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RESEARCH ARTICLE

Green Innovation in GTR Membranes: Combining Astaxanthin, *Aloe vera* and Chitosan for Periodontal Therapy- An *In Vitro* Study

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Abstract

Background: Periodontitis leads to tissue deterioration, prompting the need for effective regenerative therapies. Conventional barrier membranes, face challenges in mechanical strength and support. This study explores a novel formulation combining Astaxanthin's antioxidant and anti-inflammatory properties, *Aloe vera's* healing effects, chitosan nanoparticles biocompatibility and antimicrobial benefits to enhance periodontal tissue regeneration. **Aim:** This study aimed to develop a Guided Tissue Regeneration (GTR) membrane incorporating Astaxanthin, *Aloe vera*, and chitosan nanoparticles and evaluate its *in vitro* properties. **Methods:** Astaxanthin and *Aloe vera* extracts were dissolved in deionized water and boiled for complete incorporation. Chitosan nanoparticles were created by dissolving chitosan in acetic acid, followed by the drop wise addition of sodium tripolyphosphate while stirring. The nanoparticles were collected *via* centrifugation, washed, and re-suspended in deionized water. A chitosan solution was then prepared with glycerol as a plasticizer, and a cross-linking agent was added for stabilization. Finally, the chitosan nanoparticles and bioactive extracts were blended to form the membrane and later the same was analyzed for Scanning Electron Microscopic (SEM), Fourier Transform Infrared Spectroscopy (FTIR), cytotoxic, anti-oxidant, and degradation test activities. **Results:** The GTR membrane demonstrated a homogeneous nanoparticle distribution and initial degradation signs in SEM analysis, while FTIR confirmed the integration of Astaxanthin, *Aloe vera*, and Chitosan. Cytotoxicity tests showed low toxicity, with significant MG-63 cell viability at 15, 20, and 25 µg/mL. Antioxidant activity was dose-dependent, peaking at nearly 50% scavenging at 25 µg/mL, though still lower than ascorbic acids 65%. The degradation test revealed an increasing rate with concentration, reaching approximately 40% at 25 µg/mL. **Conclusion:** Thus the formulated GTR membrane effectively integrates Astaxanthin, *Aloe vera*, and chitosan nanoparticles, demonstrating promising *in vitro* properties for periodontal tissue regeneration with low cytotoxicity and significant antioxidant activity suitable for regenerative therapies.

Major Findings: The developed GTR membrane successfully incorporated with Astaxanthin, *Aloe vera*, and chitosan nanoparticles, exhibited favourable *in vitro* characteristics for periodontal tissue regeneration. It demonstrated minimal cytotoxicity and notable antioxidant activity, highlighting its potential for periodontal therapeutic applications.

Keywords: Astaxanthin, *Aloe vera*, Chitosan Nanoparticles, Green Synthesis

1. Introduction

Periodontitis deteriorates periodontal tissues, leading to tooth movement and loss. Conventional therapy focuses on plaque removal and inflammation control but struggles to restore original tissues[1]. Regenerative techniques aim to replace lost tissues but often fall short. Effective barrier membranes must offer mechanical support, biocompatibility, and neovascularization. Non-resorbable membranes are effective but require removal, while resorbable ones often lack mechanical strength, highlighting the need for innovative approaches in periodontal regeneration[2].

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Astaxanthin, a potent antioxidant from microalgae, yeast, and seafood, exhibits strong anti-inflammatory, anti-apoptotic, and immunomodulatory effects[3]. It promotes wound healing, protects against UV damage, and has superior free radical scavenging abilities compared to vitamins C and E[4]. These properties make it a promising candidate for regenerative periodontal therapies, where oxidative stress and inflammation are key concerns.

Aloe vera offers potent anti-inflammatory, antimicrobial, and healing properties, making it valuable in periodontal therapy. It promotes tissue regeneration, enhances collagen synthesis, and aids in wound healing. Its antioxidant effects help reduce oxidative stress in inflamed periodontal tissues, supporting overall gingival health[5].

