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Original Article

Biological and Safety Evaluation of Green-Synthesized Calcium Oxide Nanoparticles Using *Hedyotis diffusa*

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ABSTRACT

Background: Dental caries and periodontal diseases are among the most prevalent oral disorders worldwide, necessitating the development of safe and effective therapeutic approaches. Green-synthesized nanoparticles derived from medicinal plants offer enhanced biocompatibility and therapeutic potential. *Hedyotis diffusa*, a traditional medicinal herb with established antimicrobial and anti-inflammatory properties, represents a promising candidate for nanoparticle synthesis. **Objectives:** This study aimed to formulate a dental varnish containing *Hedyotis diffusa*-mediated Calcium Oxide Nanoparticles (CaONPs) and evaluate its antibacterial, anti-inflammatory, cytotoxic, and embryonic safety profiles for potential oral healthcare applications. **Materials and Methods:** CaONPs were synthesized using *Hedyotis diffusa* extract and incorporated into a resin-based dental varnish. Antibacterial activity was assessed using agar well diffusion and time-kill kinetic assays. Anti-inflammatory activity was evaluated through albumin denaturation and membrane stabilization assays. Cytotoxicity was determined using the brine shrimp lethality assay, while embryonic safety was investigated using *Artemia* cysts and zebrafish embryo models. **Results:** The CaONP-based dental varnish exhibited significant antibacterial activity, demonstrating notable zones of inhibition and rapid microbial reduction. Anti-inflammatory studies revealed dose-dependent inhibition, reaching 86.7±1.7%. Cytotoxicity analysis indicated low toxicity within the tested concentration range. Embryonic toxicity assessments showed normal hatching, survival, and development of both *Artemia* cysts and zebrafish embryos, confirming favorable biocompatibility. **Conclusion:** The *Hedyotis diffusa*-mediated CaONP dental varnish demonstrated promising antibacterial and anti-inflammatory properties with minimal cytotoxicity and acceptable embryonic safety. These findings support its potential as a sustainable nanoformulation for oral disease management, warranting further *in vivo* and clinical investigations.

Keywords: Antimicrobial activity, Anti-inflammatory, Cytotoxicity, Dental varnish, Embryonic toxicity, Hedyotis diffusa, Nanobiomaterials.

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INTRODUCTION

Oral diseases are still one of the health concerns of the world with two of the most critical oral health disorders being dental caries and periodontal diseases. Oral tissue lesions and other conditions such as pharyngeal and oral cancers are other crucial problems. In addition to its functions of the craniofacial structure, oral health plays a crucial role in the wellbeing, and it influences quality of life (Anushri *et al*., 2015). Besides, oral and general health are also associated as bad dental health has been found to increase the risk of heart diseases. Regular oral health monitoring is essential since underlying medical disorders like diabetes can exacerbate gingivitis and periodontal disease. Pathogenic microorganisms, including *Streptococcus mutans, Lactobacillus* species, and opportunistic fungi like *Candida albicans*, play a central role in enamel demineralization and periodontal tissue destruction, while the host inflammatory response further exacerbates tissue damage and disease progression. You should inform a doctor in case you have some concerns, and brushing and flossing every day will help you to check the state of your mouth, tongue, and gums using these procedures. With early

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detection and management, the probability of preventing issues and achieving an effective course of treatment is significantly high (Odell *et al*., 2024; Dubey *et al*., 2020). Conventional dental varnishes, widely employed for caries prevention and enamel protection, mainly rely on fluoride or synthetic antimicrobial agents; however, their efficacy may be limited by short retention time, suboptimal antimicrobial spectrum, development of microbial resistance, and potential adverse effects associated with prolonged use. In order to reduce gingival inflammation and encourage oral tissue repair, there is a growing need for sophisticated dental varnish formulations that have both long-lasting antibacterial action and anti-inflammatory qualities. Incorporation of bioactive agents with dual antimicrobial and anti-inflammatory functions into dental varnishes represents a promising therapeutic strategy to enhance preventive and adjunctive dental care (Natarajan *et al*., 2025). Such multifunctional varnish systems can potentially improve oral health outcomes by suppressing pathogenic microbial growth, modulating inflammatory pathways, and offering improved biocompatibility, thereby addressing the limitations of conventional dental varnish formulations. Because of their special physicochemical characteristics that improve therapeutic performance, the use of nanoparticles in dental materials has attracted a lot of interest. Owing to their nanoscale size, nanoparticles exhibit improved penetration into enamel microporosities, dentinal tubules, and biofilm matrices, allowing for more effective interaction with pathogenic microorganisms and deeper delivery of active agents (Saafan *et al*., 2018). A novel approach to treating and preventing dental infections may be offered by Nanoparticles (NPs) (Magalhaes *et al*., 2016). Due to their high charge density and high surface area, NPs are capable of interacting more forcefully with the negatively charged surface of bacterial cells, enhancing their antibacterial activity (Cao *et al*., 2028). It was also discovered that NPs used with polymers or coated on the surface of biomaterials had better antibacterial effects in the oral cavity (Saafan *et al*., 2018). Metal and organic NPs have found a variety of applications in the field of dentistry because of their wide-spectrum capability in bacteria (Magalhaes *et al*., 2016). For a stronger antibacterial action, smaller NPs may release more of their corresponding ions. Numerous studies that examined the antibacterial qualities of NPs demonstrated that they had better antibacterial action against drug-resistant microorganisms (Kasraei *et al*., 2014; Fernandes *et al*., 2018; Arif *et al*., 2022). Therefore, using nanoparticles in dentistry may be especially beneficial. Because of its established antibacterial and anti-inflammatory qualities, Calcium Oxide Nanoparticles (CaONPs) have become a promising bioactive nanomaterial for dental applications. By rupturing microbial cell membranes, generating reactive oxygen species, and creating an alkaline environment that hinders oral pathogen development and survival, CaONPs have strong antibacterial action. In addition to their antimicrobial efficacy, by preventing protein denaturation and lowering the production of pro-inflammatory mediators, CaONPs have shown the capacity to regulate inflammatory responses, which is particularly beneficial in managing gingival and periodontal inflammation (Kazi *et al*., 2024). Furthermore, calcium-based nanomaterials exhibit high physicochemical stability and favorable safety profiles, as calcium is a naturally occurring and biologically relevant element in oral hard tissues. The controlled release of calcium ions from CaONPs may also support enamel remineralization, further enhancing their suitability for dental varnish formulations (Khan *et al*., 2023). These combined attributes make CaONPs an attractive and multifunctional candidate for the development of advanced dental varnishes with improved therapeutic efficacy and biocompatibility. In Traditional Chinese Medicine (TCM), these oral disorders are commonly associated with "heat-toxicity," microbial stagnation, and impaired tissue regeneration, which necessitate therapeutic strategies that combine antimicrobial action with inflammation control. TCM has long emphasized the use of medicinal herbs with detoxifying, anti-inflammatory, and tissue-protective properties to restore oral and systemic balance, highlighting the relevance of plant-based interventions for managing oral inflammatory conditions. *Hedyotis diffusa*, also known as Bai Hua She She Cao, is a well-researched medicinal herb in Chinese medicine. It has been traditionally used due to its anti-inflammatory, antibacterial, antioxidant, and wound-healing effect. It is also underpinned as being capable of curing infectious and inflammatory diseases (Bai *et al*., 2024). It is also rich in bioactive phytoconstituents, such as flavonoids, phenolic compounds, iridoids, and polysaccharides that have been reported to regulate the inflammatory pathway as well as prevent the growth of microbes. These phytochemicals are used in the environmentally friendly synthesis of calcium oxide nanoparticles as the so-called natural reducing and stabilizing agents, which motivates the formation of nanoparticles with enhanced surface functionality and lower toxicity. The phytochemical capping of CaONPs synthesized using *Hedyotis diffusa* may enhance bio-interaction by improving nanoparticle stability, cellular compatibility, and adhesion to oral tissues, while also contributing synergistically to antimicrobial and anti-inflammatory effects (Zhu *et al*., 2022). Consequently, *Hedyotis diffusa*-mediated CaONPs represent a biologically favorable and sustainable nanomaterial platform with enhanced therapeutic potential for dental varnish applications. Despite the growing interest in nanoparticle-based dental materials, existing studies have largely focused on the antimicrobial properties or isolated biological effects of calcium-based nanoparticles without translating these findings into clinically relevant dental formulations (Wu *et al*., 2018; Khan *et al*., 2026).To date, no comprehensive investigation has reported the formulation of a calcium oxide nanoparticle-based dental varnish integrated with a systematic evaluation of its biological performance. In particular, critical aspects such as combined antimicrobial and anti-inflammatory efficacy,

