Anti-Inflammatory Activity of Coleus Amboinicus Incorporated Chitosan Against Enterococcus Faecalis - an in Vitro Study

K P Sowmya1 , M.Mukundh1,a)

1Sowmya Medical Centre, Chennai, Tamilnadu, India

**Corresponding Author:** a)[mukundhmadhav900@gmail.com](mailto:mukundhmadhav900@gmail.com)

**ABSTRACT:** Bacterial eradication is crucial for the success of endodontic treatments, as bacteria are responsible for pulpal and periradicular inflammation and their byproducts. Although effective irrigation and proper canal instrumentation can significantly reduce the bacterial load, *Enterococcus faecalis* remains difficult to eliminate completely from the root canal system. *Preparation of Coleus Amboinicus Incorporated Chitosan:* This in vitro study was carried out in a private educational institution's laboratory. The Coleus Amboinicus powder used in the study was commercially obtained. To prepare the solution, 100 mL of distilled water was heated to boiling, and 5 g of Coleus Amboinicus powder was added. The mixture was boiled for 30 minutes and then filtered using Whatman filter paper. The filtered extract was re-boiled for an additional 10 minutes and then concentrated to 10 mL. To prepare the final solution, 10 mL of the Coleus Amboinicus extract was combined with a chitosan solution and stirred using a magnetic stirrer for 24 hours. This formulation was then tested for its anti-inflammatory activity against *Enterococcus faecalis*.The results demonstrated that the plant-based irrigant formulation exhibited superior anti-inflammatory effects when compared to the standard diclofenac used in the study. The varying concentrations of the solution inhibited protein denaturation by 55%, 64%, 69%, 72%, and 81%, respectively. The combination of Coleus Amboinicus extract and chitosan has shown significant anti-inflammatory properties in root canal treatments, especially for primary teeth. In addition to its established antimicrobial effectiveness and low cytotoxicity, the synergistic effects of these two compounds may enhance the treatment of various oral lesions.

