



ANTIBACTERIAL EFFECT OF PROPOLIS MODIFIED AND NON PROPOLIS MODIFIED COLLAGEN MEMBRANE AGAINST P.GINGIVALIS AND S. MUTANS – AN IN VITRO STUDY

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ABSTRACT

Background: Collagen Membranes used in Guided tissue regeneration, are prone to microbial colonization, which can reduce their therapeutic efficiency. Propolis, a naturally occurring antibacterial agent, may be added to enhance the clinical results and strengthen antimicrobial qualities.

Aim: To evaluate and compare the antibacterial efficacy of propolis modified and non propolis modified collagen membrane against Streptococcus mutans and Porphyromonas gingivalis

Materials & Methods: An in vitro comparative study was conducted using sterile collagen membrane discs. An ethanolic extract of propolis (EEP) was added to test membranes. The agar diffusion assay (zone of inhibition) and colony forming unit (CFU) measurement were used to evaluate the antibacterial efficacy. Propolis extract alone serves as the reference control

Results: Propolis extract alone demonstrated the highest antibacterial activity. Unmodified collagen membranes exhibited negligible inhibition. Propolis-modified membranes showed measurable antibacterial effects against both microorganisms. Zones of inhibition were more pronounced against P. gingivalis. CFU analysis revealed substantial bacterial reduction with propolis treatment, with the modified membranes demonstrating intermediate efficacy compared to propolis extract alone. These differences were statistically significant (P <0.05)

Conclusion: propolis incorporation strengthen the antibacterial capability of collagen membranes, and may increase their performance during periodontal regeneration

Key words: Guided tissue regeneration, Biomaterial modification, Oral biofilm, Ethanolic extract of propolis, Colony-forming units (CFU)

INTRODUCTION

Periodontitis is a chronic multifactorial inflammatory disease characterized by progressive destruction of the periodontal ligament and alveolar bone, ultimately leading to tooth loss if untreated (1) .Successful periodontal therapy necessitates the eradication of harmful microorganism and the prevention of microbial recolonization throughout the healing phase, especially in regenerative procedures. Guided tissue regeneration (GTR) is extensively employed to rehabilitate missing periodontal tissues. Collagen membranes are widely utilized biomaterials in regenerative medicine because to their biocompatibility, resorbability, and capacity to replicate the extracellular matrix, thus promoting tissue integration and cellular migration (1) Despite these advantages, collagen membranes lack intrinsic antibacterial capabilities and are susceptible to bacterial colonization and breakdown. Infections linked to biomaterials continue to be a major obstacle to regenerative periodontal therapy. An intricate microbial environment that can quickly colonize implanted biomaterials is found in the oral cavity. Microorganisms may develop structured biofilms that offer defense against antimicrobial agents and host immunological responses once they adhere to biomaterial surfaces. Therefore, bacterial contamination of regenerative membranes can lower the predictability of periodontal regeneration and impair healing results (2,3,4,5) Microbial contamination continues to pose a significant barrier in regenerative periodontal surgery. Bacterial adherence to membrane surfaces can result in biofilm development, irritation, and compromised regeneration outcomes. The subgingival microbiota is pivotal in the advancement of periodontal disease, with certain pathogens facilitating immune dysregulation and tissue degradation (2). *Porphyromonas gingivalis* is regarded as a keystone bacteria significantly linked to periodontal disease(3). The virulence factors generated by this gram-negative anaerobe can modify host immune responses and facilitate tissue damage. Alongside periodontal infections, early colonizers like *Streptococcus mutans* significantly contribute to the production of dental biofilms and the persistence of microbes (4) . The formation of biofilm on biomaterial surfaces can jeopardize membrane integrity and diminish the efficacy of restorative interventions. Prior research has indicated that collagen-based membranes are prone to microbial colonization, underscoring the necessity for biomaterial modification approaches. (5) The incorporation of antimicrobial compounds into regenerative biomaterials has gained significant attention in recent years. Despite the efficacy of synthetic antimicrobial medicines, apprehensions about cytotoxicity and antibiotic resistance have prompted the investigation of natural bioactive alternatives. Propolis is a natural resin produced by bees, recognized for its antibacterial, anti-inflammatory, antioxidant, and immunomodulatory characteristics. The antibacterial efficacy of propolis is primarily attributed to its abundant constituents of flavonoids, phenolic acids, aromatic esters, and terpenoids. These chemicals have demonstrated the ability to disrupt bacterial cell membranes, modify membrane permeability, and inhibit essential enzymatic pathways involved in bacterial metabolism. Moreover, propolis has been reported to inhibit bacterial adhesion and biofilm formation, which are critical steps in the pathogenesis of oral infections (6, 7, 8, 9) Several studies have demonstrated that propolis is effective against bacteria that cause infections in the oral cavity . Propolis extracts have shown significant inhibitory effects against *Streptococcus mutans*, a key contributor to the formation of dental biofilms (5). Propolis has also been reported to alter the membrane structure of *Porphyromonas gingivalis*, there by suppressing bacterial growth and virulence(7) Because of these characteristics, propolis has gained significant attention as a natural antibacterial agent for integration into dental biomaterials. Functionalizing collagen membranes with propolis may offer dual advantages by maintaining the regenerative characteristics of collagen while concurrently improving resistance to microbial colonization. Changes to biomaterials like these could make periodontal regeneration therapy more predictable and successful in the long run.(8,9) Although the antimicrobial characteristics of propolis are extensively documented,limited information is available regarding its interaction with collagen membranes and its ability to enhance antibacterial activity when integrated into such biomaterials. Therefore, the aim of the present study was to evaluate the antibacterial efficacy of propolis-modified collagen membranes compared with unmodified collagen membranes against *Porphyromonas gingivalis* and *Streptococcus mutans*, using zone of inhibition and colony-forming unit (CFU) assays.