cytotoxicity, and embryonic safety have not been simultaneously assessed within a single varnish system. The lack of holistic safety profiling represents a significant barrier to the clinical translation of CaONPs in dental applications. Therefore, the development of a CaONPs-incorporated dental varnish with thorough biological and safety evaluation remains an unexplored and essential area of research. The current study's goal was to create a dental varnish using pre-synthesised Calcium Oxide Nanoparticles (CaONPs) and assess its biological efficacy. The study's specific objective was to evaluate the CaONP-based dental varnish's antibacterial efficacy against a few chosen oral infections, examine its anti-inflammatory potential, and determine its cytotoxicity and embryonic safety using appropriate *in vitro* and *in vivo* model systems. This evaluation was undertaken to establish the preliminary safety and therapeutic applicability of the CaONPs-based dental varnish for dental biomedical applications. Detailed synthesis, physicochemical characterization, and optimization of the CaONPs used in this study are reported separately

MATERIALS AND METHODS

Calcium Nanoparticles Synthesis

Calcium Oxide Nanoparticles (CaONPs) were produced using *Hedyotis diffusa* aqueous extract as a stabilizing and reducing agent, which were then centrifuged, cleaned, and dried. The formation of CaONPs was confirmed by previously standardized characterization techniques (e.g., XRD, SEM), ensuring nanoscale morphology and phase purity. The pre-synthesized CaONPs were subsequently incorporated into a resin-based dental varnish for biological evaluation (Gopalakrishnan *et al*., 2026). Calcium Oxide Nanoparticles (CaONPs) synthesized using *Hedyotis diffusa* extract were incorporated into a resin-based dental varnish matrix for biological evaluation. The formation of CaONPs was previously confirmed using XRD and SEM to ensure nanoscale morphology and phase purity.

Formulation of Dental Varnish

The synthesized *Hedyotis diffusa*-mediated Calcium Oxide Nanoparticles (CaNPs) were incorporated into a dental varnish base to develop a functional therapeutic formulation. One gram of colophony resin was dissolved in 10 mL of ethanol while being gently heated and continuously stirred to create a transparent, uniform solution that served as a good varnish foundation. To this, a measured quantity of synthesized CaNPs (typically 1-2% w/v) was gradually added and mixed thoroughly to ensure uniform dispersion of nanoparticles throughout the varnish matrix. Glycerol and a small amount of peppermint oil were added as plasticizer and flavoring agent, respectively, to improve the varnish's adherence and patient acceptability. The finished mixture was kept in sterile, airtight containers at room temperature for additional biological testing after being constantly mixed to create a smooth and uniform texture (Sureendar *et al*., 2025; Lokeshwar *et al*., 2025).

Antimicrobial Activity

The *Hedyotis diffusa*-mediated calcium nanoparticle dental varnish's antibacterial efficacy against common oral infections, Agar disc diffusion method was used to test such as *Candida albicans, Lactobacillus* species, *Streptococcus mutans, Staphylococcus aureus* and *Enterococcus faecalis*. Fresh cultures of the test organisms were inoculated into nutrient broth and cultured after 24 hr at 37°C. To create a grass culture, Mueller-Hinton Agar (MHA) plates were produced and consistently swabbed with the standardized microbial solutions. After being impregnated with 20 µL of the CaONP-containing dental varnish formulation, sterile filter paper discs (6 mm in diameter) were carefully positioned on the agar surface. Positive controls were discs loaded with common antibiotics (such as ampicillin or nystatin), and negative controls were discs with a simple varnish base. The plates were left to incubate completely (24 hr) at 37°C to ascertain the antibacterial activity. Millimeters were used to measure the zones of inhibition (Sivalingam *et al*., 2024).

Time-Kill Kinetics - CFU reduction over time

The bactericidal and fungicidal activity of the *Hedyotis diffusa*-mediated calcium nanoparticle dental varnish against specific oral pathogens. The time-kill kinetics experiment was used to assess a variety of bacteria, including *Candida albicans, Lactobacillus* species, *Streptococcus mutans, Staphylococcus aureus*, and *Enterococcus faecalis*. In order to prepare standardized microbial suspensions, the optical density of the respective organisms was adjusted to be 0.5 McFarland standard or about 1 x 10 2 CFU/mL. In sterile tubes, 1 mL of the dental varnish extract containing the calcium nanoparticles was incubated with 1 mL of the microbiological solution for every test organism. Static circumstances were used to keep the tubes at 37°C at the designated times of 0, 2, 4, 6, 8, and 24 hr. 150 µL of each tube were aliquotted. After serial dilutions of these aliquots with sterile Phosphate-Buffered Saline (PBS), 100 µL of the respective dilutions were plated on spread plate method either on Mueller-Hinton agar (bacteria) or Sabouraud Dextrose agar (*Candida albicans*). To determine the number of viable cells (CFU/mL) at each time point following a 24-hr incubation period at 37°C, the colonies were counted. The CFU count in death was used to determine the kinetics of microbial death by plotting the CFU count change versus time on a logarithmic scale (Ngene *et al*., 2024).

Anti-inflammatory Assays

Bovine Serum Albumin denaturation assay (BSA assay)

The BSA test was used to evaluate the anti-inflammatory properties of the green-produced calcium oxide nanoparticles using the following doses (10, 20, 30, 40 and 50 µg/mL) of bovine serum albumin mixed with the calcium oxide nanoparticles mediated

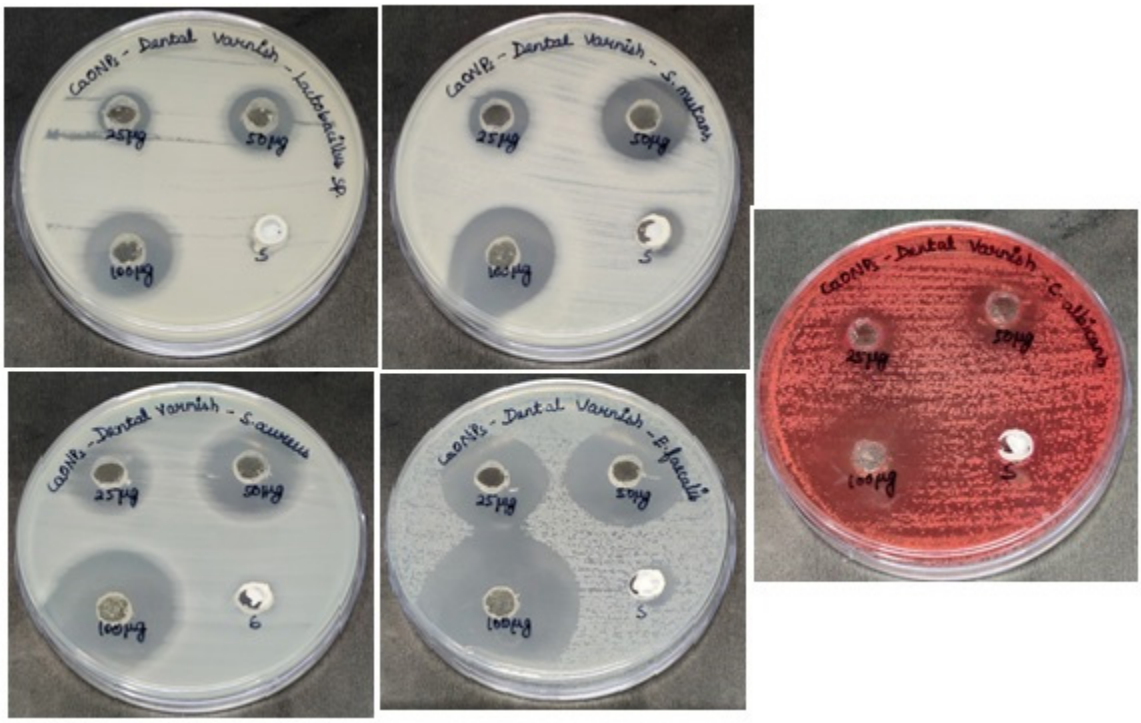

Figure 1: Antimicrobial activity of *Hedyotis diffusa* mediated calcium oxide nanoparticles against clinical pathogens *E. faecalis, S. aureus, S. mutans, Lactobacillus* sp., and *C. albicans*.

with the *Hedyotis diffusa*. The final pH was 6.3. It was left to stand at room temperature then incubated in a 55°C water bath after 10 min. The control group and the standard group were dimethyl sulfoxide and diclofenac sodium respectively. Measurements of the samples at 660 nm were then done by spectrophotometry (Gopalakrishnan *et al*., 2026).

