

Adhatoda vasica and Tetracycline Coated Type I Collagen Membrane for Periodontal Regeneration: An In-vitro Study

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ABSTRACT

Introduction: Periodontitis is a chronic inflammatory disease that leads to the destruction of periodontal tissues and alveolar bone loss. The lost periodontium has been restored using regenerative materials such as collagen membrane. Enhancing these membranes with antibiotics or bioactive agents can improve their antimicrobial and physicochemical properties.

Aim: This in-vitro study aimed to analyse the antibacterial and physicochemical properties of herbal agent *Adhatoda vasica* and tetracycline coated type I collagen membrane.

Materials and Methods: The present in-vitro study was conducted at Central Research Laboratory Meenakshi Ammal Dental College and Hospital, Chennai, Tamil Nadu, India. The study was carried out over a period of four months, from December 2024 to March 2025. Type I collagen membranes devoid of pre-existing antimicrobial agents and exhibiting no structural defects were included for analysis. A total 40 membrane samples were used and divided equally into four groups (n=10 per group) to ensure uniform distribution for analysis. *Adhatoda vasica* extract was prepared using ethanol and coated onto Type I collagen membranes via dip coating, alone and in combination with tetracycline. The study comprised four groups: Group 1: Type I collagen membrane, (CM), Group 2: *Adhatoda vasica* (Av) coated CM (Av+CM), Group 3: Tetracycline coated CM, (T+CM) and Group 4: Av and T coated CM (Av+T+CM). Antibacterial efficacy

was assessed through culture tests, while physicochemical properties such as membrane integrity was checked using Fourier-Transform Infrared Spectroscopy (FTIR), hydrophilicity via Ossila goniometer, surface roughness via Atomic Force Microscopy (AFM), drug release was measured via a plate reader and degradation rate was determined by electronic weighing balance machine. Data were analysed using Statistical Package for Social Sciences (SPSS) version 25.0. One-way Analysis of Variance (ANOVA) was used for intergroup comparisons. A p-value <0.05 was considered statistically significant.

Results: Group 4 (Av+T+CM) showed the highest antibacterial activity with zone of inhibition significantly greater than other groups (p<0.001). Surface roughness was significantly increased in Group 2 (Av+CM) and Group 4 (p<0.01) indicating enhanced topography. Hydrophilicity varied significantly with Group 2 showing the lowest contact angle (p<0.01). FTIR analysis showed no significant spectral shifts confirming structural integrity. Group 4 exhibited burst release during the initial three hours followed by sustained release upto 48 hours and exhibited slow rate of degradation.

Conclusion: The present in-vitro study suggested that *Adhatoda vasica* and tetracycline when used synergically as a coating on Type I collagen membrane, provides excellent antibacterial and physicochemical properties and hence can be used as a therapeutic material for preventing infections and promoting periodontal regeneration after periodontal surgery.

Keywords: Antibacterial activity, Biocompatible materials, Drug delivery systems, Guided tissue regeneration, Periodontitis

INTRODUCTION

Periodontitis is a chronic inflammatory disease affecting the gingiva and supporting tissues, leading to destruction of the periodontal structures thereby causing alveolar bone loss. The key periodontal pathogens and persistent development of plaque biofilm spreads subgingivally, making it extremely challenging to maintain good oral hygiene and leads to development of periodontitis [1]. The lost periodontium has been successfully restored through the regenerative materials such as collagen membranes which facilitate Guided Tissue Regeneration (GTR) by acting as a barrier that prevents the migration of epithelial cells, allowing for the growth of new bone and periodontal ligament [2]. While collagen membranes are effective scaffolds for tissue growth, they lack inherent antimicrobial properties, which limit their ability to prevent or combat bacterial infections during the healing process. To address this limitation, antibiotics such as amoxicillin, tetracycline and ciprofloxacin have been incorporated into collagen membranes. Coating collagen membranes with these antibiotics enhances their antimicrobial activity, helping to reduce bacterial load and improve the healing of periodontal tissues. The inclusion of these antibiotics not only supports tissue regeneration by preventing bacterial interference but also accelerates the healing process by reducing

the risk of infection. This combination approach, using regenerative materials like collagen membranes alongside antimicrobial agents, holds significant promise in improving the outcomes of periodontal therapies and enhancing the overall effectiveness of tissue regeneration in the treatment of periodontitis [3,4].

Tetracycline potent antibacterial properties help to suppress periodontal pathogen growth, thereby enhancing periodontal tissue healing and improving overall regenerative outcomes. Additionally, tetracycline coated collagen membranes can slowdown their degradation rate extending their functional lifespan during tissue regeneration. Moreover, tetracycline promotes fibroblast attachment and proliferation, which are essential for tissue regeneration, while its anti-inflammatory properties help control inflammation at the treatment site. By inhibiting matrix Metalloproteinases (MMPs), tetracycline preserves the extracellular matrix, ensuring structural integrity during regeneration. The coating also allows for a localised and sustained release of the drug, enhancing its efficacy and minimising systemic side effects. Together, these properties improve the regenerative outcomes of periodontal procedures, making tetracycline coated collagen membranes a highly effective tool in clinical practice [3,5].

Chemical medications are known to cause several side-effects such as localised toxicity, adverse reactions or cytotoxicity which potentially hinders the healing process. As a consequence, herbal agents or combination therapies are being preferred to mitigate the risks and to enhance the efficacy of antimicrobial strategies in periodontal regeneration. The synergy between herbal agents and antibiotics on collagen membranes improve both the safety and effectiveness of infection control during the healing of periodontal, bone or soft-tissue defects, while supporting regenerative outcomes in a more holistic and sustainable manner. Herbal agents, such as curcumin, provide antimicrobial effects while also offering anti-inflammatory, antioxidant and tissue-healing benefits. These properties help support the regenerative process by reducing inflammation and promoting tissue. When combined with antibiotics like tetracycline, the antimicrobial activity is further potentiated, providing an effective means of preventing infections during tissue regeneration [6].