MATERIALS AND METHODS

Study design - An in vitro comparative experimental study

Materials Used

- Sterile collagen membrane
- Ethanolic extract of propolis (EEP)

- Streptococcus mutans
- Porphyromonas gingivalis
- Brain Heart Infusion (BHI) broth
- Mueller–Hinton agar
- Anaerobic incubation system

Preparation of membrane discs - Collagen membranes were aseptically sectioned into uniform discs under sterile conditions.

STUDY GROUPS

Group 1: Collagen membrane without propolis against

1A: *S. mutans*

1B: *P. gingivalis*

Group 2: Collagen membrane with propolis against

2A: *S. mutans*

2B: *P. gingivalis*

Group 3: Propolis extract alone against

3A: *S. mutans*

3B: *P. gingivalis*

For preparation of propolis-modified membranes, collagen membrane discs were immersed in ethanolic extract of propolis to allow adsorption of bioactive compounds onto the membrane surface. The membranes were subsequently dried under sterile conditions to ensure uniform coating before antibacterial testing

Bacterial Culture : Under suitable growth conditions, the bacterial strains *Porphyromonas gingivalis* and *Streptococcus mutans* were cultured in Brain Heart Infusion (BHI) broth. *Porphyromonas gingivalis* was incubated anaerobically at 37°C using an anaerobic incubation equipment, whereas *Streptococcus mutans* was incubated aerobically at 37°C. To ensure uniform microbial density across all experimental processes, the bacterial suspensions were standardized to a 0.5 McFarland turbidity standard.

Agar Diffusion Assay : Standardized bacterial suspensions were inoculated onto agar plates. Test samples were placed on the agar surface. Zones of inhibition were measured in millimeters.

CFU Analysis : Bacterial suspensions were exposed to test materials. Serial dilutions were plated, and CFUs were enumerated. CFU analysis provides a quantitative assessment of antimicrobial efficacy by determining the number of viable bacterial colonies after exposure to the test materials.

OUTCOME MEASURES : The key outcome measures were the zone of inhibition and the percentage reduction in bacterial count

STATISTICAL ANALYSIS : The collected data were statistically analyzed using SPSS version 26.0 software. Descriptive statistics (mean ± standard deviation) were used to summarize the data. One-way ANOVA with post-hoc analysis was used for intragroup comparisons, while ANOVA was used for intergroup comparisons. Statistical significance was set at $p < 0.05$.