**KEYWORDS:** Enterococcus faecalis, chitosan, Coleus Amboinicus

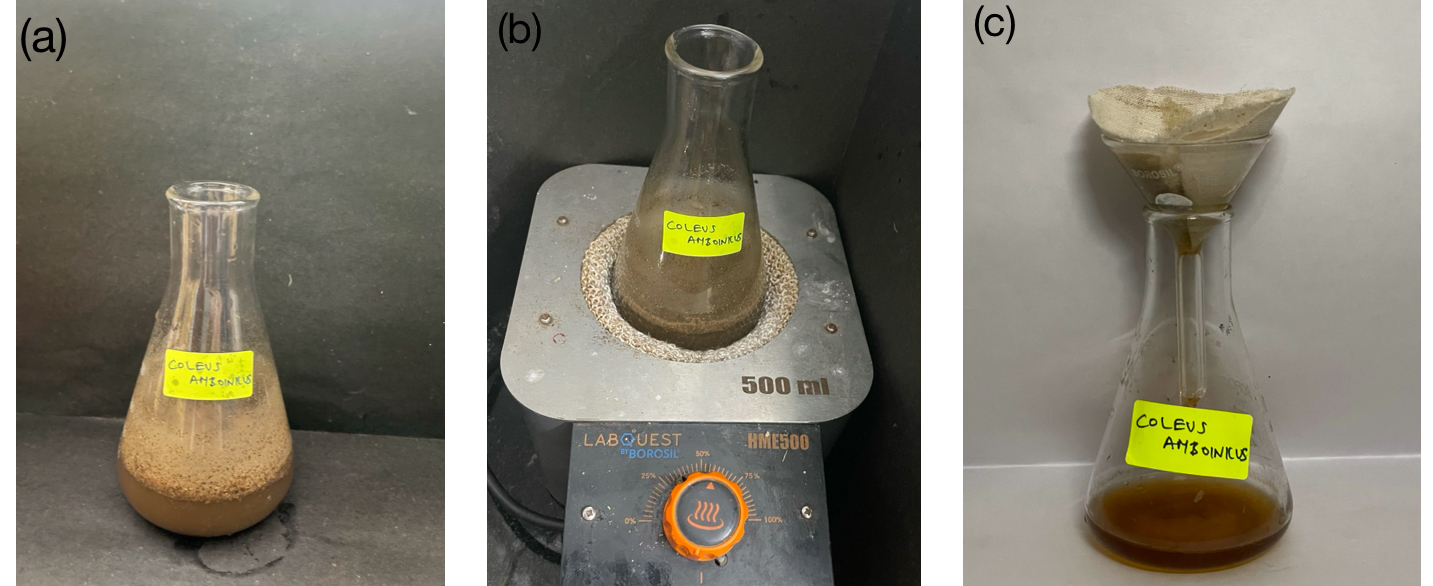
# INTRODUCTION

In endodontic treatment, it is crucial to thoroughly remove all pulpal tissue, dentinal debris, and viable microorganisms from the root canal system. The elimination of bacteria is vital for the success of endodontic procedures, as they are responsible for pulpal and periradicular inflammation and their byproducts (Aparna et al., 2021; Ganapathy, 2021). Enterococcus faecalis, a gram-positive, anaerobic bacterium, is commonly found in the human oral cavity, gastrointestinal tract, and vagina. Its ability to thrive in environments that are nutrient-rich, low in oxygen, and possess complex ecosystems allows it to adapt well to these regions (Verma & Muthuswamy Pandian, 2021). Several studies have shown that failures in endodontic treatment are often associated with a higher prevalence of E. faecalis compared to cases of primary infection. E. faecalis is the most commonly identified pathogen in cases of pain and infection following endodontic procedures, with prevalence rates reaching up to 90%. This bacterium is more frequently linked with asymptomatic conditions than symptomatic ones in primary endodontic infections (Gomes et al., 2003; Poornima et al., 2021). Despite effective irrigation with sodium hypochlorite and appropriate instrumentation, E. faecalis is difficult to completely eliminate from the root canal system (Ganapathy, 2021).Chlorhexidine, a cationic solution, is commonly used during endodontic treatments for its broad antimicrobial properties. Its ability to persist on bacterial cell membranes is due to its cationic structure (Greenstein et al., 1986). Chlorhexidine (CHX) consists of two symmetric 4-chlorophenyl rings connected by a hexamethylene chain and two biguanide groups, forming a synthetic cationic bis-guanide (Gomes et al., 2003; Khan, 2013; Mary et al., 2023). The positively charged, lipophilic nature of CHX allows it to interact with the negatively charged phospholipids and lipopolysaccharides on bacterial cell membranes, facilitating its entry into the bacterial cell through either active or passive transport (Athanassiadis et al., 2007). Its bactericidal effect occurs when the CHX molecule alters the osmotic balance of the bacterial cell, making the cell membrane more permeable and allowing the CHX to enter the cell (Garapati et al., 2022). At concentrations of 0.2%, CHX is effective in leaking out low molecular weight materials such as potassium and phosphorus. However, at higher concentrations (2%), it is bactericidal, precipitating cytoplasmic contents and leading to cell death (Delany et al., 1982).Coleus Amboinicus (CA), a widely distributed plant, is commonly used as a dietary supplement and for its medicinal properties (Merchant et al., 2022). The plant contains various bioactive compounds, such as flavonoids, quinones, tannins, phenols, terpenoids, alkaloids, glycosides, and squalene, which are known for their antioxidant effects. These compounds help neutralize free radicals and inhibit oxidative damage. The plant's active ingredients are believed to activate enzymes such as glutathione peroxidase (GPx), catalase, and superoxide dismutase (SOD), which play a role in protecting the body from oxidative stress (Chokkattu et al., 2022; Stansbury, 2019). GPx, in particular, can bind selenium and transform hydrogen peroxide (H2O2) and hydroperoxides into less harmful compounds (Ramamurthy et al., 2022). The enzyme, in conjunction with other antioxidants like SOD and catalase, neutralizes H2O2, reducing free radical levels and lipid peroxide accumulation in tissues. Coleus Amboinicus has also been found to have anti-inflammatory effects, as well as anticonvulsant and antitumor properties. Additionally, it has been shown to promote breast milk secretion and prevent kidney stones in experimental animals (Chang et al., 2010; Marya et al., 2022; Gurgel et al., 2009; Jain & Verma, 2022). Given the beneficial effects of Coleus Amboinicus, this study aims to evaluate the anti-inflammatory activity of both Coleus Amboinicus and chlorhexidine against Enterococcus faecalis in root canal treatments for primary teeth (Stansbury, 2019).

