Development of a Novel Herbal Mouthwash for Effective Inhibition of Common Oral Bacteria

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Abstract
This study explores the development and evaluation of a novel herbal mouthwash containing *neem (Azadirachta indica), shatavari (Asparagus racemosus), clove (Syzygium aromaticum), and rosemary (Rosmarinus officinalis)* extracts. These botanicals were selected for their known antimicrobial, anti-inflammatory, and antioxidant properties, offering a natural alternative to conventional mouthwashes. The herbal formulation was tested for its anti-biofilm activity against *Streptococcus mutans* and *Enterococcus faecalis*, two common oral pathogens linked to dental caries and endodontic infections. The results demonstrated significant biofilm inhibition, with higher mouthwash concentrations showing greater antimicrobial efficacy. FTIR analysis confirmed the presence of bioactive compounds in the extracts. This herbal mouthwash presents a promising natural solution for oral hygiene, potentially reducing plaque formation and improving gum health. Further research is recommended to optimize its formulation, assess long-term effects, and conduct clinical trials to validate its effectiveness compared to conventional products.
Keywords: Herbal mouthwash, biofilm inhibition, antimicrobial activity, oral pathogens, natural extracts, oral hygiene.

INTRODUCTION:

Mouthwash, also known as an oral rinse, is a widely used liquid solution designed to improve oral hygiene and maintain dental health. It is commonly employed to reduce plaque, control

gingivitis, freshen breath, and in some formulations, prevent tooth decay. There are various types of mouthwashes available, including antimicrobial, fluoride-based, whitening, and natural formulations. Antimicrobial mouthwashes typically contain agents like chlorhexidine or cetylpyridinium chloride, which are known for their ability to inhibit the growth of harmful oral bacteria. [1–3] Fluoride rinses, on the other hand, help in preventing cavities by strengthening the enamel. While mouthwashes provide significant benefits, they are meant to complement, rather than replace, regular brushing and flossing as part of a comprehensive oral care routine to ensure optimal dental health.

The oral microbiome plays a crucial role in maintaining oral and systemic health. It is a complex ecosystem consisting of bacteria, fungi, and viruses, many of which are beneficial and contribute to the overall balance within the mouth. However, certain harmful bacteria, such as *Streptococcus mutans*, can disrupt this balance, leading to dental caries, gum diseases, and halitosis. [4–7] Mechanical removal of biofilm through brushing and flossing is considered the gold standard for caries prevention. However, mouthwashes are often used as adjunctive therapies to help manage and reduce biofilm formation, particularly in hard-to-reach areas.

Chlorhexidine is one of the most commonly recommended antimicrobial mouthwashes due to its broad-spectrum efficacy against oral pathogens, particularly *Streptococcus mutans*. It is frequently used to reduce plaque and gingivitis, providing long-lasting antimicrobial effects that are beneficial in dental treatments and post-surgical care. [8,9] However, prolonged use of chlorhexidine has been associated with side effects, including taste disturbances, oral mucosal irritation, and teeth staining. [10] These drawbacks have prompted a growing interest in alternative therapies, particularly natural and herbal mouthwashes, which offer similar antimicrobial benefits without the adverse side effects associated with chemical agents.

The increasing emergence of antimicrobial resistance among oral pathogens has further driven the exploration of natural remedies. Herbal mouthwashes, particularly, have garnered attention for their potential to combat bacterial infections while offering additional anti-inflammatory and antioxidant benefits. This study focuses on the development of a novel herbal mouthwash formulated using neem (*Azadirachta indica*), shatavari (*Asparagus racemosus*), clove (*Syzygium aromaticum*), and rosemary (*Rosmarinus officinalis*). These herbs are widely recognized for their

potent antimicrobial properties and have been used traditionally to treat various infections. Neem, for example, has demonstrated significant anti-biofilm and antimicrobial activity, particularly against drug-resistant pathogens. [11] Clove, with its active component eugenol, has shown strong efficacy against a wide range of bacteria, fungi, and viruses, making it an ideal candidate for use in oral care products. [12,13] Similarly, Shatavari and rosemary have been noted for their antimicrobial effects against several pathogenic bacteria. [14,15]

Herbal mouthwashes are gaining popularity as natural alternatives to chemical-based oral care products. Preliminary studies suggest that herbal formulations can reduce plaque accumulation, gingivitis, and halitosis. However, many of these studies are limited by small sample sizes and short durations, leading to inconclusive evidence regarding their long-term efficacy. [16]Therefore, more rigorous and well-designed clinical trials are needed to validate these findings and assess the safety of herbal mouthwashes.