RESULT

The antibacterial efficacy of propolis extract, unmodified collagen membrane, and propolis-modified collagen membrane was evaluated against *Porphyromonas gingivalis* and *Streptococcus mutans* using zone of inhibition and CFU analysis, with both intragroup and intergroup statistical comparisons conducted to ascertain significance ($p < 0.05$). Overall, the three experimental groups showed dramatically different levels of antibacterial activity. Unmodified collagen membranes showed little inhibitory action, while propolis extract alone showed the strongest antibacterial efficiency. Moderate antibacterial activities were demonstrated by propolis-modified collagen membranes, suggesting that propolis was successfully incorporated into the collagen matrix.

Propolis Extract Alone had the most significant antibacterial efficacy against both *Porphyromonas gingivalis* and *Streptococcus mutans*. This was demonstrated by the most extensive zones of inhibition noted in the agar diffusion testing (Tables 1 and 2; Figures 2 and 4) and the most significant decrease in bacterial counts in the CFU analysis (Figure 5). Intragroup comparison (within propolis extract group : 25 μ L vs 50 μ L vs 100 μ L) revealed a significant concentration-dependent enhancement in antibacterial activity (Tables 3 and 4; $p < 0.05$). Intergroup analysis (propolis extract vs unmodified collagen membrane & propolis modified collagen membrane) further validated that propolis extract demonstrated much superior antibacterial effectiveness relative to the other groups across all concentrations (Tables 5–7; $p < 0.05$).

Unmodified Collagen Membranes exhibited little or no antibacterial action against either bacterium. There were no zones of inhibition seen in most concentrations (Tables 1 and 2; Figures 2 and 4), and CFU analysis (Figure 5) showed that the number of bacteria did not change much. Comparing concentrations within the same group (25 μ L vs 50 μ L vs 100 μ L) showed no statistically significant differences (Tables 3 and 4; $p > 0.05$). Intergroup analysis (collagen membrane vs propolis extract & propolis modified collagen membrane) revealed markedly decreased antibacterial activity in comparison to propolis-containing groups (Tables 5–7; $p < 0.05$).

Collagen Membrane With Propolis exhibited moderate antibacterial efficacy against both *P. gingivalis* and *S. mutans*. Measurable zones of inhibition were detected, increasing with concentration. (Tables 1 and 2; Figures 2 and 4). The CFU analysis (Figure 5) showed a big drop in the number of bacteria. Intragroup analysis (25 μ L vs 50 μ L vs 100 μ L) demonstrated a statistically significant concentration-dependent enhancement in antibacterial activity (Tables 3 and 4; $p < 0.05$). Intergroup comparisons (propolis modified membrane vs collagen membrane & propolis extract) demonstrated that propolis-modified membranes exhibited significantly enhanced antibacterial efficacy relative to unmodified collagen membranes, although displayed diminished activity in contrast to propolis extract alone (Tables 5–7; $p < 0.05$). These findings suggest that incorporation of propolis into collagen membranes may enhance resistance to microbial colonization without compromising the biomaterial's structural integrity.

DISCUSSION

Collagen membranes are widely employed in guided tissue regeneration because of their biocompatibility and ability to promote cellular proliferation and tissue integration. However, a major limitation of these membranes is their lack of intrinsic antibacterial capability, which makes them very vulnerable to microbial colonization and biofilm formation in the oral environment. This can compromise membrane stability and negatively affect regeneration outcomes. Previous research has highlighted that, while collagen membranes serve as suitable scaffolds, their clinical effectiveness is restricted by their inability to withstand bacterial contamination (10,11,12). To overcome this limitation the current study evaluated the incorporation of propolis, a naturally occurring bioactive compound with proven antibacterial qualities. Flavonoids and phenolic chemicals, which are abundant in propolis, have antibacterial properties by rupturing bacterial cell membranes, changing the permeability of membranes, and blocking vital metabolic processes. Przybyżek and Karpiński, as well as Silva-Carvalho et al. have thoroughly documented these processes and propolis's broad-spectrum antibacterial action (13). The findings of the current study demonstrated that unmodified collagen membranes displayed minimal antibacterial activity against both *Porphyromonas gingivalis* and *Streptococcus mutans*, with no significant zones of inhibition (Tables 1 and 2; Figures 2 and 4). Intragroup analysis indicated no significant changes across concentrations ($p > 0.05$), hence confirming the lack of intrinsic antibacterial action. The findings of this study were in accordance with Bottino et al. and Sheikh et al., which demonstrated that collagen membranes exhibit no antibacterial characteristics and are susceptible to bacterial colonization in the oral environment (10,11). On the other hand, propolis extract alone demonstrated the greatest reduction in CFU counts and the largest zones of inhibition, indicating the highest antibacterial effectiveness (Figure 5). Intergroup analysis demonstrated considerably higher efficacy compared to other groups ($p < 0.001$), whereas intragroup comparison showed a statistically significant concentration-dependent increase in antibacterial activity ($p < 0.05$) (Tables 3–7). These findings are supported by Cho et al. and Preethi and Dharan, who discovered that propolis greatly suppressed *Streptococcus mutans*. In addition, Lisbona-González et al. demonstrated that ethanolic preparations of propolis are effective against periodontal diseases. (5,6)