Egg Albumin denaturation assay (EA assay)

To perform the EA assay, the egg albumin denaturation test was performed using 2.8 mL of 1X phosphate buffer and 0.2 mL of fresh egg albumin. Nanoparticles of calcium oxide mediated by *Hedyotis diffusa* were introduced in the reaction mixture at different concentrations (10, 20, 30, 40, and 50 µg/mL). The final pH was 6.3. 10 min of time at room temperature were followed by incubation in a water bath at 55°C in 30 min. The control group and standard group were dimethyl sulfoxide and diclofenac sodium respectively. The samples were then measured at 660 nm spectrophotometrically (Shanmugam *et al*., 2024).

Membrane stabilization assay

In vitro membrane stabilization assay is one of the methods that are commonly employed in assessing the ability of artificial and natural materials in stabilizing membranes. It is an experiment that assesses the ability of a compound to maintain cell membrane integrity and avoid the leaking out of intracellular components of the cell by preventing membrane rupture. The items are human Red Blood Cells (RBCs), centrifuge tubes, Phosphate-Buffered Saline (PBS), Tris-HCl buffer (50 mM, pH 7.4), various doses of calcium nanoparticles (10, 20, 30, 40, and 50 µg/mL), and a UV-vis spectrophotometer. The *in vitro* membrane stabilization assay is one of the common methods that have been used to identify the ability of a substance to stabilize membranes. It can be applied on natural and human-made substances. This is an experiment that measures the capacity of a compound to stop the escape of intracellular components, maintain integrity of the cell membrane, and avoid rupturing of the membrane. These are centrifuge tubes, UV-vis spectrophotometer, Phosphate-Buffered Saline (PBS), 10, 20, 30, 40, and 50 µg/mL amounts of calcium nanoparticles, and Tris-HCl buffer (50 mM, pH 7.4). The centrifuge tubes were filled with 1 mL of the RBC suspension using a pipette. Different quantities of calcium oxide nanoparticles were then added into each tube (10, 20, 30, 40, and 50 µg/mL). The tubes were swirled and incubated at 37°C over a period of 30 min. Pellet the RBCs centrifuge at 2500 RPM for 5 min at room temperature. The absorbance of the supernatant at 560 nm is measured using a UV-vis spectrophotometer. The absorbance of the test RBC suspension is referred to as the OD sample, and the absorbance of the RBC suspension without the test chemical or compounds is referred to as the OD control (Kirubakaran *et al*., 2026).

Cytotoxicity Assay

Brine Shrimp Lethality Assay - LC_{50} determination

2 g of the iodine-free salt that had been weighed were dissolved in 200 mL of purified water. Saline water 10 to 12 mL of water was added to six well plates. A 5 µL, 10 µL, 20 µL, 40 µL, 80 µL

and 100 µL saline solution of naupoles was added to every well. The addition of CaONPs nanoparticles was then done depending on the level of concentration. The plates were incubated during a complete day (Vallinayaki *et al.*, 2024). The plates were observed after 24 hr and the count of living nauplii was counted, and the outcome was calculated using the following formula:

Number of dead nauplii/number of dead nauplii + number of live nauplii × 100

Zebrafish embryonic toxicology evaluation of Calcium Oxide nanoparticles (Dental Varnish)

Fish maintenance and CaO NPs exposure

The wild-type zebrafish (*Danio rerio*) was procured through the local Indian dealers and maintained in different tanks with controlled PH (6.8-8.5), light/dark cycle (14:10 hr) and temperature (28±2°C). The fish were fed on the finest commercial food twice a day. Female and three males were crossed in breeding tanks to get zebrafish embryo. The viable eggs were then rinsed with fresh made E3 medium at least three times. As part of the investigation, fertilized eggs were put onto six-well culture plates. Ten embryos in a 2 mL solution are present in each well. Freshly made *Hedyotis diffusa*-CaO NPs (Dental Varnish) were directly applied to the E3 medium in five different concentrations to set up the experimental treatment. Between 24 and 96 hr after fertilization, healthy fertilized embryos were exposed to different CaO NPs concentrations (Dental Varnish) of between 5 and 80 mg/dL. There were also control groups in the trial. The number of dead embryos was removed in every 12 hr in the groups that were subjected to the nanoparticles. All the experimental plates were maintained at a temperature of 28°C.

Zebrafish embryo evaluation

The post fertilization phase of embryo exposure to the developmental stages of zebrafish was checked using a compound microscope. The embryos were subjected to different concentrations of CaONPs nanoparticles (5, 10, 20, 40 and 80 0 g/mL) in 24-78 hr. Hatching and embryonic mortality was recorded after every 24 hr. One of the study objectives included the hatching rate, embryo and hatchling death, detection and recording of any malformation of the larvae and embryo in the treatment and control groups. COSLAB-Model: Pictures of deformed embryos were taken with A COSLAB-Model: HL-10A light microscope, and the percentage of aberrant embryos recorded at every 24 hr (Shruthilaya *et al.*, 2025).

Ethical Considerations

All biological assays were conducted according to standard laboratory guidelines. Brine shrimp and *Artemia* cyst assays are non-regulated and did not require ethical approval. Zebrafish embryo experiments were performed during early developmental stages, which are exempt from formal ethics approval according to institutional guidelines.

RESULTS

Antimicrobial zone of inhibition

The antibacterial activity of dental varnish containing CaONPs at the concentration of 25, 50 and 100 µg/mL was tested on five oral pathogens, namely *E. faecalis, S. aureus, S. mutans, Lactobacillus* sp. and *Candida albicans*. The zone of inhibition was measured using millimeters (mm) to determine the antibacterial activity (Figures 1 and 2). Antimicrobial activity increased in a dose-dependent manner. At 100 µg/mL, CaONPs were the most inhibitive to *E. faecalis* (41 mm), followed by *S. aureus* (33 mm),

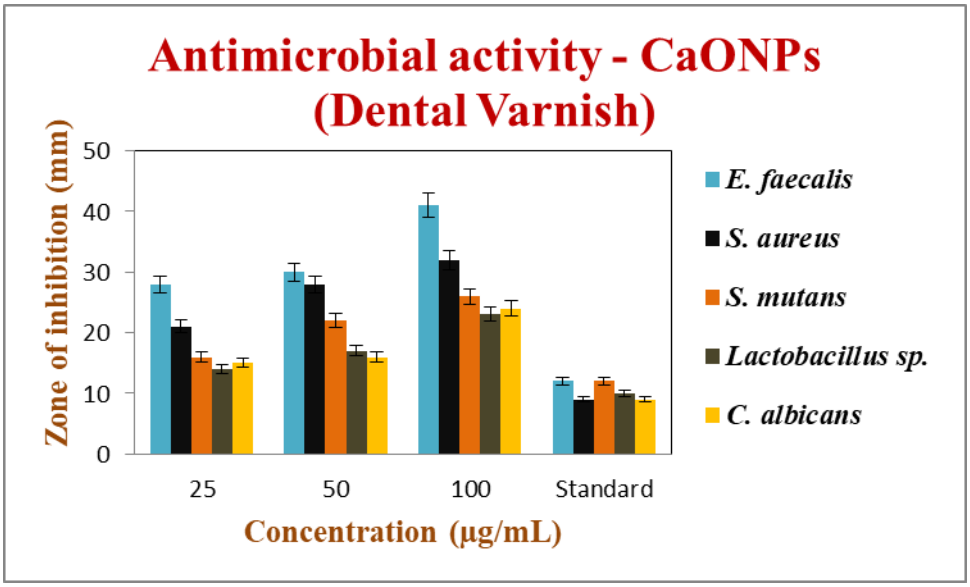

Figure 2: The antimicrobial activity of *Hedyotis diffusa*-mediated CaONPs against clinical pathogens – Comparison.

