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Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

EFFICACY OF *AZADIRACHTA INDICA* (NEEM) EXTRACT EMBEDDED ONTO GUIDED TISSUE REGENERATION MEMBRANE AGAINST *NEISSERIA SP.* CDK-10 AND *MICROCOCCUS SP.* CDK-23 ISOLATED FROM PERIODONTAL PLAQUE

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ABSTRACT

Periodontitis is the major oral disease after dental caries observes predominantly in developing countries like India. Severe inflammation of the periodontal ligament along with destruction of alveolar bones and teeth supporting tissue are the major complications associated with it. The central part of periodontal treatment is to restrict the progression of disease along with restoration of damaged tissues. Guided Tissue Regeneration (GTR) membrane has been shown to be a potential treatment practice for tissue restoration. However, microbial contamination on GTR membrane is a major risk associated with treatment using GTR membrane. Increasing antibiotic resistance is another major concern associated with infection control. Thus, the current study was designed to enhance treatment efficacy of GTR membrane by coating of natural antibacterial agent (neem extract) onto GTR membrane. Previously collected sample of a chronic periodontitis patient was used for the isolation of periodontal pathogens. Out of total 25 bacterial isolates, CDK-10 and CDK-23 showed highest resistance to ciprofloxacin were consider for further studies. The 16S rRNA ribotyping identifies strains CDK-10 and CDK-23 as *Neisseria* sp. and *Micrococcus* sp. respectively. Results showed that 50mg/ml Neem extract was capable to inhibit the complete growth of selected bacterial isolates. Suggesting, the sterile conditions in the periodontal pockets after phase-I therapy of Periodontitis can be maintained by coating GTR membrane with Neem extract.

Keywords: Neem extract, Periodontitis, GTR membrane, *Neisseria sp.*, *Micrococcus sp.*

1. INTRODUCTION

Periodontal disease is an infectious disease causing inflammation of teeth supporting tissues such as gingival, periodontal ligament, cementum and alveolar bone leading to tissue destruction and tooth loss [1]. More than 700 diverse strains of bacteria reside in oral cavity are accountable for various oral diseases including periodontitis [2]. Major putative pathogens of periodontal disease are *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Actinobacillus actinomycetemcomitans, Streptococcus mutans, Prevotella intermidia, Bacteroides forsythus* and *Treponema* denticola [3-11]. Also, microorganisms like *Staphylococci, Pseudomonads,* enteric gram-negative rods, yeasts and fungus were also reported from obstinate periodontitis lesions that are not considered a normal oral microbiota [12-15].

The ultimate goal of periodontal treatment is to prevent further disease progression in order to reduce the risk of tooth loss and to restore the tissues that have been lost as a result of periodontitis. In many clinical studies, Grafted Tissue Regeneration (GTR) membrane has been shown to be a victorious treatment modality for the regeneration of damaged tissues [16, 17]. GTR membrane prevents the apical migration of the gingival epithelium and allows the periodontal cells to repopulate in the area of the denuded root surface. Moreover, human biopsies GTR have also demonstrated a new attachment level with bone fill [16-19].

As GTR membrane is highly nutritious and vital medium, capable to boost the tissue regeneration process; contradictory, it also promotes the growth of survived pathogens. Markman, et al., 1995 [20] observed that within few minutes of GTR procedure there was bacterial colonization on the membrane. *A. actinomycetemcomitans* and P. *gingivalis* are most common periodontal pathogens found on GTR membrane [19]. Numerous studies on conjugation of antibiotics with GTR showed drastic reduction in the colonization of periodontal pathogens [16, 20-21]. However, with ever increasing resistance to antibiotics and a need to

minimize antibiotic use has triggered immense interest in the search for new antimicrobial alternatives of plant origin. Incorporation of plant based natural antimicrobial agents onto GTR membrane not only prevent this surface colonization, but also have potential to reduce inflammation, oxidative stress and enhance regeneration process.