Propolis-modified collagen membranes demonstrated a significant enhancement in antibacterial efficacy relative to collagen membranes alone, indicating effective functionalization. Intragroup analysis revealed a substantial concentration-dependent enhancement in antibacterial activity ($p < 0.05$), although intergroup comparisons demonstrated greater efficacy compared to unmodified membranes (Tables 3–7; Figure 5). These findings align with research by Chen et al., which indicated that the integration of antimicrobial agents into collagen membranes enhances their antibacterial characteristics, consequently enhancing their functional effectiveness in regenerative applications (12). However, propolis-modified membranes exhibited weaker antibacterial activity than propolis extract alone. This could be due to the controlled release and limited diffusion of bioactive chemicals from the collagen matrix. Cho et al. and Nakao and Senpuku found similar findings, implying that inclusion into biomaterials may affect active component bioavailability and diffusion. (5,7) The most remarkable finding of this study was that it had a higher antibacterial impact on *P. gingivalis* than on *S. mutans*. This can be attributed to the structural properties of gram-negative bacteria, which are more prone to membrane disruption. Nakao and Senpuku found that propolis affects the membrane integrity of *P. gingivalis*, reducing its growth (7). Koo et al. suggest that *S. mutans*' capacity to build biofilms may account for its low inhibition. (4) From a therapeutic point of view, adding propolis to collagen membranes has two benefits: it helps the body heal and protects against bacteria. This method might assist lower the number of bacteria that grow during healing and make the results of periodontal regeneration better. Chen et al. and Ivanovski et al. have both stressed how important it is to use multifunctional biomaterials in regeneration therapy that give both structural support and protection against bacteria. (12, 20) However, the study had certain limitations. The in vitro experimental design does not fully replicate the complex oral environment, which includes host immune responses, saliva, and dynamic biofilm interactions. Furthermore, the long-term release kinetics, biocompatibility, and clinical efficacy of propolis-modified membranes were not assessed. As a result, more in vivo and clinical studies are required to validate these findings and identify their therapeutic implications..

CONCLUSION

Within the limitations of this in vitro study, unaltered collagen membranes exhibited negligible antibacterial activity, while propolis extract alone showed the highest antibacterial efficiency. Propolis-modified membranes shown enhanced antibacterial efficacy, especially against *Porphyromonas gingivalis*. Incorporating propolis into collagen membranes may be an effective strategy to enhance biomaterials for periodontal regeneration therapy. However, further in vivo and clinical studies are required to confirm their long term safety and effectiveness

CONFLICT OF INTEREST - The author declares that there is no conflict of interest regarding the publication of this study

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ETHICAL APPROVAL - This study was conducted entirely in vitro and did not involve human participants, animal subjects, or human/animal-derived cells; therefore, ethical approval was not required.

DATA AVAILABILITY - The authors confirm that the raw data that support the findings are available from the corresponding author upon request

AUTHOR CONTRIBUTION (Credit Statement)

Sreeja Gopika [Author 1] : Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – original draft.

John Mathew [Author 2]: Conceptualization, Supervision, Project administration, Writing – review & editing.

ChandraMohan Sabari [Author 3]: Investigation, Data curation.

Sudhakaran Aswathy [Author 4]: Investigation, Validation.

Soman Divya [Author 5]: Data curation, Formal analysis, Visualization.

