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ISOLATION AND IDENTIFICATION OF *STREPTOCOCCUS MUTANS* FROM PATIENTS WITH DENTAL CARIES: EVALUATING THE ANTI-BACTERIAL EFFICACY OF THE *AZADIRACHTA INDICA* EXTRACTS FOR THE TREATMENT OF PERIODONTITIS

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Keywords:

Periodontitis, *Streptococcus mutans*, *Azadirachta indica*, Anti-bacterial analysis, Minimum inhibitory concentration

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ABSTRACT: Periodontitis is a common infection occurring among a wide range of population globally. *Streptococcus mutans* is found to be the predominant pathogen responsible for dental caries. The present study concentrates on isolation and identification of the predominant pathogen from patients suffering from dental caries. The isolates were grown on various selective and differential media. Biochemical tests were performed to identify the pathogen. Further antibiogram assay was done to determine the sensitivity and resistance of the pathogen to several drugs. Aqueous, methanolic, and chloroform extracts of *Azadirachta indica* were prepared. The anti-bacterial efficacy and MIC of the extracts were evaluated. Samples were collected from 5 patients suffering from dental caries. From the morphological and biochemical observations, all the predominant isolates were found to be *Streptococcus mutans*. The isolates were sensitive to chloramphenicol, ciprofloxacin, Tetracycline, Gentamycin, and Methicillin. Resistance was observed for erythromycin, Penicilin, Vancomycin, Rifampicin, and cephalothin. Methanolic extracts (15 mm) showed higher inhibitory zones than the water (13 mm) and chloroform (11 mm). The MIC of the aqueous extract was found to be 7 mg/ml, whereas MIC of the methanol and chloroform extracts was found to be 5 mg/ml and 8 mg/ml. Methanolic extracts had higher anti-bacterial activity against *Streptococcus mutans*. Therefore, it is confirmed that *Azadirachta indica* extracts can be used for the treatment of periodontitis.

INTRODUCTION: Periodontitis is one of the most prevalent oral diseases in Tamil Nadu, inflicting a major burden on many segments of the population.

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This dental caries is the result of the growth of organisms on the smooth enamel and the surrounding tissues forming the biofilm.

Streptococcus mutans has been implicated in animal and human dental caries as a key etiological agent and is also involved in plaque development and accumulation of many toxins. The key factors contributing to the production and establishment of cariogenic biofilms are the acidogenic and aciduric (associated with acid tolerance) properties of

Streptococcus mutans' ability to synthesize extracellular glucans [1]. Anton van Leeuwenhoek, who first saw plaque bacteria under the micro-scope, made proposals for the possible presence of microorganisms in dental caries in the 16th century. The probable causal involvement of microorganisms with this disease was also proposed by some other early investigators following this research. Miller proposed the chemico-parasitic theory of the growth of caries in the late 1800s.

Microorganisms in the oral cavity also caused dietary carbohydrates to break down due to the action of their enzymes, according to Miller. They developed, and this pattern led to the production of acid and demineralization of enamel. All bacteria in the mouth were considered by Miller to be potentially cariogenic, a term is now known as the non-specific plaque hypothesis.

The first study of the role of streptococci from human carious lesions in the etiology of dental caries was by Clarke (1923). Streptococci with distinctive features were isolated by Clarke and named *Streptococcus mutans* [2].

Indian science has recognized the medicinal characteristics of neem (*Azadirachta indica*) since time immemorial. Neem is alleged to have a response to many incurable illnesses. Neem is appropriately referred to as sarvarogaghna. Since time immemorial, Indians have studied and used neem for environmental enhancement in various ways for both personal and community health [3]. Neem continued to remain a friend amid all the vicissitudes that India has gone through over the centuries. It is time for Indian heritage to be recognized, and our indigenous skills and expertise are aging on rising scales as a low-cost, effective ingredient for the achievement of the lofty target of wellbeing for all in the promotional and health care movements [4].

There are antiseptic ingredients in neem twigs. This shows how these individuals are able to maintain healthy teeth and gums. Neem is defined by Ayurveda as a herbal drug used to generate teeth and maintain dental hygiene. Apart from brushing teeth and massaging gums, neem is also used in the form of powder. The neem tree (*Azadirachta indica*) originates from the Indian subcontinent, and they were grown in more than 50 tropical countries around the world mostly in the dry regions [5, 6].