Medical plants have been used by traditional medical experts since immemorial time. One of such plant is *Azadirachta indica*, habitually it is known as Neem in India. Because of its abundant availability and has great medicinal values it is commonly consider as "Village Pharmacy" [22]. Neem sticks, aqueous leaves extract and its essential oils have been largely used in the treatment of several oral diseases like dental caries, gingivitis, and periodontitis [23]. Chemical components derived from Neem have been recognized for their various pharmacological actions such as antiviral, antifungal, antimicrobial, antibacterial, antipyretic, anti-inflammatory, antitumor, analgesic, antihelminthic, anticarcinogenic, antioxidant activity [24]. Hence, incorporation of neem extract onto GTR membrane not only provides antibacterial property but it also enhances the regeneration process. Thus, the aim of the present study was, i) to isolates and characterization of periodontal pathogens, and ii) to assess the efficacy of natural antibacterial agent (Neem extract) incorporated onto GTR membrane against the selected periodontal pathogen.

2. MATERIAL AND METHODS

2.1. Isolation of periodontal pathogens

Previously collected and stored sample of a chronic periodontitis patient was used for the isolation of periodontal pathogen. 1 ml of periodontal plaque sample stored in Reduced Transport Fluid (RTF) medium [25] was diluted to 10 ml sterile RTF medium and shaken for 10 minutes at 130 rpm in orbital shaker. An aliquot was taken and serially diluted with sterilized normal saline. All diluted aliquots (100 µl) were spreaded on Triptic soya blood (5%) agar plates and incubated at 37°C for 48 hours in anaerobic chambers. Morphologically distinct colonies were selected and screened for maximum level of ciprofloxacin resistance.

2.2. 16S rRNA gene sequence analysis and Phylogenetic tree

Two bacterial isolates CDK-10 and CDK-23 that showed the highest level of ciprofloxacin resistance were selected for further identification by 16S rRNA gene sequencing. The genomic DNA was purified by Bacterial DNA Purification Kit (GeNeiPure™, India). Amplification of 16s rRNA genes were carried out by Polymerase Chain Reaction (PCR) using universal primer set 8F (5'-AGAGTTTGATCCTTGGCTC) and 1492R (5'-GGTTACCTTGTTACGACTT). The reaction mixtures composed of 5 µl 10XPCR buffer, 1 µM each primer, 0.2 mM dNTPs, 2.5 units T*aq* DNA polymerase, 5 µl DNA template and sterile MilliQ water to a final volume of 50µl. PCR products were purified (GenElute, PCR clean-Up kit, Sigma) and sequenced at Xcelris laboratory, Ahmadabad, India. Initially, 16S rRNA gene sequences were analyzed at NCBI server (http://www.ncbi.nlm.nih.gov/) using Blast (blastn) tool. Phylogenetic analysis was performed using MEGA 5.10 software, and the Phylogenetic tree was constructed using the neighbor-joining distance method [26].

2.3. Optimization of growth medium

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 the determination of most favorable growth medium. The optical density was measured at 600nm using spectrophotometer (Octa-1 Plus, Beacon, India).

2.4. Preparation of Neem extract

Neem extract was prepared according to the method of Harjai et al., 2013 [27] with some modifications. Fresh plant leaves of Neem were obtained from our campus. 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. 10 g of Neem powder was taken and grounded in mortar with 10 ml sterile distilled water. The mixture was allowed to stand for 4 h, and the liquid homogenate was filtered (0.22µm membrane filter, Sartorius A G, Goettingen, Germany) and used as a stock solution (1 g/ml).

2.5. Incorporation of natural antibacterial agent onto GTR membrane

Neem extract was used as a natural antibacterial agent, while ciprofloxacin was used as control antibacterial agent. Both the antibacterial agents were membrane sterilized (0.22µm membrane filter, Sartorius A G,

Goettingen, Germany). Sterile reconstituted Type-I collagen was used as a guided tissue regenerated membrane. The membrane was aseptically sliced and separated into two distinct groups. Group A was embedded with 50 mg/ml Neem extract, while Group B was embedded with 50 mg/ml Ciprofloxacin. Embedded pieces of GTR membrane were allowed to air dried for 10-15 minutes and used to assess antibacterial efficacy test [1].

2.6. Efficacy of antimicrobial agents embedded GTR membrane against periodontal pathogens

Air dried pieces of antimicrobial agents were aseptically placed at the center position in pathogens spreaded plates and incubated at 37°C for 48 hours. After incubation, the zone of inhibition was measured using Vernier Caliper. The disc diffusion values of Neem extract and Ciprofloxacin against selected bacterial isolates 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 was established at $P < 0.1$ level.