Nair Vinita [Author 6]: Methodology, Resources, Writing – review & editing.

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FIGURES AND TABLES

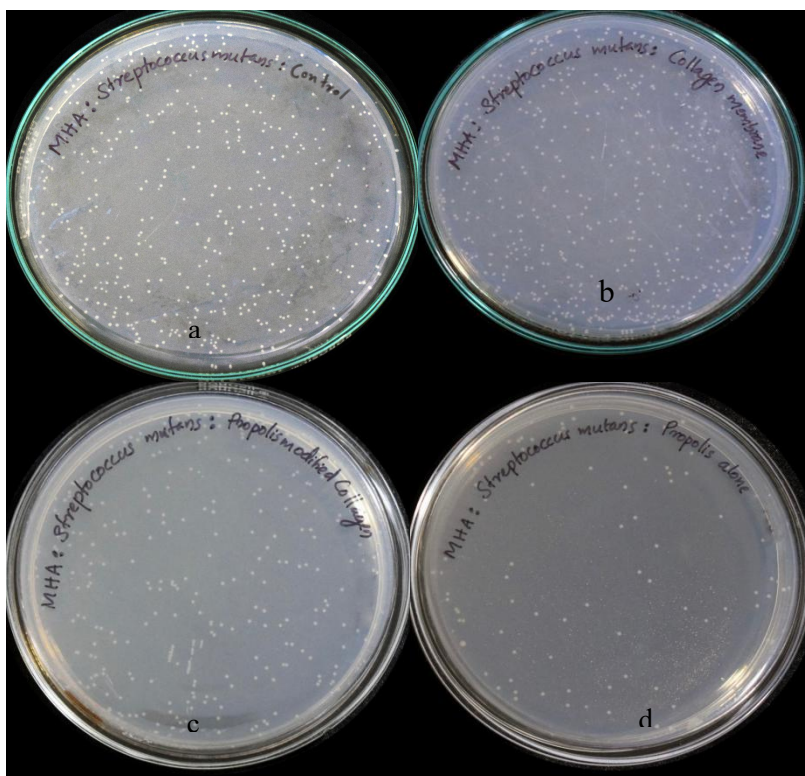


Figure 1 – Experimental group used for antibacterial evaluation against S.mutans .

- 1a Control group
- 1b Collagen Membrane Alone
- 1c Propolis Modified Collagen Membrane
- 1d Propolis Alone

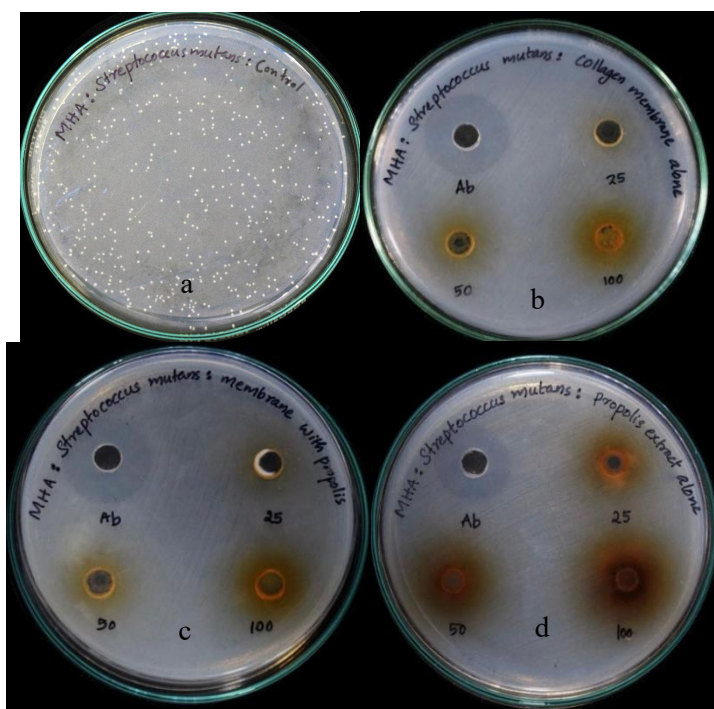


Figure 2 - Antibacterial activity of test materials assessed by Agar Well Diffusion Assay against S.mutans