Among the various herbal agent *Adhatoda vasica* a traditional Ayurvedic herb, is renowned for its rich array of bioactive compounds such as vasicine and vasicinone, which offer potent antimicrobial, anti-inflammatory and antioxidant properties. These attributes make it highly effective in the treatment of various systemic ailments, including asthma, respiratory infections, arthritis and wound healing. The plant is utilised in diverse forms, including its leaves, roots, flowers and bark while leaves are the most commonly used. Despite its well-documented benefits in other fields, its potential in addressing dental issues, particularly periodontitis remains largely unexplored [7-9].

the present study tends to explore the effect of *Adhatoda vasica* in periodontal regeneration. The null hypothesis stated that *Adhatoda vasica*, alone or in combination with tetracycline, has no significant effect on periodontal regeneration or antimicrobial activity compared to the uncoated collagen membrane. On the other hand, according to alternate hypothesis, *Adhatoda vasica*, alone or in combination with tetracycline, significantly enhances periodontal regeneration and exhibits antimicrobial activity compared to the uncoated collagen membrane. The present in-vitro study hypothesised that *Adhatoda vasica*, alone or in combination with tetracycline, offered several potential benefits in periodontal regeneration.

Adhatoda vasica's natural antimicrobial action may help in reducing the bacterial load while simultaneously enhancing tissue regeneration due to its anti-inflammatory and antioxidant properties. Additionally, its immune-modulatory effects could complement tetracycline's antibacterial activity by supporting tissue healing and immune responses. The combination of these two agents could potentially lead to synergistic effects, where the antibacterial activity of tetracycline is enhanced by the natural healing properties of *Adhatoda vasica*, promoting more effective periodontal regeneration. Moreover, the dual action of this combination may reduce the need for prolonged use of antibiotics, thereby mitigating the risks of antibiotic resistance, which is a growing concern in modern medicine. Furthermore, the inclusion of a natural herbal compound in combination with an established drug could provide a more holistic, sustainable approach to periodontal therapy, improving patient outcomes with fewer side effects. This in-vitro study holds promise not only in enhancing the therapeutic efficacy of collagen membranes for periodontal regeneration but also in advancing the integration of herbal agents with conventional pharmaceuticals in dental treatments. Therefore, the aim of the current study was to evaluate the antibacterial and physicochemical properties of *Adhatoda vasica* and tetracycline coated Type I collagen membranes. Objectives of the study were to evaluate the antibacterial property, membrane integrity, hydrophilicity, surface roughness, drug release and to evaluate rate of degradation of herbal agent *Adhatoda vasica* and tetracycline coated Type I collagen membrane.

MATERIALS AND METHODS

The present in-vitro study was conducted at the Central Research Laboratory, Meenakshi Ammal Dental College, Chennai, Tamil Nadu, India. after obtaining ethical clearance (IEC No: MADC/IEC/1/07/2024). The study was carried out over a period of four months, from December 2024 to March 2025.

Sample size calculation: The sample size was determined based on prior similar in-vitro studies evaluating the antimicrobial efficacy and physicochemical properties of coated collagen membranes. Assuming an effect size of 0.4, a significance level (α) of 0.05 and a power ($1-\beta$) of 0.80, the minimum sample size required was calculated to be 10 samples (membranes) per group using one-way ANOVA. Hence a total of 40 samples were included, divided equally into four groups ($n=10$ per group) considering the samples drop out which may occur in in-vitro studies due to factors such as contamination, sampler degradation, procedural errors during preparation or handling or loss during analysis, such occurrences are common in laboratory settings and were minimised through strict protocol adherence. All samples were documented and data analysis was performed based on the laboratory analysis.

Inclusion and Exclusion criteria: (1) Commercially available, sterile Type I collagen membranes (bovine-derived); (2) Membranes with intact physical structure and uniform thickness; (3) Membranes not previously coated or treated with any antimicrobial or chemical agents; (4) Membranes stored under appropriate sterile, dry conditions prior to coating and experimentation; (5) Only membranes within the manufacturer's expiry period and specifications were included for the investigation. While procuring the membranes, certain precautions were taken. Collagen membranes with visible defects, damage, contamination, or discoloration, membranes with any pre-existing drug, herbal, or antibiotic coating, showing non-uniform dimensions or inconsistent surface morphology, expired or improperly stored membrane materials and membranes exposed to moisture prior to experimental use were excluded from the study.