This study aims to formulate and evaluate the antimicrobial efficacy of a herbal mouthwash composed of neem, shatavari, clove, and rosemary extracts. By assessing its action against key oral pathogens, particularly *Streptococcus mutans* and *Enterococcus faecalis*, this research seeks to explore the potential of this herbal formulation as a natural, effective alternative for preventing biofilm formation and promoting overall oral health.

MATERIALS AND METHODOLOGY:

Collection and Preparation of Plant Samples

Fresh plant material from *Azadirachta indica* (Neem leaves), *Syzygium aromaticum* (Clove buds), *Rosmarinus officinalis* (Rosemary leaves), and *Salvia officinalis* (Sage leaves) was collected. Each sample was thoroughly washed with distilled water to remove any surface contaminants and then air-dried in the shade to eliminate moisture while preserving bioactive compounds. After sufficient drying, 20 grams of each plant material were weighed and ground using a mortar and pestle to obtain a fine powder. The grinding process was crucial to increase the surface area and improve the efficiency of the subsequent extraction process.

Extraction of Plant Compounds

To extract bioactive compounds, 100% ethanol was chosen as the solvent due to its ability to dissolve both polar and non-polar substances. Twenty grams of each powdered plant sample were extracted separately by suspending the powder in ethanol at a constant temperature of 50°C for 1 hour. This process ensured optimal extraction of phytochemicals from the plant material. Once the extraction was complete, the solutions were filtered using Whatman filter paper to remove particulate matter and cooled to room temperature. The ethanolic extracts of Neem, Clove, Rosemary, and Sage were then combined in equal proportions to prepare a composite mixture (1:1:1:1) for further testing.

FTIR analysis

Infrared spectra were acquired within the range of 4000 to 500 cm^{-1} utilizing a Fourier Transform Infrared (FTIR) spectrometer to identify the functional groups present in the sample. A total of 32 scans were conducted, each with a resolution of 4 cm^{-1}, to ensure accurate and comprehensive information regarding the composition of the sample.

Anti-Biofilm Activity Assay

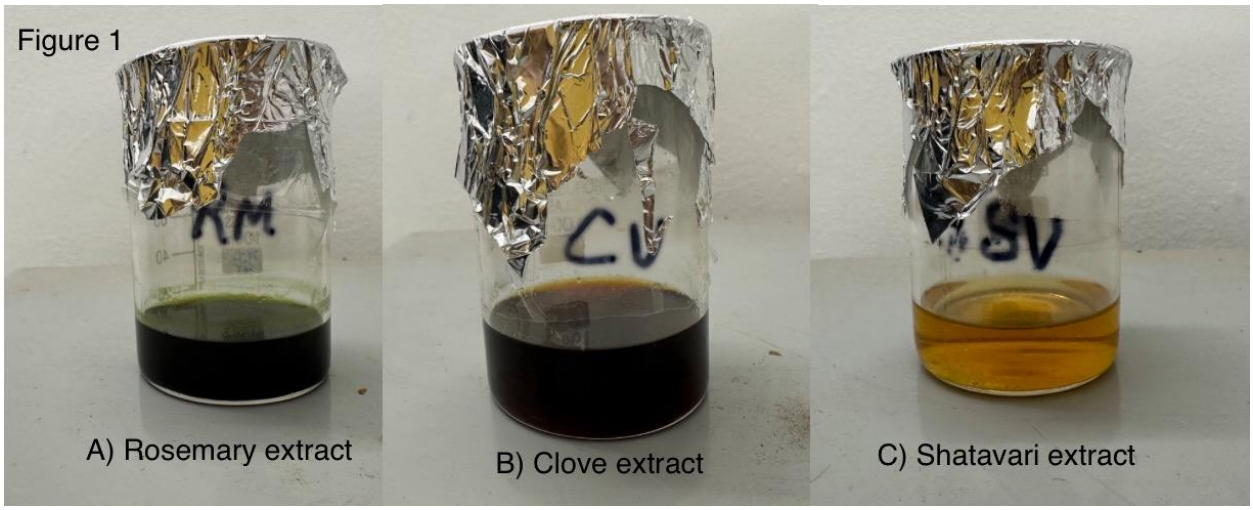
The anti-biofilm activity of the prepared extracts was tested against two bacterial species known for their biofilm-forming ability: *Streptococcus mutans* and *Enterococcus faecalis*. Both strains are implicated in dental plaque and endodontic infections. Initially, overnight cultures of *S. mutans* and *E. faecalis* were grown in suitable growth media at 37°C with continuous shaking to promote bacterial growth. The bacterial cultures were diluted to an optical density (OD600) of 0.1, ensuring consistent bacterial concentrations across all samples.

