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Original Research Article

Efficacy of Tulsi (*Ocimum sanctum*) Extract Incorporated onto Guided Tissue Regeneration (GTR) Membrane against Periodontal Pathogens

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ABSTRACT

Periodontitis is one of most common oral disease after dental caries in developing countries. The core part of periodontal treatment is to prevent further development of disease in order to restore the damaged tissues. Guided Tissue Regeneration (GTR) membrane has been shown to be a successful treatment modality; however, bacterial contamination on GTR membrane is the major associated concern. Thus, the present study was designed to increase treatment modality by embedding natural antibacterial agent (Tulsi extract) onto GTR membrane. A subgingival microbial sample of chronic periodontitis patient was collected and processed for bacterial isolation on ciprofloxacin embedded plates. Out of total 25 bacterial isolates, CDK-6 and CDK-13 showed maximum ciprofloxacin tolerance were selected for further studies. The 16S rRNA ribotyping identifies strains CDK-6 and CDK-13 as *Streptococcus* sp. and CDK-13 as *Rhodococcus* sp. respectively. Results showed that 50µg/ml Tulsi extract was competent to inhibit the complete growth of both the bacterial isolates. Suggesting, the sterile conditions in the periodontal pockets after phase-I therapy of Periodontitis can be maintained by coating GTR membrane with Tulsi extract.

Key-words: Tulsi, Periodontitis, GTR membrane, Streptococcus sp., Rhodococcus sp.

INTRODUCTION

Oral hygiene is the prime concern for healthy living. And after human microbiome project it has been known that several different species of microorganisms reside in various parts of our digestive tract imparting healthy as well as unhealthy effects. Microorganisms reside in the mouth are also responsible for the certain oral disease, amongst gingivitis and periodontitis are the most common. Periodontitis is a chronic infectious disease causing inflammation of teeth sustaining tissues (gingival, periodontal ligament, cementum and alveolar bone) leading to tissue destruction and tooth loss. [1]

Oral infections, including periodontitis have been long known to be related with oral pathogens (aerobic as well as anaerobic). More than 700 different types of bacteria domicile in oral cavity and are responsible for various oral diseases. [2] Major putative pathogens of periodontal disease are *Aggregatibacter actinomycetem-comitans, Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Streptococcus mutans, Prevotella intermedia, Treponema denticola* and *Bacteroides forsythus.* [3-11] Moreover, microorganisms such as enteric gram-negative rods, *Pseudomonads, Staphylococci,* and *yeasts* were also reported from obstinate periodontitis lesions that are not considered a normal oral microbiota. [12-14]

The core plan of periodontal treatment is to prevent further disease development in order to restore the damaged tissues. GTR has been shown to be a successful treatment modality for periodontal regeneration in many clinical studies. [15,16] The GTR technique prevents the apical migration of the gingival epithelium and allows the periodontal cells to repopulate in the area of the denuded root surface. Some of the human biopsy GTR studies have demonstrated new attachment level with bone fill. [15-17] The most common periodontal pathogens to inoculate on the GTR membrane are *A.* actinomycetemcomitans and *P. gingivalis.* [18] Markman, et al., 1995 [19] reported that within 3 min of the procedure there is bacterial contamination on the GTR membrane. Hence, incorporating antimicrobial agents in the GTR membrane may help in preventing the surface colonization. Several studies also showed drastic reduction in the colonization of periodontal pathogens on antibiotics conjugated GTR membrane. [15, 19, 20]

Currently, antimicrobial agents such as Doxycycline, Amoxicillin, Ciprofloxacin and chlorhexidine (mouthwash) are commonly used therapeutic agents against periodontal pathogens. The appearance of drug resistance along with undesirable side effects of antibiotics and chlorhexidine, [21] has triggered immense interest in the search for new antimicrobial alternatives of plant origin. [10] The most important advantage demanded for therapeutic use of medicinal plants in various diseases is their effectiveness also being economical, safe, and easy availability. [22]

Medical plants are used widely by traditional medical experts in their day-to-day practice. Tulsi (*Ocimum sanctum*) has been the pillar of holistic health system in India. Since time immemorial, Tulsi have been used broadly in the treatment of several diseases like bronchitis, upper respiratory infections, skin diseases, and malaria. Enteric pathogens, *Proteus, Klebsiella, Candida albicans, E. coli,* and *Staphylococcus aureus* are the microorganisms against which the antimicrobial property of Tulsi has been tested. [23, 24] Tulsi has long been recognized as possessing anti-inflammatory properties, antioxidant properties, [25] as a COX2 inhibitor [26] and also provides protection from radiation. [27, 28]

GTR membrane incorporated with natural antimicrobial agents may be beneficial to control membrane-associated infections in GTR therapy. The aim of the present study was, i) the isolation and characterization of periodontal pathogens and ii) to assess the efficacy of natural antibacterial agent (Tulsi extract) incorporated onto GTR membrane against the selected periodontal pathogen.