Chitosan Nanoparticles (NPs) are derived from chitosan, a natural biopolymer obtained from chitin, found in *Crustaceans* exoskeletons. These nanoparticles exhibit excellent biocompatibility, biodegradability, and antimicrobial properties, making them highly suitable for biomedical applications[6]. They also promote wound healing, and tissue regeneration, and have a mucoadhesive property. The nanoparticles ability to modulate immune responses and inhibit bacterial growth further enhances their potential in regenerative medicine and periodontal therapies[7].

Thus, a product with multiple benefits and synergistic properties was conceived and developed. This innovative formulation leverages Astaxanthin's antioxidant potential, *Aloe vera's* healing effects, and chitosan nanoparticles antimicrobial and regenerative properties. The membrane's significance lies in its ability to enhance periodontal health through excellent biocompatibility and antimicrobial activity, providing a targeted solution for tissue regeneration and advancing clinical outcomes in periodontal therapy. Thus, our study aimed to develop a GTR membrane incorporating Astaxanthin, *Aloe vera*, and chitosan nanoparticles and to evaluate its *in vitro* properties.

2. Materials and Methods

The study was conducted in Saveetha Dental College and Hospitals between August 2024 and September 2024. The institutional scientific research board [SRB/SDC/UG-2094/24/PERIO/282] was obtained before the start of the study.

2.1 Formulation of the GTR Membrane

2.1.1 Preparation of Astaxanthin Solution

In the first step, 10 g of Astaxanthin powdered extract was added to 100 mL of deionized water. The mixture was heated until it reached a boil, ensuring complete dissolution of the Astaxanthin. Once dissolved, the solution was allowed to cool down to room temperature before further use.

2.1.2 Preparation of Aloe vera Solution

In the second step, 10 g of fresh *Aloe vera* extract was added to 100 mL of deionized water. The solution was heated until boiling to incorporate the *Aloe vera* extract fully. Afterward, the solution was left to cool to room temperature.

2.1.3 Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared by dissolving 2-3% (w/v) chitosan in a 1% (v/v) acetic acid solution, stirring continuously for several hours until fully dissolved. A solution of 0.5-1% (w/v) Sodium Tripolyphosphate (TPP) in deionized water was prepared, and the TPP solution was added dropwise to the chitosan solution while stirring, with a TPP-to-chitosan ratio between 1:4 and 1:10, depending on the desired nanoparticle size. For better dispersion, an ultrasonicator was optionally used, and the mixture was stirred for several more hours to allow complete nanoparticle formation.

2.1.4 Collection and Purification of Nanoparticles

The nanoparticles were collected by centrifuging the solution at 12,000-15,000 rpm for 30 minutes. The nanoparticles were then washed with deionized water and centrifuged again to purify them. Finally, the purified nanoparticles were re-suspended in deionized water for further use. A chitosan solution was prepared separately by dissolving 2-3% (w/v) chitosan in 1% acetic acid, to which glycerol was added as a plasticizer to enhance membrane flexibility. A cross-linking agent, such as glutaraldehyde or genipin, was introduced to stabilize the membrane.

2.1.5 Incorporation of Nanoparticles and Bioactive Components

The prepared chitosan nanoparticles were gradually incorporated into the chitosan solution, stirring thoroughly to ensure uniform distribution. The

previously prepared Astaxanthin and *Aloe vera* solutions were then slowly added to the chitosan-nanoparticle mixture, and the final GTR membrane was formed (Figure 1).

2.2 Characterization of the Formulated GTR Membrane

2.2.1 Morphology of the GTR Membrane- SEM Analysis

The membrane's microstructure was analyzed using a Scanning Electron Microscope (SEM), specifically the FEI Quanta FEG 650 SEM (JSM IT-800, JEOL Ltd., Akishima, Tokyo, Japan). The SEM operated at an accelerating voltage of 2,000 kV. Images of the membrane's surface were captured at magnifications of 500x.

2.2.2 FTIR Analysis

The cross-linking behavior of the GTR membrane, which incorporates Astaxanthin, *Aloe vera*, and chitosan nanoparticles, was examined using a Bruker FTIR spectrometer. Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) spectra were obtained from dry films. These films were prepared by pouring hydrogels containing alginate, gelatin, Astaxanthin, *Aloe vera*, and chitosan nanoparticles into a polystyrene petri dish and allowing them to dry for three days. Following the drying process, the films underwent FTIR analysis to identify characteristic absorption bands.