Therefore, the present study focuses on the identification and confirmation of predominant bacteria in dental caries, *i.e.*, *Streptococcus mutans*. The use of neem for dental caries through anti-bacterial examination is also investigated.

MATERIALS AND METHODS:

Sample Collection: The clinical sample from patients attending the outpatient ward at K.S.R Institute of Dental Science and Research, Tamil Nadu, India, were collected. The case history and socioeconomic of the patient is also recorded and presented in **Table 1**; the samples from the patients showing periodontitis were collected and analyzed for the study. The sterile cotton swabs were used for sample collection. These swabs were used for further analysis.

TABLE 1: CASE HISTORY OF PATIENTS WITH DENTAL CARIES

Sample	Source	Gender	Periodontitis	Years of Incidence	Depth of caries
1	Patient 12 years	F	Positive	3	Upto pulp
2	Patient 22 years	M	Positive	8	Upto pulp
3	Patient 24 years	M	Positive	5	Upto pulp
4	Patient 26 years	F	Positive	1	Upto dentine
5	Patient 32 years	F	Positive	14	Upto pulp

Isolation of Bacteria Responsible for Dental Caries: The swabs were preliminarily streaked on Nutrient agar for the cultivation of microbes and incubated at 37 °C for 24 h. Then the predominant microorganisms were streaked on Mitis-Salivarius bacitracin agar, Sucrose blood agar, Blood agar, bile aesculin agar aesculinhydrolysis agar.

Identification of Bacterial Isolates: The isolates were identified on the basis of their morphological, physiological, and biochemical characteristics. Biochemical characteristics include Gram staining, carbohydrate fermentation (mannitol, sorbitol, sucrose, lactose, rafinose, and inulin), and H_2S production.

Antibiotic Sensitivity Test: Antibiotic sensitivity test was carried out in Mueller- Hinton agar enriched with 5% blood. Himedia antibiotic discs were used. After the inoculum had dried, single discs were applied with a sharp needle. Penicillin G (10 unit), amoxycillin/ clavulanic acid (30 mcg), gentamicin (10 mcg), amikacin (30 mcg), teracycline (30 mcg), erythromycin (15 mcg), co-trimaxazole (23.75 mcg), cefuroxime (30 mcg), cefoperazone (75 mcg), ciprofloxacin (5 mcg), netillin (30 mcg), bifampicin (5 mcg), vancomycin (30 mcg), metronidazole, and methicillin. (Bacitracin 25 mcg) were the drugs used to determine the sensitivity profile of the isolated organism.

Collection and Extraction of Bioactive Compounds from the Neem: The neem leaves were collected from a neem tree at Kadachanullur, Namakkal District, Tamil Nadu, India. The leaves were dried completely for two days. This extraction was done for the separation of organic compounds from natural plant materials. 10 grams of dried plant powder were packed in a filter paper and placed in a cylinder of soxhlet apparatus and to successfully extract with all the three solvents (one solvent at a time) chloroform, water, and methanol, no plant residues were left. Before extraction of the bioactive compounds, the samples were flushed twice with respective solvents. Then the solvent was added into the soxhlet extractor at the ratio of 1:15 into each of the samples than the extract was concentrated by evaporation in a hot air oven at 400 C to 50 ºC, and they were stored at 4 ºC for further use.

Determination of Anti-bacterial Activity of Neem Leaves: Soxhlet extraction was done as shown below to obtain methanolic, chloroform, and water extracts from the dried leaves. The anti-bacterial activity was determined using the well diffusion method. The identified pathogen was streaked over the plates, and wells were cut. 100 ul of solvent extracts were added to the wells and incubated at 37 ºC for 24 h. The diameter of the zones of inhibition that resulted was measured.

