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Original Article

Antimicrobial, Anti-inflammatory and Cytotoxicity Evaluation of a Novel Formulated Nano-Colloidal Mouthwash: An *in vitro* Study

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ABSTRACT

Background: Adjunctive mouthwashes are widely used in periodontal care to control plaque and gingival inflammation. Conventional agents such as chlorhexidine are effective but associated with adverse effects including tooth staining, altered taste, and mucosal irritation. Herbal and nanoparticle-based formulations are being explored as safer alternatives with dual antimicrobial and anti-inflammatory properties. **Aim:** To formulate a nano-colloidal mouthwash incorporating clove (*Syzygium aromaticum*) extract, stevia (*Stevia rebaudiana*) extract, and green-synthesized Silver Nanoparticles (AgNPs), and to evaluate its antimicrobial, anti-inflammatory, and cytocompatibility profiles against common oral pathogens. **Materials and Methods:** Clove and stevia extracts were prepared by Soxhlet and hot-water extraction, respectively. AgNPs were synthesized using a green reduction method with plant extracts as reducing agents. Characterization was performed using Field-Emission Scanning Electron Microscopy (FE-SEM) and Energy-Dispersive X-ray spectroscopy (EDAX). Antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis*, and *Candida albicans* was assessed using the agar well diffusion method. Anti-inflammatory activity was evaluated by protein denaturation and membrane stabilization assays. Cytocompatibility was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay on human gingival fibroblasts. **Results:** FE-SEM revealed spherical AgNPs with an average size of 20-40 nm, and EDAX confirmed elemental silver peaks. The nano-colloidal mouthwash produced inhibition zones ranging from 12 mm (*Candida albicans*) to 18 mm (*E. faecalis*). Protein denaturation was inhibited by >60%, and membrane stabilization was comparable to standard anti-inflammatory agents. MTT assay showed 79.8% cell viability at working concentration, confirming acceptable cytocompatibility. **Conclusion:** The formulated nano-colloidal mouthwash combining clove, stevia, and AgNPs exhibits potent antimicrobial and anti-inflammatory activity with good biocompatibility. It holds promise as a natural and biocompatible alternative to conventional mouthwashes. Further *in vivo* and clinical studies are recommended to validate safety and efficacy in periodontal therapy.

Keywords: Anti-inflammatory assay, Antimicrobial activity, Clove extract, Disease, Stevia, Health, Innovation, Nano-colloidal mouthwash, Periodontal pathogens, Research, Silver Nanoparticles (AgNPs).

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INTRODUCION

Cloves are the aromatic flower buds of *Syzygium aromaticum* (family Myrtaceae), native to the Maluku Islands in Indonesia, and are widely used as spices, flavoring, or fragrance components in consumer products such as toothpaste, soaps, and cosmetics (Bharath and Priyangha, 2025; Merr, 2021). Beyond culinary use, clove essential oil has long been utilized in traditional medicine as an anodyne (analgesic), especially for dental emergencies and other disorders (Uchibayashi, 2001; Yun, 2018). A major bioactive compound in clove is eugenol, which has proven antimicrobial and anti-inflammatory properties. Evidence shows that eugenol, when combined with zinc oxide, is effective as an analgesic and for the management of alveolar osteitis, while also inhibiting *Enterococcus faecalis*, a pathogen associated with root canal treatment failure (Uchibayashi, 2001).

Stevia (*Stevia rebaudiana*) is a natural sweetener and sugar substitute derived from the leaves of the plant, native to Brazil and Paraguay (Stevia, 2013a). Its active components, steviol glycosides such as stevioside and rebaudioside, are reported

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to be 50-300 times sweeter than sucrose, heat- and pH-stable, and non-fermentable (Is Stevia a Good Sugar Substitute?, 2021; Stevia, 2013b). Unlike sugar, these glycosides are not metabolized by the human body, thus contributing no calories and posing no cariogenic risk (Cardello *et al*., 1999). Stevia has been traditionally used for centuries by the Guaraní peoples of South America to sweeten local teas and medicines, and is now widely incorporated into modern sugar- and calorie-reduced products (Faq, 2004; Jayanandan *et al*., 2025).

