

ORIGINAL RESEARCH ARTICLE

Antibiofilm Formation Activity of *Terminalia bellerica* Plant Extract Against Clinical Isolates of *Streptococcus mutans* and *Streptococcus sobrinus*: Implication in Oral Hygiene

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Received 11 May 2012; Revised 08 Aug 2012; Accepted 17 Aug 2012

ABSTRACT

Dental caries is a localized, transmissible pathological infectious disease which results in destruction of hard dental tissue. This begins with the formation of dental plaques which is a structurally and functionally organized biofilm. *Streptococcus mutans* is the most important bacterium in the formation of dental plaque and dental caries. Several antibiotics are available to treat oral infections but these have several undesirable side effects. Thus there is a need for alternative prevention and treatment options that are safe, effective and economical. Hence the search for alternative products continues and natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals. The ethanolic extract of a plant *Terminalia bellerica* (common name = Baheda) was tested for its antimicrobial activity against the oral plaque forming bacteria *Streptococcus mutans*. It was found to significantly inhibit biofilm formation. In the present study it was found that the extract from *Terminalia bellerica* showed strong activity against *Streptococcus mutans*. The extract also prevents the formation of biofilm by the bacteria. The study suggests possible benefits of this herbal preparation which inhibit the biofilm formation by streptococci, a oral pathogens.

Key words: *Terminalia bellerica*, *Streptococcus mutans*, *Streptococcus sobrinus*, dental caries, plaques.

INTRODUCTION

Dental caries is an infectious microbial disease that results in localized dissolution and destruction of the calcified tissues of the teeth [3]. *Streptococcus mutans* is known as the causative bacteria in the formation of dental plaque and dental caries. This oral microorganism adheres to an acquired pellicle formed on the enamel surface of the tooth surface, aggregates with oral bacteria and initiates plaque formation by synthesizing glucan from sucrose which is catalyzed by glucosyl transferases. *S. mutans* in dental plaque metabolizes the carbohydrates contained in foods releasing organic acid metabolites that demineralize tooth surfaces, resulting in dental caries [15].

Several agents are commercially available, for example the antibiotics commonly used to treat oral infections i.e. Penicillins and Cephalosporins, Erythromycin, Tetracycline and derivatives, &

Metronidazole have been documented [2]. These chemicals can alter oral microbiota and have undesirable side effects such as vomiting, diarrhea, and tooth staining [9]. Other antibacterial agents used in the prevention and treatment of oral diseases including Cetylpyridinium chloride, Chlorhexidine, amine fluoride or products containing such agents are reported to exhibit toxicity, cause staining of teeth or in the case if ethanol (commonly found in mouth washes) have been linked to oral cancer [5]. Given the incidence of oral disease, increased resistance by bacteria, increased resistance by bacteria to antibiotics, adverse affects of some antibacterial agents currently used in dentistry and financial considerations in developing countries, there is a need for alternative prevention and treatment options that are safe, effective and economical. Hence the search for alternative products

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continues and natural phytochemicals isolated from plants used in traditional medicine are considered as good alternatives to synthetic chemicals [10]. 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 in prevention and treatment of dental caries. The general antimicrobial activities of medicinal plants and plant products, such essential oils, have been reviewed previously [4]. *Terminalia bellerica* commonly known as Baheda belongs to family Combretaceae is an important plant used in the popular Indian herbal preparation – “triphala”. Antibiotic activity against a wide variety of microorganisms- pathogenic and non-pathogenic Gram positive and Gram negative bacteria, yeast and fungi has been reported of the fruit of *Terminalia bellerica* [12, 1]. Usually *Terminalis bellerica* is used in combination with other plants. Present work investigates the potential of this plant alone in inhibition of clinically isolated *Streptococcus*.

MATERIALS AND METHODS

1. Maintenance media

For the maintenance of selected isolate of streptococci Brain Heart Infusion Agar (BHI) medium was used.

2. Medium for antimicrobial assay

For antimicrobial assay Muller Hinton Agar (MHA) was used.