Determination of the Minimum Inhibitory Concentration (MIC) of the Neem Extract: The inoculum, prepared from a 24 h old *Streptococcus mutans* culture contained approximately 106 CFU/ml plant exacts were diluted 1:10 in physiological saline (PS). These primary dilutions were further diluted in double concentrated BHI with 2% sucrose (BHIS2X) in a series of eleven 5-fold dilutions in the wells of a 2 well tissue culture micro-titer plate. Ten different concentrations of the solvent extracts were used. The plates were incubated for 48 h at 37 °C. The turbidity of the wells was measured at 690 nm using multi micro-titre reader and growth percentage was calculated to identify the MIC values. Plates were incubated at 48 h in anaerobic conditions.

RESULTS:

Collection of Specimens: In this study of dental caries, outpatients attending treatments in K.S.R Institute Dental Science & Research Tiruchengode were analyzed. The teeth swab sample collected in a patient infected by periodontitis. Deep teeth swab was collected from 5 different age-old patient (12 years, 22 years, 24 years, 26 years, 32 years). Samples from the patients showing the destruction of cementum and dentine were only analyzed.

TABLE 2: BIOCHEMICAL CHARACTERISTICS OF THE ISOLATES

S. no.	Test	Result obtained
1	Haemolysis	α
2	Bile esculin agar	+
3	VogesProskauer	+
4	Sensitivity to bacitracin	-
5	H_2s Production	-
6	Manitol	+
	Sorbitol	+
	Sucrose	+
	Lactose	+
	Rafinose	+
	Inulin	+

Identification of Bacterial Pathogen and Biochemical Parameters: The collected infected teeth Sample for Processed by plating into the Nutrient agar. After incubation, the predominant microorganism was plated on Mitis – Salivarious Bacitracin agar. All the colonies were streaked on Mitis – Salivarious Bacitracin agar, which on incubation of around 48 h appeared light blue to black color with, raised, glass or burned – sugar appearance. Also, from the biochemical tests, the isolated pathogens were confirmed to be *Streptococcus mutans*. **Table 2** shows the biochemical characteristics of the isolated pathogens.

The isolates were inoculated on blood agar for observing their hemolytic pattern, and that was identified as alpha-haemolytic. **Fig. 1** shows the growth of *Streptococcus mutans* on MSB agar, alpha hemolysis was observed on blood agar **Fig. 2**, aesculin was hydrolyzed aesculin hydrolysis agar **Fig. 3**, bile aesculin was hydrolyzed on bile aesculin agar **Fig. 4**.

