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RESEARCH ARTICLE

DETECTION OF BIOFILM FORMATION AND ANTIBIOFILM ACTIVITY OF MEDICINAL PLANTS ON DENTAL CARIOGENIC BACTERIA AND ITS IMPLICATION IN ORAL HYGIENE.

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Abstract

Dental caries, one of the globally affecting diseases of the oral cavity is still prevalent in today's era despite knowledge of most advanced sciences and technologies in dental practice. There has been constant effort to focus on interception and correction of this disease entity but today our horizon has broadened the approach and goal remain to prevent the disease process rather than to correct it. Medicinal plants have been documented for prevention and cure of many systemic diseases since ancient times. With advancements in science and scientific procedures, it is now known that plants have a potential curative action for oral diseases such as dental caries. The usage of these herbal extracts in clinical practice can miraculously benefit the overall health of the patient. Medicinal plants are known to produce chemicals, which interfere with biofilm production. We have screened some of the medicinally important plants for its activity to inhibit the biofilm production. Inhibition of biofilm by natural inhibitors may lead to the development of new therapy in treating dental caries. Hexane, ethyl acetate, ethanol and methanol extracts of *Morus alba*, *Citrus limon*, *Psidium guajava*, *Azadirachta indica*, *Acacia nilotica* were tested against dental caries causing bacteria which are isolated from caries of infected patients. Based on the biochemical analysis and partial sequencing of 16S rRNA 2 bacterial strains were identified, as follows: *Bacillus cereus* and *Streptococcus constellatus* from infected tooth sample. The sequences from selected caries bacteria (2 isolates) showed high degrees of similarity to those of the NCBI reference strains (about 99%).

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Introduction:-

Dental caries is an infectious microbial disease that results in localized dissolution and destruction of the calcified tissues of the teeth. Natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals. It has been well documented that medicinal plants confer antimicrobial activity towards oral bacteria. Plant extracts or phytochemicals that inhibit the growth of oral pathogens, reduce the development of biofilms and dental plaque, influence the adhesion of bacteria to surfaces and reduce the symptoms of oral diseases can serve as alternatives to prevention and treatment of dental caries. [7]

Oral biofilms are the major contributor to dental caries. Cariogenic pathogens from the dental plaque ferment the different sugars and lower the pH which results in demineralization of tooth enamel leading to dental caries.

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Medicinal plants are known to produce chemicals, which interferes with biofilm production. We have screened some of the medicinally important plants for its activity to inhibit the biofilm production. Inhibition of biofilm by natural inhibitors may lead to the development of new therapy in treating dental caries.

Dental caries, otherwise known as tooth decay, is one of the chronic diseases of people worldwide and individuals are susceptible to throughout their life. Dental plaque, a unique microbial biofilm ecosystem comprising a diverse microbial community has a primary role in its pathogenesis. Medicinal plants found in the environment harboring high bacterial cell density were long suspected to have protective mechanisms against microbial infections and offer a large and attractive phytochemical repertoire for the discovery of novel microbial disease control agents. In spite of the fact that several traditional medicinal plants contain active constituents which are known to have antibacterial activity against various microorganisms, including dental caries causing bacteria, earlier studies exclusively focused only on their effects on planktonic bacteria with little emphasis on the highly resistant biofilm. [3]

The present studies were therefore undertaken to investigate and identify dental cariogenic bacteria. The technique of 16s rRNA partial sequencing is also used for identification of the bacterial species. Studies were also performed on biochemical characterization.

Materials And Methods:-

Collection of the infected tooth sample:-

The infectious tooth sample was taken from Kavediya Dental Clinic, Pune under aseptic conditions. The sample is incubated for 24 hrs at 37°C in saline and nutrient broth respectively. After the incubation time, spread plate and streak plate was done. In next step, the tooth sample crushed in the saline and the same protocol was followed. Only the isolated bacteria which are common in these 3 steps are selected and identified by colony characterization.