To assess biofilm formation, 200 µL of the diluted bacterial cultures were pipetted into each well of a sterile 96-well microtiter plate and incubated at 37°C for 24 hours, allowing for the formation of biofilms on the well surfaces. After biofilm formation, the non-adherent, planktonic cells were carefully removed by aspiration, ensuring that only the biofilm-bound bacteria remained in the wells. Subsequently, 200 µL of the combined plant extracts at various concentrations (ranging from 50 mg/mL to 0.009 mg/mL) were added to the wells and incubated for an additional 24 hours at 37°C.

Following incubation, the wells were gently washed three times with phosphate-buffered saline (PBS) to remove any residual planktonic cells. The adherent biofilms were stained with 0.1% crystal violet solution for 15 minutes, followed by a PBS wash to remove excess stain. The wells were allowed to air dry, and the biofilm-bound dye was then solubilized by adding 200 µL of 95% ethanol or acetone. The optical density (OD) of the solubilized dye, which correlates with the biomass of the biofilm, was measured at 570 nm using a microplate reader.

Data Analysis

The percentage inhibition of biofilm formation was calculated by comparing the absorbance values of the treated wells to those of the control wells, which contained no plant extracts. All assays were performed in triplicate to ensure reproducibility and reliability of the results. Appropriate positive and negative controls were included to validate the experimental outcomes, and statistical analysis was conducted to assess the significance of the observed biofilm inhibition.



RESULTS:

FTIR Analysis of Extracts

The FTIR analysis of five extracts—Clove (CV), Clary Sage (SG), Rosemary (RM), Neem (NM), and a composite mixture of all four (MX)—revealed the presence of significant functional groups associated with bioactive compounds (Fig. 2). A prominent broad peak around 3400 cm^{-1}, corresponding to hydroxyl (O-H) stretching, was observed in all extracts, indicating the presence of phenolic compounds and alcohols. This peak was especially prominent in Clove and Rosemary, suggesting that these extracts are particularly rich in phenolic compounds such as eugenol in Clove and rosmarinic acid in Rosemary. Both compounds are well-known for their antioxidant and antimicrobial properties. Moderate O-H stretching was also seen in Clary Sage and Neem, indicating the presence of alcohols and potential phenolic content, though at lower intensities than those found in Clove and Rosemary.

The aliphatic C-H stretching vibrations typically found between 2850 and 2950 cm^{-1}, were present in all extracts, indicating the presence of alkane chains likely derived from terpenoids, which are commonly found in essential oils. The intensity of these peaks was moderate across all extracts, suggesting a uniform distribution of aliphatic components.

C=O stretching vibrations, observed around 1700 cm^{-1}, were most pronounced in Rosemary and Neem, indicating the presence of carbonyl-containing compounds, such as esters, aldehydes, and carboxylic acids. In Rosemary, these carbonyls likely stem from carnosic acid, a potent antioxidant, while in Neem, they may originate from triterpenoids, which are recognized for their antimicrobial activity. A similar C=O peak was identified in the composite mixture (MX), suggesting contributions from these individual extracts.