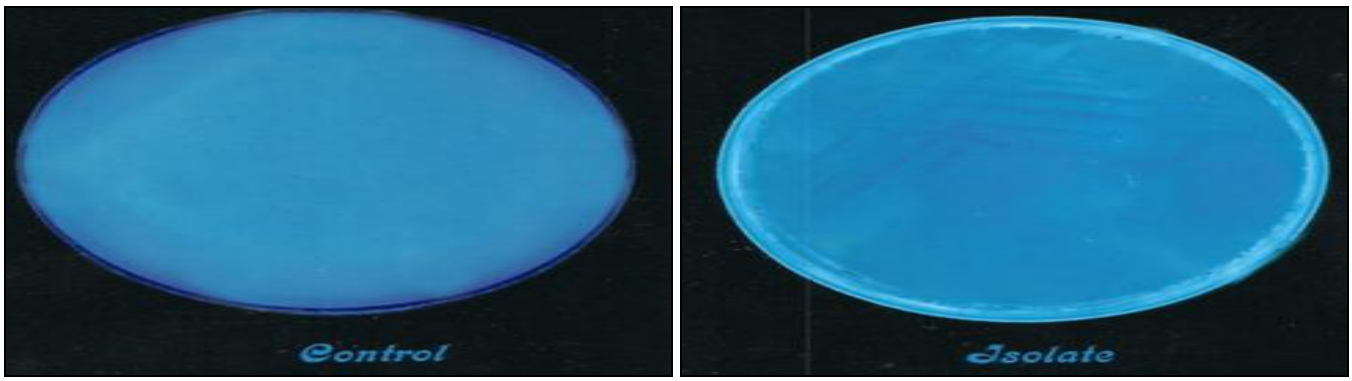

FIG. 1: GROWTH OF *STREPTOCOCCUS MUTANS*ON MSB AGAR

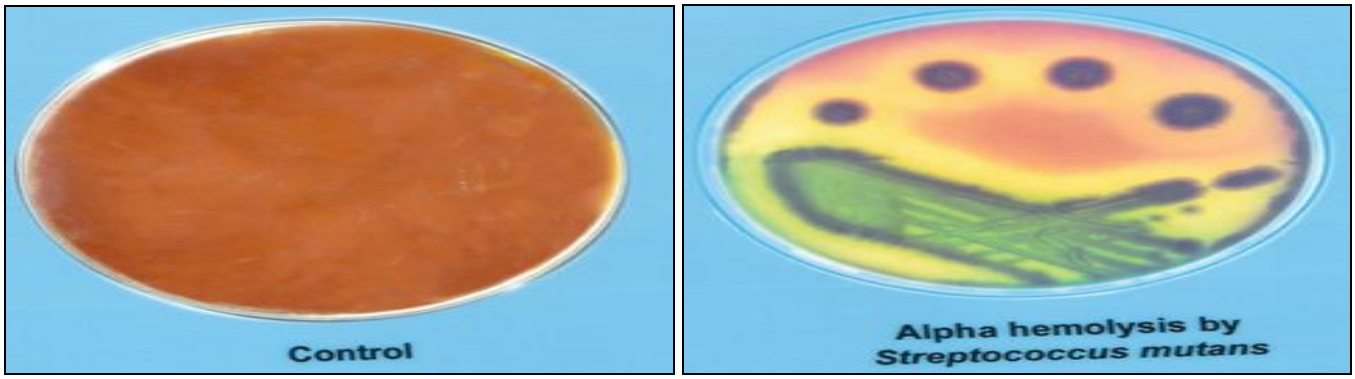

FIG. 2: ALPHA HEMOLYSIS OF *STREPTOCOCCUS MUTANS*ON BLOOD AGAR

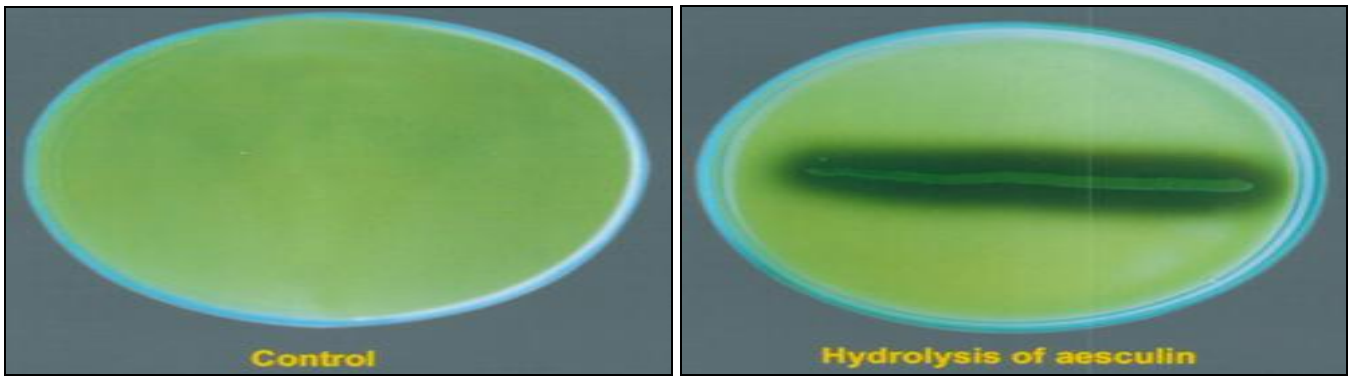

FIG. 3: GROWTH OF *STREPTOCOCCUS MUTANS* ON AESCULIN HYDROLYSIS AGAR

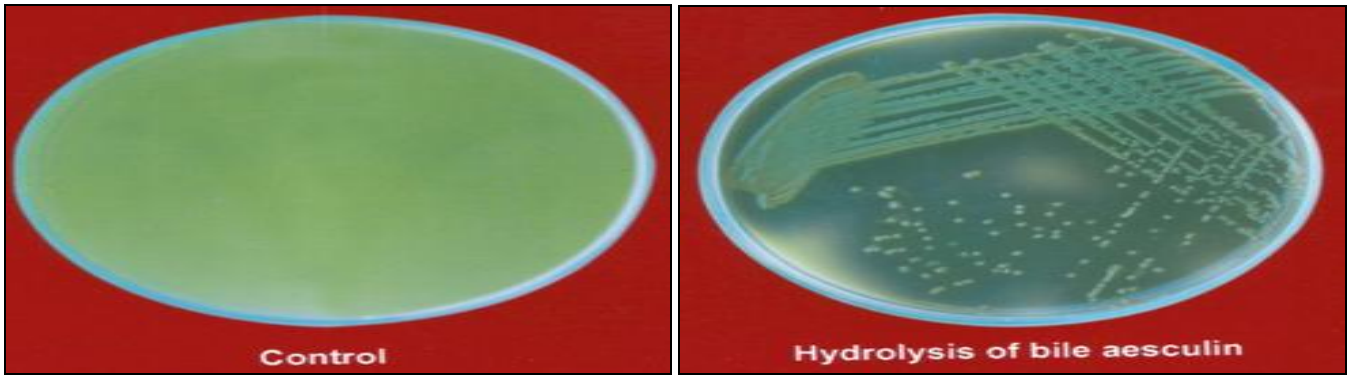

FIG. 4: GROWTH OF *STREPTOCOCCUS MUTANS* ON BILE AESCULIN AGAR

Antibiogram Assay: The positive teeth swab sample isolate of *Streptococcus mutans* was tested for their susceptibility against different antibiotics as shown in **Table 3**. The positive isolate was sensitive to chloramphenicol (30 mcg). The other sensitive antibiotics, ciprofloxacin (5 mcg), Tetracycline (30 mcg), Gentamycin (10 mcg), and Methicillin (5 mg) and resistant to erythromycin (15 mcg) Penicillin (10 mcg), Vancomycin (30 mcg), Rifampicin (5 mcg) and cephalothin (30 mcg) show the **Table 3**.

TABLE 3: ANTIBIOGRAM PROFILE

Sl. No	Antibiotics		Zone of inhibition	Result
	Name	Concentration		
1	chloramphenicol	30 mcg	21 mm	Sensitive
2	Ciprofloxacin	5 mcg	20 mm	Intermediate
3	Tetracycline	30 mcg	19 mm	Intermediate
4	Gentamicin	10 mcg	19 mm	Intermediate
5	Vancomycin	30 mcg	13 mm	Resistant
6	Methicillin	5 mcg	10mm`	Resistant
7	Penicillin G	10 mcg	Nil	Resistant

Anti-bacterial Activity and Minimum Inhibitory Concentration (MIC) Determination: Anti-bacterial analysis showed significant inhibitory zones against isolated *Straptococcus mutans*.

Methanolic extracts (15 mm) showed higher inhibitory zones than the water (13 mm) and chloroform (11 mm). Hence the order of anti-bacterial activity was observed to be methanol > water > chloroform.

The MIC of the aqueous extract was found to be 7 mg/ml, whereas MIC of the methanol and chloroform extracts were found to be 5 mg/ml and 8 mg/ml. Methanolic extracts had higher anti-bacterial activity against *Streptococcus mutans*. **Fig. 5** shows the growth of the pathogen on treatment with various concentrations of neem extracts.