Biochemical characterization:-

Gram staining:-

The diluted suspensions of the bacteria were smeared on clean slides, air dried, heat fixed by passing over a flame for 2 to 3 times. The slides were flooded with crystal violet solution for one minute, washed with water and flooded with Gram's iodine for one minute. The slide was washed with water and decolorized with 95% ethyl alcohol dropped from a dropping bottle until no violet color was visible from drain off solution. The slides were washed with water and counterstained with Safranin stain for about 30 seconds and washed with water. The slides were air dried and examined under a microscope using 100x objectives using a daylight filter. Cells were then identified by the color observed purple for Gram-positive and pink or red for the Gram-negative cell.

Colony morphology:-

Shape, size, color, elevation and margin of colony and appearance are observed in overnight plate culture on nutrient agar media and noted down.

Cell morphology:-

The gram stained cells were viewed under the light microscope under 100x oil immersion to determine the shape and size of the cells.

Motility test:-

It is used to check the ability of bacteria to migrate away from the line of inoculation. The bacteria were inoculated into motility media, i.e., mannitol agar with a needle by stabbing the culture in a straight line and were observed after 24-48 hours incubation. If the test organism migrates away from the line of inoculation, the bacteria is motile.

Effect of pH on growth of isolates:-

Effect of pH on growth of isolated microorganisms was observed by inoculating the isolates in test tubes containing nutrient broth (Hi-media) having a range of pH 1 to 14. Tubes were incubated at 37°C for 24 hours.

Effect of temperature on growth of isolates:-

Effect of temperature on growth of isolates was determined by inoculating the isolates in nutrient broth and incubated at a different temperature that is at 4°C, 28°C, 37°C, 50°C for 24 hours.

Studies on biofilm forming ability of isolates:-

Tube method:-

A qualitative assessment of biofilm formation was determined by tube method. Nutrient broth (10mL) was inoculated with loopful of microorganism from overnight culture plates and incubated for 24 hours at 37°C. The tubes were decanted and washed with PBS9 (pH 7.3) and dried tubes were stained with crystal violet(0.1%). Excess stain was removed and tubes were washed with deionized water. Tubes were then dried in an inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong. Experiments were performed in triplicate and *S.mutans* was used as a control. [4]

Air-Liquid interface assay:-

A sterile, flat-bottom, 6-well plate adjusted so that it sits at a 30° to 50° angle from horizontal. Isolates were grown overnight till stationary phase comes. Stationary-phase cultures were diluted 1:100 in nutrient broth. Aliquot (3 ml) of each diluted culture was inoculated into a separate well in the angled 24-well plate such that the upper edge of the aliquot just reaches the center of the bottom of the well. Plate covered with a lid and incubated at temperature 37°C for 72 hours. Cultures were aspirated from the wells and gently washed twice, each time by adding 5ml of sterile medium and then aspirating. After completing these two washes, 2ml of medium (i.e. enough to cover the bottom of the well) was added to each well. The plate was observed under an inverted microscope and focused just on the center of the bottom of the well to observe bacteria at the air-liquid interface. Along with all the pure cultures in respective wells, one well was inoculated mixed culture of isolate 1 and isolate 2 and *S.mutans* were used as a control. [4] The amount of biofilm formation was scored as 0-absent, 1-weak, 2-moderate or 3-strong.

Time and biofilm formation by Coverslip assay:-

A sterile flat bottom 6 well plate is inoculated with 5ml of diluted (1:100) overnight grown culture and coverslips (4 in each well) were kept at an angle 90° to the bottom of the well. Plate covered with a lid and incubated at temperature 37°C. After each time interval of 4hrs, 8hrs, 24hrs and 48hrs one coverslip was removed aseptically from each well and washed twice in PBS and dried so as to remove all the nonadherent cells from coverslip. Dried coverslips were stained with crystal violet(0.1%) for 10 min. Excess stain was removed by gentle washes of distilled water. Airdried coverslips were observed under a microscope and *S.mutans* were used as a control.

Screening of medicinal plants for anti-biofilm activity:-

Collection of plant material:-

Five plants were selected for the studies are *Morus alba*, *Citrus limon*, *Psidium guajava*, *Azadirachta indica*, *Acacia nilotica*. The stem part and leaves part were brought to the laboratory and thoroughly washed in running tap water to remove debris and dust particles and then rinsed with distilled water. Then stem and leaves dried in shade and ground by using a grinder. The dried powder was stored in airtight containers prior to extraction.