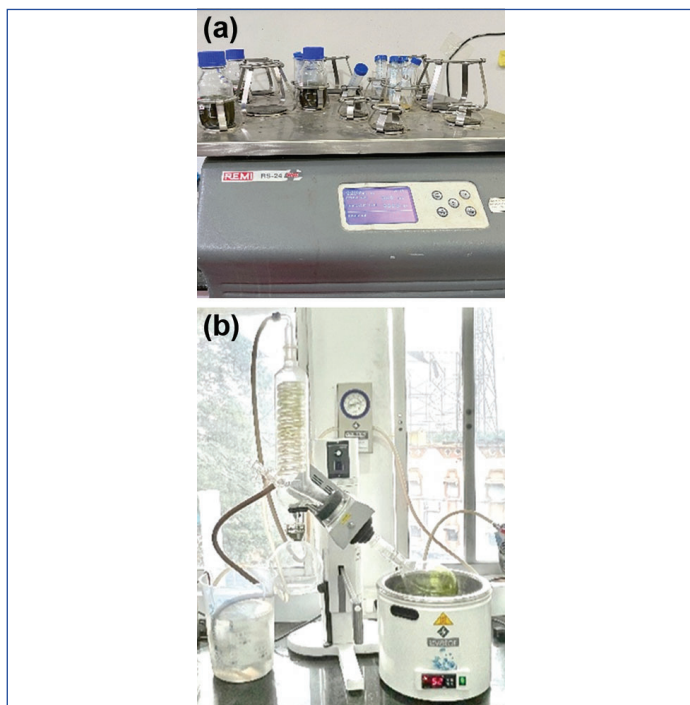
Study Procedure

The procedure was done under the Biosafety Protocol. The Type I Collagen membrane HEALIGUIDE® (20 x 30 mm), a bovine-derived xenograft, was procured from Advanced Biotech Products (P), Chennai, Tamil Nadu, India. This collagen membrane was selected for its biocompatibility and suitability in periodontal regeneration applications. The herbal agent *Adhatoda vasica* was sourced in powdered form from the leaves of the plant, purchased from FOODHERBS® in Coimbatore, Tamil Nadu, India, with a quantity of 200 grams. This herbal powder was used for its antimicrobial and anti-inflammatory properties. The drug tetracycline Resteclin® (250 f), in the form of capsule was obtained from Apollo pharmacy in Chennai. Tetracycline, a broad-spectrum antibiotic was chosen for its well-documented ability to control bacterial growth, particularly in periodontal infections. These materials were combined in the study to create bioactive dual-drug coated collagen membranes aimed at enhancing periodontal healing and infection control.

In this in-vitro study, Type I collagen membranes were subjected to antibacterial and physicochemical tests following coating with varying combinations of the herbal agent *Adhatoda vasica* and/or tetracycline. The following groups were prepared to assess the impact of each coating combination: Group 1: Type I Collagen membrane (CM) Group 2: *Adhatoda vasica* coated Type I Collagen membrane (Av+CM) Group 3: Tetracycline coated Type I Collagen membrane (T+CM) Group 4: *Adhatoda vasica* and tetracycline coated Type I Collagen membrane (Av+T+CM).

Preparation of the *Adhatoda vasica* extract: Preparation of the *Adhatoda vasica* extract involved the extraction of the herbal agent from powdered *Adhatoda vasica* using ethanol as the solvent. In order to obtain pure form of *Adhatoda vasica*, 300 mL of ethanol was added in 150 grams of powdered *Adhatoda vasica*

and placed in an orbital shaker at room temperature (37°C) for three days [Table/Fig-1a]. After the extraction period, the ethanol solvent was removed using a rotary evaporator, which was set to a pressure of 200 mbar and a temperature of 52°C to facilitate the evaporation of ethanol, leaving behind the concentrated extract shown in [Table/Fig-1b] [10].



[Table/Fig-1]: a) Orbital shaker. b) Rotary evaporator.

Coating of *Adhatoda vasica* and tetracycline on Type I collagen membrane:

The dip coating method was employed to coat *Adhatoda vasica* and tetracycline (T) on Type I collagen membranes ensuring uniform deposition of the bioactive compounds [11]. Group 1 (CM) served as the control, consisting of an uncoated Type I collagen membrane. Group 2 (Av+CM) coating the membrane with 1.6 mg of pure *Adhatoda vasica* dissolved in 1 mL of ethanol, allowing adsorption onto the surface. Group 3 (T+CM) involved coating the membrane with 0.243 mg of tetracycline dissolved in 1 mL of ethanol, facilitating antibiotic incorporation. A higher dose of *Adhatoda vasica* was necessary due to its complex phytochemical composition and comparatively lower per-milligram antimicrobial activity, whereas tetracycline, being a potent antibiotic with high efficacy at low concentrations, required only a minimal dose to achieve effective membrane incorporation and antibacterial action. This optimised balance ensured efficient surface adsorption without compromising membrane integrity or drug stability [5,9].

In Group 4 (Av+T+CM), a combination of 0.8 mg of *Adhatoda vasica* and 0.121 mg of tetracycline was used, ensuring a dual-drug coating strategy. The concentrations were adjusted to half the dose used in individual groups (Group 2 and Group 3) to maintain a balanced total drug load on the membrane and to evaluate potential synergistic effects with combined doses. This strategy is commonly used in combination therapies to reduce the risk of cytotoxicity while preserving or enhancing antimicrobial efficacy.

The coated membrane was left undisturbed for 1 hour, allowing sufficient interaction and adherence of the bioactive agents onto the membrane surface. Subsequently, the membranes were removed and dried for one minute, ensuring solvent evaporation while retaining the coated compounds. The processed membranes were then subjected to antibacterial and physicochemical analyses to assess their structural integrity, hydrophilicity, surface roughness, drug release against *Staphylococcus aureus* (Gram-positive), *Streptococcus mutans* (Gram-positive) and *Escherichia coli* (Gram-negative) bacterial colonies. *Staphylococcus aureus* and *Escherichia*

coli were procured from clinical isolates, while *Streptococcus mutans* was obtained from the Microbial Type Culture Collection (MTCC 890), Chandigarh, India.

Brain Heart Infusion (BHI) broth inoculum was prepared and each microorganism was separately inoculated into the broth. The cultures were incubated at 37°C for 24 hours to allow bacterial growth. After incubation, a 10:8 dilution of the inoculum was prepared and the culture was plated using Mueller Hinton Agar for *Staphylococcus aureus* and *Escherichia coli* and Mannitol Salt Agar for *Streptococcus mutans*. Following the plating process, the groups (CM, Av+CM, T+CM and Av+T+CM) were applied onto the culture plates and left at room temperature for two hours to ensure proper interaction between the membrane and the bacterial cultures. After this incubation period, the plates were further incubated at 37°C for another 24 hours. The primary outcome of interest was the measurement of the zone of inhibition, which would indicate the antibacterial effect of the various treatments. The effectiveness of the antimicrobial agents, both individually and in combination, was assessed by comparing the size of the zones of inhibition surrounding the membrane on the culture plates. The study aimed to assess the broad-spectrum antimicrobial activity of the coated membranes, evaluating the synergistic effects of the herbal agent *Adhatoda vasica* and antibiotic tetracycline coating collagen membrane in inhibiting bacterial growth.