# MATERIALS AND METHODS

## Preparation of Coleus Amboinicus incorporated chitosan

The present in vitro study was designed and conducted in the institutional study setup. The Coleus Amboinicus powder was procured from the local market of Chennai for the preparation of solution. 100 mL of distilled water was set to boil, to which 5 gm of Coleus Amboinicus powder was added. The boiling was carried out for half an hour, followed by filtration through Whatman filter paper. The prepared extract was re-boiled for 10 minutes and finally concentrated to 10 mL. To prepare 10 mL Coleus Amboinicus extract add a chitosan solution and keep it in a magnetic stirrer for 24 hours. Coleus Amboinicus incorporated chitosan solution was used to examine anti-inflammatory activity against Enterococcus faecalis.



**FIGURE 1:** 5 gm of Coleus Amboinicus powder in 100 mL of distilled water (a), boiling Coleus Amboinicus extract (b), concentrated 10 mL of Coleus Amboinicus extract (c ).

## Anti-inflammatory activity

## Bovine serum albumin denaturation assay

The green synthesized silver nanoparticles was tested for its anti-inflammatory activity using two assays such as Bovine serum albumin denaturation assay and Egg albumin denaturation assay. 0.45mL of bovine serum albumin was mixed with 0.05 mL of different concentrations (10-50 µg/mL) of Coleus Amboinicus extract and chlorhexidine. The pH was adjusted to 6.3. Then it was kept at room temperature for 10 minutes followed by incubation in a water bath at 55°C in a water bath for 30 min.Diclofenac sodium was used as the standard group while dimethyl sulphoxide was used as control. Then, the samples were measured spectrophotometrically at 660nm.

Percentage of protein denaturation was determined utilizing following equation,

% inhibition= Absorbance of control- Absorbance of sample×100

Absorbance of control

## Egg Albumin denaturation assay

To perform, Egg albumin denaturation assay, 0.2mL of fresh egg albumin was mixed with 2.8 mL of phosphate buffer. Different concentrations (10-50 µg/mL) of Coleus Amboinicus extract and chlorhexidine were added to the reaction mixture. The pH was adjusted to 6.3. Then it was kept at room temperature for 10 minutes followed by incubation in a water bath at 55°C in a water bath for 30 min. Diclofenac sodium was used as the standard group while dimethyl sulphoxide was used as control. Then, the samples were measured spectrophotometrically at 660nm.

Percentage of protein denaturation was determined utilizing following equation,

% inhibition= Absorbance of control- Absorbance of sample×100

Absorbance of control

## Membrane stabilization assay

The in vitro membrane stabilization assay is a widely used technique for evaluating the membrane stabilizing properties of natural and synthetic compounds [(Wadhwani et al., 2022)](https://paperpile.com/c/RGvBlP/aAhB). This assay measures the ability of a compound to stabilize the cell membrane by preventing its disruption and subsequent release of intracellular contents. The materials include Human red blood cells (RBCs),Phosphate-buffered saline (PBS),Tris-HCl buffer (50 mM, pH 7.4),Different concentrations of Coleus Amboinicus extract and chlorhexidine (10-50 µg/mL),Centrifuge tube, UV-Vis spectrophotometer.