Silver has also played a pivotal role in dentistry. In the nineteenth century it was a component of dental amalgams, though later replaced by esthetic resins (Rai *et al*., 2015). With the advancement of nanoscience, Silver Nanoparticles (AgNPs) have gained renewed importance owing to their potent antimicrobial activity against bacteria, fungi, and viruses (Rai *et al*., 2014; Rai *et al*., 2015). Their broad bioactivity is attributed to nanoscale size, increased surface area-to-volume ratio, and their ability to interact with microbial membranes and nucleic acids (Besinis *et al*., 2015; Padovani *et al*., 2015; Wei *et al*., 2015). AgNPs have been applied in various dental materials, including prosthetic coatings, adhesives, and implant surfaces, to prevent biofilm formation and enhance healing (Rai *et al*., 2014; Rai *et al*., 2015; Wei *et al*., 2015).

Mouthwashes are widely used adjuncts to mechanical plaque control and are valued for their antiseptic and anti-inflammatory effects (Nedumaran and Rajasekar, 2025; Parham *et al*., 2020). Conventional chemical formulations, such as chlorhexidine-based rinses, are effective in reducing plaque and gingivitis but are often associated with undesirable side effects including tooth staining, taste alteration, and mucosal irritation (Nedumaran and Rajasekar, 2025; Rajendiran *et al*., 2021; Refaey *et al*., 2024) This has encouraged research into herbal and nanoparticle-based alternatives. Herbal extracts such as clove, neem, and tea tree oil possess inherent antimicrobial and anti-inflammatory properties (Bansal *et al*., 2019; Talebi *et al*., 2022), while AgNPs provide additional broad-spectrum antimicrobial effects through unique physicochemical interactions (Khaldoun *et al*., 2025). Given this context, combining clove and stevia extracts with silver nanoparticles in a single nano-colloidal mouthwash formulation may provide a safe, effective, and biocompatible adjunct for periodontal therapy. The present study was therefore undertaken to formulate such a mouthwash and to evaluate its antimicrobial, anti-inflammatory, and cytocompatibility profiles against common oral pathogens associated with periodontal disease.

MATERIALS AND METHODS

Clove buds (*Syzygium aromaticum*) and stevia leaves (*Stevia rebaudiana*) were selected as the herbal components for the nano-colloidal mouthwash formulation. Clove buds were sourced from a local herbal supplier, thoroughly washed, shade-dried, and ground into fine powder. The powdered clove was subjected to Soxhlet extraction using ethanol as the solvent. The obtained extract was filtered, concentrated with a rotary evaporator, and stored at 4ºC until further use. Stevia leaves were similarly washed, dried, and powdered. An aqueous extract was prepared by hot-water infusion, followed by filtration and concentration.

Silver Nanoparticles (AgNPs) were synthesized using a green synthesis approach. Aqueous plant extract served as both the reducing and stabilizing agent for Silver Nitrate ($AgNO_3$). Upon the addition of $AgNO_3$ solution to the plant extract, the reaction mixture was stirred at room temperature under controlled conditions. A visible color change indicated nanoparticle formation. The colloidal solution was centrifuged and repeatedly washed with distilled water to remove unreacted materials. The final nanoparticle pellet was redispersed in distilled water for subsequent use in formulation. The mouthwash was prepared by incorporating the ethanolic clove extract, aqueous stevia extract, and synthesized AgNPs into a distilled water base. The pH of the formulation was adjusted to 6.8-7.2 to ensure oral compatibility. No synthetic preservatives were added. The formulation was stored in amber-colored bottles at room temperature until use.

Characterization of synthesized silver nanoparticles was performed to confirm morphology and elemental composition. Field Emission Scanning Electron Microscopy (FE-SEM) was used to assess particle size and shape. Energy-Dispersive X-ray analysis (EDAX) was employed to confirm elemental silver and exclude contamination. Visual observation of color change and stability in colloidal suspension were also monitored throughout storage. Antimicrobial activity of the mouthwash was evaluated by agar well diffusion method. Standard microbial strains, including *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis*, and *Candida albicans*, were cultured on Mueller-Hinton agar. Sterile wells were made in the agar plates, and the mouthwash formulation was introduced into each well. Plates were incubated under aerobic conditions, and the zones of inhibition were measured in millimeters.

The anti-inflammatory potential of the formulation was determined by *in vitro* protein denaturation and erythrocyte membrane stabilization assays. The ability of the mouthwash to inhibit heat-induced protein denaturation and to stabilize red-blood-cell membranes against hemolysis was assessed and compared to standard anti-inflammatory controls. Cytotoxicity and biocompatibility were evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on cultured human gingival fibroblast cell lines. Cells were treated with the mouthwash formulation at working concentration, followed by incubation with MTT reagent. Mitochondrial dehydrogenase activity was quantified to assess cell viability, which was expressed as a percentage compared with untreated control cells.