Physiochemical properties: Bruker Alpha II FTIR was utilized to assess the membrane integrity of all the four groups [Table/Fig-2]. The FTIR technique is a powerful method for identifying molecular structures and functional groups within materials. Using this equipment, all the groups were exposed to infrared light after being placed in the Diamond Crystal Attenuated Total internal Reflectance (ATR) accessory which allows for the measurement of the infrared light absorption by the membrane. When exposed to infrared light, the molecular vibrations within the membrane interact with the light, producing a spectrum that reflects the presence of specific functional groups in the material.

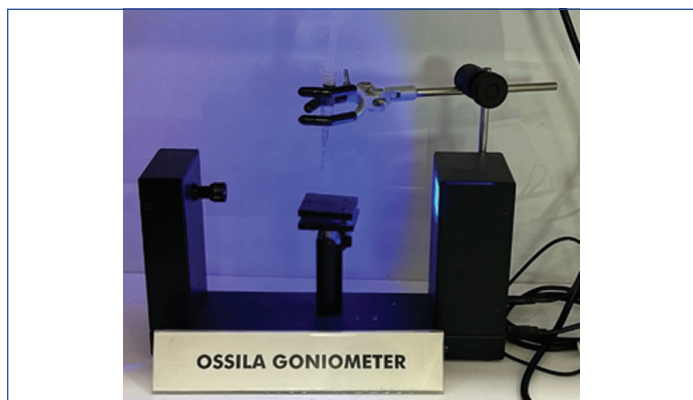
By analysing the resulting FTIR spectra, the membrane integrity could be evaluated, particularly looking for any changes or additions of new functional groups that may indicate chemical interactions. Any shifts in peak positions or the appearance of new peaks would suggest the presence of newly introduced functional groups due to the coating process. This analysis provided the valuable insight into how the addition of *Adhatoda vasica* and tetracycline affects the chemical structure of the collagen membrane, helping to understand the potential interactions between the herbal agent, antibiotic and collagen matrix. This approach ensures that the membrane's structural integrity is maintained while potentially enhancing its antimicrobial properties.



[Table/Fig-2]: Bruker Alpha II Fourier-Transform Infrared Spectroscopy (FTIR).

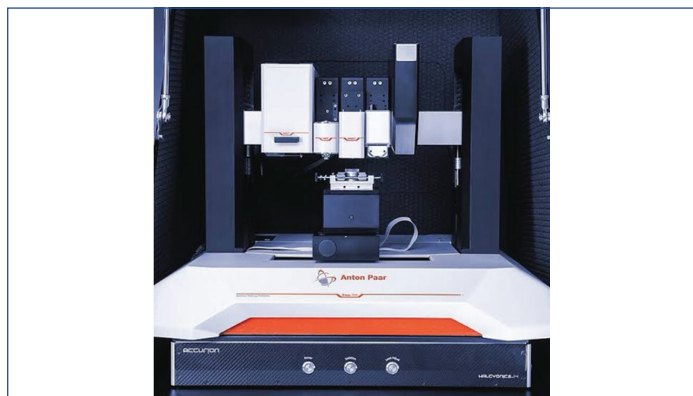
The hydrophilicity of the all the four groups was determined using an Ossila goniometer to explore that how well a material interacts with water thus influencing its effectiveness in biological applications such as wound healing and tissue regeneration [Table/Fig-3].

A 3 μ L of distilled water (H_2O) was dropped onto the surface of the collagen membranes in all the four groups. The membranes were then kept at room temperature to ensure stable conditions for the measurement. The Ossila goniometer was then used to measure the angle formed between the water droplet and the surface of the membrane. A lower contact (<90) angle would suggest a more hydrophilic surface, indicating that the water spread easily across the membrane, while a higher contact angle (>90) would indicate a more hydrophobic surface, with water beads remaining on top of the membrane. The findings of this test were tabulated providing insight into how the addition of *Adhatoda vasica* and tetracycline might alter the surface properties of the Type I collagen membrane. Changes in the hydrophilicity of the membrane could influence its interaction with surrounding tissues, its ability to absorb fluids and its overall effectiveness in supporting periodontal regeneration.



[Table/Fig-3]: Ossila goniometer to check hydrophilicity.

The surface roughness of all the four groups was assessed using Atomic Force Microscopy (AFM) [Table/Fig-4].



[Table/Fig-4]: Atomic Force Microscopy (AFM).

The surfaces of the membrane were investigated by Anton Paar Step 700 (Nanosurf, Liestal, Switzerland) with Stat0 2LAuD cantilever (tip diameter 1 nm) in the static mode. Image sizes covered areas of $20\text{ }\mu\text{m} \times 20\text{ }\mu\text{m}$, the lateral resolution was $\sim 100\text{ nm}$. Approach parameters were optimised according to these surfaces, setpoint 20 nN, P-gain 1000- 1500, I-gain 1000-1300 and D-gain 100-500, where the setpoint denoted the oscillation amplitude of the cantilever, while P, I and D denoted the proportional, integral and differential part of a common PID controller, respectively.

Drug release of Group 2, Group 3, Group 4 was quantified by measuring the absorbance of the release media using a plate reader [Table/Fig-5].

