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# Research Article
# Bactericidal and Anti-biofilm Activity of Tannin Fractions Derived from *Azadirachta* against *Streptococcus mutans*

[1]Malla Sudhakar and [2]B. Venkata Raman

[1]Department of Biotechnology, Indian Academy Degree College, Bharathiar University, Coimbatore, India
[2]Department of Basic Sciences, Audisankara College of Engineering and Technology, Gudur, SPSR Nellore, India

## Abstract

**Background and Objective:** Ethnobotanical herbs are an important source for producing treatment cures for various oral infections. There is a huge demand in terms of using medicinal plants against many of the ailments which are both chronic and acute. The aim of our study was to determine the antibacterial activity of the *Azadirachta* plant tannin fractions especially on the oral bacteria *Streptococcus mutans*. It studied the tannin fractions on the bacterial growth and its antibiofilm activity along with the down-regulation of the genes responsible for the biofilms. **Materials and Methods:** Constituent major phytochemicals were screened from the plant extracts and tannin fractions were precipitated and purified. Antibacterial kinetics and antibiofilm assays were performed on the *S. mutans* by using the purified tannin fractions. The strain was treated with the fractions to screen for the down regulation of the Sortase A (*srtA*), Antigen I/II (*AgI/II*) and Competence D (*comD*) genes and was quantified using real time PCR. A *recA*, house keeping gene was used as normalize in the study. **Results:** Chloroform extract was shown to have more antibacterial activity and used for further purification for the tannin fraction. From the antibacterial kinetic study, it was confirmed that 2MIC values were almost similar to the positive control and helped in prolonging the lag phase for exactly 24 h and thereafter with a small log phase. **Conclusion:** This study on the possible inhibitory role of the tannin fractions of neem extract on the biofilm inducing proteins was confirmed and further its is planned to purify the tannin fractions to study the antibiofilm activity in detail.

Key words: *Streptococcus mutans*, real time PCR, sortase A, biofilm assay, *Azadirachta*

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Corresponding Author: Malla Sudhakar, Department of Biotechnology, Indian Academy Degree College, Bharathiar University, Coimbatore, India



Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Ethnomedicine deals with the use of traditional medicines which do have relevant written evidences and has been transmitted over the centuries orally to the upcoming generations both literally and practically. The neem tree (*Azadirachta indica*) was known for its wondrous medicinal properties in the Indian subcontinent. It is of most important globally as it aids in offering invaluable solutions to many of the ailments prevailing now[1].

Dental caries is now a multifactorial oral disease which has infested majority of the human population round the world. Bacterial plaque is the leading cause for these pathogenesis and infections of the disease. Current researches are showed that the properties of bacteria associated with a surface in a biofilm can be markedly different than those of the same cells growing in liquid broth (planktonic cells). Plaque mostly found on the protected and stagnant surfaces and poses a great threat of the oral disease. Oral health generally influences the overall quality of life which results in poor oral health eventually leading to chronic conditions. A very close association exists between the oral infections and the oral microbes and the past reports stated more than 750 species of bacteria inhabit the oral cavity[2].

*Azadirachta indica*, commonly called as neem has gained more attention in the recent years owing to its vast range of medicinal properties. Neem is being used widely in Ayurveda, Unani and Homoeopathy since it processes wide array of biologically active compounds which were diverse chemically and structurally[3]. Almost all the parts of the neem tree (leaves, flowers, seeds, fruits, roots and bark) were being used in treating many chronic and acute ailments like inflammation, infections, skin diseases and dental caries. Till date more than 140 compounds were found to be reported from different parts of the neem of which many were characterized structurally.

As stated neem was used traditionally since ages as immunomodulant, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral and anticarcinogenic[4]. Wollinsky *et al.*[5] have reported that some of the active components from the neem bark were containing phytochemical components which could inhibit the virulence factors of the oral streptococci associated dental plaque formation. It was thus the plant was kept at the zenith keeping in view of its valuable components which can be used in designing contemporary drugs.

