



## Anti- Cariogenic Activity of Piper Betel Leaf Extracts Against Streptococcus Mutans and Streptococcus Oralis By in Vitro

Dinesh M.D<sup>1</sup>, Anjana. J. C<sup>2</sup>, Neethu George<sup>1</sup> Nithya Jayan<sup>3</sup>,  
Sharannya Mohan<sup>4</sup>, Meenatchisundaram.S<sup>5</sup>.

<sup>1</sup>Department of Microbiology, Pazhassiraja College, Pulpally, Wayanad, Kerala, South India

<sup>2</sup>Department of Microbiology, SNGIST Arts And Science College, Mannakapady, Kuramallor Cochin, Kerala,

<sup>3</sup>Department of Microbiology, Sreenarayana College, Alathur, Palakkad, Kerala, South India

<sup>4</sup>Department of Biochemistry, Dr. N.G.P. Arts And Science College, Coimbatore, Tamil Nadu, South India.

<sup>5</sup>Department of Microbiology, Nehru Arts And Science College, Thirumalayampalayam, Coimbatore – 641 105, Tamil Nadu, South India.

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**ABSTRACT:** In our study we assessed the anti- cariogenic activity of Piper betel leaf extract on Streptococcal dental caries. The aqueous extracts (Hot and cold) were judged by the agar well diffusion method and their actions further determined by Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assesses. The extracts were fractionated by Thin Layer Chromatography (TLC). Column chromatography was used to separate the active compounds from the fusion and GC-MS (Methanolic extracts) was used to categorize the phyto components of the fractions. The inhibition zone diameters of the extracts ranged from 12 and 11 mm respectively. The crude methanolic extract on GC MS showed the presence of 12 active phyto- compounds. The results took after these studies substantiate the use of Piper betel leaf as traditional medicine for the development of effective antimicrobial.

**Keywords:-** Piper betel, Minimum Inhibitory concentration (MIC), Minimum bactericidal concentration (MBC), GC- MS.

### I. INTRODUCTION

Oral health is a fundamental component of health the whole time life. Poor oral health and untreated oral diseases and environment can cover a significant impact on eminence of life. They can influence the most basic human requirements, as well as the ability to eat and drink, swallow, maintain proper nutrition, smile, and communicate. The mouth is the doorway to our body and its first procession of defence. It performs more necessary functions than any other part in the body like it add protection from harmful bacteria getting into our blood stream and lungs, lubricates itself to permit us to swallow and to speak, holds the implements we need to chew and commence the digestive practice for our food and Participates in the breathing process. Poor oral hygiene can direct to a multiplicity of dental and medical problems such as gum disease, infection, bone loss, heart disease, strokes and more. Dental caries and oral cancer are emerging disease and concurrent with oral hygiene.

Dental caries or cavity in teeth is one of the multifactorial disease in humans be deficient in of oral hygiene. Dental caries is a lifetime disease, with a possibility of new lesions that continue to befall humanity. The highest priority risk group is between 11–14 years of age (1). Females have usually demonstrated higher decayed, missing and filled scores than males of the same age. It was long argued that certain races (such as Africans & Asians) enjoy greater caries-resistance (compared to Europeans and Americans). However, today it is believed that an environment with its typical culture, socioeconomic status, life style & dietary pattern can have a greater impact on caries resistance or development than the so-called inherent racial attributes (2, 3). Regrettably oral hygiene exercise is very meagre in our society. As a result dental caries is highly prevalent in developing countries. In India, caries are standstill exceedingly widespread in nature. Various healths related programmes and strict oral hygiene practice make decline in the prevalence of dental caries industrialized countries.