Materials and Methodology

2.1 Chemicals and Materials

Sodium chloride, Pyrogallol, Resazurin, EDTA, Acetic acid, Hydrochloric acid, and Potassium dichromate were procured from Loba-Chemie, India. Tris Buffer, Trisaminomethane Hydrochloride (Tris-HCl) was procured from SRL, India. Tryptic soya agar, Brucella agar with Hemin and Vitamin K1, Nutrient agar, Nutrient broth, Tryptic soya broth, Brucella broth with Hemin and Vitamin K1, Citrate agar, Urea broth, Triple sugar Iron agar, Boric acid, Ethidum bromide, Bromophenol blue, antibiotics and Gram staining Kit were procured from HiMedia, India, PCR Master Mix, Taq DNA polymerase, Magnesium chloride, 10X PCR buffer without MgCl2, dNTPs, PCR molecular weight markers, DNAase free water, and Agarose were procured from Sigma, U.S.A. All the chemicals used in study were of analytical grade.

2.2 Sample collection and transportation:

A sample of chronic periodontitis patient was collected from B. N. Patel Dental hospital, Anand, Gujarat. An in-vitro experimental design was formulated for the study. The experimental procedures were undertaken with the understanding and written consent of the patient. The study

was approved by institutional ethical committee. The sub gingival microbial sample was collected from the pockets with help of sterile paper point (Antaeos Munich, Germany). Sample was immediately stored in membrane sterilized (0.22µm, Sartorius A G, Goettingen, Germany) Reduced Transfer Fluid (RTF), [29] and transported in cold (~4°C) temperature to the laboratory for further studies.

2.3 Isolation of bacteria from collected sample

After arrival, sample was immediately processed for the bacterial isolation. For the isolation of bacteria, 1ml of sample was diluted in 9 ml of sterile RTF. The aliquots were serially diluted and plated on Tryptic soya blood agar. All plates were incubated in anaerobic jar (Pyrogallol was used to scavenge oxygen) at 37ºC. After 48 hours of incubation, well-defined isolated colonies were selected on the basis of colonial morphological characteristics.

2.4 Identification of selected Periodontal Pathogens

Selected bacterial isolates were identified by 16S rRNA gene sequencing. The DNA from bacterial isolates CDK-6 and CDK-13 were extracted by Bacterial DNA Purification Kit (GeNeiPure™, India). Polymerase Chain Reaction (PCR) was carried out in reaction mixture contained 25 µl 2X ReadyMix (Sigma, USA). Amplification of 16s rRNA genes were carried out using universal primer set 8F (5'-AGAGTTTGATCCTTGGCTC) and 1492R (5'-GGTTACCTTGTTACGACTT). Purified PCR products were sequenced using internal overlapping primers. [30] Sequences were initially analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using Blast (blastn) tool, and corresponding sequences were downloaded. Phylogenetic analyzes were performed using MEGA 5.10 software, and the Phylogenetic tree was constructed using the neighbor-joining distance method. [31]

2.5 Determination of Optimal growth medium

The selected bacterial isolates were grown in 1) Brucella, 2) Brucella Blood (1%), 3) Tryptic Soy, 4) Tryptic Soy Blood (1%), 5) Nutrient, and 6) Nutrient Blood (1%) agar/broth mediums for determination of most favorable growth medium. The optical density was measured at 600nm using spectrophotometer (Octa-1 Plus, Beacon, India).