The oral disease mainly includes bacteria like streptococci species (*Streptococcus mutans*, *Streptococcus sanguis*, *Streptococcus sobrinus* and *Streptococcus oralis*) and *Lactobacillus* spp.[6]. Among these strains, the Gram-positive bacterium *S. mutans* was considered superior in creating dental caries among the humans[7]. It is also widely known in causing infective endocarditis by infecting the endothelial cells of the heart[8].

*Streptococcus mutans* lodges two types of adhesion mechanisms like sucrose-independent and sucrose-dependent during infection. In the sucrose dependant mechanism, glucosyltransferases which are closely associated with cell wall convert the extracellular sucrose into glucan which then binds to the glucan-binding proteins (Gbps) of the bacteria and aids in cell-cell aggregation leading to the formation of dental biofilms[9].

On the other hand, in the absence of sucrose, the adhesion mechanism is mediated by surface adhesions like streptococcal protein antigen P (SpaP, also known as antigen I/II or P1). This protein is primarily involved in binding to the Salivary Agglutinin Glycoprotein (SAG)[10]. Studies done so far on the primates stated that the SpaP was found to be diminished in terms of adhesion activity which are immunized with antigen I/II[11].

Other studies also suggested that SpaP along with wall-associated protein A (WapA) aid in mediating *S. mutans* to bind to collagen[12]. Moreover, glucan-binding proteins A (GbpA) and C (GbpC) are also found to be associated with biofilm formation on the tooth along with SpaP and WapA[13]. The surface adhesion proteins are usually fixed within the bacterial cell surface through the sortase A (SrtA) which recognized the cell sorting signal of the surface proteins. This signal was found to contain a highly conserved LPXTG motif (where, X represents any amino acid) at the carboxy-terminal end of the protein which cleaves the peptide bonds. Such released carboxy-terminal ends with threonine are anchored to the pentaglycine of lipid II-surface protein which is then affixed to the cell wall peptidoglycan by means of transglycosylation and transpeptidation reactions[14].

Several studies reported that *SrtA*-deficient *S. mutans* failed in anchoring the proteins into the surface of the bacteria and as such showed diminished biofilm activity[15]. Thus, *SrtA* was found to be the primary factor in forming dental caries through regulating the sorting of the adhesion proteins and aids as a promising target for novel drug development in treating dental caries. Inhibiting the adherence of bacteria could be an effective strategy to fight against the biofilm-related infections as it can stop the biofilm without even changing the ecological balance of the oral cavity. Many *SrtA* inhibitors were reported and they ranged from synthetic small molecules[16], peptide-analogs[17] and natural plant products[18]. Among all of them, flavonoids extracted from

the medicinal plants source were found to be ideal in terms of inhibitory activity against the *SrtA*. Among them quercetin to inhibit the *S. aureus SrtA*, epigallocatechin gallate to inhibit the *S. pneumoniae SrtA*[19] and formononetin to inhibit *S. mutans SrtA*[20]. Morin, a flavonoid from the Chinese herbs could restrain the *SrtA* of *S. mutans*, thus inhibiting the adhesion biofilm formation[18].

Considering the above and previous studies, it is aimed at screening the antibacterial and anti biofilm activity. In addition to the biofilm studies at the *in vitro* level, this study was designed to screen the upregulation and down regulation of the biofilm inducing genes (*SrtA*, *Agl/II* and *comD*) under the treatment of the proposed plant extract.

## MATERIALS AND METHODS

**Area of study:** The study was done at Department of Biotechnology, Indian Academy Degree College, Bangalore, India, from February, 2018 to December, 2019.

**Neem plant extraction:** Neem plant stems were collected washed with tap water, rinsed with distilled water and shade dried for over 1-2 months. The parts were then pulverized and further filtered by using muslin cloth to pass through 100 mm sieves. The processed powder was then used for extraction procedures.

Fifty grams of sample powder was added to 500 mL of conical flask and soaked in 200 mL of methanol with continuous stirring for 2-3 days[21]. The contents are then filtered by using Whatman filter paper No. 41 and the clear supernatant obtained was used for preliminary screening for antibacterial and biofilm studies. The extraction was performed with ethanol and chloroform also and used in the preliminary screening.