RESULTS

The ethanolic extract of *Syzygium aromaticum* (clove) displayed a characteristic dark brown color indicating the presence of concentrated phytochemicals such as eugenol. The aqueous extract of *Stevia rebaudiana* showed a lighter coloration consistent with the extraction of natural glycosides like stevioside. When combined with green-synthesized Silver Nanoparticles (AgNPs), the final nano-colloidal mouthwash appeared as a light brown homogenous solution that remained physically stable without visible sedimentation during storage demonstrating adequate miscibility of herbal extracts and nanoparticles in the aqueous medium.

Characterization of silver nanoparticles confirmed successful synthesis. FE-SEM revealed predominantly spherical particles with a uniform distribution, ranging in size from 20 to 40 nm (Figure 1). Energy Dispersive X-ray Analysis (EDAX) further confirmed the elemental composition, showing a strong silver peak at approximately 3 keV, with minimal contamination from other elements (Figure 2). The nanoscale size and purity of AgNPs ensured a high surface area, supporting their role in enhancing antimicrobial activity.

Biocompatibility of the mouthwash formulation was evaluated using the MTT assay on human gingival fibroblast cells. The formulation demonstrated 79.82% cell viability at the working concentration (Figure 3), which falls within the acceptable range of cytocompatibility according to ISO standards (>70%). This indicates that the mouthwash is safe for potential oral application without inducing significant cytotoxic effects on gingival tissues.

Antimicrobial efficacy, assessed by agar well diffusion, demonstrated clear zones of inhibition against all tested organisms. As summarized in (Table 1) *Enterococcus faecalis* exhibited the greatest susceptibility with an inhibition zone of 18 mm, followed by *Escherichia coli* (17 mm), *Staphylococcus aureus* (15 mm), and *Candida albicans* (12 mm). Photographic documentation (Figure 4) illustrates the distinct clearance zones, confirming the broad-spectrum antimicrobial activity of the formulation. These findings highlight the combined antimicrobial effects of clove-derived eugenol and silver nanoparticles, which act synergistically against Gram-positive, Gram-negative, and fungal pathogens.

The anti-inflammatory activity of the nano-colloidal mouthwash was demonstrated through protein denaturation and erythrocyte membrane stabilization assays. As shown in (Table 2) the formulation inhibited protein denaturation by more than 60%, while membrane stabilization values were comparable to those achieved by standard anti-inflammatory agents. This suggests that the mouthwash not only controls microbial growth but also

Table 1: Antimicrobial activity of the nano-colloidal mouthwash against tested microorganisms (zone of inhibition in mm).

Microorganism	Zone of inhibition (25 µL)	Zone of inhibition (100 µL)
Escherichia coli	17±0.5	18±0.6
Staphylococcus aureus	14±0.4	15±0.5
Enterococcus faecalis	7±0.3	8±0.2
Candida albicans	6±0.2	8±0.3