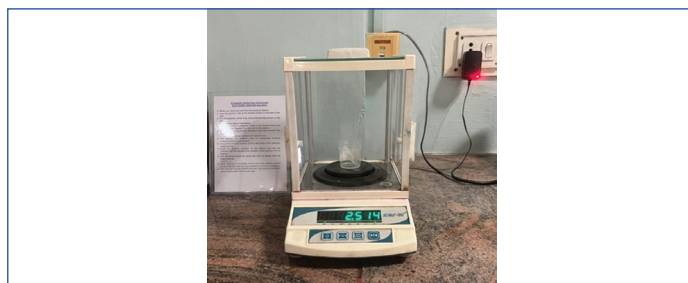
A $1 \times 1\text{ mm}$ diameter of collagen membranes were dipped separately in 1mL of Stimulated Body Fluid (SBF). SBF solution was then collected at 0, 3, 6, 12, 24, 48 hours and it was refrigerated at 4°C .



[Table/Fig-5]: Plate reader.

Later the collected SBF solution was transferred into plate reader and the standard curves for drug concentration of *Adhatoda vasica* and tetracycline in SBF solution were measured.

To check the rate of degradation, $1 \times 1\text{ mm}$ in diameter of Group 1, Group 2, Group 3 and Group 4 were separately hydrolysed in 1 mL of SBF. The membranes were taken out on 1st, 3rd, 7th, 11th, 14th, 18th, 21st and 28th day and finally 'dry weight' after degradation was measured using an electronic weighing balance machine [Table/Fig-6].



[Table/Fig-6]: Electronic weighing balance machine to check percentage of weight loss in all four groups.

The degradation ratio was expressed as the percentage of weight loss calculated using the following equation: Weight loss (%) = $(W_0 - W_d)/W_0$, where W_0 is the actual weight before degradation and W_d is the dry weight after degradation [9].

STATISTICAL ANALYSIS

Data was entered and analysed using SPSS Statistics version 25.0. Descriptive statistics were presented as means $\pm$ standard deviation. For inter-group comparisons of quantitative variables (zone of inhibition, surface roughness, contact angle and membrane integrity), One-way ANOVA was used. A p-value <0.05 was considered statistically significant, indicating that the observed differences among groups.

RESULTS

Antibacterial property: The zone of inhibition was checked and measured. Group 4 (Av+T+CM) exhibited the highest antibacterial efficacy against *Staphylococcus aureus*, *Streptococcus mutans* and *Escherichia coli* colonies of bacteria followed by Group 3 (T+CM), Group 2 (Av+CM) and Group 1 (CM) [Table/Fig-7]. Statistical analysis was performed using One-way ANOVA and p-value <0.001 was considered statistically significant. Antibacterial activity against *Staphylococcus aureus*, *Streptococcus mutans* and *Escherichia coli* was significant with p-value <0.001 [Table/Fig-8].



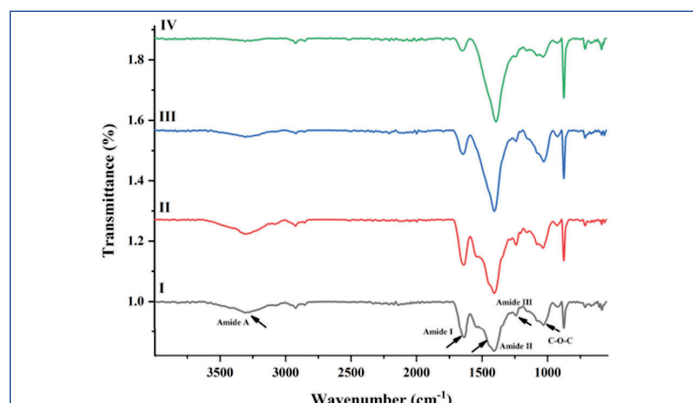
[Table/Fig-7]: Antibacterial activity of all four the groups against *S. aureus*, *S. mutans* and *E. coli*.

Groups	Zone of inhibition			FTIR	Hydrophilicity	Atomic Force Microscopy (AFM)
	<i>Staphylococcus aureus</i> (mm)	<i>Streptococcus mutans</i> (mm)	<i>Escherichia coli</i> (mm)	Wavenumber (cm ⁻¹)	Average Angle (°)	Surface Roughness (nm)
Group 1 (CM)	-	-	-	1636.66±0.40	79.41±1.6	105.02±11.2
Group 2 (Av+CM)	4.10±0.10	4.06±0.15	3.12±0.20	1635.43±0.50	73.21±1.8	368.03±12.5
Group 3 (T+CM)	6.25±0.12	6.03±0.18	5.00±0.14	1641.74±0.38	88.41±2.1	264.61±10.2
Group 3 (Av+T+CM)	9.03±0.10	8.18±0.12	5.60±0.17	1644.83±0.45	84.53±1.7	382.06±11.8
p-value	<0.001	<0.001	0.003	NS	< 0.001	< 0.001

[Table/Fig-8]: Evaluating physicochemical and antimicrobial assessments of *Adhatoda vasica* and tetracycline coated Type I collagen membranes.

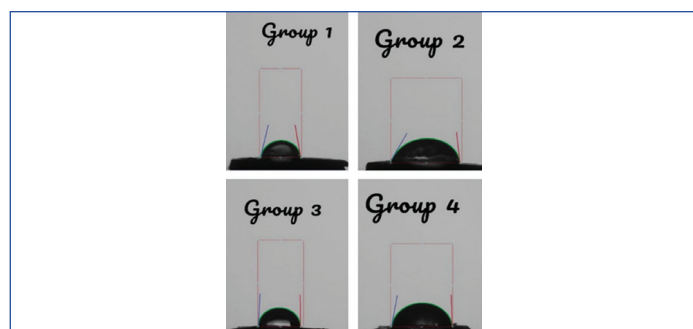
Data were analysed using SPSS version 25.0. One-way ANOVA was used for intergroup and intragroup comparisons; *S. aureus* F (3,36) = 16867.17, *S. mutans* F (3,36) = 6985.80, *E. coli* F (3,36) = 2867.77, Hydrophilicity F (3,36) = 132.29, Surface roughness F (3,36) = 1245.03, FTIR F (3,36) = 1020.80. A p-value <0.05 was considered statistically significant NS: Not significant.