Source of all media/component was Hi-Media Laboratories Pvt. Limited Mumbai.

3. Test organism

The *S. mutans* and *S. sobrinus* clinical isolates that were used source in the study along with the source are given in (Table 1).

Table1: List of the test organisms* and its source used in the study

Bacteria	Isolate number	Source
<i>S. mutans</i>	S2, S3, S11(b), S12(b), S14, S15, S22(1), S24, S25, S26, S28, S29, S31(b)	Clinical isolates from dental plaques and caries
<i>S. sobrinus</i>	S14	Clinical isolates from dental plaques and caries
<i>S. mutans</i>	MTCC 890	Microbial Type Culture Collection

(Source *: [9])

4. Plant material

The plant used in the study is *Terminalia bellerica*. The description of the plant is given in (Table 2).

Table 2: List of plant used in the study and its description

Plant name	Vernacular name	Family	Parts Used
<i>Terminalia bellerica</i>	Kadukai, baheda	Combretaceae	Dry fruit

source: a local ayurvedic shop.

METHODS

Clinical samples were isolated and these isolates were used for:

1. Identification of streptococci from isolated colonies.

Streptococci were identified on the basis of morphological studies and biochemical testing.

2. Morphological identification

Morphological characteristics were identified through Gram staining.

3. Catalase test

To perform catalase test production test, a loop full culture from suspected isolated streptococcal colonies were taken on a slide and added small quantity of hydrogen peroxide on the culture. The result was observed on the basis of appearance of bubbles that is seen in case of catalase positive microorganism and absent in case of catalase negative.

4. Preparation of plant extract

All the plant material was dried in open air protecting that area from direct exposure to sunlight. The dried plant parts were grounded. 100 mg of each powdered plant material was extracted with 95% ethanol by dipping in it for 3-5 days and resulted liquid was filtered using Whatman No.1 filter paper (Whatman Ltd. England). The ethanol was removed by evaporation using rotary vacuum evaporator under pressure and temperature 55°C. The ethanolic extract was kept at room temperature for drying. The crude extract was prepared by dissolving known amount of the dry extracts in DMSO (Dimethyl sulfoxide), to have a stock solution of 100 mg/ml.

5. Preparation of multi-strains inoculum

Prepared 3ml of BHI broth and add 1% dextrose in the 4 screw cap tubes each. Sterilized the broth by autoclaving at 121°C, 15 lbs, 15 min. With the help of loop transferred colonies of strains S15, S26, S14 and MTCC 890 to the sterilized broth aseptically. The tubes were incubated at 37°C for 24 h.

6. Detection of biofilm formation and acid production in streptococci.

BHI broth containing 1% D-glucose was prepared and 3 ml of the broth was transferred into screw

cap tubes. The broth was then sterilized by autoclaving at 121°C, 15 lbs, for 15 min. the screw cap tubes were then inoculated with 30µl of overnight grown multi (the multi-strains used in the study were of the strains S15, S26 & MTCC 890 of *S. mutans* and the S14 strain of *S. sobrinus*) strains culture. The tubes were then tilted at an angle of 30° and incubated at 37°C for 18 h. After incubation the supernatant was carefully decanted without disturbing the adhering cells. pH was also noted at this time. Washed the tube containing biofilm with saline (0.85% NaCl). Then added 3 ml saline and mixed well to separate the cells which adhered with glass surface. Then O.D. was recorded at 550 nm [7].

7. Biofilm inhibition assay.

BHI broth containing 1% D-glucose was prepared and 3ml of the broth was transferred into screw cap tubes. The broth was then sterilized by autoclaving at 121°C, 15lbs, for 15 min. The screw cap tubes were then inoculated with 30µl of overnight grown culture of *S. mutans* MTCC 890 strain. The plant extracts were added in different concentration to give desired conc. of extract from stock conc. of extract in DMSO (100 mg/ml). The tubes were then tilted at an angle of 30° and incubated at 37°C for 18 h. After incubation the supernatant (non adherent cells) was carefully decanted without disturbing the adhering cells. pH was also noted at this time. Wash the tube containing biofilm with saline (0.85% NaCl). Then added 3ml saline and mixed well to separate the cells which adhered with glass surface. Then the change in O.D. was recorded at 550 nm [7].