2a Control Group

2b Collagen Membrane Alone

2c propolis Modified Collagen Membrane

2d propolis Alone

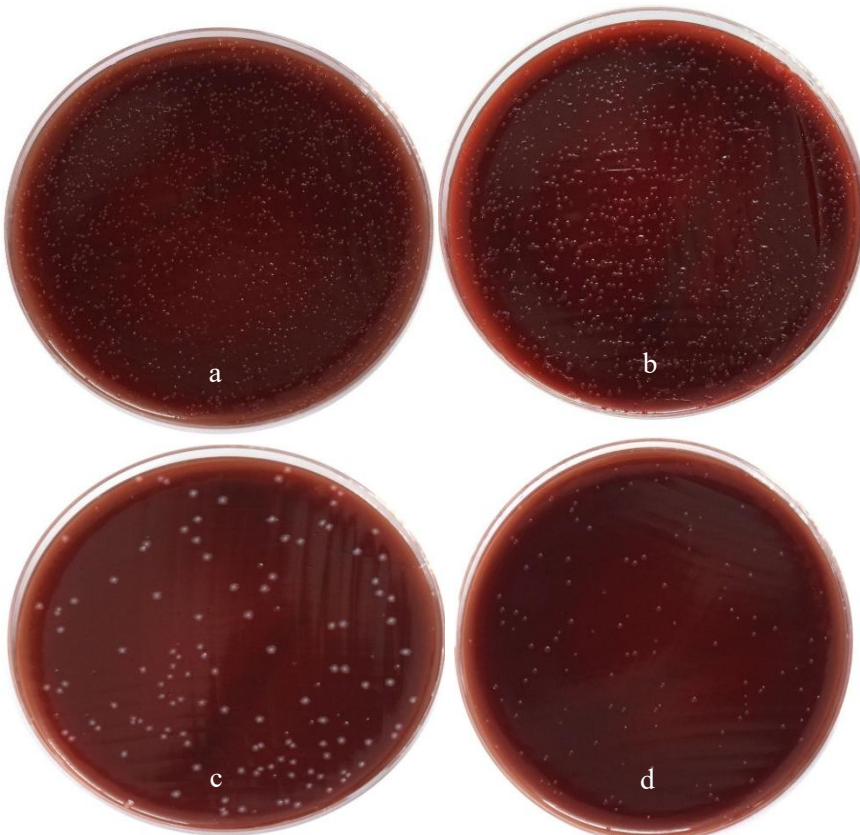


Figure 3- Experimental Group used for antibacterial evaluation against *P.gingivalis*

3a Control Group

3b Collagen Membrane Alone

3c Propolis Modified Collagen Membrane

3d Propolis Alone





Figure 4 - Antibacterial activity of test materials assessed by Agar Well Diffusion

Assay against *P.gingivalis*

- 4a Control Group
- 4b Collagen Membrane Alone
- 4c Propolis Modified Collagen Membrane
- 4d Propolis Alone