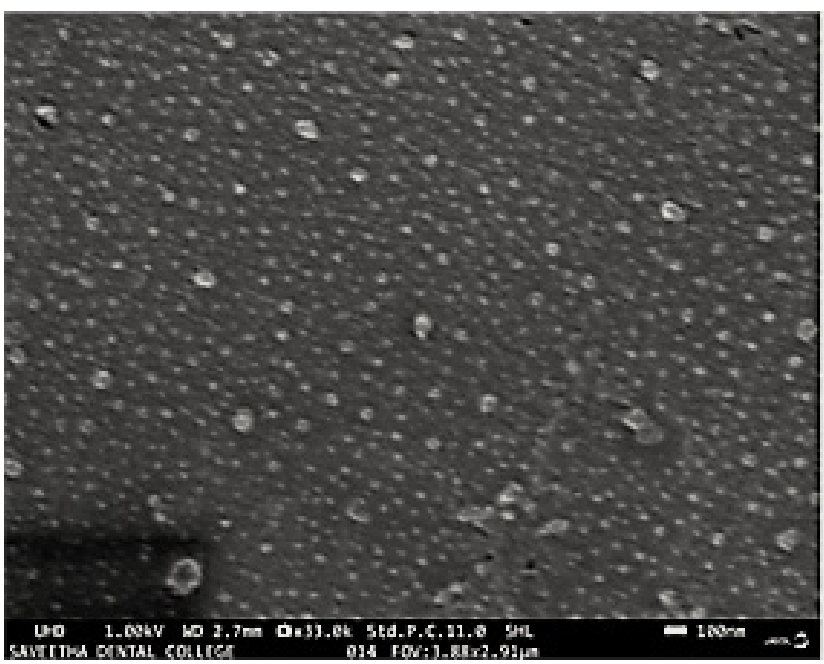

Figure 1: Field-Emission Scanning Electron Microscopy (FE-SEM) micrograph of synthesized silver nanoparticles showing spherical morphology (20-40 nm).

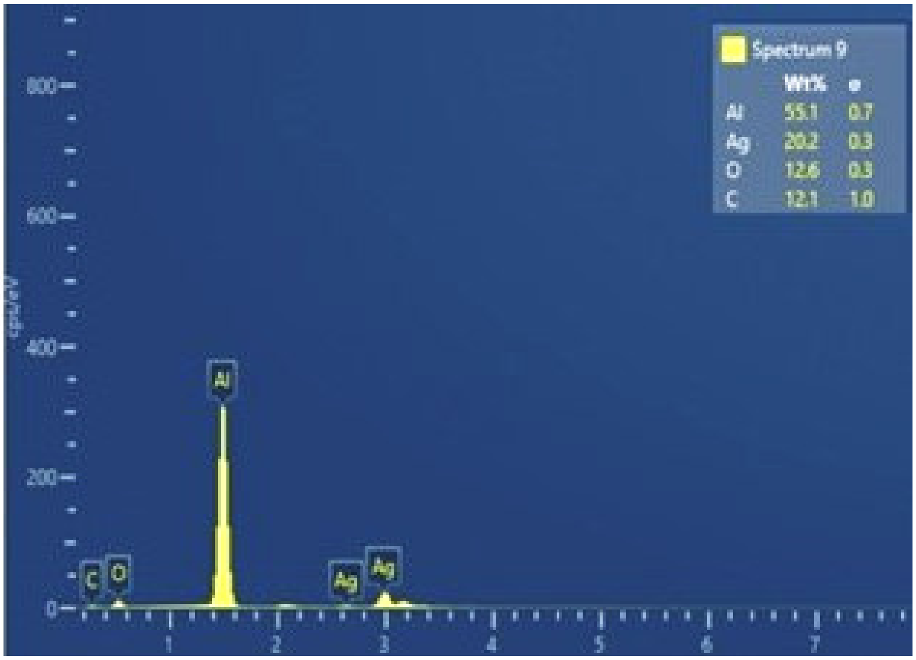

Figure 2: Energy Dispersive X-ray Analysis (EDAX) spectrum of silver nanoparticles confirming strong silver peak with minimal impurities.

has the potential to alleviate gingival inflammation, a key factor in periodontal disease management.

DISCUSSION

The present study demonstrated that a nano-colloidal mouthwash formulated with clove (*Syzygium aromaticum*) extract, stevia (*Stevia rebaudiana*) extract, and green-synthesized Silver Nanoparticles (AgNPs) exhibits significant antimicrobial, anti-inflammatory, and cytocompatible properties. The results agree with previous studies that have highlighted the potential of green-synthesized AgNPs as effective antimicrobial agents against oral pathogens. Emmanuel *et al.*, reported that silver nanoparticles synthesized using *Justicia glauca* leaf extract inhibited *Streptococcus mutans, Enterococcus faecalis, Escherichia coli*, and *Candida albicans* at low concentrations, confirming their role as potent antimicrobial agents in dental applications (Emmanuel *et al.*, 2015). Our findings, with inhibition zones ranging from 12 to 18 mm, support these observations and demonstrate broad-spectrum efficacy of biosynthesized AgNPs.

The contribution of clove extract in the formulation further enhances antimicrobial potential. Clove contains eugenol, a phenolic compound with established antimicrobial and anti-inflammatory activity. Lakshmeesha *et al.*, showed that clove-mediated AgNPs inhibited *S. mutans, S. aureus*, and *C. albicans*, with nanoparticles in the size range of 4-16 nm (Jardón-Romero *et al.*, 2022). This aligns with our results, where FE-SEM revealed spherical AgNPs of 20-40 nm that demonstrated strong inhibition, particularly against *E. faecalis*. The synergy between clove phytochemicals and AgNPs may account for the enhanced antimicrobial activity observed in our formulation.