7. Minimum Inhibitory Concentration (MIC) of plant extracts

Minimum Inhibitory Concentration (MIC) of plant extract was determined by microtitre plate broth dilution method. Plant extract was taken from stock by micropipette and added into 1st well of microtitre plate containing 300 µl broth. 150 µl broth and plant extract was transferred from 1st well to 2nd well by two fold serial dilution method. Similarly 150 µl of broth and extract were transferred from next well up to 7th well. The last well (8th well) of microtitre plate was considered as control (containing no test compound only broth present). 10 µl of the bacterial culture (0.5Mc Farland turbidity) was added in each well. The microtitre plates were incubated 24h at 37°C. The MIC was determined by the absence of visible growth of the organism in presence of lowest concentration of test compound extract.

RESULTS

1. Identification of streptococci

The streptococci were isolated from dental caries/plaques and identified on the basis of their morphology and catalase test.

2. Morphological identification by Gram staining.

Gram staining of all the isolates were carried to observe their morphology and to differentiate Gram positive cocci from others. The isolates were found to be Gram positive cocci arranged in chains/pairs

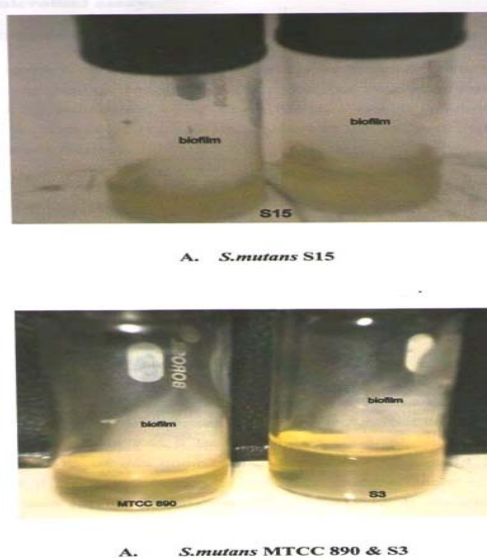
3. Catalase test

The streptococcal strains were found to be catalase negative.

4. Determination of biofilm formation in streptococci

The different streptococcal isolates were found to form biofilm on the glass surface (Fig 1). The ability of biofilm formation in 14 strains was determined spectrophotometrically. It was observed that all strains formed biofilm on glass surface after 18 hr of incubation. The biofilm formation was quantified by taking the OD (Optical Density) at 550 nm. It was found that strain S15 and S24 showed maximum turbidity, (maximum OD.). The less O.D. was shown by strains S12 (b), S14, S31 (b) and S26, thus these strains have less adhesion ability. Along with biofilm formation the strains also showed decrease in pH of the broth as shown in the table below. Thus streptococcal isolates tend to produce acid along with biofilm formation (Table 3).

Fig 1: Screw cap tube showing biofilm formation by streptococcal isolates.



The streptococcal isolates formed biofilm on glass surface. It was observed that all 14 strains formed biofilm on glass surface after 18 hr of incubation. The ability of biofilm formation in 14 strains was determined spectrophotometrically. The biofilm formation was

quantified by taking the OD (Optical Density) at 550 nm. The Fig 1(A) shows the biofilm formation of by the *Streptococcus mutans* isolate S15. The Fig 1(B) shows biofilm formation by *S. mutans* MTCC 890 and S3 strain.

Table 3: Biofilm formation and acid production in *S. mutans* and *S. sobrinus*

Strains	pH	O.D
S2	6.0	1.058
S3	6.0	2.844
S11(b)	6.0	2.226
S12(b)	6.0	0.581
S14	6.0	0.166
S15	6.0	3.416
S22(1)	6.0	1.264
S24	6.0	3.816
S25	6.0	1.450
S26	6.0	0.698
S28	6.0	1.902
S29	6.0	1.089
S31(b)	6.0	0.589
MTCC 890	6.0	2.732

5. Antistreptococcal activity of plant extract.

The ethanolic plant extract of *Terminalia bellerica* was screened against the 14 streptococcal isolates. *Terminalia bellerica* extract showed strong activity against all the strains of *Streptococcus mutans* and *S. sobrinus* strain S14 with the zone of inhibition of 8 mm to 14 mm (Table 4 & Fig 2).