Preparation of methanol, ethanol, ethyl acetate, hexane extracts of leaves and stem.

For the preparation of these four solvent extracts, 1 g of the dried and powdered leaves and stem materials were added separately to the 4 solvents respectively for 48 hrs at room temperature, followed by filtration through several layers of muslin cloth. Extracts were concentrated under reduced pressure. The concentrated products were kept at 4°C before testing its antibiofilm activity.

Antibiofilm activity of plant extracts:-

Biofilm formation was assessed. An overnight culture of *B. cereus* and *S. constellatus* was inoculated into fresh nutrient broth (2 ml) with plant extract with respective controls. After incubation for 48hrs at 37°C, media and unattached cells were decanted from the 24 well plates. The remaining planktonic cells were removed by gentle rinsing with sterile water. Plates were then set on a shaker for 5 min to allow full release of the biofilm. Biofilm formation was quantified by measuring optical density at 570nm by spectrophotometer.

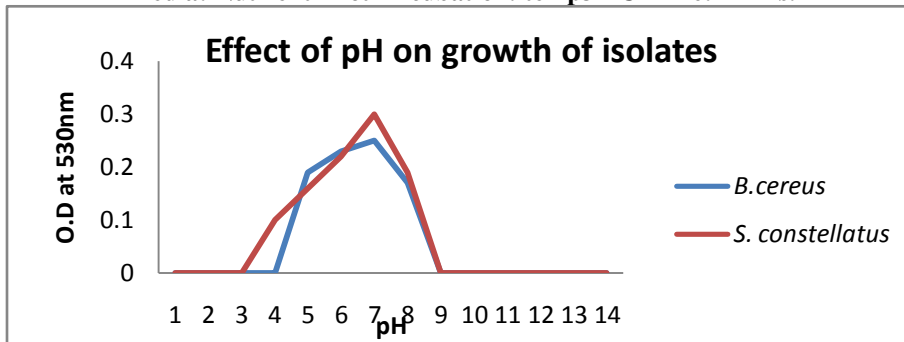
Observations And Results:-

Table No 1:-Morphological characterization of Bacterial Isolate 1 and 2

Character	<i>Bacillus cereus</i>	<i>Streptococcus constellatus</i>
Size	1mm	2mm
Shape	Circular	Circular
Colony margin	Irregular	Regular

Margin	Undulate	Entire
Elevation	Flat	Convex
Color	Yellowish white	Yellowish white
Opacity	Opaque	Opaque
Consistency	Moist	Moist
Motility	Motile	Non -motile
Gram Staining	Gram positive bacillus	Gram positive cocci

Graph 1:-Effect of pH on growth of isolates
Media: Nutrient Broth Incubation: temp 37°C Time: 24 hrs.



Graph 2:- Effect of temperature on growth of isolates
Media: Nutrient Broth Incubation: temp 37°C Time: 24 hrs

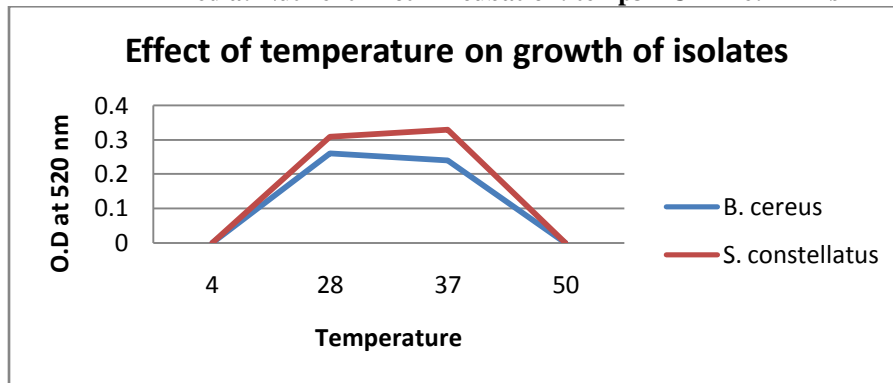


Table No 2:-Detection of biofilm production using tube method.
Media: Nutrient agar Incubation: Temp 37°C Time 24 hr.