Accumulations of bacteria especially streptococcal species play a major role while causing cariogenic lesions. The principle causative agent of dental caries are a group of Streptococcal species collectively referred

\*Corresponding Author: Dinesh M.D,

<sup>1</sup>Department Of Microbiology, Pazhassiraja College, Pulpally, Wayanad, Kerala, South India

to as the *mutants*, of which *Streptococcus mutans* and *Streptococcus mitis* are the most important agents of human dental caries. *Streptococci* are gram positive cocci arranged in chains. They are widely distributed in nature. Many of them are normal bacterial flora in humans. A few of them are pathogenic. Viridians *streptococci* are among the first bacteria to colonize the human mouth after birth and generally the predominant colonizing strains are *S. mitis* and *S. oralis*. Dental caries is a localized, transmissible, pathological infectious process that ends up in the destruction of hard dental tissue (4).

Antibiotics are constantly in use for treating dental caries, it has been used in aspire of iradication or inhibition of biofilm in the oral cavity. Penicillin, methicillin, ampicillin, erythromycin, cephalothin, chlorhexidinedigluconate and many other antibiotics have been reported to effectively prevent dental caries more than ever viridians streptococci in humans and animals. These antibiotics are never used clinically because of many adverse effects such as hypersensitivity reactions, supra infections and teeth staining. A high concentration of antibiotics causes coagulation of the intracellular constituents. As a result, the cytoplasm becomes congealed, with a resulting reduction in leakage. These drawbacks defend further research and development of natural antibacterial plant extracts that are safe for the host or specific for oral pathogens. The World Health Organisation (WHO) estimates that 80% of the people living in developing countries almost exclusively use traditional medicines. This means approximately 3300 million people use medicinal plants on a regular basis. Medicinal plants used in traditional medicine should therefore be studied for safety and efficacy. Hence in our present study we investigate the anti- cariogenic activity of Betel leaf extracts against *Streptococcal* dental caries.

## II. MATERIALS AND METHODS

### 2.1. Collection of Streptococcal dental caries

*Streptococcus mutans* (MTCC- 497) and *Streptococcus oralis* (MTCC – 2696) were obtained from MTCC- IMTEC Chandigarh in lyophilized form was used for our present study and brought to pure culture on Todd Hewitt agar and Blood agar plates and maintained at 4°C.

### 2.2. Collection of Medicinal Plants and preparation of plant extracts

*Piper betel* leaves were obtained from the region of Wayanad District, Kerala, South India and the extracts (cold and hot extracts) were prepared by the method of Uhegbuet *al.* (2005) using distilled water as the solvent. About 20 g of powdered sample of the herb was extracted by soaking in 180 mL of distilled water in a beaker, stirred for about 6 min and left overnight. Thereafter, the solution was filtered using filter paper (Whatman No. 1) and the extracts were evaporated to dryness under reduced pressure below 40°C. The crude plant extract was subjected to phytochemical analysis for detecting the chemical compounds in it (7, 8).

### 2.3. Antimicrobial activity of Betel leaf extracts against clinical Streptococcal Dental caries -Well diffusion method

The agar well diffusion method was adopted according to (8) Kavanagh, (1972) to assess the antibacterial activity of the prepared extract. A loop full of bacterial stock suspensions was thoroughly mixed with 100 ml of sterile nutrient agar and kept for overnight incubation. 0.1ml of overnight culture was spread on the surface of Muller Hinton agar plates and wells were cut. The wells were filled with extract of various concentrations of about 20µl to 80 µl. After 24 hours at 37°C, the agar plates were examined for the zone of inhibition and the zones were measured in millimeters. The zones were measured, averaged and the mean values were tabulated.

### 4.4. Minimum Inhibitory Concentration (MIC) – Dilution Method

Different concentrations of plant extract ranging from 1mg to 10mg/ml were added into test tubes which containing 1ml nutrient agar. To this 50µl of an overnight broth culture of the test organisms were inoculated and incubated the tubes for 24 hours at 37°C. A tube containing 5 ml sterile nutrient broth was inoculated with the drop of an overnight broth culture and kept at 4°C in a refrigerator overnight, to be used as standard for the determination of complete inhibition.