In the region of 1600–1500 cm^{-1}, significant peaks corresponding to C=C aromatic stretching were observed, particularly in Clove and Rosemary, indicative of aromatic compounds typically found in phenolic acids and flavonoids. The strong peaks in Clove can be attributed to its high eugenol content, while Rosemary's rich phenolic profile contributes to its notable aromatic stretching. Neem and Clary Sage exhibited moderate peaks in this range, suggesting the presence of aromatic compounds, albeit at lower concentrations than in Clove and Rosemary. The MX sample demonstrated strong peaks in this region, reflecting the cumulative contribution of aromatic compounds from all four extracts.

C-O stretching vibrations between 1200 and 1000 cm^{-1} were pronounced in Clary Sage and Rosemary, indicative of esters, ethers, or alcohols. The presence of linalool, terpene alcohol in Clary Sage, and esters and phenolic acids in Rosemary likely contributed to these peaks. The C-O stretching vibrations were also prominent in the MX sample, confirming the retention of these functional groups in the combined extract.

The FTIR spectra of the individual extracts revealed a diverse array of bioactive compounds. Clove and Rosemary were particularly rich in phenolic compounds, while Clary Sage and Neem contributed notable amounts of terpenoids, esters, and triterpenoids. The composite extract (MX) integrated these properties, exhibiting broad peaks corresponding to hydroxyl, carbonyl, aromatic, and ether groups, indicating a potential synergistic effect for applications in antioxidant, antimicrobial, and anti-inflammatory treatments.

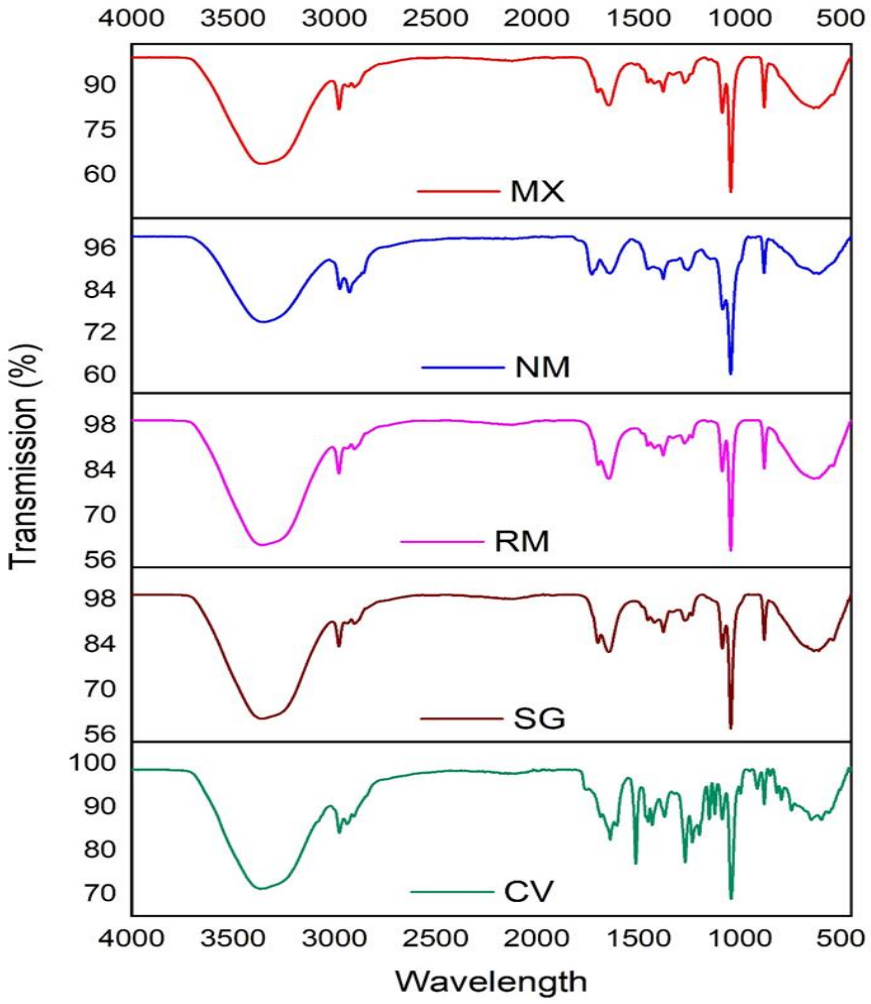


Figure 2 shows the FTIR spectra of various herbal extracts, with each spectrum representing a different extract. MX Stands for mixture, NM stands for neem extract, RM stands for rosemary extract, SG stands for Shatavari extract, CV stands for Clove