Sr No	Isolate Name	Biofilm production
1	<i>Bacillus cereus</i>	+++
2	<i>Streptococcus constellatus</i>	++++
3	Positive control	+++
4	Negative control	----

+ Very weak biofilm, ++ weak biofilm, +++ moderate, ++++ strong, ---- biofilm absent

Image 1:-Biofilm detection by tube method

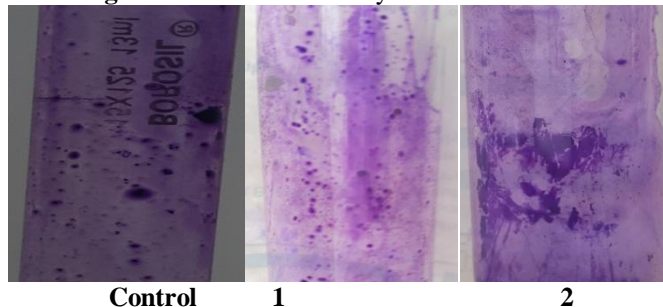


Table No. 3: Air-liquid interface assay for biofilm detection.
Media: Nutrient agar Incubation: Temperature-37°C Time- 24 hrs.

Sr No	Isolate Name	Biofilm production
1	<i>Bacillus cereus</i>	+++
2	<i>Streptococcus constellatus</i>	+++
3	<i>Bacillus cereus</i> + <i>Streptococcus constellatus</i>	++++
4	Positive control	+++
5	Negative control	----

+ Very weak biofilm, ++ weak biofilm, +++ moderate, ++++ strong, ---- biofilm absent