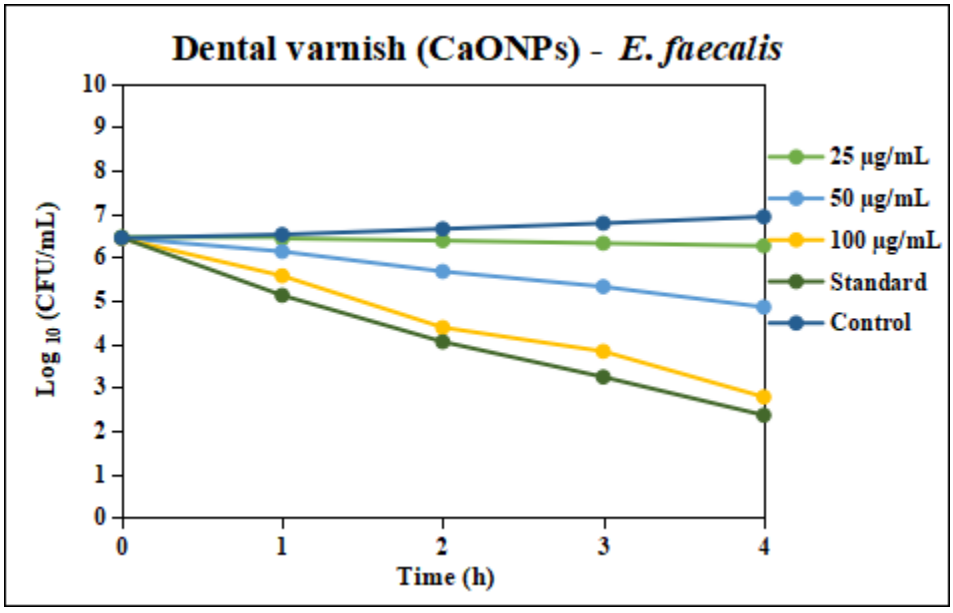

Figure 3: Time-Kill Kinetics analysis of *Hedyotis diffusa* mediated CaONPs against clinical pathogen *E. faecalis*.

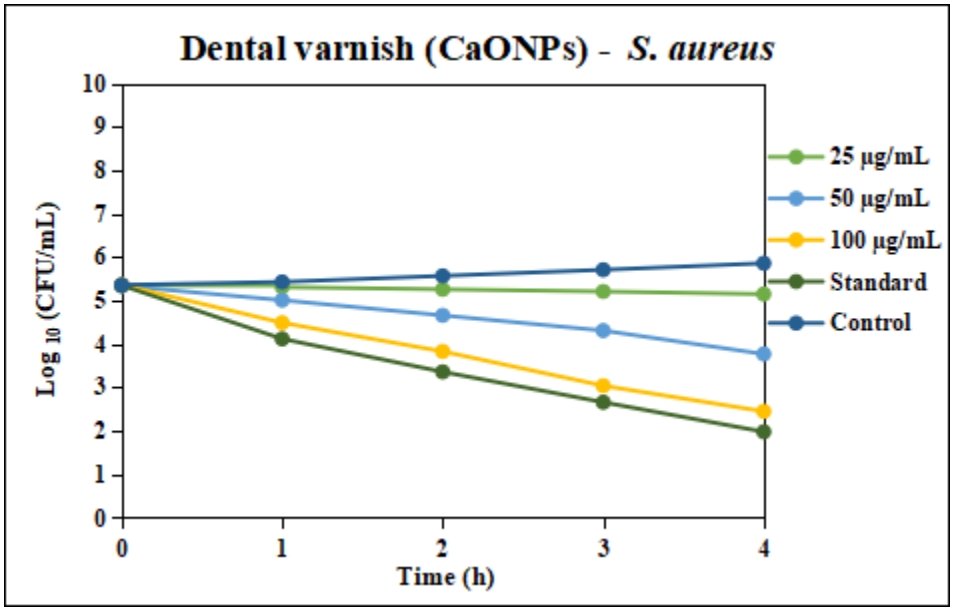

Figure 4: Time-Kill Kinetics analysis of *Hedyotis diffusa* mediated CaONPs against clinical pathogen *S. aureus*.

S. mutans (27 mm), *Lactobacillus* sp. (25 mm), and *C. albicans* (24 mm). Even at lower concentrations (25 and 50 µg/mL), significant inhibition was seen, with *E. faecalis* consistently showing the highest sensitivity. When compared with the standard treatment, CaONPs at 100 µg/mL outperformed the standard antimicrobial agent in most cases, particularly against *E. faecalis* and *S. aureus*. These findings suggest that CaONPs incorporated into dental varnish provide potent and broad-spectrum antimicrobial effects, making them promising agents for oral care applications.

Time-kill kinetics

The time-kill kinetics study demonstrated a concentration- and time-dependent antimicrobial effect of CaONPs-based dental varnish against all tested oral pathogens (Figures 3-7). Among them, *E. faecalis* exhibited the highest sensitivity, with bacterial counts decreasing from $\log_{10}$ 6.8 CFU/mL to approximately $\log_{10}$ 3.1 CFU/mL within 4 hr at 100 µg/mL concentration, closely matching the standard treatment which reduced it further to $\log_{10}$ 2.6 CFU/mL. Similarly, for *S. aureus*, the 100 µg/mL concentration significantly lowered the bacterial count to $\log_{10}$ 3.0 CFU/mL, showing strong antimicrobial potential. In the case

of *S. mutans*, a gradual reduction in CFU count was observed with the highest concentration achieving a final value of $\log_{10}$ 3.4 CFU/mL. *Lactobacillus* sp. showed moderate susceptibility, with CaONPs at 100 µg/mL reducing the count to $\log_{10}$ 4.3 CFU/mL, while the standard control achieved a further reduction. Notably, *C. albicans* also responded well to the treatment, with a substantial decline in viability to $\log_{10}$ 4.0 CFU/mL at 100 µg/mL, indicating promising antifungal activity. Across all tested organisms, the 100 µg/mL concentration of CaONPs consistently outperformed the lower concentrations (25 and 50 µg/mL), and approached the efficacy of the standard antimicrobial agent. These findings reinforce the broad-spectrum antimicrobial potential of CaONPs dental varnish, making it a viable candidate for clinical oral applications.

Anti-inflammatory activity

Bovine serum albumin denaturation assay

The anti-inflammatory effect of the Calcium Oxide Nanoparticle (CaONP) dental varnish with the *Hedyotis diffusa* as an anti-inflammatory system was determined and compared with that of a traditional anti-inflammatory medication using the Bovine Serum Albumin (BSA) denaturation assay. In the case of CaONP dental varnish and the standard, the concentration (10-50 µg/mL) of the solution increased the percentage of protein denaturation inhibition (Figure 8). The CaONP varnish at 10 µg/mL had 45.3±1.2% inhibition and this value gradually rose to 78.5±1.6% at the concentration of 50 µg/mL. The normal had a slightly higher inhibitory profile, which ranged between 47.2±1.1% and 82.1±1.4% at 10 µg/mL and 50 µg/mL respectively. Although it can be said that the standard was a little more active at all concentrations. The CaONP varnish was also efficient, which suggests that it has a high anti-inflammatory potential. According to these findings, the dental varnish synthesized has dose-dependent anti-inflammatory activity, which might be due to the presence of phytoconstituents of *Hedyotis diffusa* and based on the physicochemical characteristics of calcium nanoparticles.