Anti-biofilm Activity

The anti-biofilm activity of the extracts was assessed against *Streptococcus mutans* and *Enterococcus faecalis* over a concentration range of 50 mg/mL to 0.009 mg/mL, with a positive control used for comparison (Table 1). In the case of *S. mutans*, the highest inhibition

percentages were observed at 50 mg/mL, with values of 88.50% and 85.89%. The inhibitory effect remained high at 25 mg/mL, with inhibition rates of 88.44% and 88.50%. As the concentration decreased, the inhibition gradually declined, with rates of 84.70% and 83.60% at 12.5 mg/mL, and dropping to 43.34% and 43.28% at the lowest concentration of 0.009 mg/mL. The positive control exhibited inhibition rates of 93.50% and 99.50%, indicating strong antimicrobial efficacy at all tested concentrations.

For *E. faecalis*, the inhibition rates were lower overall compared to *S. mutans*, starting at 77.50% and 77.24% at 50 mg/mL and decreasing to 74.46% and 73.07% at 25 mg/mL. The inhibitory effect continued to decrease with lower concentrations, reaching values of 24.87% and 25.51% at 0.009 mg/mL. The positive control for *E. faecalis* demonstrated inhibition rates of 81.00% and 81.57%.

Overall, the results indicated a concentration-dependent reduction in anti-biofilm activity for both bacterial strains, with higher concentrations of the extracts leading to greater inhibition. The extracts showed strong potential for use in antimicrobial applications, particularly at higher concentrations, where they exhibited significant inhibition of *S. mutans* and *E. faecalis* biofilms.

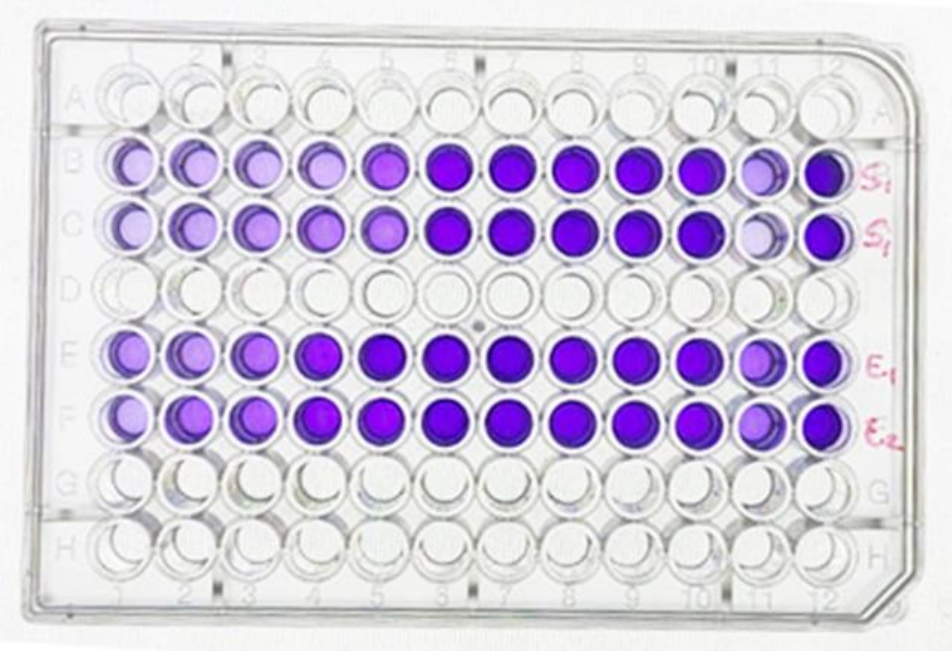


Fig. 3. Anti-biofilm activity against two bacterial pathogens.