Table 2: Anti-inflammatory activity of the nano-colloidal mouthwash assessed by protein denaturation and membrane stabilization assays.

Concentration (µL)	Anti-inflammatory activity (%)
200	−10.55
300	−11.18
400	24.73
500	38.21

The inclusion of stevia extract in the formulation is noteworthy. Although primarily recognized as a natural sweetener, stevia contributes to the biocompatibility and palatability of the mouthwash. Herbal-nanoparticle combinations have been previously reported to exhibit synergistic antimicrobial effects. Lee *et al.*, demonstrated that nano-encapsulated clove oil combined with thymol exerted enhanced inhibitory effects on cariogenic bacteria compared to individual components (Lee *et al.*, 2020). Similarly, our results confirm that the integration of phytochemicals with AgNPs broadens the antimicrobial spectrum and supports their role in disrupting biofilm-associated pathogens relevant to periodontal infections. The anti-inflammatory activity observed in this study is consistent with prior reports (Kumar *et al.*, 2025). Inhibition of protein denaturation exceeded 60%, while membrane stabilization activity was comparable to standard anti-inflammatory controls. Such outcomes are in line with the findings of Khan *et al.*, who demonstrated that green-synthesized AgNPs are capable of inhibiting protein denaturation and stabilizing cellular membranes (Krishnappan *et al.*, 2024). This anti-inflammatory potential is particularly relevant to periodontal therapy, where modulation of host inflammatory response is as critical as microbial suppression.

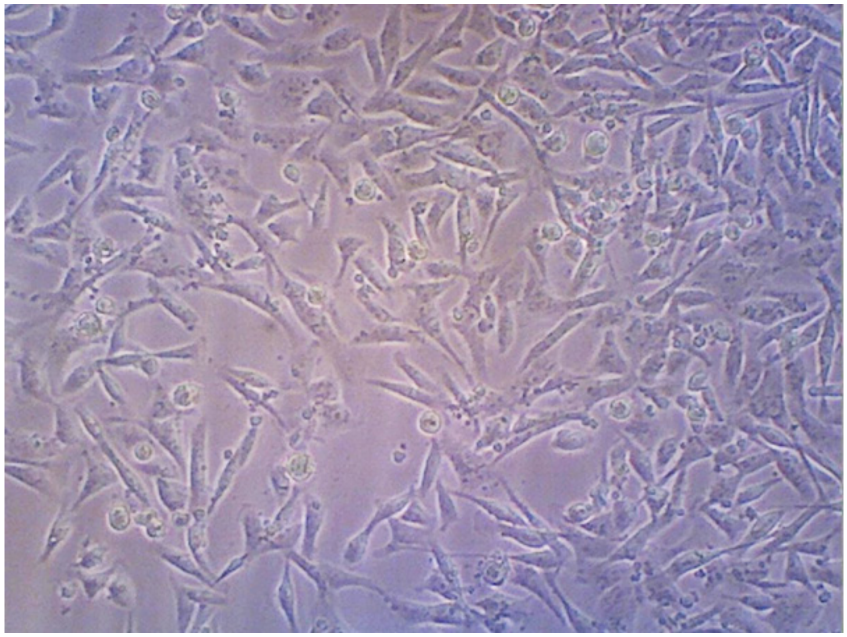

Figure 3: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay results on human gingival fibroblasts showing 79.82% cell viability at working concentration.