2.2.3 Cytotoxic Activity

Cytotoxic analysis was conducted using fluorescence microscopy, an imaging technique that employs fluorescent dyes or proteins to visualize and quantify cellular structures. In this method, green fluorescence indicates cell viability, with stronger fluorescence correlating to higher levels of cell health and integrity. Additionally, fluorescence microscopy enables the differentiation between live and dead cells, providing valuable insights into the biological activity of the compounds under investigation.



Figure 1. The formulated GTR membrane containing Astaxanthin, *Aloe vera*, and chitosan nanoparticles.

2.2.4 Antioxidant Activity-2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The antioxidant activity of the formulated GTR membrane containing chitosan nanoparticles was evaluated using a DPPH radical scavenging assay. DPPH, a stable free radical, reacts with antioxidants by accepting hydrogen atoms, causing a colour change from purple to light yellow, measurable at 517 nm. Test tubes were prepared with varying concentrations (5-25 µg/mL) of the formulated GTR membrane containing chitosan nanoparticles, 2 mL of methanol, 0.1 mM DPPH solution, and 50% methanol as a buffer. After a 30-minute incubation in the dark, absorbance readings were taken at 517 nm. Methanol served as the blank, while ascorbic acid as the standard. The membranes were then evaluated for their antioxidant capacity at various concentrations.

2.2.5 Degradation Test

Five uniform-sized GTR membrane samples, each weighing 5g, were cut and placed in the wells of a 24-well plate. Each sample was fully submerged in Phosphate Buffered Saline (PBS) and treated with varying concentrations of a test solution (5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, and 25 µg/mL) by adding the appropriate volume of the solution to each well containing the GTR membranes. The samples were then incubated at 37° C to simulate physiological conditions. After the incubation period, the membrane samples were carefully removed from the PBS solution. The membranes were blotted dry to remove surface PBS and subsequently placed in a drying oven at 50° C ensuring the complete removal of any residual moisture.

The fully dried membranes were then evaluated for their degradation rates. This calculation was performed for each concentration to assess the impact of concentration on degradation rates. The results were analyzed to determine the relationship between concentration levels and degradation percentages.

3. Results

3.1 SEM Analysis

The SEM image of the formulated GTR membrane showed a homogenous distribution of nanoparticles, indicating excellent integration of Astaxanthin, *Aloe vera*, and chitosan, Signs of initial degradation such as fragmentation and increased porosity were observed. The well-structured membrane exhibited appropriate porosity and roughness, and the smooth or gel-like appearance in areas of the membrane was also noticed (Figure 2).

3.2 FTIR Analysis

The FTIR analysis of the GTR membrane confirmed the presence of Astaxanthin, *Aloe vera*, and chitosan, each contributing distinct vibrational signatures. The peak at 2881 cm^{-1} indicates C-H stretching from aliphatic chains in Astaxanthin, while peaks at 954 cm^{-1} and 840 cm^{-1} confirm its unsaturated and aromatic nature.

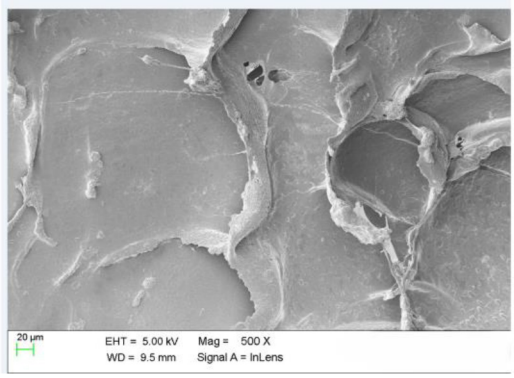


Figure 2. Scanning electron microscopic image of the formulated GTR membrane.