2.6 Tulsi extract preparation:

Fresh plant leaves of Tulsi were obtained from local market. Leaves were separated from stem, washed thoroughly with tape water followed by sterile distilled water. Leaves were air dried under shaded condition at room temperature. The dried leaves were coarsely powdered until a homogenous powder was obtained. Ethanolic extract was prepared from the powder obtained using "Cold extraction method". [10]

2.7 Efficacy of Natural antibacterial agent incorporated onto GTR membrane against Periodontal Pathogen

Tulsi extract was used as a natural antibacterial agent and ciprofloxacin was used as a control antibiotic. Sterile reconstituted Type-I collagen was used as a guided tissue regeneration membrane. The membrane was aseptically sliced into 1x0.5 cm pieces and divided into two groups. Group 1 membrane pieces were soaked for 15 minutes with 5% Ciprofloxacin and group 2 with 5% Tulsi extract. The GTR membrane pieces were allowed to air dried for five minutes and placed at the center position in pathogen spreaded plates in aseptic condition. Plates were incubated at 37°C and after 48 hours the diameter of inhibition zone was measured using a Vernier Calliper. Statistical analysis was done using the software Statistical Package for Social Sciences (SPSS, version 12.0; SPSS Inc., Chicago, IL, USA). The disc diffusion values of Tulsi extract and Ciprofloxacin against CDK-6 and CDK-13 were entered in the SPSS software for statistical analysis. Descriptive statistics was retrieved, and data were analyzed using one-way analysis of variance (ANOVA), and

Tukey *post-hoc* test was used for comparison within the group. Statistical significance level was established at $P < 0.05$.

RESULTS

3.1 Isolation and Characterization of organisms

Bacterial isolates were characterized depending on their morphology using gram staining procedure, which showed that both bacterial isolates were gram positive. Strain CDK-6 and CDK-13 observed cocci and rod shape cells respectively.

8F and 1492R of primer set was used to amplify 16s rRNA gene (1.5kb fragment) using PCR amplification in selected isolates. The bands having sufficient concentration of DNA were eluted and purified (Fig.1). The nucleotide sequences of 16S rRNA gene were compared with known sequences in the NCBI database using Nucleotide Blast (blastn) to identify the most similar sequence alignment. The sequences of 16S rRNA gene of CDK-6 (1026-bp) and CDK-13 (1433-bp) were showed 96% and 99% homology with 16s rRNA gene sequences of *Streptococcus sp*. Strain CDK-6 (MH128327.1) and *Rhodococcus sp*. Strain KKDK-9 (KF741285) respectively.

The 16SrRNA gene sequence data of CDK-6 and CDK-13, and other related species were used to construct Phylogenetic tree. Mega 5.10 software package, a neighbor-joining distance method was used to align the sequences with the other known bacterial 16S rRNA gene sequences, and tree was generated and presented in Fig. 2.

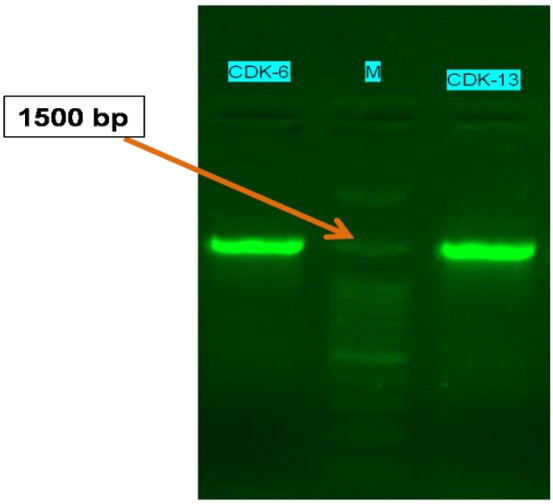


Fig. 1: Agarose Gel Electrophoresis of 16s RNA gene of CDK-6 and CDK-13. (M- Marker)

3.2 Comparative growth on different nutritive media

Both CDK-6 and CDK-13 were inoculated in six different nutrient mediums (1) Brucella Agar/broth medium, (2) Brucella Blood (1%) Agar/broth medium, (3) Tryptic Soya Agar/broth medium, (4) Tryptic Soya Blood (1%) Agar/broth medium, (5) Nutrient Agar/broth medium and (6) Nutrient Blood (1%) Agar/broth medium and next day following results were observed (Fig. 3). Bacterial isolates CDK-6 and CDK-13 comparatively grown well in Tryptic-soya broth and Brucella broth medium respectively. CDK-6 showed negligible growth in Nutrient broth with/without blood.