Table 4: Zones of inhibition (mm) formed by the plant extract against the 14 streptococcal isolates

Plant	Zone of inhibition against the streptococcal isolates (mm)													
	S2	S3	S11(b)	S12(b)	S14	S15	S22(1)	S24	S25	S26	S28	S28	S31(b)	MTCC 890
<i>Terminalia bellerica</i>	11	9	8	10	14	8	10	11	11	9	10	10	10	11

6. Determination of inhibition of multi-strains biofilm formation (using 1% dextrose) by *Terminalia bellerica* plant extract

The active plant extracts of the plant showed positive antiadherence effect on the multi-strains biofilm formation on the screw cap tube glass surface in the presence of 1% dextrose.

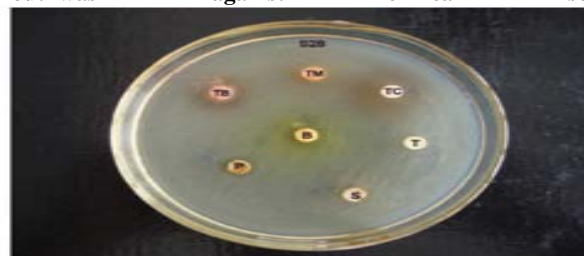
These active plant extract was found to inhibit the multi-strains biofilm formation on the glass surface and showed decrease in turbidity when the OD was taken at 550 nm (Table 5 & Fig 3).

Fig 3: Inhibition of multi-strains biofilm formation (using 1% dextrose) by *Terminalia bellerica* plant extract



A

Fig 2: Antibacterial activity of different plant extract and mouthwash against clinical isolates.



A



B

The Petri-plates show growth of Streptococcal isolates on Mueller Hinton Agar medium and zones of inhibition are formed by the plant extracts. Fig 2 (A) Antibacterial activity of different plant extracts including *Terminalia bellerica* against *S. mutans* strain S28. The zone of inhibition is formed by the different plant extracts and it has been found that *Terminalia bellerica* has strong antistreptococcal activity. Fig 2(B) Antibacterial activity of different plant extracts including *Terminalia bellerica* against *S. sobrinus* strain S14. Abbreviations:-

TB-*Terminalia bellerica*; S-*Symplocos racemosa*; TM-*Thunbergia mysorensis*; TC-*Terminalia chebula*; T-*Tribulus terrestris*; P-*Psoralea corylifolia*; B-*Berberis aristata*



B

Fig 3(A): The multiple clinically isolated streptococcus inoculated in brain heart infusion broth forming biofilm on the screw cap tube glass surface. Fig3 (B): Inhibition of Biofilm formed by multiple clinically isolated *Streptococcus* by *Terminalia bellerica*

Table 5: Multi-strains biofilm inhibitions by plant extract (Change in pH and OD with 1% dextrose)

Plant extract	Concentration	pH	OD	Inhibition %
<i>Terminalia bellerica</i>	(a) 250 µg/ml	6	0.146	92.2
	(b) 125 µg/ml	6	0.192	89.8
*Control	-	6	1.89	-

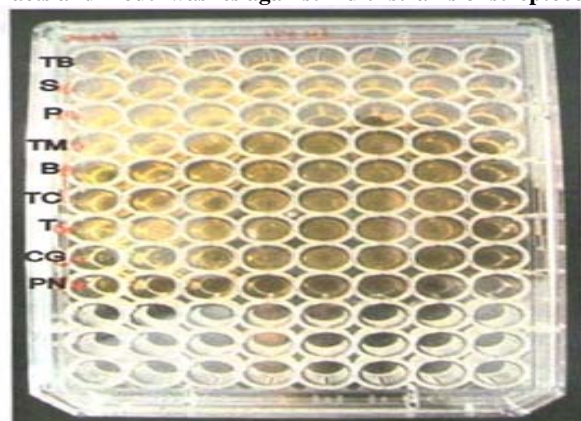
*Control contains only the bacterial culture treated with DMSO only.