## Preparation of RBC suspension

Collect fresh human blood in a sterile tube containing anticoagulant(Rafi et al., 2024). Centrifuge the blood at 1000 g for 10 minutes at room temperature to separate the RBCs from other blood components. Remove the supernatant and wash the RBCs three times with PBS. Resuspend the RBCs in Tris-HCl buffer to obtain a 10% (v/v) RBC suspension.

## Assay procedure

Pipette 1mL of the RBC suspension into each centrifuge tube.Then different concentrations of Coleus Amboinicus extract and chlorhexidine was added to each tube.Mix gently and incubate the tubes at 37°C for 30 minutes. Centrifuge the tubes at 1000 g for 10 minutes at room temperature to pellet the RBCs. Measure the absorbance of the supernatant at 540 nm using a UV-Vis spectrophotometer.

Calculate the percentage inhibition of hemolysis using the following formula:

% inhibition = [(OD control – OD sample) / OD control] x 100

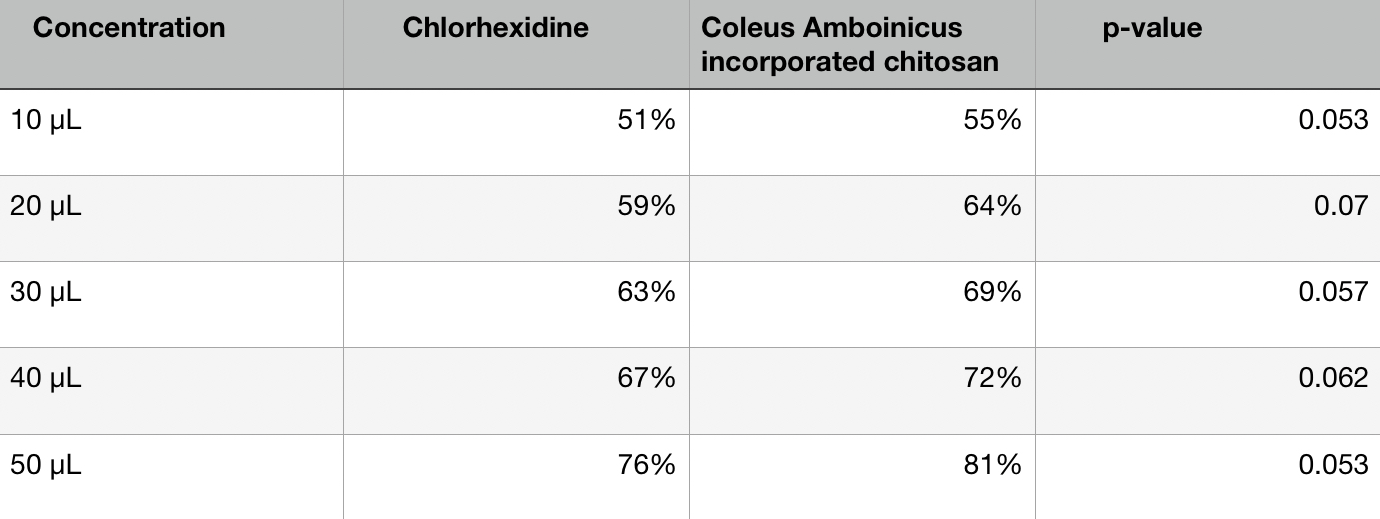
where OD control is the absorbance of the RBC suspension without the test compound(s) and OD sample is the absorbance of the RBC suspension with the test compound.