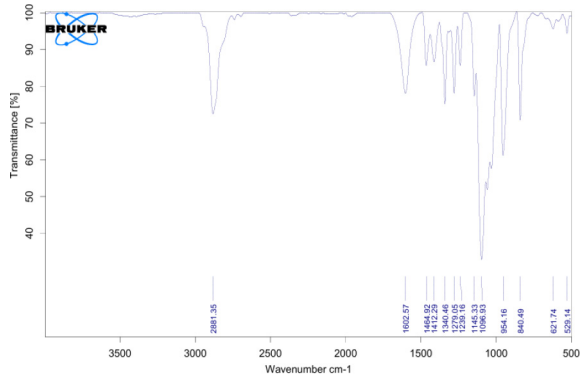


Figure 3. FTIR analysis of the formulated GTR membrane.

Aloe vera's characteristic polysaccharide structure is identified by C-O and C-OH stretching peaks at 1279 cm^{-1}, 1239 cm^{-1}, 1145 cm^{-1}, and 1096 cm^{-1}, alongside the Amide I band at 1602 cm^{-1}, indicating protein-like structures. Chitosan is confirmed through amide and amine functional groups, highlighted by the peak at 1602 cm^{-1} and additional peaks at 1464 cm^{-1}, 1412 cm^{-1}, and 529 cm^{-1}. These findings demonstrate the successful integration of all three components in the membrane (Figure 3).

3.3 Cytotoxic Activity

The bar graph (Figure 4) presents a cytotoxicity analysis of the formulated GTR membrane tested on MG-63 cells, with concentrations ranging from 5 µg/mL to 25 µg/mL and a control group. The cytotoxicity analysis shows that cell viability increases with higher concentrations of the membrane. At 5 µg/mL, moderate viability is observed, but at 10 µg/mL, a slight variation in viability occurs. As the concentration increases to 15, 20, and 25 µg/mL, the cell viability significantly improves, with the highest absorbance recorded at 25 µg/mL, which indicates optimal cell survival. Thus, the formulated GTR membrane demonstrates low cytotoxicity, especially at higher concentrations (15, 20, and 25 µg/mL), promoting better MG-63 cell viability than the control.

The X-axis represents the different concentrations of standard used in µg/mL and the Y-axis represents the absorbance at 570nm.

The fluorescence microscopy images (Figure 5) showed MG-63 cells treated with various concentrations

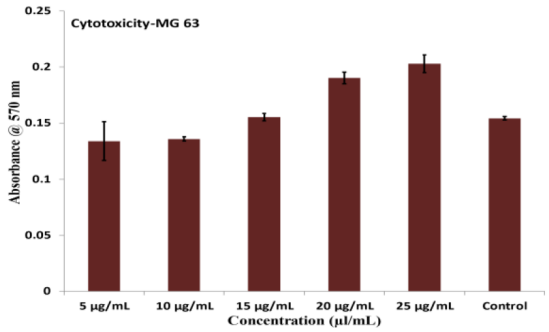


Figure 4. The bar graph depicts the cytotoxicity analysis of the formulated GTR membrane tested on MG-63 cells, with concentrations ranging from 5 µg/mL to 25 µg/mL and with a control.

of the formulated GTR membrane, with live or viable cells highlighted by a green fluorescent stain. The images revealed a clear dose-dependent effect on cell viability. In the Control group: (a) Where no GTR membrane was applied, the lowest fluorescence intensity was observed, indicating a smaller number of viable cells. At 15 µg/mL; (b) The GTR membrane showed a moderate increase in fluorescence, suggesting a slight improvement in cell viability compared to the control. At 30 µg/mL; (c) The fluorescence intensity increased further, indicating a greater number of viable cells. Finally, cells treated with 50 µg/mL; (d) Exhibited the most intense green fluorescence and the highest cell density, suggesting that this concentration supported the greatest cell viability. Overall, these results demonstrated that increasing concentrations of the GTR membrane positively affected cell viability, with the strongest effect observed at 50 µg/mL.