Table 1 Zone of Inhibition in (cm) By CDK-6 and CDK-13 supplemented with Tulsi Extract and Ciprofloxacin (Average ± SD)

GTR membrane embedded with	Zone of Inhibition	
	CDK – 6	CDK – 13
Tulsi Extract (50 µg/ml) (Conjugated)	0.14 ± 0.007	2.56 ± 0.05
Ciprofloxacin (50 µg/ml) (Control)	0.25 ± 0.05	2.36 ± 0.08

Table 2 Efficacy of Tulsi extracts against periodontal pathogens (ANOVA and Post-hoc-Tukey)

Organism	Tulsi Extract (50µg/ml)			Ciprofloxacin (50µg/ml)			*P* Value	*Post-hoc Test*
	Mean	SD	SEM	Mean	SD	SEM		
Streptococcus sp. Strain CDK-6	0.140	0.007	0.003	0.250	0.052	0.021	< 0.0001*	Ciprofloxacin > Tulsi †
Rhodococcus sp. Strain CDK-13	2.558	0.058	0.023	2.358	0.080	0.032	< 0.0001*	Tulsi > Ciprofloxacin ††

*Significant at 0.01 level; † Ciprofloxacin showing significant larger zone of inhibition; †† Tulsi extract showing significant larger zone of inhibition compare to Ciprofloxacin