### 2.5. Minimum Bactericidal Concentrations (MBC)

Dilutions and inoculations were prepared in the same manner as described for the determination of MIC. The control tube without plant extract is immediately sub cultured (Before incubation) along with the tubes with plant extract at 37°C overnight. The MIC of the control organism was read to check that the drug concentrations are correct. The growth was compared with control tube, which represents the original inoculum. If similar number of colonies presents it indicates bacteriostatic only. A reduced number of colonies-indicates a partial or slow bactericidal activity and if no growth was observed then the whole inoculum has been killed. The highest dilution showing at least 99% inhibition is taken as Minimum Bactericidal Concentrations (MBC).

### 2.6. Thin layer Chromatography

Prepared silica (Silica gel G, Hi Media of 60 to 120 meshes) were spotted with 10 µL extracts using capillary tubes. The solvent system used as mobile phase was Ethanol, Methanol, Ethyl acetic acid (50:50:1µl). Following Thin Layer chromatography bands appeared and was visualized in UV- chamber at wavelength of 254 nm. The spot are scraped using sterile spatula and dissolved in solvent system. After centrifugation the supernatant which containing the plant compounds was collected and performed well diffusion method for assessing inhibition properties of separated compounds. (9, 10)

### 2.7. Column Chromatography

After assessing the suitable solvent system for getting maximum separation via TLC examination, [Ethanol, Methanol, Ethyl acetic acid (50:50:1µl)] was used to grain the silica packs tightly. The *T. Chebula* extracts dissolved in solvent (same as in the column) was added to the column using a Pasteur pipette. The solvent was continually added to the top of the column until each band resolves and was carefully collected. The collected fractions were examined for inhibition properties of eluted fractions.

### 2.8. GC-MS analysis

Based on the inhibition properties of fractions collected after coloumn chromatography, fraction 1 and 8 were subjected for GC MS analysis. Chromatographic separation was carried out with Agilent Technologies-7890 GC System, 5975C inert MSD. The column used was Agilent 190913-433; 325°C capillary column measuring 30mX250µm with a film thickness of 0.25µm. The carrier gas used was Helium at a flow rate of 1.1 ml/min. 1µl sample injection volume was utilized. The inlet temperature was maintained as 100 - 250°C. The mass spectra of compounds in samples were obtained by electron ionization (EI) at 70 eV, and the detector operated in scan mode from 20 to 600 amu. Identifications were based on mass spectral matching with standard compounds in NIST and Wiley libraries. The essential chemical constituents were identified by matching mass spectra with spectra of reference compounds in mass spectral library of National Institute of Standards and Technology (NIST 147). The relative amounts of individual components were expressed as percent peak areas relative to total peak area.

## III. RESULT AND DISCUSSION

Dental caries causing Streptococcal species (*Streptococcus mutans* and *Streptococcus oralis*) were obtained from IMTECH, Chandigarh. The organisms were subcultured on Todd Hewitt agar. The medicinal plants were collected from in and around Wayanad region and extracts were prepared using distilled water as solvent. Preliminary screening and identification of bioactive chemical element present in the *Piper betel* leaf were carried out. Within the present study we investigated the presence of Tannin, Phenols, sugars, Reducing sugars, proteins (Table- 3).

The hot extract of concentration 80µl of hot extract produced 12mm and 11mm zone respectively whereas the cold extract of *Piper betel* extract produced 4mm, 3.2mm zones correspondingly (Table – 1 & Fig 1 and Fig 2). When the concentration of the extracts were decreased which showed slight decrease in inhibition zones.

Based on the TLC results *Piper betel* leaf extracts were subjected for column chromatography using silica gel as adsorbent. The sample was eluted using the same solvent later used in TLC 15 fractions were collected at the flow rate of 0.6ml/minute and fractions were concentrated at room temperature. The fraction 6 showed the inhibition properties against Streptococcal dental caries. These fractions were further subjected to GCMS analysis. The quantitative determination of the chemical compounds was based on the comparison of peak areas of samples with those in GC MS library.