A-Control,b-At 15 µg/mL,c.At 30 µg/mL,d.At 50 µg/mL

3.4 Antioxidant Activity

The antioxidant activity of the formulated GTR membrane containing chitosan nanoparticles was evaluated using a DPPH radical scavenging assay, as shown in the bar graph (Figure 6). Ascorbic acid, used as a positive control, demonstrated the highest antioxidant activity at around 65%. In comparison, the nanoparticles at 5 µg/mL exhibited the lowest activity, around 25%. As the concentration of nanoparticles increased, so did the antioxidant activity, with 10 µg/mL and 15 µg/mL reaching approximately 35% and 40%, respectively. The activity continued to rise with higher concentrations, reaching around 45% at 20 µg/mL and close to 50% at 25 µg/mL. Although the GTR membrane displayed dose-dependent antioxidant properties, its activity remained lower than that of ascorbic acid, suggesting moderate effectiveness in scavenging DPPH radicals.

The X-axis represents the different concentrations of standard and various concentrations of the membrane extract added in microlitres (µg/ml), Y-axis represents the total percentage of antioxidant activity.

3.5 Degradation Test

The degradation test results for the GTR membrane showed a progressive increase in degradation rates with higher concentrations (Figure 7). At 5 µg/mL, the membrane exhibited a degradation rate of approximately

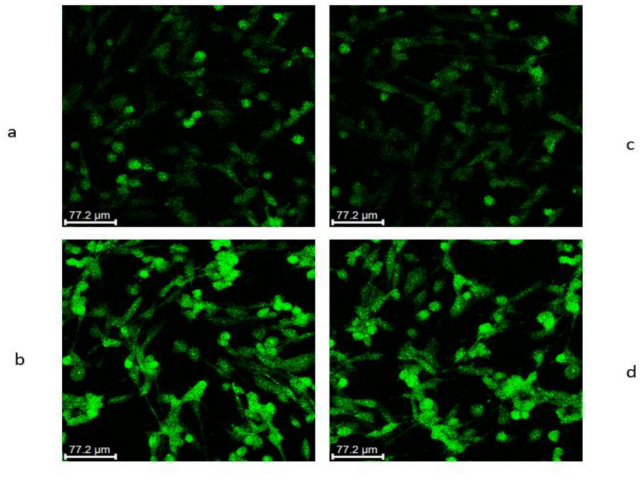


Figure 5. The fluorescence microscopy images show MG-63 cells treated with varying concentrations of the GTR membrane, including control, at 15 µg/mL, 30 µg/mL, and 50 µg/mL.

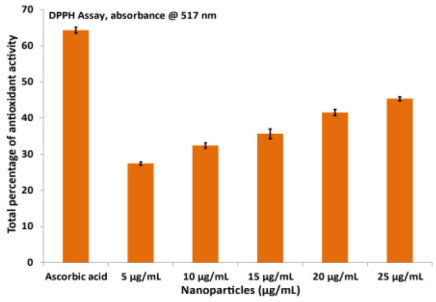


Figure 6. The bar graph depicts the antioxidant activity of the formulated GTR membrane (DPPH assay absorbance at 517nm).

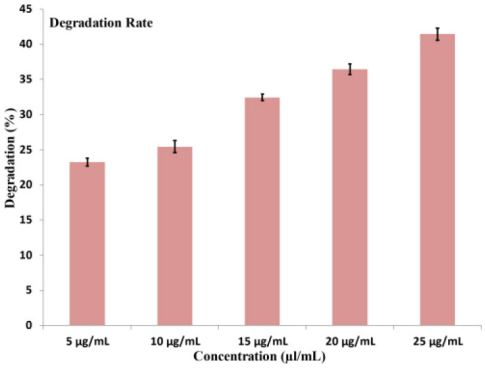


Figure 7. The bar graph depicts the degradation rate of the formulated GTR membrane.

22%. As the concentration increased to 10 µg/mL, the degradation rate rose slightly to around 25%. At 15 µg/mL, the degradation rate further increased to about 28%, indicating a steady rise. At 20 µg/mL, the membrane showed a more significant degradation rate of approximately 32%. The highest concentration, 25 µg/mL, resulted in the greatest degradation, with a rate of around 40%. Overall, the results demonstrated that the GTR membrane degraded more extensively as the concentration increased.