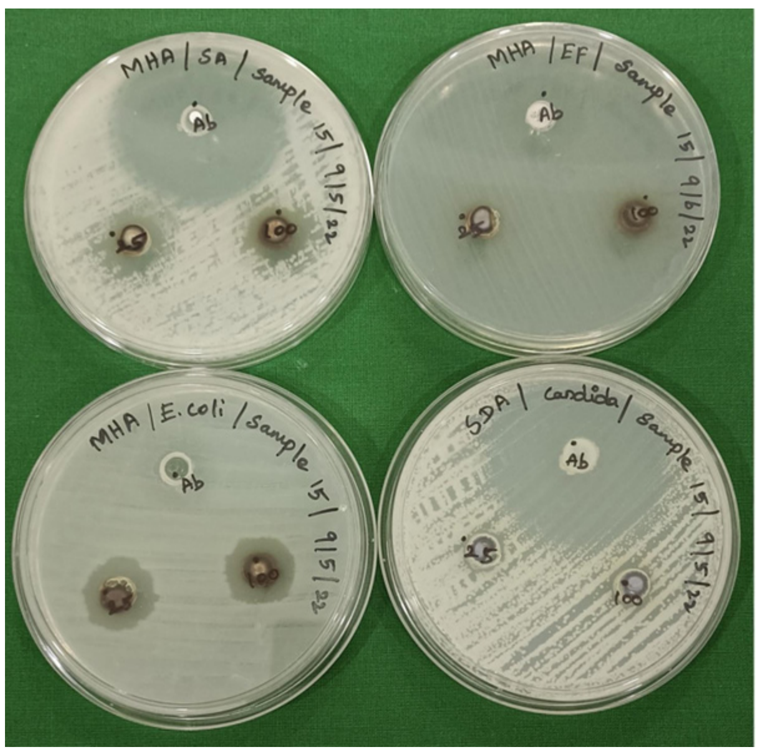

Figure 4: Agar well diffusion assay showing zones of inhibition against *E. coli*, *S. aureus*, *E. faecalis*, and *C. albicans*.

Cytocompatibility is a vital consideration for any oral formulation. The MTT assay in this study revealed ~80% cell viability at the working concentration, confirming acceptable safety margins. These results parallel those of Deena Dayal *et al*., who reported that *Phaseolus lunatus*-mediated AgNP mouthwash displayed low toxicity in both cell culture and zebrafish embryo models (Dutt *et al*., 2023). Our findings further support the potential for safe application of such formulations in the oral cavity.

While the present findings are promising, certain limitations must be acknowledged. This study was restricted to *in vitro* assays, and therefore, the actual clinical efficacy of the mouthwash in reducing plaque accumulation, gingival inflammation, or microbial load in patients remains to be evaluated. Moreover, the study did not assess the long-term stability, sensory acceptability, or potential side effects such as mucosal irritation under clinical conditions.

The absence of replicate measurements for statistical analysis also limits the generalizability of the results.

Future research should focus on well-designed *in vivo* and clinical trials to validate these findings. Assessment of plaque index, gingival index, microbial colony-forming units, and patient acceptability will be essential. Additionally, evaluating the formulation against multispecies biofilms rather than individual pathogens would provide a more realistic picture of its efficacy in the complex oral environment. Optimization of formulation stability, taste masking, and comparison with conventional agents such as chlorhexidine should also be prioritized.

Taken together, this study highlights the therapeutic potential of a nano-colloidal mouthwash combining clove, stevia, and AgNPs. The formulation demonstrated broad-spectrum antimicrobial effects, notable anti-inflammatory activity, and acceptable cytocompatibility, making it a promising natural alternative to chemical mouthwashes. With further refinement and clinical validation, such herbal-nanoparticle formulations could represent an effective adjunct in periodontal therapy.

CONCLUSION

This study formulated and evaluated a nano-colloidal mouthwash incorporating clove (*Syzygium aromaticum*), stevia (*Stevia rebaudiana*), and green-synthesized silver nanoparticles. The formulation demonstrated broad-spectrum antimicrobial efficacy, with notable inhibition against *Enterococcus faecalis, Escherichia coli, Staphylococcus aureus*, and *Candida albicans*. In addition, it exhibited significant anti-inflammatory potential, as evidenced by inhibition of protein denaturation and membrane stabilization, and showed acceptable cytocompatibility with nearly 80% viability of human gingival fibroblasts at working concentration.

These findings collectively suggest that the mouthwash has the potential to act as a natural, biocompatible alternative to conventional chemical rinses such as chlorhexidine, which are often associated with side effects including staining, taste alteration, and mucosal irritation. By integrating phytochemicals with nanotechnology, the formulation offers a dual advantage of antimicrobial and host-modulatory benefits. Nevertheless, the present study is limited to *in vitro* assays. Well-designed *in vivo* and clinical trials are essential to validate its therapeutic efficacy, long-term safety, and patient acceptability. Future investigations should also assess its stability, palatability, and comparative performance against established agents. Within these limitations, the formulation represents a promising adjunct in periodontal therapy, combining natural extracts with nanoscale innovation.

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ABBREVIATIONS

AgNPs: Silver nanoparticles; **FE-SEM:** Field emission scanning electron microscopy; **EDAX:** Energy dispersive X-ray analysis; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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