3. RESULTS

3.1. Isolation and characterization of periodontal pathogens

The present study was undertaken to isolate periodontal pathogens from previously collected sample. Total 25 morphologically distinct bacterial colonies were selected and further screened for maximum level of ciprofloxacin resistance. Among them, two bacterial isolates CDK-10 and CDK-23 were found to resist highest concentration of ciprofloxacin. Both the isolates were characterized initially by gram staining procedure. Gram staining showed that isolate CDK-10 was gram negative and cocci shape. While, CDK-23 was gram positive cocci shaped cells.

The 16S rDNA sequence data showed that CDK-10 (1009-bp) and CDK-23 (1041-bp) were homologous with 16s rRNA gene sequences of *Neisseria subflava* strain KCOM: 2639 (KX096321.1) and *Micrococcus* sp. strain Ulm33 (KC618505.1) respectively. The 16S rDNA sequences of CDK-10 and CDK-23, 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. 1.

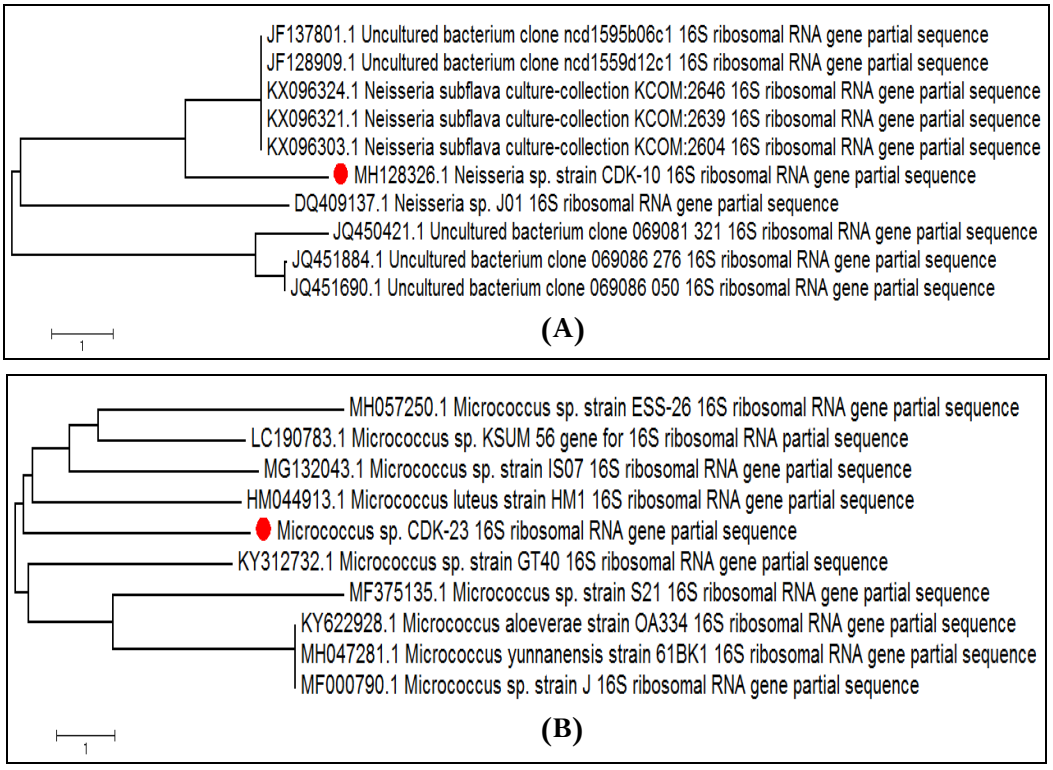
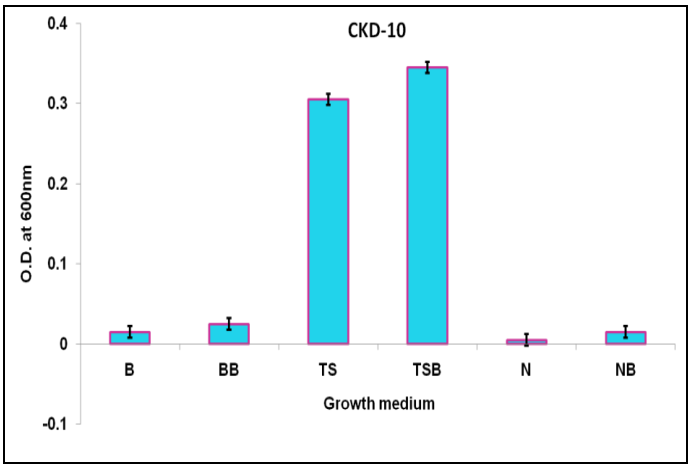