Egg albumin denaturation assay

The anti-inflammatory properties of the *Hedyotis diffusa*-mediated Calcium Oxide Nanoparticle (CaONP) dental varnish were evaluated using the Erythrocyte membrane stabilisation (EA) assay. The findings, which are shown in (Figure 9), indicate that the CaONP formulation increases the percentage of membrane stabilisation in a dose-dependent manner. At 10 µg/mL, the dental varnish showed 52.6 ± 1.3% inhibition of hemolysis, which increased steadily to 79.2 ± 1.5% at 50 µg/mL. At every concentration, the typical anti-inflammatory drug showed somewhat greater activity, reaching 83.7 ± 1.4% at the highest dose. Nevertheless, the CaONP dental varnish exhibited consistent and comparable efficacy, indicating strong protective effects against hypotonicity-induced hemolysis. These findings validate the membrane stabilizing and anti-inflammatory capability of the formulated varnish, which may help in managing inflammation in oral environments.

Membrane stabilization assay

The anti-inflammatory effectiveness of the *Hedyotis diffusa*-mediated Calcium Oxide Nanoparticle (CaONP) dental varnish was assessed using the membrane stabilization assay. Effective membrane stability was demonstrated by the CaONP varnish's dose-dependent increase in the percentage suppression of hemolysis, as seen in Figure 10. The CaONP varnish showed 54.3±1.6% inhibition at 10µg/mL, which gradually rose to 81.5±1.8% at 50µg/mL. With a maximal inhibition of 86.7±1.7%

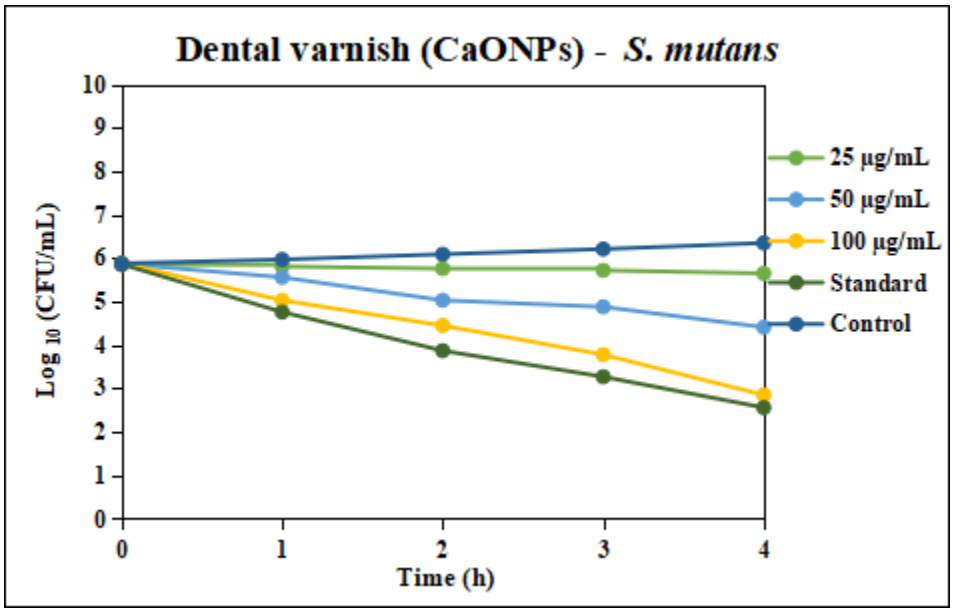

Figure 5: Time-Kill Kinetics analysis of *Hedyotis diffusa* mediated CaONPs against clinical pathogens *S. mutans*.

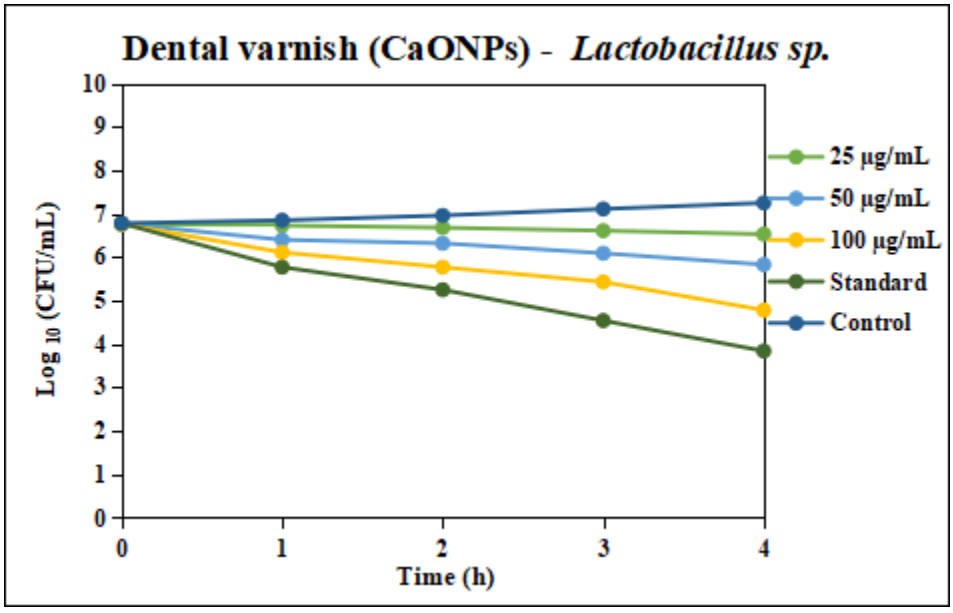

Figure 6: Time-Kill Kinetics analysis of *Hedyotis diffusa* mediated CaONPs against clinical pathogens *Lactobacillus* sp.

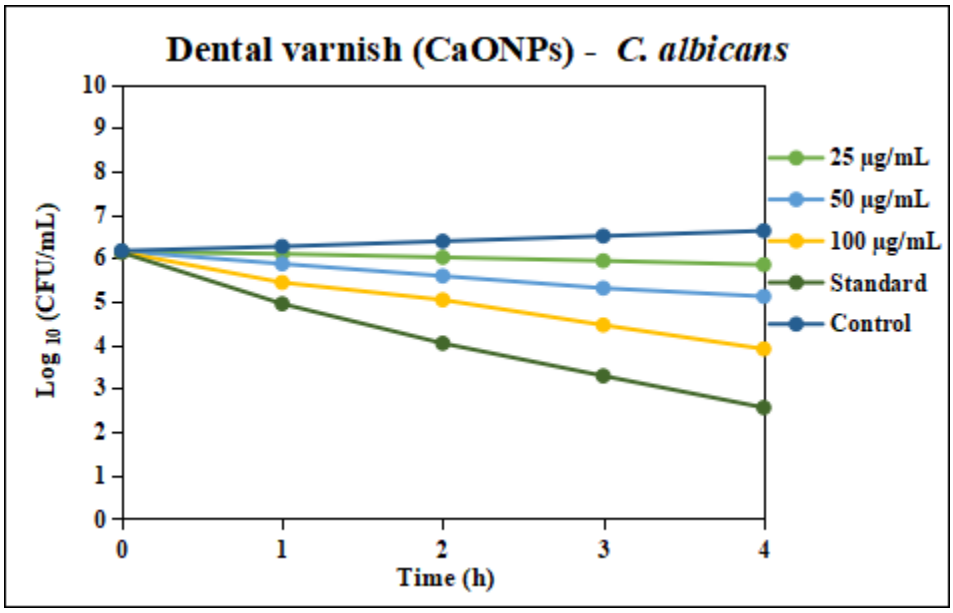

Figure 7: Time-Kill Kinetics analysis of *Hedyotis diffusa* mediated CaONPs against clinical pathogens *C. albicans*.

at the highest dose tested, the conventional anti-inflammatory drug had somewhat greater action at all concentrations. The CaONP varnish showed substantial protective benefits against membrane lysis despite the slight difference, indicating its potential as an anti-inflammatory dental treatment agent.