Miswak sticks (*Salvadora persica* L.) were procured from GKVK, Bangalore and were used as positive control in the preliminary studies. Their antibacterial activities in regard to dental caries were confirmed by many biologists[22]. The initial preliminary screening via disc diffusion method and biofilm by ethanol acetone method confirmed that methanol extract showed more significant activity when compared to chloroform and ethanol extracts. The activity was also significant in relation to the positive control methanolic extract.

**Bacterial culture:** *Streptococcus mutans* (MTCC 890) culture was procured from MTCC, Chandigarh, India. The organism MTCC890 was immediately revived according to the rules specified in the information sheet. The subculturing was done by using the brain heart infusion broth.

**Phytochemical screening:** The plant extracts were screened for phenols, tannins, glycosides, coumarins, carbohydrates, saponins, phlobatannins, steroids, alkaloids and flavonoids using the specified protocols. The highly positive components present in methanol were used for further study. Preliminary qualitative phytochemical screening was carried out[23]. The plant extracts were screened for phenols, tannins, glycosides, coumarins, saponins, phlobatannins, steroids, alkaloids and flavonoids by using the specified protocols.

Presence of alkaloids was done by Alkaloids Wagner's test, presence of flavonoids by flavonoids Shinoda test, presence of steroids by Steroids Liebermann Burchard test, presence of cardiac glycosides by Glycosides Keller-Killani test, presence of terpenoids of Terpenoids Salkowski test, presence of phenol by Phenols Liebermann's test, presence of tannins by Tannins Modified Prussian blue test and saponins by Forth test.

**Antimicrobial testing:** The antimicrobial activity of the plant extracts along with positive control (miswak extracts) was carried out by using the agar diffusion and Minimal Inhibitory Concentration (MIC) methods.

**Agar diffusion method:** The antimicrobial testing was done on LB agar plates using the agar diffusion method. In brief, about 100 µL of the bacterial culture was spread on the agar plates. The wells (about 3 mm in diameter) were cut using a sterile glass capillary tube at the peripheral side and in the centre. The wells were added with 50 µL of extracts (methanol, chloroform and ethanol) made from neem stems (100 mg $mL^{-1}$). Vancomycin (10 mg $mL^{-1}$) was used as positive control throughout the study. Miswak methanol extract was also used as secondary positive control. The plates were then incubated at 37°C for about 24-48 h. Following incubation, the mean diameter of the growth inhibition zone (mm) was measured and recorded. All the tests were done in triplicates and done under the same experimental conditions.

**Minimal Inhibitory Concentration (MIC):** The MIC of the neem plant extracts were determined by the microdilution method using 96 multi-well microtiter plates as described by Panda *et al.*[24]. In brief the dissolved extracts were further diluted to final concentration of 100 mg $mL^{-1}$ and 50 µL of each extract (Methanol, chloroform and ethanol) were pipetted into their respective wells containing 180 µL of Mitis salivarius broth. Lastly, 10 µL of the bacterial suspension was added to all the wells except for the negative control. Vancomycin and Miswak extract (methanol) were used as positive controls. The plates were incubated at 37°C for about 18-24 h. The least concentration at which turbidity occurred

was less was taken as the MIC value. The plates were analyzed to determine the MIC. All the tests were done in triplicates. Following incubation the plates were read for optical density under PLATE reader (Genetix Ltd) at 600 nm and also screened for CFU $mL^{-1}$.

**Antibacterial activity kinetics:** The extracts positive for the disc diffusion and broth dilution were alone used for antibacterial kinetic studies as described by Sim *et al.*[25]. It is used to estimate the effect of the extracts (at concentrations of 1/4× MIC, 1/2× MIC, MIC and 2× MIC) upon *S. mutans* according to the incubation time. Following incubation the optical density was measured at 600 nm at 0, 3, 6, 9, 24, 30, 48 and 52 h. Sterile distilled water was used as negative control and Vancomycin (10 mg $mL^{-1}$) (Sigma-Aldrich) was used as positive control. The results are expressed as mean with Standard Deviation (SD) of 3 wells for each extract concentration and for each incubation time.