Fig. 1: Phylogenetic Tree (A) CDK-10 and (B) CDK-23

3.2. Comparative growth on different nutritive media

Both CDK-10 and CDK-23 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 optical density was observed. Both the isolates showed optimum growth in Tryptic-soya broth and Tryptic-soya blood broth medium (Fig. 2).



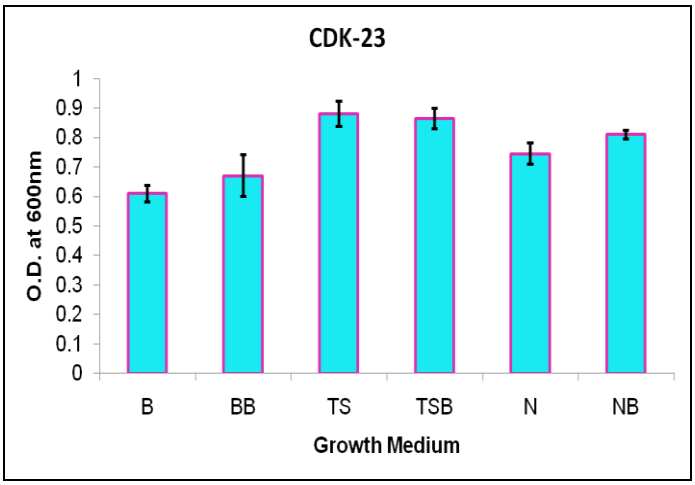
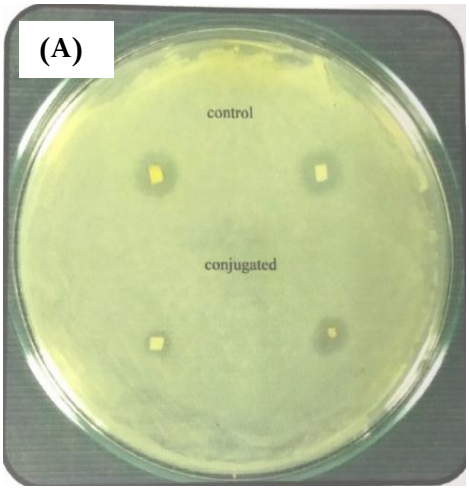


Fig. 2: Growth comparison of CDK-10 And CDK-23 on different broth mediums

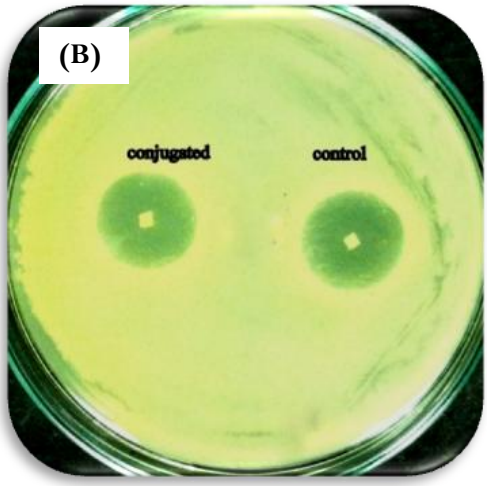
B- Brucella broth, BB- Brucella blood (1%) broth, T- Tryptic soya broth, TB- Tryptic-soya blood (1%) broth, N- Nutrient broth, NB- Nutrient blood (1%) broth

3.3. Efficacy of Neem extracts against strain CDK-10 and CDK-23

Aqueous neem extract in this study have showed to possess antibacterial activities against CDK-10 and CDK-23. However, the level of susceptibility against neem extract and ciprofloxacin was different in both the organisms. The zone of inhibition obtained in GTR membrane coated with neem extract and antibiotic is depicted in Fig. 3.