Cytotoxicity Assay

The cytotoxic effect of Calcium Oxide Nanoparticles (CaONPs) in the presence of *Hedyotis diffusa* incorporated in dental varnish was assessed within two days using the Brine Shrimp Lethality Assay. Figure 11 shows the percentage of living nauplii in different concentrations of 5 µg/mL up to 80 µg/mL compared to the control. CaONP dental varnish recorded less than 95% survival rates at every dosage on the first day. Similarly, there was no significant decrease in the percentage of live nauplii on Day 2 at the doses up to 40 µg/mL. However, at 80 µg/mL, a moderate cytotoxic effect was noted, with the survival rate dropping to approximately 42%, indicating a dose-dependent response at higher concentrations. These findings suggest that the CaONP dental varnish exhibits negligible cytotoxicity at therapeutic concentrations, supporting its biosafety for potential biomedical applications.

Zebrafish embryonic toxicology

The hatching and viability rate of *Artemia* cysts were found to respond concentration-dependently to dental varnish of CaONPs of various concentrations (5, 10, 20, 40 and 80 µg/mL) (Figures 12 and 13). Hatching rates of the lower concentrations (5-20 µg/mL) and the control group were both above 98% which shows the non-toxic and biocompatible characteristics of CaONPs in these concentrations. The higher the dose, however, the more apparent was the decline in hatching success, the lower the resulting hatching rates, 80 per cent at 40 µg/mL, and 60 per cent at 80 µg/mL. This suggests that elevated concentrations may exert a harmful effect on embryonic development, possibly through oxidative stress or disruption of key metabolic pathways. Similarly, the viability of hatched nauplii remained nearly 100% at lower concentrations and even at 40 µg/mL, indicating that the survival of hatched larvae was not significantly impacted up to this point. At 80 µg/mL, however, a marked reduction in viability to approximately 60% was noted, implying cytotoxic stress at this concentration. The decreased viability may result from nanoparticle-induced physiological disturbances affecting motility, respiration, or osmoregulation in the larvae. Overall, these findings confirm that CaONPs are safe at lower concentrations but may induce toxicity at higher doses (Figure 14).

DISCUSSION

The antimicrobial efficacy of Calcium Oxide Nanoparticles (CaONPs) is primarily attributed to their unique physicochemical properties at the nanoscale, which enable effective interaction with microbial cells. Through direct contact, CaONPs are known to compromise the integrity of microbial cell membranes, increasing membrane permeability, ultimately leading to cell death by letting internal components leak out. Moreover, CaONPs

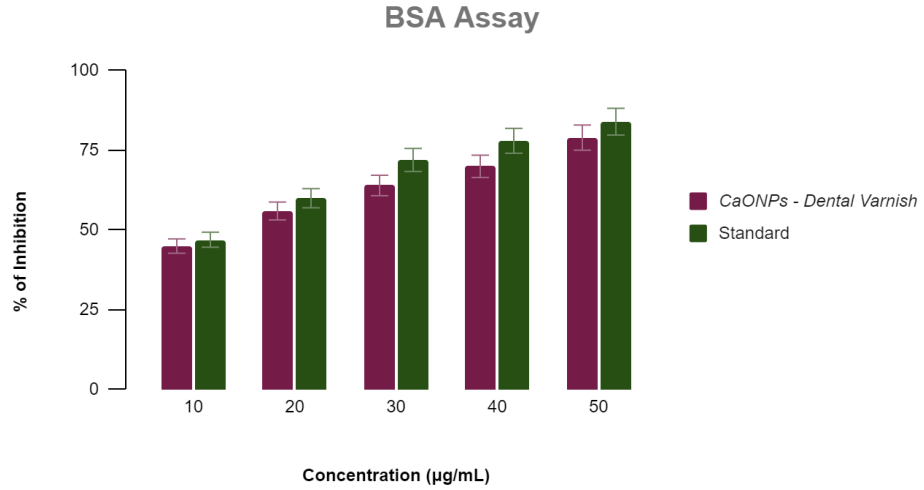

Figure 8: Percentage inhibition of protein denaturation by CaONPs-Dental Varnish and Standard at various concentrations (10-50 µg/mL) using BSA assay. Data represented as mean±SD.

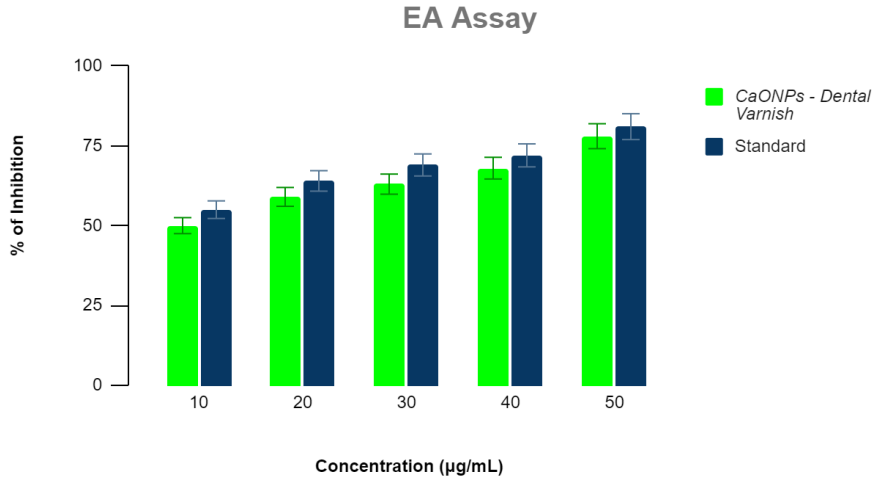

Figure 9: EA assay indicating the percentage inhibition of hemolysis at concentrations ranging from 10-50 µg/mL for CaONPs-Dental Varnish and Standard.

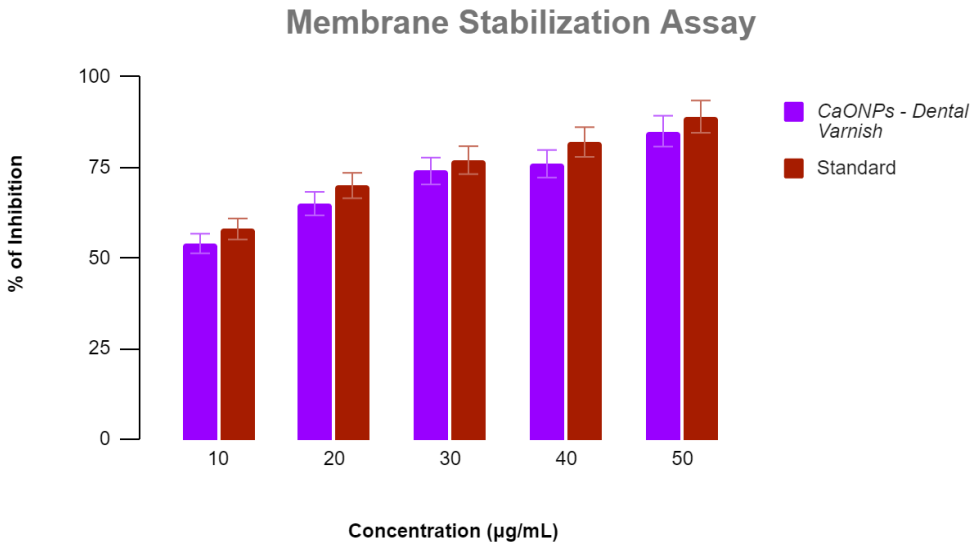

Figure 10: The Percentage inhibition of hemolysis at various concentrations (10-50 µg/mL) for CaONPs-Dental Varnish and Standard.

