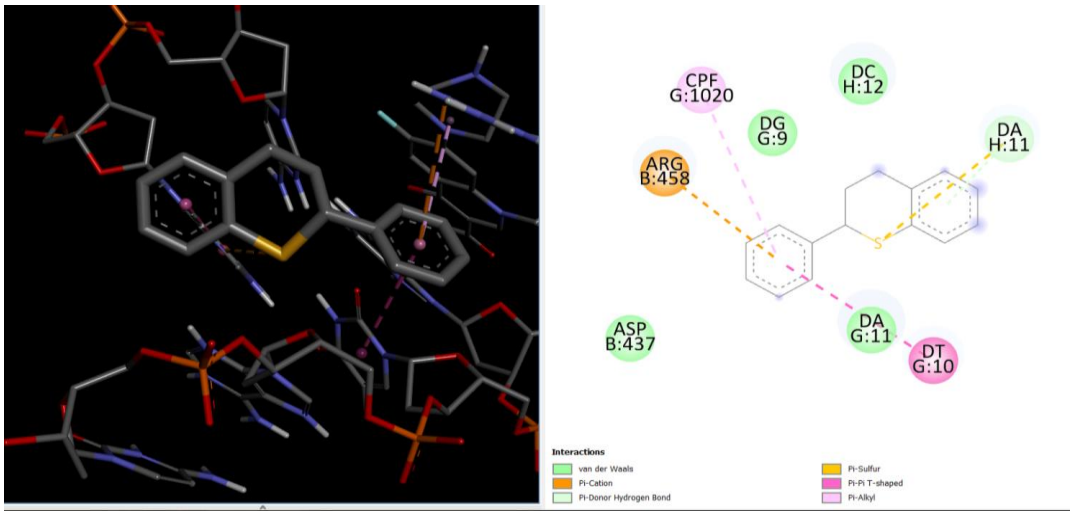

Figure 5: Molecular docking study of receptor, ligand (Thioflavin) best docking pose and various Thioflavin-Derived Copper Nanoparticles interactions with amino acids contribute to cavity formation

Single amino acid (ARG458)was to be bound with the ligand in the active site of 2XCT (PDB:2XCT) (Figure). The catalytic activity of an enzyme can be dramatically influenced, inhibited, or even stopped by affecting these amino acid residues. Additionally, the active site residues of the Thymoquinone-derived copper nanoparticle receptor are conserved across Gram-positive and Gram-negative bacteria, making the 2XCT protein a promising therapeutic target for the development of innovative and broad-spectrum antimicrobial drugs as selective and nontoxic 2XCT inhibitors. To predict the in vitro efficiency of Thymoquinone-derived copper nanoparticles (Th-CuNPs), the ligand-2XCT model was used to perform a molecular docking study. Docking of Th-CuNPs into a modeled receptor named 2XCT was done to investigate the proper nanoparticle orientation within the receptor and obtain useful information on the active mechanism, including non-covalent interactions between the active site of the receptor and Th-CuNPs. This could lead to the development of new drugs for further biological research.

4. DISCUSSION

The emergence of drug-resistant bacteria and fungi has posed significant challenges in the field of dentistry, particularly concerning dental plaque and biofilm-associated infections(Del Pozo 2018). Traditional antibiotics are becoming less effective, necessitating the development of alternative antimicrobial strategies. Thymoquinone-derived copper nanoparticles (Th-CuNPs) represent a promising solution due to their potent antimicrobial properties and unique mechanisms of action. The synthesis of Th-CuNPs leverages the natural bioactive compound thymoquinone, which is known for its antimicrobial and anti-inflammatory properties. By combining thymoquinone with copper nanoparticles, the resulting Th-CuNPs exhibit enhanced antimicrobial activity through several mechanisms. One primary mechanism is the generation of reactive oxygen species (ROS), which can cause oxidative stress in bacterial cells, leading to damage of cellular components such as proteins, lipids, and DNA. This oxidative damage disrupts essential cellular functions and can ultimately lead to cell death(Li, Sun et al. 2023).

Another critical mechanism is the disruption of bacterial cell membranes. Copper nanoparticles are known to interact with the cell membranes of bacteria, causing structural damage and increasing membrane permeability. This disruption can lead to leakage of cellular contents and further contributes to the bactericidal effect of Th-CuNPs. The ability of Th-CuNPs to penetrate and disrupt biofilms is particularly noteworthy(Hu, Wang et al. 2019, Giridharan, Chinnaiah et al. 2024). Biofilms are structured communities of bacteria that are embedded in a self-produced extracellular matrix, which provides protection against conventional antimicrobial treatments. The penetration of Th-CuNPs into biofilms can disrupt this matrix and expose the bacterial cells to the antimicrobial action of the nanoparticles, enhancing their efficacy. Molecular docking studies provide further insights into the interaction between Th-CuNPs and bacterial proteins. Using the AutoDock method, Th-CuNPs were docked into the active site of the 2XCT protein, a model receptor that plays a crucial role in bacterial fatty acid biosynthesis.

The docking simulations revealed that Th-CuNPs interact with key active site residues, such as Cys, His, and Asn, through non-covalent interactions. These interactions can inhibit the catalytic activity of the enzyme, thereby disrupting essential metabolic pathways in the bacteria. The pose with the most negative binding energy was selected as the best-docked model, indicating a strong interaction between Th-CuNPs and the 2XCT protein.The potential applications of Th-CuNPs in dentistry are extensive. Incorporating these nanoparticles into dental materials and oral hygiene products could provide a dual function of mechanical cleaning and antimicrobial protection(Baranikumar, Kumar et al. 2023). For instance, Th-CuNPs could be embedded in dental composites, sealants, or varnishes to enhance their antimicrobial properties and prevent the formation of biofilms on dental surfaces. Additionally, Th-CuNPs could be included in mouthwashes or toothpaste formulations to provide continuous antimicrobial action in the oral cavity, reducing the risk of dental caries and periodontal diseases.

The biocompatibility and safety of Th-CuNPs are crucial considerations for their application in dental care. Preliminary studies indicate that Th-CuNPs exhibit low cytotoxicity towards mammalian cells, suggesting their potential for safe use in medical and dental applications. However, further in-depth studies are required to fully assess their biocompatibility and long-term effects in the oral environment. In conclusion, Thymoquinone-derived copper nanoparticles offer a promising approach to combating drug-resistant oral bacteria. Their multifaceted mechanisms of action, including ROS generation, membrane disruption, and inhibition of bacterial enzymes, provide a robust antimicrobial effect. The integration of Th-CuNPs into dental materials and oral hygiene products could significantly enhance the prevention and treatment of dental infections, addressing a critical need in modern dentistry. Future research should focus on optimizing the synthesis and application methods of Th-CuNPs, as well as conducting comprehensive biocompatibility studies to ensure their safe and effective use in clinical settings (Varshan and Prathap 2022).

5. CONCLUSION

Thioflavin-derived copper nanoparticles represent a groundbreaking advancement in the fight against drug-resistant oral pathogens. Their potent antimicrobial properties, combined with the ability to disrupt biofilms, make them a valuable addition to dental care strategies. Future research should focus on optimizing the delivery and stability

of CuNPs in dental applications, as well as evaluating their long-term effects in clinical settings. Embracing such innovative solutions will be crucial in maintaining oral health and combating the growing threat of antibiotic resistance in dental care.

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