(A) Zone of inhibition in CDK- 10



(B) Zone of inhibition in CDK-23

Fig. 3: Efficacy of Neem extract incorporated onto GTR membrane against A) CDK-10 and B) CDK-23

Considerable increment in zone of inhibition was observed in ciprofloxacin compared to the neem conjugated GTR (Table 1). One-way ANOVA revealed that the mean zones of inhibition against Strains CDK-10 and CDK-23 was statistically significant at the level of $P < 0.001$ and $P < 0.1$ respectively. *Post-hoc* tests revealed the significant increments in the antimicrobial efficacy of Ciprofloxacin compared to neem extract against *Neisseria* sp. Strain CDK-10 and *Micrococcus* sp. Strain CDK-23.

Table 1: Efficacy of neem extract and ciprofloxacin coated GTR memrane against periodontal pathogens (ANOVA and Post-hoc-Tukey)

Isolated periodontal pathogens	Zone of inhibition (cm) GTR membrane supplemented with Neem extract (50µg/ml) Control			Ciprofloxacin (50µg/ml) Conjugated			Level of significance *P* value	Post-hoc Test
	Mean	SD	SEM	Mean	SD	SEM		
Neisseria sp. Strain CDK-10	0.40	0.06	0.02	0.67	0.02	0.01	< 0.01	Ciprofloxacin > Neem †
Micrococcus sp. Strain CDK-23	1.81	0.20	0.08	2.03	0.16	0.06	< 0.1	Ciprofloxacin > Neem †

† *Treatment with Ciprofloxacin showing significantly larger zone of inhibition*

4. DISCUSSION

Bacterial contamination on GTR membrane is a major problem adversely associated with periodontitis treatment [19] that enhances the rate of recurrence [16]. Incorporation of antibacterial agents on to GTR membrane definitely reduces this colonization. Several studies on antibiotic coated GTR membrane have been reported to minimize the chances of colonization of periodontal pathogens [27, 28]. However, the appearance of antibiotic resistance along with undesirable side effects triggered immense interest in the search for new alternatives or adjuncts. Numerous plant products such as neem, tulsi, green coffee, lemon, and others have been tested for their antimicrobial properties in the past with substantial success [1, 10, 30-34].

In present study, we attempted to get insight on the antimicrobial efficacy of Neem coated GTR membrane against periodontal pathogens isolated from chronic periodontal plaque. Interest on use of neem was based on its proven properties like antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, antipyretic, analgesic, and immunostimulant [23, 35]. Numbers of Studies have been reported the antimicrobial efficacy of Neem, particularly against periodontal pathogens [35, 36, 37]. Botelho et al., 2008 [38] in their experiments concluded that *Azadirachta indica* was highly efficacious in the treatment of periodontal disease thus exhibiting its biocompatibility with human PDL fibroblast. Incorporation of neem extract on to GTR membrane not only restricts the growth of pathogens but it also promotes the regeneration process. Neem leaves are tremendously rich in a large variety of secondary metabolites, possessing antioxidant properties that can modulate inflammation [24].

In present study, total 25 different bacterial isolates were separated, out of which 2 bacterial isolates CDK-10 and CDK-23 showed highest ciprofloxacin tolerance were selected for further studies. 16s rRNA gene sequence data of CDK-10 and CDK-23 identifies them as the members of *Neisseria* and *Micrococcus* genus respectively. Results of this *in vitro* study showed that 50 mg/ml aqueous Neem extract was competent to inhibit the growth of both the pathogens. Major mechanisms of action of Neem have been proposed by many authors earlier. Heyman, et al., 2017 [35] proposed the antibacterial activity of neem by virtue of its polyphenolic and polycationic compounds. These compounds basically reduce the adherence capacity of bacteria to cellular surfaces [35]. A study by Chava, et al., 2012 observed that 50% neem extract was effective to inhibit the growth of *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus sanguis* [37]. Results of current study also showed that ciprofloxacin was the superior treatment agent against both the organisms compared to neem extract. However, surplus properties such as antioxidant and anti-inflammatory with safety, abundant availability and cost effectiveness will surly signify the neem extract as a better candidate for GTR based treatment of periodontitis.

5. ACKNOWLEDGMENTS

The authors acknowledge B.N. Patel Institute of Paramedical and Science (Paramedical Division), Anand, Gujarat, India for financial support.

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