**Biofilm inhibition assay:** Bacterial biofilm inhibition assay was done[26]. In brief, 200 µL of Brain heart infusion broth was added to all the wells of a 96 well of a microtiter plate and 20 µL of bacterial culture was inoculated into all the wells except the last column which serves as sterile control (containing only 200 µL of Brain heart infusion broth). Vancomycin was sued as positive control. The microtiter plates were incubated at 37°C for 24-48 h and following incubation, the medium was discarded and the wells were washed thrice with Phosphate Buffered Saline (PBS). The cells which are supposed to be adhered to the wells were stained with 0.1% crystal violet and then washed thrice with PBS (pH 7.4). The cell-bound dye was then eluted with about 200 µL of ethanol:acetone (80:20) and the absorbance of the eluted solution was read at 595 nm by using Plate reader (Genetix Ltd.).

**Tannins precipitation:** Tannins are polyphenolic compounds with relatively large molecular weight and can be precipitated by alkaline ethanol precipitation method[27]. Chloroform extract which showed positive results for antibacterial and biofilm inhibition assay was alone used for the study. In brief, 0.5 mL of chloroform extract was taken in a centrifuge tubes and added with 5 mL of 100% ethanol and kept in an orbital shaker for 1 h. The contents were then centrifuged at 5000 rpm for 5 min and the supernatant collected was added with 5 mL of ethanol. The contents were mixed thoroughly to repeat the earlier process. To the supernatant collected about 1 mL of 1% NaOH was added. The contents were freezed for 10 min and again centrifuged at 5000 rpm for 5 min. The precipitated tannin pellets were then collected and re-suspended in 0.1 mL of sterile distilled water and stored for future use.

**Isolation of tannins:** The crude extract (2 g) was dissolved in about 20 mL of ethanol and was applied onto a column (1.5×40 cm) packed with Sephadex LH-20 gel[28]. Ethanol being the first eluent removed most of the low phenolic compounds following ethanol elution, about 500 mL of 50% acetone (v/v) was added to the column to elute the tannins. The fractions obtained were then placed in rotary evaporated to remove excess solvent. Then lyophilisation was done to remove the excess water.

**Tannin estimation:** Quantitative estimation of tannins was determined by Folin-Denis method[29]. In brief, about 1 mL of the extract and standard solution of tannic acid (100-800 µg $mL^{-1}$) were made upto 7.5 mL with distilled water. To the samples 0.5 mL of Folin-Denis reagent was added and the contents were mixed thoroughly. Almost 0.5 mL of sodium carbonate was added and made upto 10 mL with distilled water and the absorbance was measured at 700 nm by using double beam UV-visible spectrophotometer (Shimadzu-190). The total tannic acid content was expressed as mg of tannic acid equivalent per gram of extract.

**Antibacterial activity with purified tannin compounds:** All the fractions with equal optical density values were pooled together and all the fractions collected from the column were concentrated on the water bath at 45°C. The concentrated samples were then freeze dried and stored for further use. The tannin fractions were dissolved in sterile distilled water and were used for the antibacterial activity determination by well diffusion method, broth dilution method and biofilm inhibition assays.

**RNA extraction**

**Treatment:** To 50 mL of media about 20 µL of bacterial suspension was added and mixed thoroughly. About 10 µL of tannin fraction was added to the tube labeled treatment and 10 µL of the vancomycin (20 mg $mL^{-1}$) was added to the tube labeled positive control. Tube with no treatment serves as control. The contents are vortexed properly and incubated at 37°C for 24-48 h. Following incubation the cultures were removed and used for the RNA extraction procedure.

Table 1: Primer details of the genes used in the study

| Names | Length | Tm (°C) | GC (%) | Sequence | Product length |
|---|---|---|---|---|---|
| **Sortase A** | | | | | |
| FW | 20 | 60.04 | 55 | CACAACAAGGCTGCCCATTC | 470 |
| RV | 20 | 60.11 | 55 | ATTGTTCTAGCAGTCGCCCC | |
| **Antigen I/II** | | | | | |
| FW | 20 | 58.98 | 50 | ACAGTTCTTGCCGGTTCAAC | 490 |
| RV | 20 | 59.09 | 50 | AGGGGCTGCTTCAAGACTAG | |
| **comD** | | | | | |
| FW | 20 | 58.98 | 55 | GCGATTGGAGCCTTTAGTGG | 217 |
| RV | 20 | 58.91 | 55 | GCCTGAGATGGAGTTGCTTG | |
| **recA** | | | | | |
| FW | 20 | 59 | 55 | ATCTCCGTCAATCTCCGCAC | 382 |
| RV | 20 | 59.97 | 50 | ACGCGCTGAACAAAAGGTTC | |