**Table – 1:** Antibacterial activity of cold and hot aqueous extracts of Piper betel leaf extract against S.oralis and S.mutans - Well cut Method

Plant extract	Conc. µg/ml	Hot Extract (Zone in mm)		Cold Extract (Zone in mm)	
		S.mutans	S.oralis	S.mutans	S.oralis
Piper betel	80	12	11	4.0	3.2
	40	10.2	9.8	3.1	2.8
	60	7.2	6.4	2.6	1.9
	20	4.9	3.9	1.8	1.1

**Table 2 -** Minimal inhibitory concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of Piper betel extract against S.mutans and S.oralis

Plant extracts	MIC/MBC	Hot Extract - MIC (mg/ml)
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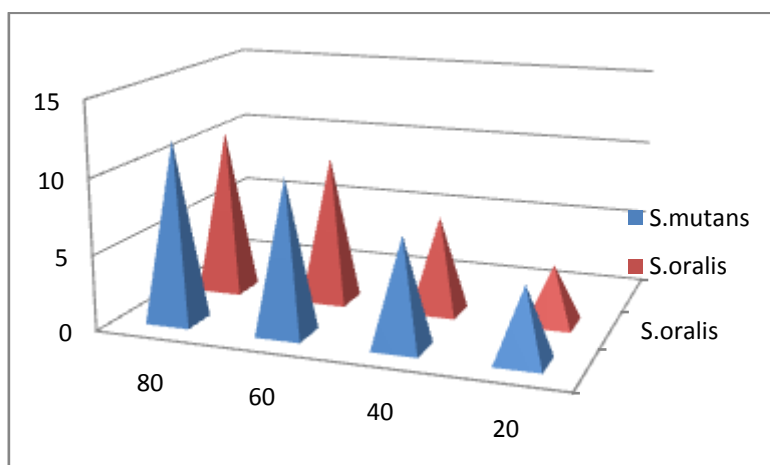
		S.mutans	S.oralis
Piper betel	MIC	2	3
	MBC	5	6

**Table: 3 - Phytochemical result**

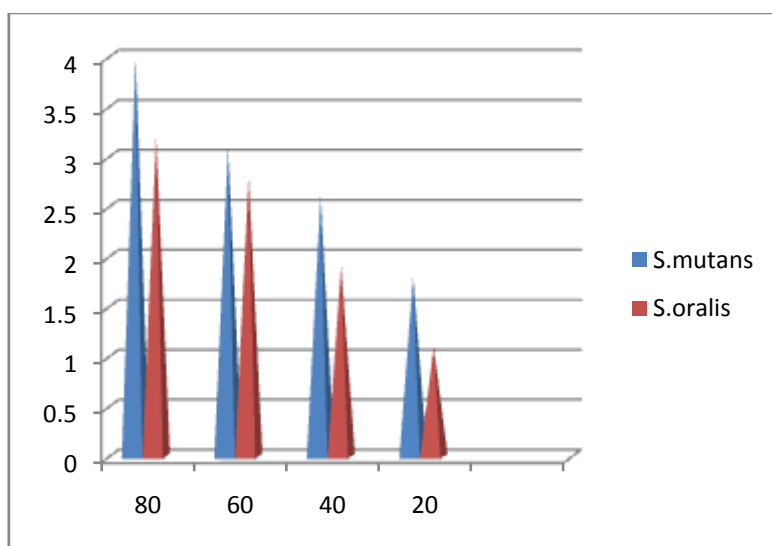
Tests	Result
Saponin	-
Tannin	+
<b>Anthraquinone</b>	-
<b>Flavanoid</b>	-
Salkowsky	-
<b>Phenol</b>	+
Amino acids	-
<b>Sugars</b>	+
<b>Reducing sugars</b>	+
<b>Proteins</b>	+