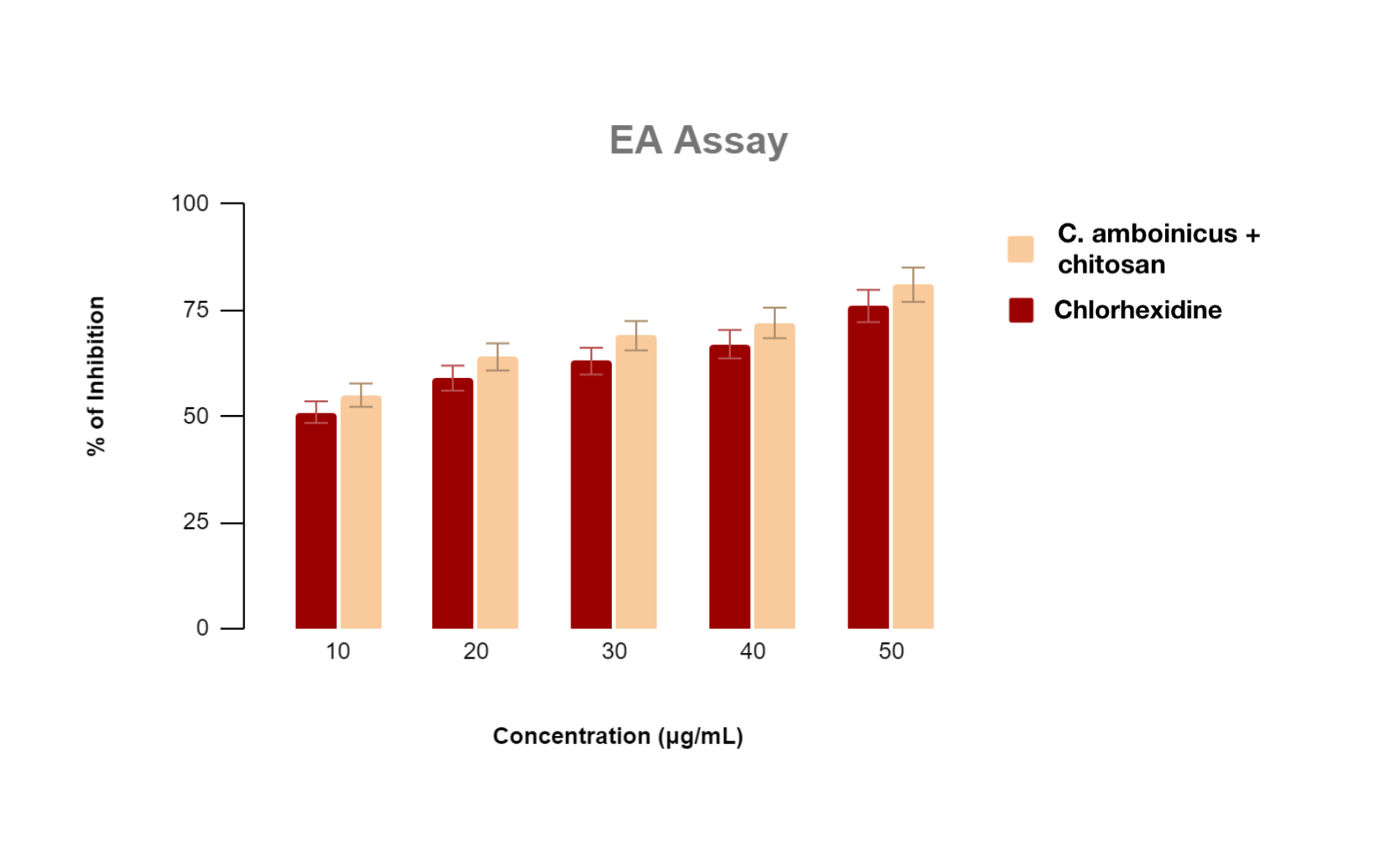
# RESULTS

## Anti-inflammatory activity

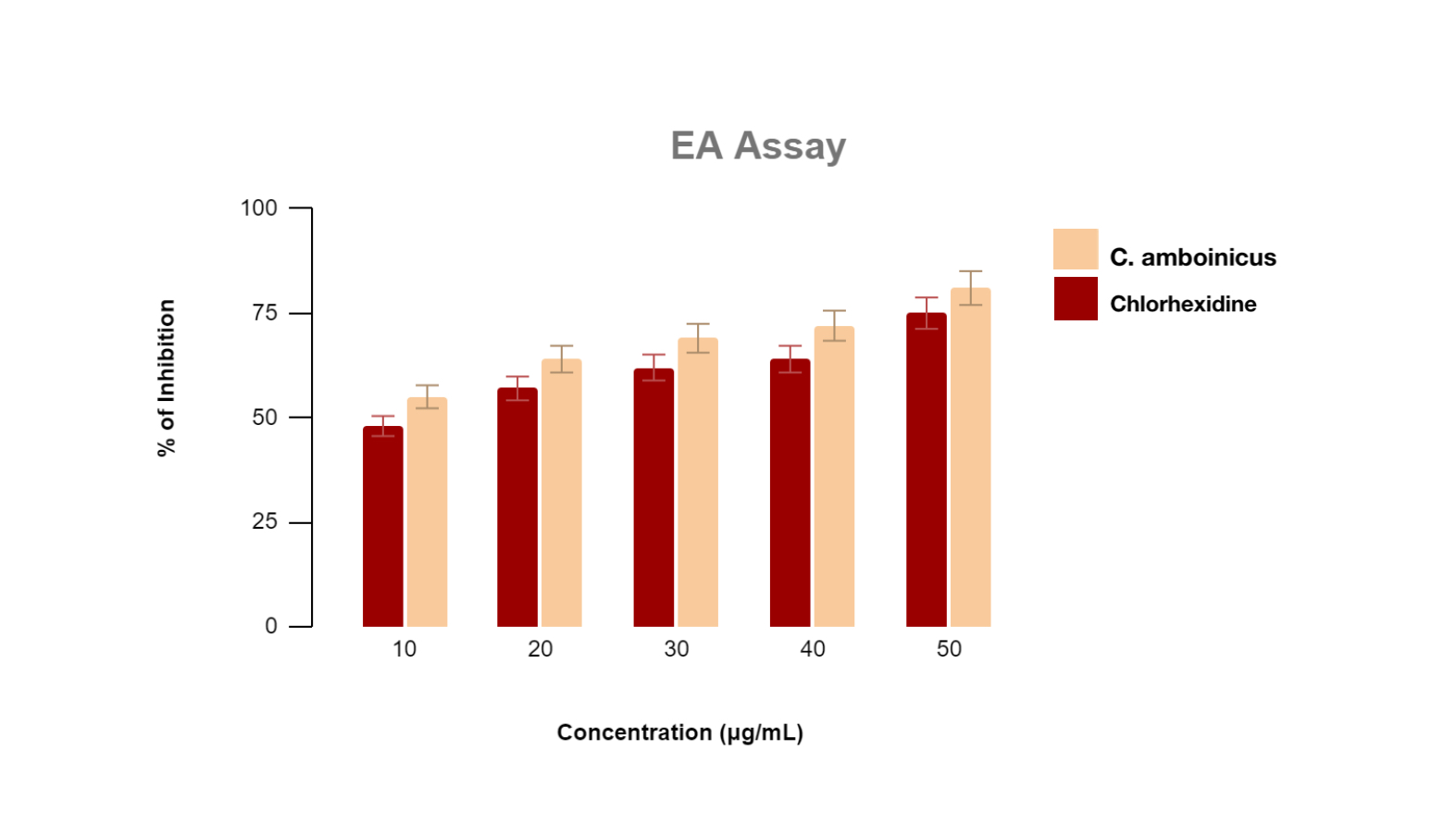
The results indicated that the plant-based irrigant formulation demonstrated a stronger anti-inflammatory effect compared to the standard chlorhexidine used in the study (Tuluwengjiang et al., 2024). The different concentrations of the formulation inhibited protein denaturation at values of 55%, 64%, 69%, 72%, and 81% (Figure 1), which were comparable to the commercial irrigant. The maximum anti-inflammatory effect was observed at a concentration of 50 μL, reaching 81%. Even at the lowest concentration of 10 μL, the formulation still exhibited 55% anti-inflammatory activity. At concentrations of 10 μL, 20 μL, and 30 μL, the herbal oral formulation showed significantly higher anti-inflammatory activity than the control (p<0.05). While the activity at 40 μL and 50 μL was also higher, the differences were not statistically significant.