Membrane integrity: FTIR analysis revealed that all four groups (CM, Av+CM, T+CM, and Av+T+CM) exhibited similar characteristic vibrational peaks at approximately 1635-1645 cm⁻¹ corresponding to the amide I band, which is a key marker of collagen's molecular integrity. No additional functional groups or shifts in the major absorption bands were observed in the coated groups compared to the uncoated CM group. These consistent wavenumber values (CM: 1636.66±0.40; Av+CM: 1635.43±0.50; T+CM: 1641.74±0.38; Av+T+CM: 1644.83±0.45). Statistical analysis for membrane integrity of the collagen membrane was performed using one-way ANOVA and p-value was not statistically significant [Table/Fig-8] indicate that the coating process did not chemically alter the collagen matrix. Therefore, the lack of peak shift or formation of new peaks confirmed that the structural integrity of the collagen membrane was retained following coating with *Adhatoda vasica* and tetracycline [Table/Fig-9].



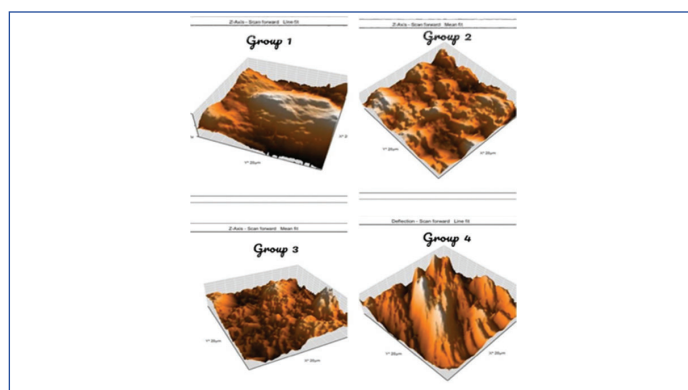
[Table/Fig-9]: Functional group expression via Fourier-Transform Infrared Spectroscopy (FTIR).

Hydrophilicity: Group 2 (Av+CM) exhibited more hydrophilic property followed by Group 1 (CM), Group 4 (Av+T+CM) and Group 3 (T+CM) exhibited less hydrophilic property [Table/Fig-10]. Statistical analysis for hydrophilicity of the collagen membrane was performed using one-way ANOVA. A p-value <0.001 was considered statistically significant [Table/Fig-8] which indicates that all four groups show hydrophilic property.



[Table/Fig-10]: Contact angle of collagen membranes (Group 1, 2, 3 and 4).

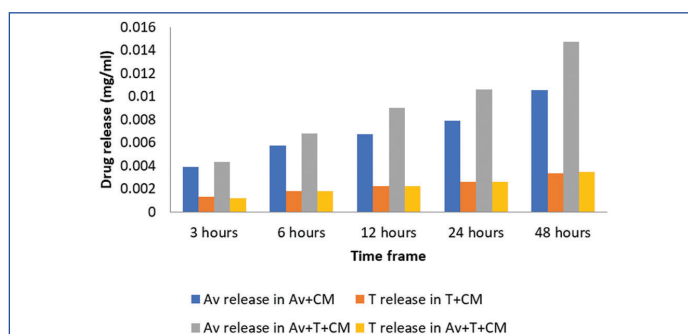
Surface roughness: On testing the surface roughness using AFM, Group 4 (Av+T+CM) exhibited high surface roughness followed by Group 2 (Av+CM) Group 3 (T+CM) and Group 1 (CM) [Table/Fig-11]. Statistical analysis for surface roughness of the collagen membrane was performed using One-way ANOVA. A p-value <0.001 was considered as statistically significant [Table/Fig-8].



[Table/Fig-11]: Surface roughness of collagen membranes (Group 1, 2, 3 and 4).

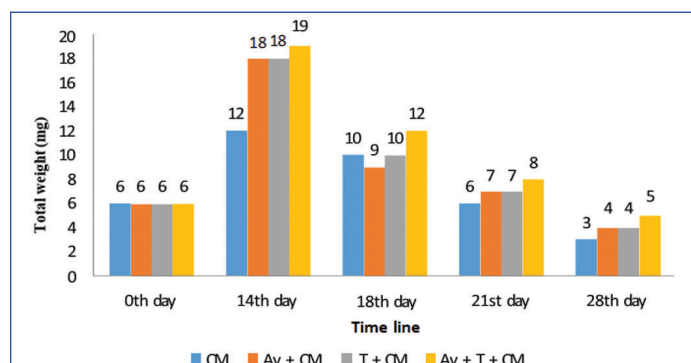
Drug release profile was evaluated in Group 2 (Av+CM), Group 3 (T+CM) and Group 4 (Av +T+CM) and all of them equally exhibited burst release during the initial 3 hours, followed by sustained release up till 48 hours. Group 1 (CM) was not taken into consideration since it was plain Type I collagen membrane without any drug coating.

To further analyse the contribution of each component in the dual-coated membrane Group 4 was subdivided to independently quantify the release of *Adhatoda vasica* and tetracycline over time. The drug release profile revealed that *Adhatoda vasica* exhibited a higher cumulative release compared to tetracycline within the same group. Moreover, the release of *Adhatoda vasica* in Group 4 was greater than its release observed in Group 2 (Av+CM), suggesting that the combination coating may have enhanced its diffusion or retention properties. All coated groups (Groups 2, 3, and 4) demonstrated an initial burst release within the first three hours, followed by a sustained release up to 48 hours. Group 1 (CM) was excluded from this analysis as it did not contain any active agent [Table/Fig-12].