Table 1 Anti-biofilm activity of herbal extract mouthwash against S. mutants and E. faecalis

	Concentration of sample in mg/ml										
Pathogens	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.56**	**0.78**	**0.39**	**0.19**	**0.009**	**PC**
S. mutants	**88.50%**	**88.44%**	**84.70%**	**81.16%**	**69.77%**	**56.91%**	**55.67%**	**52.65%**	**46.09%**	**43.34%**	**93.50%**
S. mutants	**85.89%**	**88.50%**	**83.60%**	**82.06%**	**70.66%**	**58.67%**	**55.18%**	**53.73%**	**47.16%**	**43.28%**	**99.50%**
E. faecalis	**77.50%**	**74.46%**	**70.23%**	**54.49%**	**49.51%**	**30.67%**	**30.28%**	**29.17%**	**25.24%**	**24.87%**	**81.00%**

E. faecalis	**77.24%**	**73.07%**	**71.49%**	**52.39%**	**49.05%**	**31.30%**	**31.92%**	**29.77%**	**25.09%**	**25.51%**	**81.57%**

DISCUSSION

The provided figures illustrate the anti-biofilm activity of a herbal extract mouthwash against *Streptococcus mutans* and *Enterococcus faecalis*, Biofilms, formed by bacteria like *Streptococcus mutans*, are a key precondition for dental caries. These microbial communities adhere to the tooth surface, producing acids that demineralize enamel and lead to tooth decay. As biofilms grow, they create a protective matrix that shields bacteria from saliva and oral hygiene measures, allowing acids to continuously damage teeth. Preventing biofilm formation is crucial in reducing the risk of caries development. [17]. There is substantial evidence supporting the horizontal transmission of *Streptococcus mutans* and its link to dental caries. [18] This research highlights how *S. mutans* can spread between individuals, contributing to the development of cavities. Understanding this transmission pathway is crucial for developing effective preventive strategies against dental caries, emphasizing the importance of oral hygiene and microbial management in both clinical and community settings to reduce the incidence of tooth decay.*Enterococcus faecalis* is a resilient oral microorganism that competes with other bacteria, invades dentinal tubules, and can withstand nutritional deprivation, making it a significant challenge in endodontic infections. Currently, chlorhexidine is considered the most effective antimicrobial agent against *E. faecalis*, providing robust antibacterial action to help control its growth and prevent reinfection. However, concerns regarding chlorhexidine's side effects have prompted interest in alternative treatments for managing this resilient pathogen in dental care. [19] Furthermore *Enterococcus faecalis* exhibits esterase-like degradative activity on dental methacrylate resin restoration materials, which may hasten the degradation of the dentin-methacrylate resin interface. This degradation can lead to increased proliferation of bacterial biofilms and enhance their penetration into the root canal system. As a result, the presence of *E. faecalis* in endodontic infections poses a significant risk, potentially compromising the integrity of dental restorations and contributing to treatment failures. [20] *Enterococcus faecalis*, an

opportunistic pathogen, is linked to secondary apical diseases and demonstrates a strong ability to resist antibiotics and various treatments due to its multiple virulence factors. This bacterium can disrupt the normal functions of immune cells, impairing the body's ability to eliminate the infection effectively. Its resilience and ability to evade immune responses complicate treatment efforts, making *E. faecalis* a significant challenge in managing endodontic infections and related dental issues. [21]