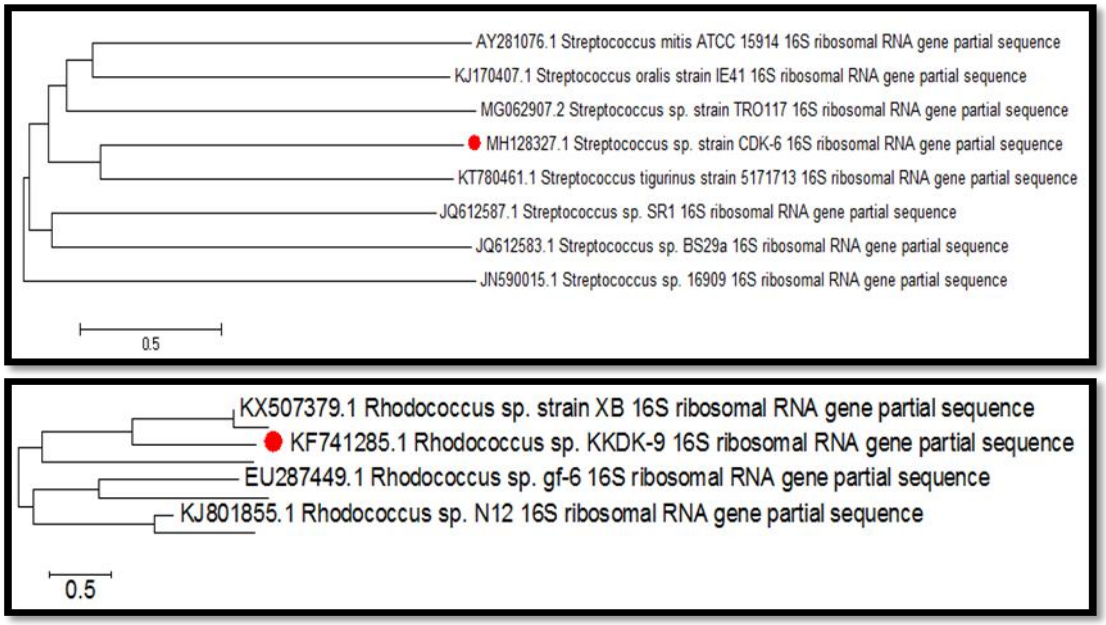


Fig. 2 Phylogenetic Tree CDK-6 and CDK-13

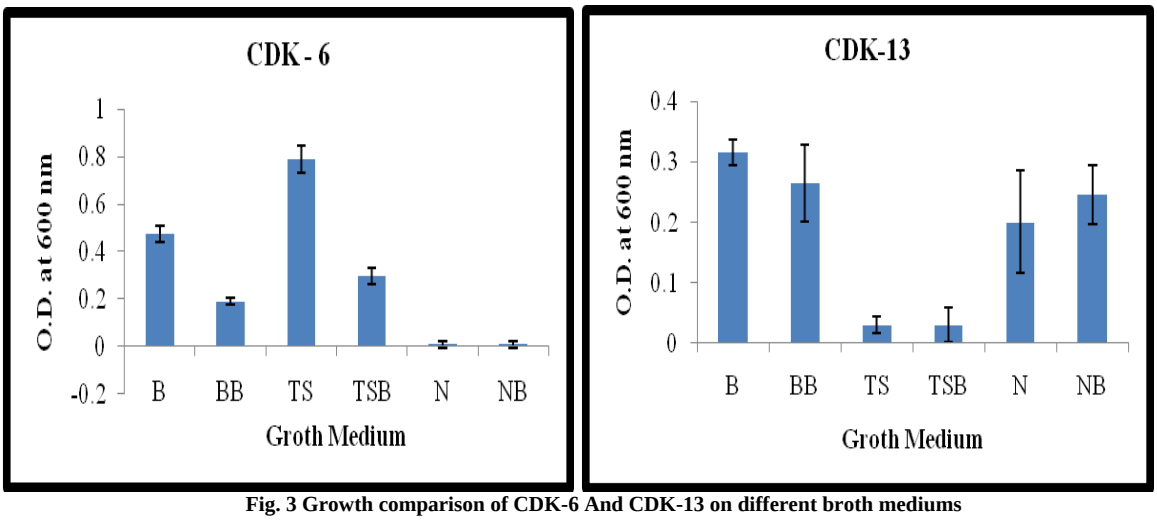
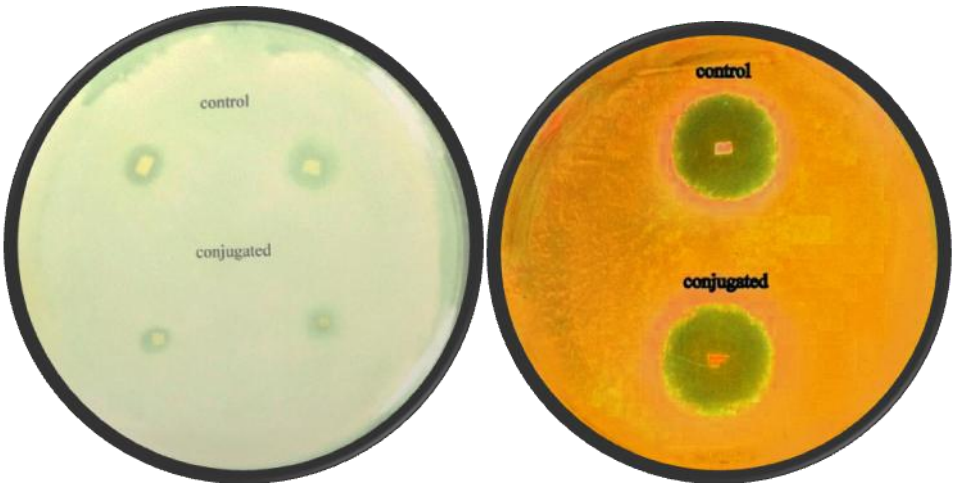


Fig. 3 Growth comparison of CDK-6 And CDK-13 on different broth mediums

B - Brucella broth, BB - Brucella blood (1%) broth, T - Tryptic soya broth, TB - Tryptic-soya blood (1%) broth, N - Nutrient broth, NB - Nutrient blood (1%) broth



A) Zone of inhibition in CDK- 6 B) Zone of inhibition in CDK-13

Fig. 4 Efficacy of Tulsi (*Ocimum sanctum*) extract incorporated onto GTR membrane against A) CDK-6 and B) CDK-13