Image 2:-Biofilm detection by Air-liquid interface assay

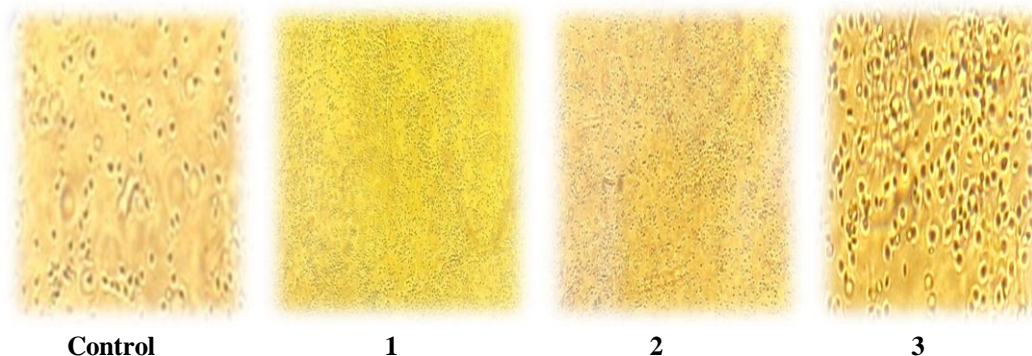
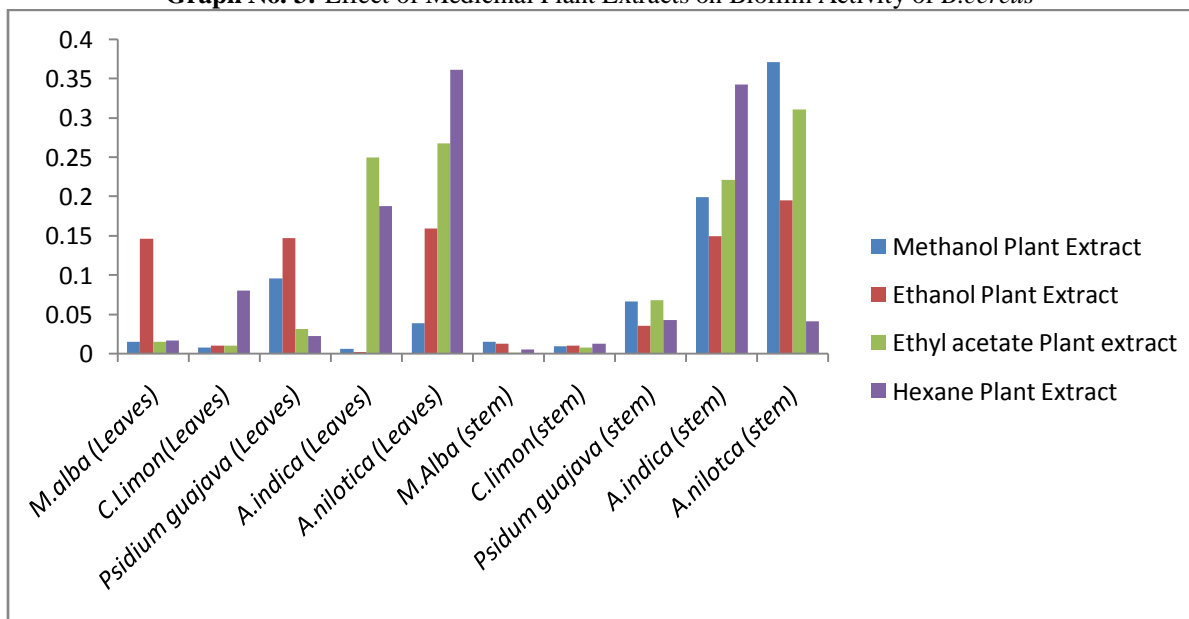
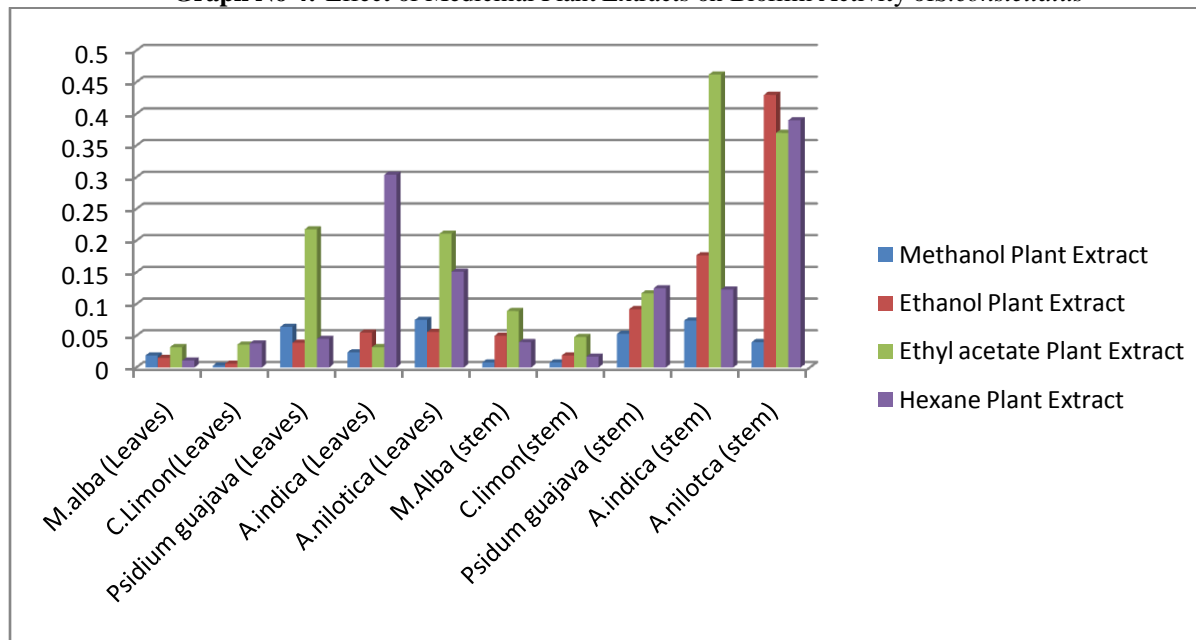


Table No. 4:-Time and biofilm formation by Coverslip assay.