The synergistic action of these herbs in the mouthwash formulation not only targets harmful bacteria but also promotes overall oral health by reducing inflammation and oxidative stress. Herbal mouthwashes, formulated from natural ingredients such as neem, clove, and rosemary, offer promising benefits in controlling plaque and inflammation and better patient compliance. [22] These plants possess potent antimicrobial and anti-inflammatory properties, making them effective against common oral bacteria like *Streptococcus mutans*, which causes plaque buildup and gum disease. Studies have shown that herbal mouthwashes can reduce bacterial growth, decrease gingival inflammation, and help prevent periodontal disease. While not a complete replacement for conventional treatments, they can serve as valuable supplements in oral hygiene routines, offering a natural alternative with fewer side effects for maintaining healthy gums and teeth. [23] The development of this herbal-based mouthwash opens avenues for further research and potential applications in oral healthcare. Herbal mouthwashes, containing ingredients like neem, clove, and rosemary, offer an effective natural alternative to the gold standard chlorhexidine mouthwash. [24]While chlorhexidine is renowned for its strong antimicrobial action, herbal 8formulations also exhibit potent antibacterial, anti-inflammatory, and plaque-reducing properties. [25] Herbal mouthwashes, with fewer side effects like staining or taste alteration, can be analogously used in maintaining oral hygiene and managing gum inflammation, making them a valuable supplement. Neem gels are effective in controlling plaque index due to their potent antibacterial properties. Neem's active compounds, such as azadirachtin and nimbin, help inhibit the growth of plaque-forming bacteria like *Streptococcus mutans*. Studies suggest that using neem-based gels can significantly reduce plaque formation and improve overall oral hygiene. With anti-inflammatory effects, neem gels offer a natural and effective solution for maintaining a low plaque index and promoting gum health. [26]. Clove toothpaste has demonstrated significant improvement in oral hygiene and gingival health due to the antimicrobial and anti-inflammatory

properties of eugenol, a key compound in clove. Regular use of clove-based toothpaste helps reduce bacterial growth, plaque formation, and gum inflammation. [27] The randomized control trial showed that individuals using clove toothpaste experience reduced gingival bleeding and swelling, leading to healthier gums and better overall oral health, making it a beneficial addition to daily oral care routines.In a study comparing rosemary toothpaste to conventional toothpaste, rosemary toothpaste effectively treated gingival bleeding. The study used the Plaque Index (PI) and Gingival Bleeding Index (GBI) to assess outcomes, showing significant improvement in reducing both plaque and gingival bleeding in the rosemary group. The antimicrobial and anti-inflammatory properties of rosemary played a key role in promoting gum health, making it a natural and effective alternative for improving gingival conditions compared to conventional toothpaste. [28]. The current study concludes that shatavari liposomes show promising potential as a formulation for topical and transdermal drug delivery, offering notable anti-inflammatory activity. This innovative delivery system enhances the bioavailability and effectiveness of shatavari's active compounds, making it an attractive option for treating various inflammatory conditions. The findings support further research into the development and optimization of shatavari liposome formulations to improve therapeutic outcomes in dermatological applications and promote better patient adherence to treatment. [29]

Despite its potential, the herbal-based mouthwash has some limitations. One significant challenge is ensuring the stability and shelf-life of the natural ingredients, which may degrade over time without preservatives. Additionally, the mouthwash's effectiveness may vary depending on the individual's oral microbiome. The taste and aroma of herbal extracts may not be universally appealing, potentially affecting user compliance. Finally, while initial studies show promise, extensive clinical trials are necessary to conclusively prove the mouthwash's efficacy compared to conventional products, and regulatory approval processes might be lengthy. Future studies could explore optimizing the formulation by varying the concentrations of neem, clove, rosemary, and Shatavari to enhance its efficacy against a broader spectrum of oral pathogens. Additionally, clinical trials on diverse populations could be conducted to validate the safety and effectiveness of this mouthwash in real-world settings. The formulation's impact on long-term oral health, including its effects on gum health, cavity prevention, and oral microbiome balance, could also be investigated. Furthermore, expanding this herbal approach to

include other beneficial herbs or natural compounds could lead to the development of a range of natural oral care products, such as toothpaste and mouth sprays.:

Conclusion:

The combination of these extracts in the mouthwash formulation likely contributes to the observed anti-biofilm activity, as each extract has its own set of bioactive compounds that work synergistically. The FTIR spectra provide a fingerprint of these compounds, illustrating the complex chemical nature of the extracts used. The results suggest that the herbal mouthwash could be a potential alternative to traditional chemical-based mouthwashes in preventing biofilm formation by oral pathogens.

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