3.3 Efficacy of Tulsi extract against Periodontal CDK-6 and CDK-13

Both the organisms showed different susceptibility against Ciprofloxacin and Tulsi embedded with GTR membrane. By knowing the concentration of conjugated Tulsi and antibiotic we obtained zone of inhibition (Fig. 4). Minor increment in the zone of inhibition size was observed in Tulsi conjugated GTR compared to control in CDK-13. While in case of CDK-6 ciprofloxacin containing GTR membrane (Control) showed significant increase in zone of inhibition compared to the Tulsi conjugated GTR membrane Table 1. One-way ANOVA also revealed that the mean zones of inhibition against Strains CDK-6 and CDK-13 was statistically significant ($P < 0.001$). Tulsi extract at 50µg/ml concentration, showed wider zones of inhibition against *Rhodococcus sp*. Strain CDK-13, which was slightly higher to Ciprofloxacin in comparison (Table 2). *Post-hoc* tests revealed a significant difference in the antimicrobial efficacy of Ciprofloxacin and Tulsi against *Streptococcus sp*. Strain CDK-6, with mean zone of inhibition obtained by Ciprofloxacin clearly exceeding the mean zones of inhibition obtained by Tulsi. While in case of *Rhodococcus sp*. Strain CDK-13 Tulsi extract showed wider mean zone of inhibition compared to ciprofloxacin.

DISCUSSION

Bacterial colonization on the GTR membrane negatively affects the outcomes of the periodontal treatment. [18] Adherence of the periodontal pathogens onto the GTR membrane may increase the rate of recurrence of periodontitis. [15] The effectiveness of systemic drug administration is not satisfactory as the drug is dissolved by dispersal over the whole body, and a minute level of the total dose actually reaches the periodontal pathogens. Moreover, it also increases the resistance power of the organisms against the antibiotic. But when the same drug is administered locally it acts only on the target area and its effectiveness also increases at low doses. [31]

The studies on GTR membrane coated with antibiotic have already been done for the release of the antibiotics which may prevent the bacterial colonization onto GTR membrane. [32,33] The market is presently laden with various popular therapeutic antimicrobial products. The quest for developing herbal remedies with an extensive array of antimicrobial properties without the side effects of synthetic medications is still going on for treating periodontal and other bacterial diseases. [34,35] However, very few literature is available that showed the effects of natural antibacterial agents coated onto the GTR membrane. Direct incorporation of the natural antimicrobial agent like Tulsi, Neem and other natural extracts on to the GTR membrane may allow more beneficial infection control, as the risk of drug resistance is always associated using traditional antibiotic treatments. Tulsi has been well-known for its antibacterial efficacy since ancient times in Indian. Essential oils extracted from Tulsi showed tremendous antibacterial activities. [36] Several studies have been reported antibacterial, antifungal, antioxidant, anti-inflammatory and immunomodulatory effects of Tulsi extract. [37-39] It has been suggested that Tulsi extract also strengthen the host immune response against infection by increasing the level of interleukin-4 and T helper cells. [38,40]

The aim of the present study is to coat the GTR membrane with Tulsi extract as it has the natural antibacterial capacity. Numbers of Studies have been reported the antimicrobial efficacy of Tulsi, particularly against periodontal pathogens is more commonly associated with initiation and progression of various periodontal diseases, especially aggressive periodontitis. [41- 48]

In the present study, we have isolated total 25 different bacterial isolates, out of which 2 bacterial isolates (CDK-6 and CDK-13) showing maximum ciprofloxacin were selected for further

studies. 16s rRNA gene sequencing of CDK-6 and CDK-13 showed the bacteria were *Streptococcus* and *Rhodococcu*s genus respectively. In vitro experiment showed that Tulsi at a concentration of 10% can effectively inhibit the growth of *A. actinomycetemcomitans*, compared to that of doxycycline. [10] Yamani, H.A., 2016 [49] reported 4.5 and 2.25 % of Tulsi essential oils completely inhibited the growth of *Staphylococcus aureus* and *E. coli* respectively. 10% concentration of green coffee extract showed no growth inhibition of periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis.* [15] In the present study, 50 µg/ml concentration of Tulsi extract was competent to inhibit the complete growth of both the organisms. Also, compare to ciprofloxacin, Tulsi extract was superior treatment agent against *Streptococcus* sp. CDK-6. While in case of *Rhodococcus* sp. CDK-13, ciprofloxacin was the best treatment agent, compared to Tulsi extract.

The current study is one of the first to assess the antimicrobial efficacy of Tulsi extract coated onto GTR membrane against isolated periodontal pathogens. Since there was limited literature available that could depict the way to increase the efficacy of treatment through GTR membrane by use of natural agents like Tulsi. The present study also encourages researchers to carry out further studies assessing toxicity, durability and other assessments followed by clinical trials to provide an insight into the activity of Tulsi against periodontal pathogens on a transient as well as a longitudinal basis to establish clear implications of Tulsi in periodontal disease management.

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