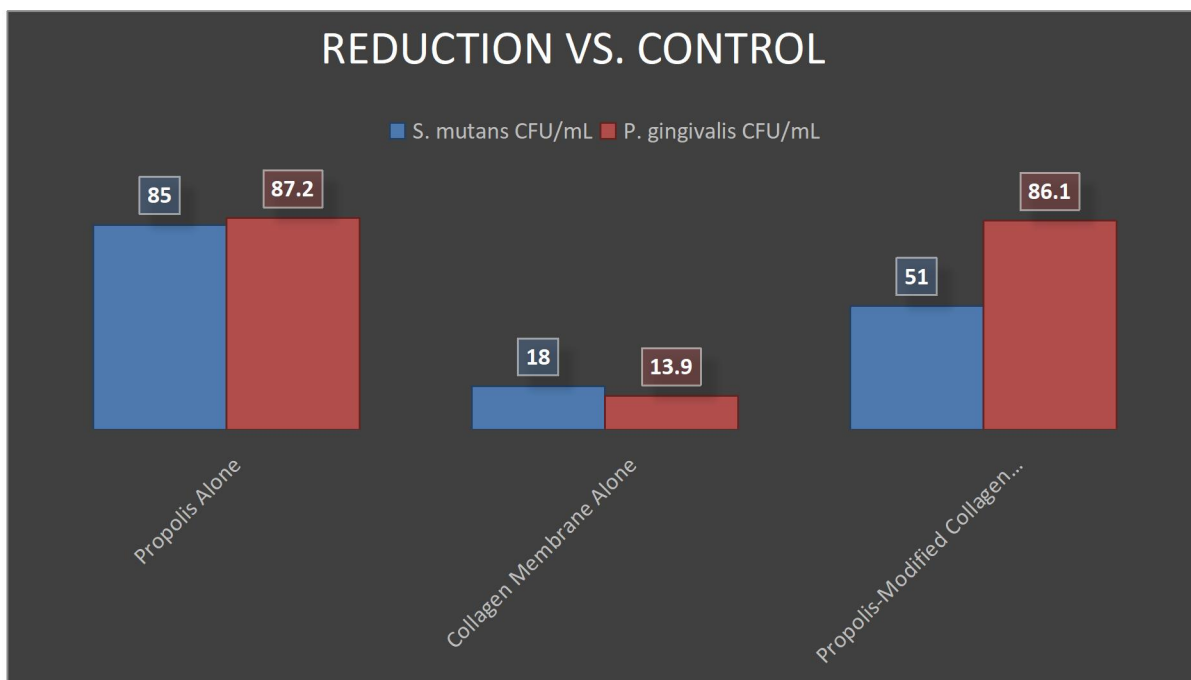


Figure 5 -Colony-forming unit (CFU) analysis showing antibacterial activity of test materials against *Streptococcus mutans* and *Porphyromonas gingivalis*.

TABLE 1: Descriptive Statistics - Zone of Inhibition (mm) Against *P. gingivalis*

Sample Type	Concentration	N	Mean \pm SD	Median	Min-Max	95% CI
Collagen Membrane Alone	25 μ L	3	0.0 \pm 0.0	0.0	0.0-0.0	0.0-0.0
	50 μ L	3	0.0 \pm 0.0	0.0	0.0-0.0	0.0-0.0
	100 μ L	3	11.0 \pm 0.5	11.0	10.5-11.5	10.2-11.8
Membrane with Propolis	25 μ L	3	0.0 \pm 0.0	0.0	0.0-0.0	0.0-0.0
	50 μ L	3	11.0 \pm 1.0	11.0	10.0-12.0	9.5-12.5
	100 μ L	3	12.5 \pm 0.5	12.5	12.0-13.0	12.0-13.0
Propolis Alone	25 μ L	3	15.0 \pm 1.0	15.0	14.0-16.0	13.5-16.5
	50 μ L	3	16.0 \pm 1.0	16.0	15.0-17.0	14.5-17.5
	100 μ L	3	18.5 \pm 0.5	18.5	18.0-19.0	18.0-19.0

TABLE 2: Descriptive Statistics - Zone of Inhibition (mm) Against *S. mutans*

Sample Type	Concentration	N	Mean \pm SD	Median	Min-Max	95% CI
Collagen Membrane Alone	25 μ L	3	0.0 \pm 0.0	0.0	0.0-0.0	0.0-0.0
	50 μ L	3	0.0 \pm 0.0	0.0	0.0-0.0	0.0-0.0
	100 μ L	3	0.0 \pm 0.0	0.0	0.0-0.0	0.0-0.0
Membrane with Propolis	25 μ L	3	7.5 \pm 0.5	7.5	7.0-8.0	6.8-8.2
	50 μ L	3	9.5 \pm 0.5	9.5	9.0-10.0	8.8-10.2
	100 μ L	3	12.0 \pm 0.5	12.0	11.5-12.5	11.2-12.8
Propolis Alone	25 μ L	3	8.0 \pm 0.5	8.0	7.5-8.5	7.2-8.8
	50 μ L	3	10.5 \pm 0.5	10.5	10.0-11.0	9.8-11.2
	100 μ L	3	13.5 \pm 0.5	13.5	13.0-14.0	12.8-14.2