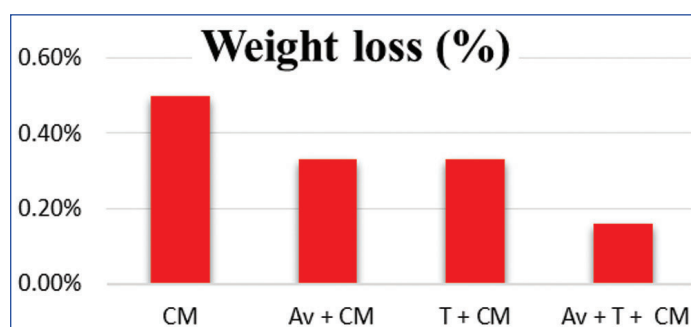
[Table/Fig-12]: Comparison of drug release of the Group 2, 3 and 4 at various time frames.

At 28th day, the rate of degradation in Group 4 (Av+T+CM) showed slowest degradation rate with a total weight 5 mg, followed by Group 3 (T+CM) and Group 2 (Av+CM) of 4 mg each and Group 1 (CM) showing the weight of 3 mg [Table/Fig-13]. The increase in collagen membrane weight on days 14, 18, and 21 was due to transient mineral deposition from calcium-phosphate ions in SBF solution onto the collagen surface. In addition, residual salt crystallisation and bound water may have contributed to the temporary rise in dry weight of the collagen membrane. Despite this, the overall trend shows progressive degradation of the membranes over 28 days.



[Table/Fig-13]: Comparison of drug release of the Group 2, 3 and 4 at various time frames.
The X-axis represent the time line in days and the Y-axis represent the total weight of the collagen membrane.

Total percentage of weight loss was measured using the formula weight loss (%) = $(W_0 - W_d)/W_0$, where W_0 is the actual weight before degradation (0th day) and W_d is the dry weight after degradation (28th day). Group 1 (CM) showed fastest rate of degradation and the percentage of weight loss observed was 0.50% followed by Group 3 (T+CM) and Group 2 (Av+CM) of 0.33% of percentage of weight loss each and slowest rate of degradation was observed in Group 4 (Av+T+CM) with percentage of weight loss observed was 0.16%, respectively [Table/Fig-14].



[Table/Fig-14]: Weight loss (%).
The X-axis represents all the four groups and the Y-axis represents the weight loss (%).

DISCUSSION

Type I collagen membranes are critical in periodontal regeneration, serving as scaffolds in GTR and mimic the extracellular matrix, creating an environment conducive to cellular activities essential for tissue repair and regeneration. Their hydrophilic and porous nature supports cell adhesion, proliferation and migration while promoting angiogenesis to ensure nutrient supply and waste removal [12].

Collagen membrane coated antibiotic shows anti-microbiological properties and improve healing of periodontal tissues [13]. Type I collagen membranes were coated tetracycline, a broad-spectrum antibiotic and *Adhatoda vasica* (Av), a medicinal herb. Tetracycline inhibits bacterial protein synthesis by binding to the 30 S ribosomal subunit, effectively targeting both Gram-positive and Gram-negative bacteria. Among these, periodontal pathogens such as *Streptococcus mutans* are particularly susceptible to its action [14]. Beyond its antimicrobial activity, tetracycline exhibits anti-inflammatory effects by inhibiting matrix Metalloproteinases (MMPs), which degrade extracellular matrix components, thereby

promoting tissue stability and healing. Its controlled release formulations further optimise localised drug delivery, minimising systemic side effects and enhancing periodontal outcomes [5].

Adhatoda vasica, a traditional Ayurvedic herb, complements tetracycline with its bioactive compounds, such as vasicine and vasicinone, which exhibit potent antimicrobial, anti-inflammatory and antioxidant properties. These compounds mitigate microbial colonisation and promote tissue repair by modulating inflammatory pathways and oxidative stress. When incorporated into collagen membranes, *Adhatoda vasica* enhances antimicrobial efficacy and supports fibroblast adhesion and proliferation, key processes for tissue regeneration. Together, tetracycline and *Adhatoda vasica* combine modern pharmacological strategies with traditional herbal medicine, creating a robust therapeutic platform for periodontal therapy [7,8].

In this study, the null hypothesis which states that *Adhatoda vasica*, alone or in combination with tetracycline, has no significant effect on antimicrobial activity or periodontal regeneration compared to the uncoated collagen membrane was rejected based on the results (antibacterial activity, hydrophilicity, surface roughness and degradation rate) of the study. A significant improvement was observed in the drug coated groups, particularly in the dual-coated (Av+T+CM) group hence accepting the alternate hypothesis. These findings highlight the added value of functionalising collagen membranes with antimicrobial agents, thereby supporting their role in enhancing periodontal regeneration.

In this study, dual-coated collagen membranes incorporating *Adhatoda vasica* and tetracycline demonstrated broad-spectrum antibacterial activity by significantly inhibiting *Staphylococcus aureus*, *Streptococcus mutans* and *Escherichia coli* colonies. The enhanced antimicrobial efficacy suggests a synergistic effect between the herbal bioactive agent and tetracycline, offering superior bacterial suppression compared to amoxicillin-impregnated membranes as reported by Cheng CF et al., and Barik B et al., [13,15]. This highlights the potential of biofunctionalised collagen membranes for infection control in periodontal and regenerative applications. The findings indicate that dual-drug coatings may overcome the limitations of single-drug coatings, which often exhibit a narrower antimicrobial spectrum [13,15].

FTIR analysis of *Adhatoda vasica* and tetracycline-coated collagen membranes revealed no additional functional groups, indicating that the coating process did not alter the chemical structure of the membranes. This confirms that the coating preserves the chemical integrity of the collagen matrix, ensuring its stability and functionality. These findings were consistent with those of Zhang J et al., and Belbachir K et al., who utilised FTIR spectroscopy to assess collagen integrity and demonstrated that structural preservation is crucial for maintaining biocompatibility and bioactivity. The absence of chemical modifications suggests that the dual coating enhances the membrane's therapeutic properties without compromising its original composition [16,17].