FW: Forward primer, RV: Reverse primer, Tm: Melting temperature

**RNA extraction:** The RNA extraction procedure was followed according to the instructions given in the manual. The cultures ($<1\times10^9$ bacteria) were centrifuged at 5000×g for about 5 min at 4°C and the supernatant obtained was discarded. To the pellet about 500 µL of Buffer RLT was added and vortexed vigorously for 5-10 sec. The contents were then centrifuged for about 10 sec at maximum speed and collected in a fresh tube. An equal volume of ethanol (70%) was added and mixed thoroughly by pipetting. About 700 µL lysate obtained was added to the RNeasy spin column placed in a 2 mL collection tube. The contents were then centrifuged for 15 sec at about 9000×g and the flow was discarded. To the column 700 µL Buffer RW1 was added and centrifuged for 15 sec at 9000×g to wash the spin column. The 500 µL of Buffer RPE was added to the RNeasy spin column and centrifuged for 15 sec at 9000×g to wash the spin column and the flow-through was discarded. Previous step was repeated twice to clean the membrane. The column was placed in a new collection tube and added with 30-50 µL RNase-free water. The contents were then centrifuged for 1 min at 9000 rpm to elute the RNA and the RNA was stored at -20°C. The RNA quality was checked by the ultraviolet spectrophotometer and used for complementary DNA (cDNA) synthesis.

## Reverse Transcription (RT) PCR

**cDNA synthesis:** The cDNA synthesis was performed by using the RT-PCR kit using SuperScriptTMII Reverse Transcriptase, 200 U $\mu L^{-1}$ (HiMedia). In brief, about 2 µg of the RNA obtained in the previous section was used as the starting reaction. The RNA concentration obtained was about 1.98 µg $\mu L^{-1}$. So, it used about 1.12 µL of the total RNA along with random primers and 1 µL of RT enzyme. The contents were mixed thoroughly and incubated at 25°C for 10 min. Following incubation at 70°C for 45 min, the cDNA obtained was then stored until further use for gene expression.

**Real-time PCR:** Primers for the real time (Table 1) were designed by using primer 3 software and were purchased from Sigma-Aldrich. The real-time PCR assay was then performed according to Kaluzna *et al.*[30] using the iQTM SYBR Green Supermix (HiMedia). The primers (600 nM) and 1 µL of the RT products were used in the PCR assay and the reaction was done in a total volume of 12.5 µL. All the reactions were carried out in triplicates and also, run in parallel with its respective negative control to confirm the positive amplification.

**Expression of HuR and HIF1-A members in samples:** Real-time quantification was performed on the samples (both control and treatment) in the Corbett Research cycler (Bio-Rad). The sortase A, Antigen I/II and comD primers (SA, AI/II, cD) of about 600 nM concentration were used in the amplification program. About 1.12 µL of the RNA products were initially used and the program was run for about 40 cycles at 92°C for 50 sec, 64°C for 45 sec and with an elongation at 72°C for 50 sec. The housekeeping gene *recA* was amplified along with the respective gene of interest to deduct a comparative analysis of the mRNA expression. The comparative analysis of the relative levels of mRNA of the test samples (including control) was quantified using ΔΔCt method. The Ct values obtained for the gene of interest were normalized to its housekeeping gene.

**Statistical analysis:** All the experiments were done in triplicates. Wherever applicable, the data was subjected to one-way Analysis of Variance (ANOVA) and differences between samples were determined by tukeys test ($p\leq0.05$) were considered statistically significant. Microsoft Excel 2010 statistical package was used throughout the study for analyses.

## RESULTS

**Phytochemical screening:** The plant extracts (methanol and chloroform) were found to contain tannins and phenols in predominance (Table 2). The highly positive components present in methanol and methanol were used for further study. Tannins were treated as the subject of study and both chloroform and methanol extract were chosen in the study.