7. Determination of MIC (Minimum Inhibitory concentration) of active plant extracts against multi strains of streptococci.

In this study ethanolic extract plant showed potential activity in disc diffusion assay. The antibacterial activity of these active ethanolic extracts was quantified in terms of MIC (Minimum inhibitory concentration) by broth dilution assay.

The multi-strains used in the study were of the strains S15, S26 & MTCC 890 of *S. mutans* and the S14 strain of *S. sobrinus*. The active ethanolic plant extract has good antistreptococcal activity in broth dilution assay. *Terminalia bellerica* showed MIC of 500 µg/ml (Fig 4).

Fig 4: Microplate assay for determination of MIC of plant extracts and mouthwashes against multi-strains of streptococci.



The microtitre plate containing 300 µl Brain Heart Infusion broth with 2 fold serially diluted different plant extracts (150 µl) along with 10 µl bacterial culture. MIC was determined by the absence of visible growth of the organism in presence of lowest concentration of test compound extract.

TB-*Terminalia bellerica*; S-*Symplocos racemosa*; P-*Psoralea corylifolia*; TM-*Thunbergia mysorensis*; B-*Berberis aristata*; TC-*Terminalia chebula*; T-*Tribulus terrestris*; CG - *Chlorhexidine gluconate*; PN- *Potassium nitrate*.

DISCUSSION

Streptococcus mutans is the most important oral bacteria which plays a major role in dental caries, bacteremia and consequently bacterial endocarditis [13]. Application of antibiotics for prevention of dental caries is not recommended, since there is risk of development of MDR (Multiple Drug Resistance) strains. The use of plants and their extracts in the treatment of diseases dates back to 460-370 BC when Hippocrates practiced the art of healing by use of plant based drugs. Different plants and their parts (flowers, bud, leaves, stem, bark, fruits, pulp, and roots) have been used for thousands of years [8]. In the present study clinical isolates of *S. mutans* and *S. sobrinus* were identified on the basis of Gram-staining and catalase test. The 14 strains were isolated from clinical samples and were tested for

their biofilm forming tendency. All strains of *S. mutans* and *S. sobrinus* were found to form biofilm on the glass surface of screw cap tube in the presence of 1% dextrose. The strain S15 and S24 was found to have maximum tendency to form biofilm. In this study plant extract of *Terminalia bellerica* was found to be active for anticariogenic potential. *Terminalia bellerica* has been previously evaluated for its antimicrobial potential in combination with *Phyllanthus emblica* and *Terminalia chebula* called as 'Triphala' [6]. Triphala has significant antimicrobial activity and thus can be employed as an effective anti-plaque agent and can be used in the prevention of dental caries [14]. The plant extract was evaluated for antistreptococcal and antibiofilm activity against clinical isolates of *S. mutans* and one isolate of *S. sobrinus*. *Terminalia bellerica* was found to be active against all the streptococcal strains and also has potential antibiofilm activity.

CONCLUSION

The aim of this study was to search antimicrobial activity and biofilm formation inhibition in ethanolic extract of plant *Terminalia bellerica* against Streptococcal isolates from dental caries. 14 isolates including the MTCC 890 strain were identified by Gram staining and catalase test. Isolated streptococcal isolates were allowed to form biofilm on the glass surface of screw cap tubes. *Terminalia bellerica* plant extract (ethanol) was screened for its antimicrobial activity against the isolates of streptococci. MIC of active plant extract against multiple clinical isolates of *Streptococcus* was determined by two fold broth dilution assay. *Terminalia bellerica* showed antibacterial activity with MIC 500 µg/ml. The plant extract was quantified for inhibition of biofilm formation and biofilms formed by all clinical isolates were inhibited by *Terminalia bellerica*.

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