can generate reactive oxygen species (ROS) on their surface, resulting in oxidative stress that damages the lipids, proteins, and nucleic acids of bacteria. The release of calcium ions from CaONPs further contributes to their antimicrobial action by altering membrane potential and interfering with essential enzymatic processes within microbial cells. Moreover, the alkaline microenvironment created by CaONPs inhibits the growth and survival of acidogenic and aciduric oral pathogens such as *Streptococcus mutans*. CaONPs' ability to penetrate microbial biofilms is enhanced by their nanoscale size, which effectively disrupts the architecture of the biofilm and inhibits bacterial colonization. The study documented the antibacterial properties of Calcium Oxide Nanoparticles (CaO-NPs), which were produced using a microwave irradiation technique and confirmed by XRD and TEM to have a crystalline structure and nanoscale size. Although they were less effective against yeast strains, Both Gram-positive and Gram-negative bacteria were effectively inhibited by the CaO-NPs in a dose-dependent manner. CaO-NPs' tiny particle size and high surface reactivity were thought to be responsible for the improved antibacterial impact because they allow for close interaction with microbial cell membranes, which disrupts the membrane and causes microbial death. This study highlighted CaO nanoparticles as promising antimicrobial agents for biomedical applications, supporting their incorporation into advanced formulations such as dental varnishes for effective control of oral pathogens (Roy *et al.*, 2013). The article has described the green synthesis of Calcium Oxide Nanoparticles (CaONPs) with the *Ficus carica* fruit extract as a reducing agent and capping agent. This yielded stable nanoparticles with an average diameter of approximately 80-85 nm as it was observed by UV-vis, FTIR, XRD, SEM, and EDX. *Pseudomonas aeruginosa* and *Staphylococcus aureus* had the greatest representatives, with the potent antibacterial activity of the biosynthesized CaONPs, which showed a significant inhibition zone against any kind of Gram-positive and Gram-negative pathogen. Importantly, these CaONPs also demonstrated potent antibiofilm activity, effectively reducing biofilm formation in a dose-dependent way, demonstrating their ability to disrupt microbial community structures in addition to preventing the proliferation of planktonic bacteria. The study highlights the potential of phyto-mediated CaONPs as eco-friendly antimicrobial and antibiofilm agents, supporting their future exploration in biomedical applications where bacterial resistance and biofilm-associated infections are of concern (Khan *et al.*, 2023). Together, these processes explain CaONPs' broad-spectrum antibacterial action and lend credence to their possible use in dental varnish formulations for the treatment and prevention of oral infections. Gingivitis, periodontitis, aphthous ulcers, and peri-implantitis are among the major oral lesions that are mostly caused by inflammation, where excessive production of pro-inflammatory mediators leads to tissue damage, pain, and delayed healing. Effective modulation of the inflammatory response is therefore essential for controlling disease progression and promoting oral tissue repair. By stabilizing cellular membranes, preventing protein denaturation, and lowering the activity of inflammatory mediators, Calcium Oxide Nanoparticles (CaONPs) have the potential to reduce tissue irritation and edema. Incorporation of CaONPs into a dental varnish matrix allows sustained local release at the site of application, which may enhance therapeutic efficacy while minimizing systemic exposure. Using a one-pot green method, calcium hydroxide nanoparticles (carob-CaNPs) mediated by *Ceratonia siliqua* (carob) fruit extract were created, and their antibacterial and anti-inflammatory qualities were assessed *in vitro*. FTIR, XRD, TEM, SEM, and EDX were used to describe the nanoparticles, confirming their crystalline structure, important

functional groups, and nanoscale size (<100 nm). With dose-dependent suppression seen across strains, the carob-CaNPs demonstrated broad-spectrum antibacterial efficacy against both Gram-positive (*Staphylococcus aureus, S. epidermidis*) and Gram-negative anaerobic pathogens (*Porphyromonas gingivalis, Fusobacterium nucleatum*). Crucially, in RAW264, carob-CaNPs preserved excellent cell viability in macrophages and did not elicit a significant pro-inflammatory response, indicating biocompatibility and lack of inflammatory activation. These findings suggest that plant-mediated calcium nanoparticle formulations can provide effective antimicrobial action while minimizing inflammatory effects, supporting their potential utility in dental and medical applications (AlDhalaan *et al.*, 2025).

Calcium Oxide Nanoparticles (CaO-NPs) were greenly synthesized using *Mentha arvensis* (mint) leaf extract, and their multifunctional properties were evaluated. Particle sizes typically ranged from 37 to 62 nm, and crystalline nanoscale shape was validated by characterizing the biosynthesized CaO-NPs using techniques such UV-visible spectroscopy, FTIR, XRD, SEM, and DLS. The substantial anti-inflammatory efficacy of mint-mediated CaO-NPs was demonstrated by protein denaturation and nitric oxide scavenging experiments, highlighting their ability to control inflammatory reactions. Additionally, they exhibited anti-cancer properties in MTT assays, indicating dose-dependent cytotoxicity against cancer cell models. Beyond biomedical activity, the CaO-NPs also showed effective photocatalytic

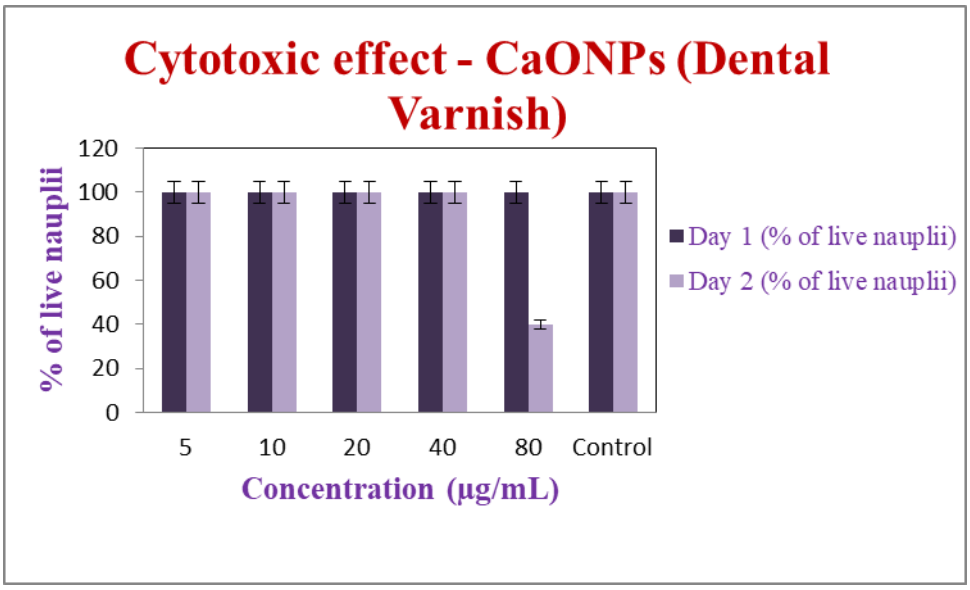

Figure 11: The percentage of live nauplii after exposure to various concentrations (5-80 µg/mL) of CaONPs-Dental Varnish over two days.

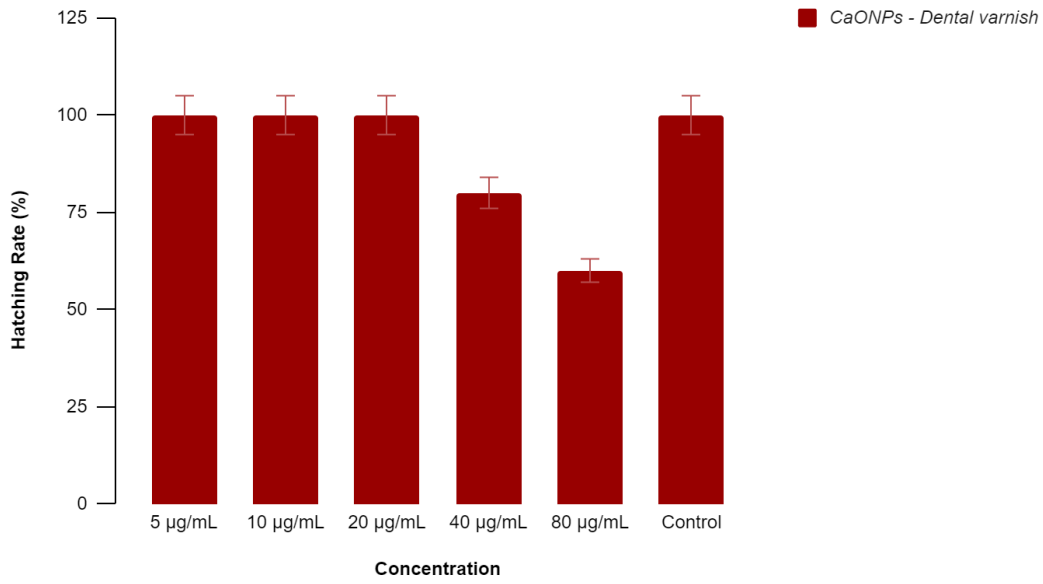

Figure 12: Zebrafish embryos-Toxicology Hatching rate.