**Abbreviation:** - + (Positive) - (Negative)

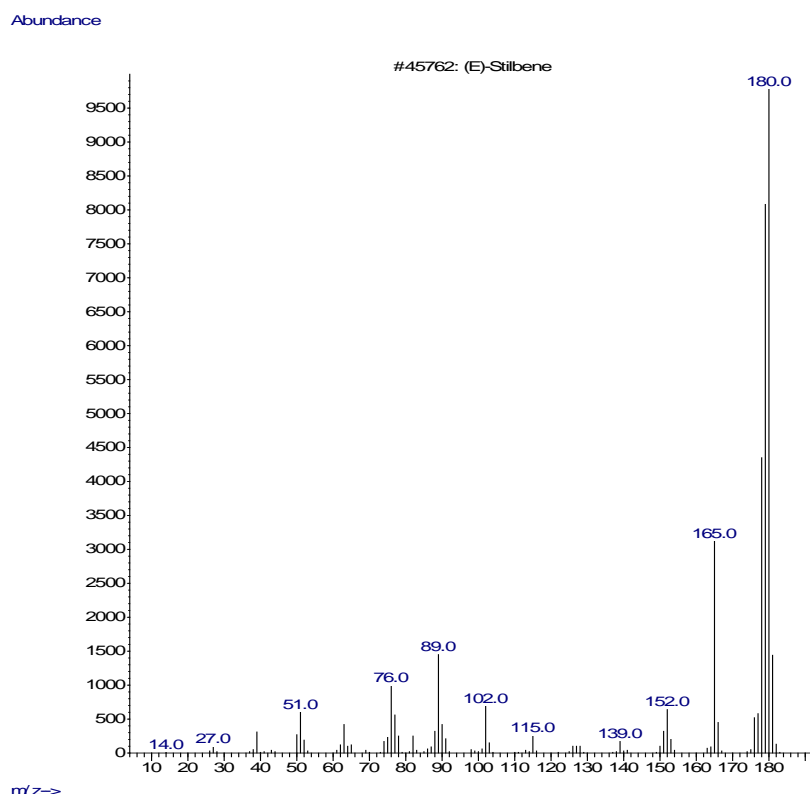
**Figure – 1** Comparison of Antimicrobial activity of Betel leaf Hot extract against *S. mutans* and *S. oralis*



**Figure – 2** Comparison of Antimicrobial activity of Betel leaf Cold extract against *S. mutans* and *S. oralis*



**Figure – 3:** Total ion chromatogram of fraction- 6(after column chromatography)



Dental caries causing Streptococcal sp. (*S.oralis* and *S.mutans*) were obtained from IMTEC, Chandigarh. Aqueous extracts (Hot and Cold) of Piper betel extract were investigated. Each plant extracts were tested at three different concentrations (20, 40 60 and 80 $\mu$ l/ml) to see their inhibitory effects against *S.orlaisand* *S.mutans*. The determination of the MIC by means of the liquid dilution method showed that Piper betel leaf extracts tested exhibited an antimicrobial effect against *S. oalisand* *S.mutans*. These results showed that the extracts (Hot extracts) from Piper betel leaf possessed antimicrobial activity against these two organisms.

The present experimental results indicate that hot extracts of Piper betel leaf exhibited an antimicrobial effect against Streptococcus oralisand *S.mutans*. The result from GC MS screening showed the presence of Stilebene which is responsible for anti- microbial. Stilebenes are biosynthesised by green plants of various taxonomic groups, including dicotyledons, monocotyledons, gymnosperms and bryophytes, and their role in defending the producers against microbial attacks is widely accepted (10). Aslamet al., 2006, reported that the Stilbenes with a free hydroxyl group were active against both bacteria and fungi with MICs in the range 25–100 mg/ml (11). Moreover based on the library search report 2 - ethyl acridine may possess anti-cancer activity. Further investigations are needed for identification and purification of the specific antimicrobial components from these plants against Streptococcus oalisand *S.mutans*.

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