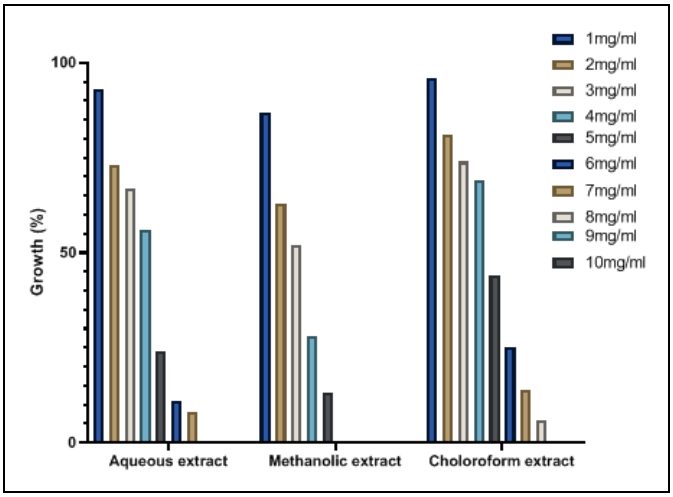

FIG. 5: MIC OF THE *AZADIRACHTA INDICA* EXTRACTS

DISCUSSION: Periodontitis is a common infection occurring among a wide range of populations globally. People with poor teeth care have high risks of periodontitis. This disease occurs by the formation of biofilms on the dentine layer of the teeth. *Streptococcus mutans*is found to be the predominant pathogen responsible for dental caries [7]. The present study concentrates on isolation of *Streptococcus mutans* from patients suffering from dental caries. Five isolates were obtained, and all the isolates were found to be *Streptococcus mutans* by morphological and biochemical characteristics. *Streptococcus mutans* isolated in this study were resistant to a number of Antibiotics like a tetracycline, Gentamycin, vancomycin, methicillin, Penicillin-G. Some of antibiotics sensitive *Streptococcus mutans* nearly (Chloro-hexcidine is current prophylaxis of dental plaque mainly relies on the use of antimicrobials.

Streptococcus mutans and *Streptococcus sobrinus* are the microorganisms most generally profound in human samples. Occurrence of this species is worldwide, and *Streptococcus mutans* is the most isolated bacterium. *Streptococcus sobrinus* is commonly found in mixed colonies with *Streptococcus mutans* and is believed to be chiefly responsible for the advancement of smooth surface caries. *Streptococcus rattus*, which was first isolated from rats [8], has also been identified from human samples [9]. The essential role of streptococci in the development of dental caries was investigated even in the early years (1924) by Clarke. Keyes (1960) had reported the direct

participation of particular microbes in dental caries using animal experiments conducted on hamsters. Out of the five specimens analyzed, four specimens showed positive for *Streptococcus mutans*. Several reports have already proved the correlation between streptococci and dental caries. VamHoute (1980) evidenced that population with a higher caries incidence have comparatively higher ranges of *Streptococcus mutans* than a population with a lower incidence of dental caries. *Streptococcus mutans* were continuously isolated from deep infections, but less frequently from tooth surrounding areas [10].

The neem (*Azadirachta indica*) tree has been commonly known as "the village pharmacy" due to the significant medicinal properties of its leaves, sap, fruit, seeds, and twigs. The neem tree has been widely used in traditional medicine. The neem has nearly 100 bioactive substances, including nimbolide, nimbidin, azadirachtin, and other triterpenoids and limonoids. The medicinal and biological uses of *Azadirachta indica* are extraordinarily diverse [4]. In India, the saps are used for the treatment of fever, general debilitation, digestive issues, and skin disorders. The bark gum is used for respiratory and other diseases. The leaves are used for digestive disturbances, intestinal parasites, and viral infections. The fruit is used for fungal infections, malaria, diabetes, bacterial infections, fevers, inflammatory diseases, debilitations, fertility prevention, and as a potent insecticide [11]. The neem plays a vital role as a systemic antibiotic if it is ingested by mouth. Therefore, in the present study neem (*Azadirachta indica*) extracts were used to determine the anti-bacterial efficacy against isolated *Streptococcus mutans*. Aqueous, methanol, and chloroform extracts were used, and methanol extracts showed higher anti-bacterial activity than the other two extracts. Hence, Neem extracts can be used for the treatment of dental caries.

CONCLUSION: Samples from patients with dental caries were collected in sterile swabs and cultivated. From the morphological and biochemical characteristics, the isolated bacteria were found to be *Streptococcus mutans*. Methanolic, chloroform, and aqueous extracts of the *Azadirachta indica* were prepared using the soxhlet apparatus. The anti-bacterial activity was determined against the isolated *Streptococcus mutans*.

Methanol extracts showed higher anti-bacterial activity than the aqueous and chloroform extracts. MIC of the methanol extract was found to be 5 mg/ml. This confirms that *Azadirachta indica* extracts can inhibit the growth of dental caries, causing *Streptococcus mutans*.

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CONFLICTS OF INTEREST: Authors declare no conflict of interest.

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