The X-axis represents the different concentrations of the membrane extract added in (µg/ml), Y-axis represents the degradation rate in percentage.

4. Discussion

Periodontitis is a chronic inflammatory condition affecting the supporting structures of teeth, including the gingiva, periodontal ligament, and bone[8]. It results from prolonged bacterial infection, typically originating from plaque. Periodontitis leads to gingival recession, pocket formation, bone loss, and in advanced cases, tooth mobility and potential loss. Treatment focuses on controlling infection, stopping disease progression, and restoring periodontal defects by using GTR membranes[9].

Periodontal regeneration aims to restore damaged tissue surrounding teeth affected by periodontal disease. To enhance biological activity during regeneration, scaffolds, cells, and growth factors are utilized[10]. Since the introduction of the Guided Tissue Regeneration (GTR) concept, various membranes have been employed[11]. The identification and isolation of cells capable of rebuilding the periodontal ligament, cementum, and connective tissue are crucial.

Eliminating epithelial cells from the periodontal defect is a primary objective, as they have a higher turnover rate than osteoblasts and fibroblasts, which are the primary targets for treatment[12]. GTR methods have successfully treated periodontal issues and stimulated bone regrowth. GTR creates a gap around defects using membranes as mechanical barriers, preventing surrounding connective tissues from competing for space and facilitating new bone formation. GTR membranes help ensure proper tissue regeneration, improve clinical outcomes, and support the overall success of periodontal treatments by guiding the natural healing process.

Astaxanthin is highly effective in reducing oxidative stress and inflammation. It also enhances cellular defence mechanisms by scavenging free radicals and protecting cells from damage. Studies have also highlighted that Astaxanthin supports immune function and helps to maintain overall cellular health, making it a valuable component in regenerative therapies[13].

Aloe vera has natural compounds that help to maintain hydration and balance within the oral environment, which is crucial for gingival health. *Aloe vera* also supports the repair of damaged tissues and enhances the resilience of the gingival tissues, contributing to a resilient periodontal structure[14].

Chitosan nanoparticles enhance periodontal treatments through their targeted delivery capabilities and controlled release of therapeutic agents. Additionally, they facilitate the regeneration of periodontal tissues by promoting cell adhesion and proliferation. The nanoparticles ability to create a protective barrier also helps in reducing infection risk and improving overall treatment outcomes[15].

Thus a GTR membrane incorporating Astaxanthin, *Aloe vera*, and chitosan nanoparticles offers a range of advantages for periodontal therapy enhancing tissue regeneration and wound healing, and improving tissue health. Both *in-vitro* and *in-vivo* studies have highlighted the individual benefits of these ingredients in managing periodontal conditions. By combining these three components into a single product, the aim is to maximize their synergistic effects.

Our study aimed to evaluate the *in-vitro* characteristics of the formulated GTR membrane with nanoparticles. SEM analysis revealed efficient integration of Astaxanthin, *Aloe vera*, and chitosan, indicating the bioactivity of the membrane. Signs of initial degradation suggest that the GTR membrane was breaking down at a rate conducive to tissue regeneration. The GTR membrane exhibited adequate porosity confirming its ability to facilitate cell migration and nutrient exchange effectively.

The FTIR analysis confirms the successful integration of Astaxanthin, *Aloe vera*, and chitosan in the GTR membrane. Astaxanthin is identified by C-H stretching and unsaturated or aromatic features, *Aloe vera* by its polysaccharide structure and protein-like amide I band and chitosan by its amide and amine functional groups. These distinct vibrational signatures

validate the presence of each component in the membrane.

The cytotoxicity assay revealed that the formulated GTR membrane on MG-63 cells shows that cell viability increases with higher membrane concentrations, with optimal survival at 20 µg/mL, indicating low cytotoxicity at higher concentrations.

Fluorescence microscopy further confirms a dose-dependent increase in cell viability, with the highest fluorescence and cell density observed at 50 µg/mL. Overall, both analyses demonstrate that the GTR membrane enhances cell viability, particularly at higher concentrations making it promising for regenerative applications. These findings align with previous research that demonstrated that Astaxanthin-loaded chitosan or polyvinyl alcohol or graphene oxide nanofiber membranes promoted periodontal bone defect repair and inhibited periodontal inflammation in animal experiments[16].