**Table 1:** Comparative analysis of the anti-inflammatory property of the prepared formulation with control; a p-value of less than or equal to 0.05 was considered significant

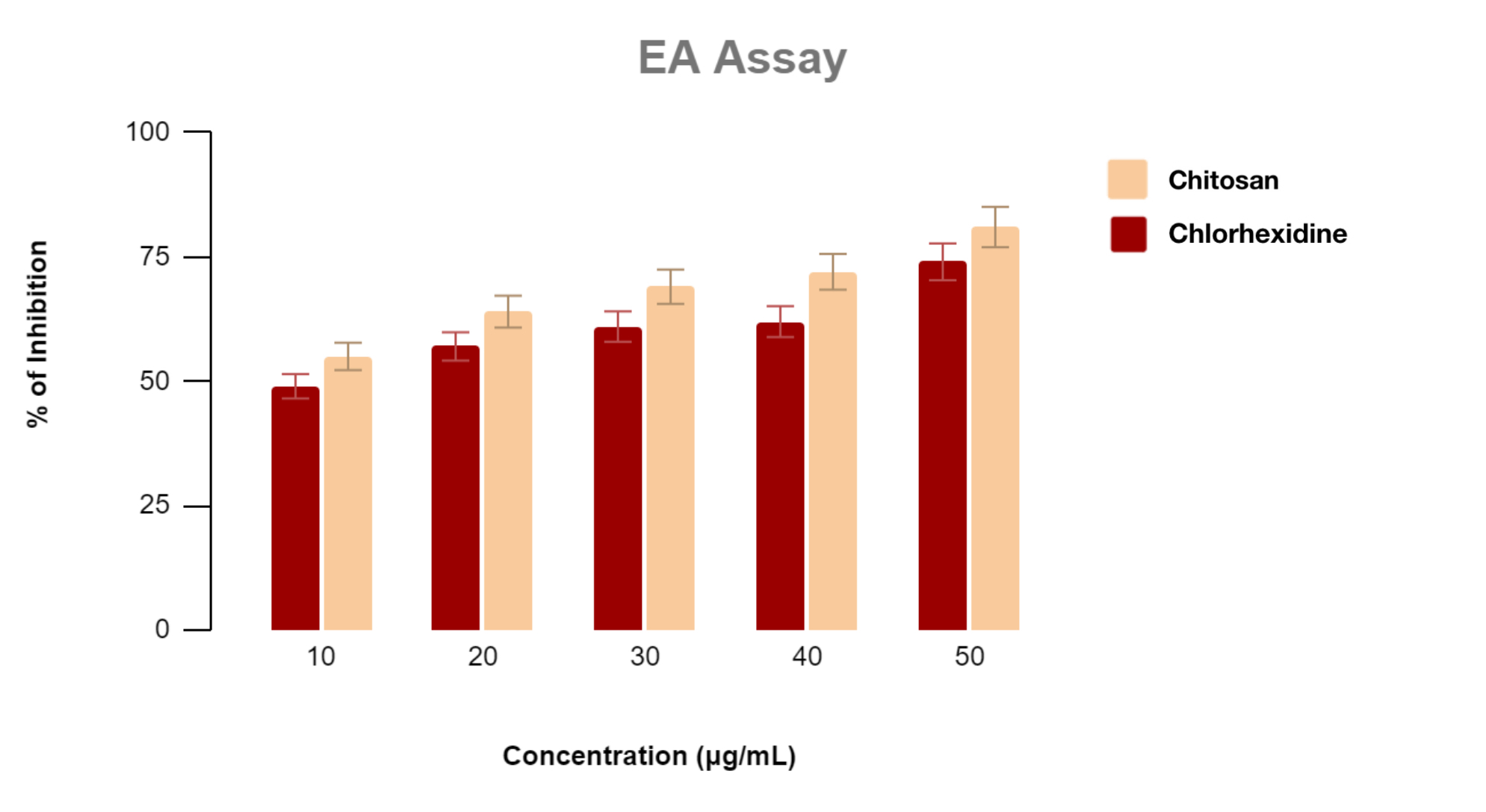




**Figure 1:** Bar graph showing Anti-inflammatory activity of Coleus Amboinicus incorporated chitosan at various concentration



**Figure 2:** Bar graph showing Anti-inflammatory activity of Coleus Amboinicus extract at various concentration



**figure 3:** Bar graph showing Anti-inflammatory activity of chitosan at various concentration

# DISCUSSION

Enterococcus faecalis is a well-researched biological indicator, particularly noted for its resilience. Numerous laboratory studies on its susceptibility to endodontic therapy have shown that this bacterium exhibits strong resistance to various antimicrobial agents (Sreevarun et al., 2023). Furthermore, E. faecalis can survive in harsh conditions, including environments with low nutrient availability and a high alkaline pH of up to 11.5 (Kayaoglu & Ørstavik, 2004). The bacterium’s ability to thrive as a mono-infection in treated root canals and form biofilms on canal walls, even without the presence of other bacteria, makes it highly resistant to root canal treatments and antimicrobial agents. Several studies have explored the link between E. faecalis and various periradicular diseases (Evans et al., 2002; Love, 2001), although fewer investigations have specifically focused on its role in endodontic therapy (Solanki et al., 2023). Given these considerations, the current study aims to evaluate the anti-inflammatory properties of Coleus Amboinicus and chlorhexidine against E. faecalis in root canal treatments for primary teeth (Muthuswamy Pandian et al., 2022; Pinheiro et al., 2003).Both Coleus Amboinicus extracts and chitosan have been shown to possess antimicrobial properties against a range of oral