This study demonstrates that *Adhatoda vasica* and tetracycline-coated collagen membranes maintain hydrophilicity, which enhances fibroblast adhesion and proliferation, facilitating periodontal regeneration. This finding aligns with the observations of Becerra J et al., and Li Z et al., who reported that collagen-hyaluronate composite membranes promote fibroblast activity and tissue repair. The retained hydrophilicity of the dual-coated membranes likely improves cellular interactions, creating a favorable microenvironment for tissue regeneration. These results highlight the potential of biofunctionalised membranes in periodontal therapy by optimising cellular attachment, migration and proliferation [18,19].

AFM analysis of *Adhatoda vasica* and tetracycline-coated collagen membranes demonstrated an increase in surface roughness, which is known to enhance cell adhesion and proliferation. This observation

aligns with the findings of Stylianou A et al., and Hallab NJ et al., who reported that increased surface roughness positively influences cellular attachment and growth on biomaterial surfaces. The enhanced roughness likely improves protein adsorption, facilitating a more favourable microenvironment for fibroblast proliferation and tissue regeneration. These results suggest that surface topography modifications play a crucial role in optimising the bioactivity of collagen membranes for periodontal applications [20,21].

Adhatoda vasica and tetracycline-coated collagen membranes demonstrated a dual-phase drug release profile, beginning with an initial burst release within the first three hours, followed by a sustained release phase over an extended period of 48 hours. This controlled release mechanism ensures a rapid therapeutic effect, which is essential for early bacterial suppression, while the prolonged release maintains consistent antimicrobial activity for enhanced periodontal healing. Based on the results from drug release kinetics and inhibition zones, Group 4 (Av+T+CM) demonstrated the greatest antimicrobial activity against *S. aureus*, *S. mutans* and *E. coli*, likely due to increased drug availability, potentially due to its influence on drug diffusion and membrane porosity, enhancing drug release and contributing to a synergistic antibacterial effect. Hence, the incorporation of *Adhatoda vasica* was observed to enhance tetracycline and this dual release system effectively addresses both immediate infection control and long-term bacterial inhibition, optimising the healing environment for periodontal regeneration. The results suggest that herbal bioactives, when combined with conventional antibiotics, may serve as potential drug carriers, improving therapeutic outcomes. This observation aligns with the findings Owen GR et al., but in contrast with Collins AE et al., who highlighted the importance of controlled-release tetracycline in sustaining optimal drug concentrations at the site of infection, reinforcing the clinical significance of prolonged antimicrobial action. By integrating herbal compounds with antibiotic-based membranes, a synergistic effect can be achieved, potentially reducing antibiotic resistance risks while maximising treatment efficacy [22,23].

Furthermore, *Adhatoda vasica* and tetracycline-coated collagen membranes exhibited a slow degradation rate, which enhances membrane stability and provides a prolonged scaffold function for cell attachment and tissue regeneration. The delayed degradation helps maintain mechanical integrity, ensuring barrier function over an extended period, which is crucial for GTR in periodontal therapy. This property facilitates a sustained wound healing process, allowing for optimal fibroblast proliferation, extracellular matrix deposition and new tissue formation. The findings were in agreement with Moses O et al., and Vallecillo-Rivas M et al., who reported that tetracycline impregnation delays collagen membrane degradation, thereby enhancing periodontal healing outcomes [5,24]. The incorporation of *Adhatoda vasica* may further contribute to membrane stability by modulating enzymatic degradation and providing additional anti-inflammatory effects, which are beneficial for soft-tissue healing [8]. The extended degradation period also ensures sustained drug release, supporting long-term antimicrobial action against periodontal pathogens. By maintaining structural integrity for a prolonged duration, these membranes create a favorable microenvironment for tissue regeneration and bone remodeling [5,22].

This study introduces an innovative approach by integrating *Adhatoda vasica*, a natural herbal agent, with tetracycline for periodontal regeneration, offering a novel antimicrobial strategy. A comprehensive evaluation of antibacterial efficacy, membrane integrity, hydrophilicity, surface roughness, drug release kinetics and degradation rate ensures a thorough assessment. The use of advanced analytical techniques such as FTIR, AFM and plate reader analysis enhances the reliability of findings. Clinically, the combination of *Adhatoda vasica* and tetracycline shows promise in improving antimicrobial properties and slowing membrane degradation, potentially benefiting periodontal regeneration. Additionally, the

study confirms that these coatings do not compromise collagen membrane integrity, supporting its biocompatibility as a potential biomaterial.

Limitation(s)

Despite these strengths, the study has few limitations. First being in-vitro, the results may not fully translate to in-vivo conditions. The study tests only three bacterial species, which may not fully represent the diverse periodontal microbiome. Cytotoxicity and long-term biocompatibility assessments were not performed, leaving safety aspects unverified. The 28-day degradation analysis may not adequately predict long-term stability. Future research should focus on in-vivo validation, long-term biocompatibility and comparative studies with other herbal agents. Future research should validate the clinical efficacy and safety of *Adhatoda vasica* coated membranes through in-vivo studies and biocompatibility assessments. Expanding the antimicrobial spectrum, optimising herbal extract standardisation and comparing it with other herbal and synthetic agents will enhance clinical relevance. Additionally, evaluating mechanical strength, handling properties and long-term drug release will improve its applicability for periodontal regeneration.

CONCLUSION(S)

This in-vitro study of Type I collagen membranes functionalised with *Adhatoda vasica* and tetracycline demonstrate broad-spectrum antimicrobial efficacy, maintain structural integrity, promote fibroblast activity and facilitate effective periodontal tissue healing and regeneration. Ongoing clinical investigations will further optimise these materials for a range of periodontal conditions, ensuring that collagen-based membranes evolve into a more effective, customisable and dependable therapeutic modality for periodontal regenerative treatments.

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