The DPPH antioxidant assay shows that the formulated GTR membrane with chitosan nanoparticles exhibited dose-dependent antioxidant activity, increasing from 25% at 5 µg/mL to around 50% at 25 µg/mL. While the activity improves with higher concentrations, it remains lower than the ascorbic acid control. These results indicate that the GTR membrane provides moderate antioxidant effectiveness in scavenging DPPH radicals. In addition, other studies have shown that the synergistic effects of *Aloe vera* and chitosan enhance overall antioxidant capacity, making them valuable components in formulations designed to improve oral health and promote tissue regeneration[17].

The degradation test showed a concentration-dependent increase in degradation rates for the GTR membrane, starting at 22% for 5 µg/mL and rising to 40% at 25 µg/mL. The results demonstrated that higher concentrations led to more extensive membrane degradation. These results align with previous research that emphasized the optimal degradation rates of membranes containing chitosan and *Aloe vera*[18].

Nanoparticle-incorporated GTR membranes have shown enhanced effectiveness in tissue regeneration due to their concentration-dependent degradation rates, aligning with tissue growth in periodontal and bone repair. In contrast, the more commonly available non-resorbable membranes like e-PTFE require surgical removal, increasing patient discomfort and procedural complexity[19]. Resorbable membranes often suffer from insufficient mechanical strength, premature degradation, or limited biocompatibility, hindering healing. Additionally, cost and ease of manipulation are important factors. These challenges highlight the potential of nanoparticle membranes incorporating Astaxanthin and *Aloe vera* as promising alternatives.

Awareness of GTR membranes incorporating nanoparticles has been growing recently, particularly due to their potential in tissue engineering applications[20]. *In-vitro* studies have shown that these membranes exhibit enhanced degradation rates and biocompatibility, making them suitable for periodontal and bone regeneration. Consequently, the use of nanoparticle-enhanced GTR membranes aims to optimize healing processes in patients with intrabony periodontal defects, offering a more effective solution for tissue regeneration and the management of periodontal conditions effectively. *Aloe vera* has been extensively researched for its role in tissue engineering, particularly for promoting wound healing and enhancing tissue regeneration[21,22].

Chitosan, known for its biocompatibility and antibacterial properties, has also been studied for its applications in wound healing and as a scaffold material in tissue engineering[23,24]. A study demonstrated the potential of chitosan as a GTR membrane material when combined with eggshells, revealing a fibrous surface and a desirable pore size suitable for periodontal applications[25]. Extensive research on green nanoparticle synthesis highlights eco-friendly methods, demonstrating the potential of natural compounds in advanced therapies. For instance, the synthesis of reduced graphene oxide nanoparticles has shown promising antimicrobial activity against common oral pathogens, coupled with minimal cytotoxic effects[26,27].

5. Conclusion

In conclusion, the formulated GTR membrane with Astaxanthin, *Aloe vera*, and chitosan nanoparticles. In conclusion, the formulated GTR membrane incorporating Astaxanthin, *Aloe vera*, and chitosan nanoparticles significantly enhances tissue regeneration by effectively promoting periodontal healing and minimizing inflammation. This innovative membrane not only demonstrates optimal degradation

rates but also exhibits low cytotoxicity. By promoting tissue regeneration it addresses the critical needs in regenerative dentistry, offering a biocompatible and effective solution for patients with intra bony defects.

6. Strength and Constraints

The study's strengths lie in its innovative formulation, which synergistically combines the properties of Astaxanthin, *Aloe vera*, and chitosan nanoparticles to address multiple aspects of periodontal regeneration, including antioxidant activity. However, the study's reliance on *in-vitro* results may not accurately reflect *in-vivo* conditions, potentially restricting the applicability of its findings to clinical practice.

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8. Author Contribution

Dr. Priyangha P.T contributed to conceptualization, data curation, manuscript writing, editing, review, and final editing. Sai Shreya Reddy was responsible for concepts, design, and literature search. All authors have read and agreed to the published version of the manuscript.

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