**TABLE 3: INTRAGROUP COMPARISON - *P. gingivalis*
Concentration-Dependent Effects Within Each Sample Type**

Sample Type	Comparison	Mean Difference	95% CI	p-value
Collagen Membrane Alone	25 μ L vs 50 μ L	0.0	0.0-0.0	0.355
	25 μ L vs 100 μ L	11.0	9.8-12.2	<0.001
	50 μ L vs 100 μ L	11.0	9.8-12.2	<0.001
Membrane with Propolis	25 μ L vs 50 μ L	11.0	9.5-12.5	<0.001
	25 μ L vs 100 μ L	12.5	11.0-14.0	<0.001
	50 μ L vs 100 μ L	1.5	0.8-2.2	0.012
Propolis Alone	25 μ L vs 50 μ L	1.0	0.3-1.7	0.021
	25 μ L vs 100 μ L	3.5	2.8-4.2	<0.001
	50 μ L vs 100 μ L	2.5	1.8-3.2	<0.001

**TABLE 4: INTRAGROUP COMPARISON - *S. mutans*
Concentration-Dependent Effects Within Each Sample Type**

Sample Type	Comparison	Mean Difference	95% CI	p-value
Collagen Membrane Alone	25 μ L vs 50 μ L	0.0	0.0-0.0	1.000
	25 μ L vs 100 μ L	0.0	0.0-0.0	1.000
	50 μ L vs 100 μ L	0.0	0.0-0.0	1.000
Membrane with Propolis	25 μ L vs 50 μ L	2.0	1.3-2.7	0.002
	25 μ L vs 100 μ L	4.5	3.8-5.2	<0.001
	50 μ L vs 100 μ L	2.5	1.8-3.2	<0.001
Propolis Alone	25 μ L vs 50 μ L	2.5	1.8-3.2	<0.001
	25 μ L vs 100 μ L	5.5	4.8-6.2	<0.001
	50 μ L vs 100 μ L	3.0	2.3-3.7	<0.001

TABLE 5: INTERGROUP COMPARISON AT 25 μ L CONCENTRATION

Comparison Between Different Sample Types at Same Concentration

Bacteria	Comparison	Mean Difference	95% CI	p-value
<i>P. gingivalis</i>	Collagen Alone vs Membrane+Propolis	0.0	0.0-0.0	1.000
	Collagen Alone vs Propolis Alone	15.0	13.5-16.5	<0.001
	Membrane+Propolis vs Propolis Alone	15.0	13.5-16.5	<0.001
<i>S. mutans</i>	Collagen Alone vs Membrane+Propolis	7.5	6.8-8.2	<0.001
	Collagen Alone vs Propolis Alone	8.0	7.2-8.8	<0.001
	Membrane+Propolis vs Propolis Alone	0.5	-0.2-1.2	0.142 (ns)

TABLE 6: INTERGROUP COMPARISON AT 50 μ L CONCENTRATION

Comparison Between Different Sample Types at Same Concentration

Bacteria	Comparison	Mean Difference	95% CI	p-value
<i>P. gingivalis</i>	Collagen Alone vs Membrane+Propolis	11.0	9.5-12.5	<0.001
	Collagen Alone vs Propolis Alone	16.0	14.5-17.5	<0.001
	Membrane+Propolis vs Propolis Alone	5.0	4.2-5.8	<0.001
<i>S. mutans</i>	Collagen Alone vs Membrane+Propolis	0.0	0.0-0.0	1.000
	Collagen Alone vs Propolis Alone	0.0	0.0-0.0	1.000
	Membrane+Propolis vs Propolis Alone	0.0	0.0-0.0	1.000

TABLE 7: INTERGROUP COMPARISON AT 100 μ L CONCENTRATION

Comparison Between Different Sample Types at Same Concentration

Bacteria	Comparison	Mean Difference	95% CI	p-value
<i>P. gingivalis</i>	Collagen Alone vs Membrane+Propolis	1.5	0.8-2.2	0.008
	Collagen Alone vs Propolis Alone	7.5	6.8-8.2	<0.001
	Membrane+Propolis vs Propolis Alone	6.0	5.3-6.7	<0.001
<i>S. mutans</i>	Collagen Alone vs Membrane+Propolis	11.0	9.5-12.5	<0.001
	Collagen Alone vs Propolis Alone	12.0	10.5-13.5	<0.001
	Membrane+Propolis vs Propolis Alone	1.0	0.3-1.7	0.021