pathogens (Chokkattu et al., 2023). Integrating these substances into root canal treatments may assist in effectively disinfecting the canal and eliminating bacteria, thereby enhancing treatment success (Anti-Inflammatory Potential Mouthwash Formulated Using Clove Ginger Mediated Zinc Oxide Nanoparticles: Vitro Study, n.d.; Laghari et al., 2023; Sedgley et al., 2006). Chitosan is known for its biocompatibility, making it an ideal material for tissue engineering and regenerative applications. When combined with Coleus Amboinicus, it could potentially aid in the regeneration of periapical tissues, thus improving the overall success rate of root canal procedures (Adel et al., 2023; Gutmann & Lovdahl, 2010; Pereira et al., 2017). Additionally, chitosan’s capacity to form nanoparticles or gels can be leveraged to encapsulate bioactive compounds from Coleus Amboinicus, allowing for sustained release and targeted delivery within the root canal system (Subramanian & Harikrishnan, 2023). This controlled release mechanism may significantly enhance the efficacy of the treatment.

# CONCLUSION

The combination of Coleus Amboinicus and chitosan has demonstrated strong anti-inflammatory effects in root canal treatments for primary teeth. Along with their proven antimicrobial properties and low cytotoxicity, the synergistic effects of these two substances could be beneficial in treating various oral lesions. As the adverse effects of modern pharmaceuticals continue to raise concerns, there has been a growing interest in natural alternatives. In the future, the use of safer and more affordable natural products may offer a viable alternative to conventional medicines. Additionally, further research into the biological benefits of anti-inflammatory herbs for treating diseases, including in vivo studies and clinical trials, is necessary.

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