Time	4 hours	8 hours	24 hours	48 hours
<i>Bacillus cereus</i>	No adhered cells	No adhered cells	Patches of cells were observed	Biofilm was observed
<i>Streptococcus constellatus</i>	No adhered cells	Patches of cells were observed	Increased cell attachment	Biofilm was observed
Positive control	Few cells adhere to surface	Increase in the adhered cell no	Patches of cells were observed	Biofilm was observed
Negative control	----	----	----	----

Graph No. 3:-Effect of Medicinal Plant Extracts on Biofilm Activity of *B.cereus*



Graph No 4:-Effect of Medicinal Plant Extracts on Biofilm Activity of *S.constellatus***Sequencing and analyses of DNA:-**

The identification of the dental cariogenic bacterial isolate strain 1 and 2 by the 16S rRNA gene partial sequencing were performed at Genombio Technologies Pvt. Ltd., Pune, India using the universal primers. The partial 16S rRNA gene of selective isolate was sequenced and presented in FASTA format. Finally 16S rRNA sequence of the dental cariogenic bacterial isolate was compared with that of other bacterial sequence by way of BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi>).

The sequence of strain 1 was found to be of 589 base pairs as follows:

>3942

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AACCGCATGGTTCGAAATTGAAAGGGCGGCTTCGGCTGTCACCTTATGGATGGACCCGCGTCGCATTAG
CTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCATAACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACCTCTGTTGTTAGGGAA
GAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACCGTAACTAACCAGAAAGCCACGGCTAACTACGT
GCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCA
GGTGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGAC
TTGAGTGCAGAAGAGGAAAGTGGAATTCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACA
CCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACTGACACTGAGGCGCGAA
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The sequence of strain 2 was found to be of 589 base pairs as follows:

>3943

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ATTTACTGCATGGTAGATGTTTAAAAGGTGCAAAATGCATCACTACCAGATGGACCTGCGTTGTATAGC
TAGTTGGTGAGGTAACGGCTCACCAAGGGCAACGATACATAGCCGACCTGAGAGGGTGATCGGCCAC
ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGGGG
AACCGTACCGAGCAACGCCGCGTGAGTGAAAGGTTTTTCGGATCGTAAAGCTCTGTTGTTAAGGAA
GAACGTGTGTGAGAGTGAAAGTTACACAGTGACGGTACTTAACCAGAAAGGGACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTATTGGGCGTAAAGCGAGCGCA
GGCGGTTAGATAAGTCTGAAGTGAAAGGCAGTGGCTCAACCATGTAGGCTTTGGAAACTGTTAACT
TGAGTGCAGAAGGGGAGAGTGGAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACAC
CGGTGGCGAAAGCGGCTCTCTGGTCTGTAAGTACTGACGCTGAGGCTCGAAA
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Conclusion and Discussions:-

In this study, we have isolated and identified two strains of bacteria viz. *Bacillus cereus* and *Streptococcus constellatus* involved in Dental caries. Many isolates were screened out of which these two were

identified. Identification was done based on the Colony and cell characteristics, Light microscopic properties, Biochemical assays and confirmed by 16s rRNA sequencing. *Streptococcus constellatus* was reported to be involved in dental caries by co-aggregating with other bacteria to bring about the maturation of biofilms [9]. *Bacillus cereus* was not identified before in oral diseases, though other strains of *Bacillus* sp. were prevalent in many. The optimum growth pH and the temperature were equal to physiological conditions for both the bacteria. Thus, the host system provides perfect growth conditions during the infection.

When tested for Biofilm formation of both the strains, both show strong Biofilm forming ability. *S. constellatus* displays strong biofilms as good as the positive control. Also, the film formation starts with 8 hours of inoculation, which is much sooner than in the case of *B. cereus*, in which the first adherent cells are observed after 24 hours of inoculation.

Once it was established that both the strains produce strong biofilms, the anti-biofilm activity of different medicinal plants in the different solvent system against both the isolates was analyzed. The plant extracts used were *Morus alba*, *Citrus limon*, *Psidium guajava*, *Azadirachta indica*, *Acacia nilotica*. *M. alba* and *C. limon* extracts show potentially higher anti-biofilm activity than other plant samples. Both the leaf and stem extract of *C. limon* causes higher detachment of cells in biofilm against both strains. Leaf samples serve as better extract than stem samples, owing to higher phytochemical content than the stem. Though in the case of *Psidium guajava*, stem samples demonstrate slightly higher activity than its leaf samples. *Azadirachta indica* and *Acacia nilotica* show the least inhibitory effect on both the samples, irrespective of the solvent used for extraction.

Hence, *Morus alba* and *Citrus limon* can be used as Biofilm inhibitors for reducing Dental caries.

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