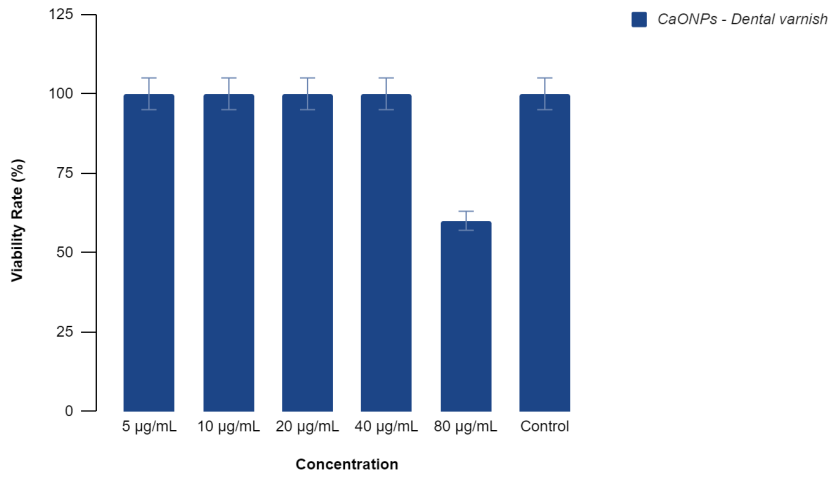

Figure 13: Zebrafish embryos-Toxicology viability rate.

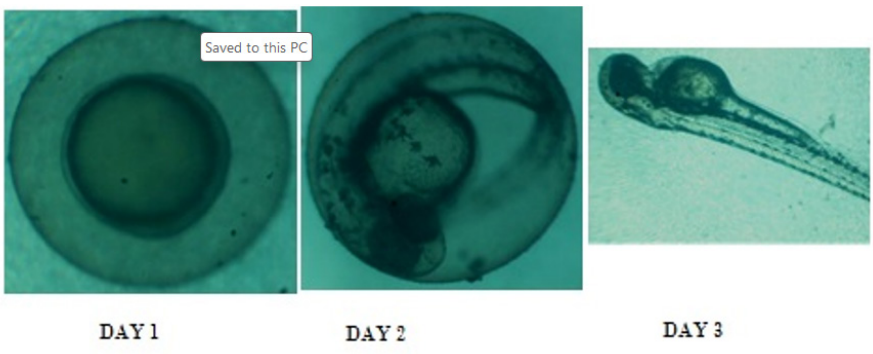

Figure 14: The progression of zebrafish embryonic development from Day 1 (fertilized egg), Day 2 (organogenesis stage), to Day 3 (hatched larva).

degradation of common azo dyes (e.g., methyl red, methyl orange, methylene blue), illustrating their utility in environmental remediation. The study underscores the versatility of plant-based CaO-NPs in therapeutic and catalytic applications, with mint-derived phytochemicals contributing to enhanced bioactivity and eco-friendly synthesis (Shanmuganathan *et al.*, 2023). The observed anti-inflammatory activity of the CaONP-based varnish suggests its relevance in managing inflamed oral tissues and supports its potential use as an adjunctive therapy in the treatment and prevention of inflammatory oral lesions. Assessment of cytotoxicity and developmental safety is essential for the clinical translation of nanoparticle-based dental materials, as these formulations are intended for prolonged contact with oral tissues. In the present study, the CaONPs-incorporated dental varnish demonstrated acceptable safety at selected concentrations, as evidenced by low toxicity in the brine shrimp lethality assay and minimal adverse effects in embryonic models. These findings suggest that CaONPs, when incorporated into a varnish matrix, exhibit controlled bioavailability and reduced cytotoxic potential. The favorable safety profile may be attributed to the biocompatible nature of calcium-based nanoparticles and the stabilizing effect of plant-derived phytoconstituents, which can mitigate nanoparticle-induced oxidative stress. Previous research using plant-mediated calcium nanoparticles has found similar findings, supporting their applicability for biological applications (Alorku *et al.*, 2020). The combined findings of cytotoxicity and embryonic safety provide credence to the potential of CaONP-based dental varnish for more *in vivo* testing and potential clinical use. Compared with widely studied dental nanomaterials such as Silver (AgNPs) (Yudaev *et al.*, 2022), Zinc Oxide (ZnO) (Puspalatha *et al.*, 2022), Copper Oxide (CuO) (Amiri *et al.*, 2017), nano-Hydroxyapatite (nHA) (Bordea *et al.*, 2020), and bioactive glass Calcium Oxide Nanoparticles (CaONPs) (Saisruthi *et al.*, 2024) offer a distinct balance of antimicrobial efficacy, biocompatibility, and mineral-supporting activity. AgNPs and ZnO are potent antimicrobials but raise concerns about dose-dependent cytotoxicity and potential development of dysbiotic oral flora; by contrast, CaONPs provide effective antimicrobial action while releasing biologically relevant calcium ions that may aid enamel remineralization and pose lower systemic toxicity. Unlike nHA and bioactive glass, which primarily support remineralization, CaONPs combine alkaline, ion-release, and ROS-mediated antimicrobial mechanisms, giving them both

preventive and antimicrobial functionality in a single agent. Plant-mediated (green) synthesis of CaONPs further improves surface functionalization and colloidal stability through phytochemical capping, which can reduce oxidative stress and improve cellular compatibility relative to chemically synthesized counterparts. In varnish formulations, CaONPs' chemical stability and ease of incorporation into resin or polymer matrices make them suitable for sustained local delivery, whereas highly reactive metal nanoparticles may require more complex stabilization. Overall, CaONPs occupy a middle ground offering multifunctional benefits (antimicrobial + remineralizing + anti-inflammatory) with a favorable safety profile making them a promising alternative or complement to existing dental nanomaterials, especially where long-term biocompatibility and mineral support are priorities. The Calcium Oxide Nanoparticle (CaONP)-based dental varnish demonstrates considerable clinical potential as a multifunctional preventive and adjunctive therapeutic agent in dentistry. By providing sustained local antimicrobial activity, the CaONP varnish may effectively reduce cariogenic and periodontal pathogens, thereby helping to prevent dental caries, gingivitis, and peri-implant infections. Its anti-inflammatory properties further support the management of inflamed oral tissues, potentially alleviating gingival irritation and promoting mucosal healing when applied to susceptible sites. The controlled release of calcium ions from CaONPs may additionally contribute to enamel remineralization and strengthening of tooth surfaces, offering benefits beyond microbial control. Importantly, the favorable cytotoxicity and embryonic safety profiles observed at selected concentrations suggest suitability for repeated topical application, which is essential for clinical dental use. Owing to its ease of application, localized action, and multifunctional therapeutic effects, CaONP-based dental varnish holds promise for incorporation into routine preventive dentistry, pediatric care, and post-procedural management, warranting further validation through *in vivo* studies and clinical trials.

CONCLUSION

Calcium oxide nanoparticles (CaONPs) can be successfully incorporated into a stable resin-based dental varnish, resulting in a multifunctional formulation with strong antimicrobial and anti-inflammatory activities. The CaONP-incorporated varnish was found to be safe at the tested concentrations, as demonstrated by cytotoxicity and embryonic toxicity assessments. These results reinforce the need for more *in vivo* research and upcoming clinical dental studies by highlighting the potential of CaONP-based dental varnishes as a viable approach for oral health applications.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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