Tannin content of the extracts (chloroform and methanol) was expressed as mg QE $g^{-1}$ dried weight. The concentration of the tannins was found to be more for chloroform extract than the methanol extract. The calibration equation for tannic acid (standard) was $y = 0.0015x$ ($R^2 = 0.9959$), where x is the tannic acid concentration in mg $L^{-1}$ and y is the absorbance reading at 700 nm. The concentration of tannin in methanol and chloroform was found to be 89.57±0.13 and 302.83±0.54, respectively. The tannins on further purification with alkaline method was found to yield more tannin content. Methanol was found to yield only one fraction, but chloroform showed two fractions (C1, C2) with equal absorbance. These fractions were also estimated for the total tannin content. The tannin concentration was found to be 213±0.23, 612±0.13 and 723±0.11, respectively for methanol and chloroform fraction 1 and fraction 2, respectively (Fig. 1).

**Antibacterial activity:** Vancomycin and miswak extract were used as positive controls and showed very high significant levels of inhibition on the growth of *Streptococcus* (Fig. 2). Chloroform extract showed more inhibition than methanol extract. The mean diameter of the zone of inhibition of the Erythromycin, Miswak extract (methanol), methanol and chloroform were found to be 24±0.45, 18±0.60, 4±0.71 and 15±0.05, respectively (Fig. 3).

**Broth dilution method:** The least concentration at which most of the bacterial growth was inhibited and used as MIC value. The lower the MIC the effective is the compound. The MIC values of the Erythromycin, methanol and chloroform were found to be significant (Fig. 4).

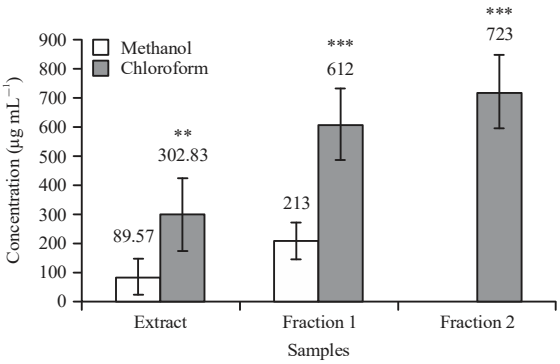


Fig. 1: Content of tannins in extracts and fractions
Results are expressed as concentration ±SE, all the values were average of triplicates, **Highly significant, ***Very highly significant

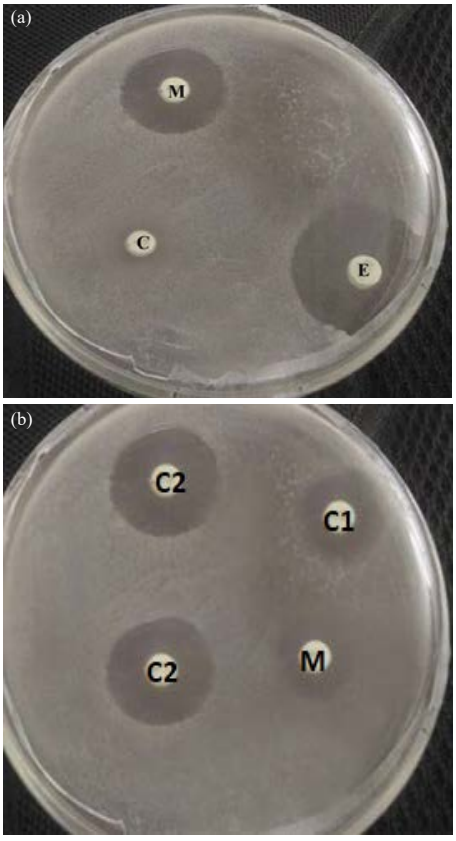


Fig. 2(a-b): Plates showing the antibacterial activity by disc diffusion method (a): C: Control, E: Erythromycin (10 mg $mL^{-1}$), M: Miswak extract and (b) C1: Chloroform fraction 1, C2: Chloroform fraction 2
M: Methanol fraction, plates were with Brain heart infusion agar

Table 2: Phytochemical screening of the plant extracts

| Samples | Tannins | Saponins | Steroids | Phlobatannins | Glycosides | Coumarins | Alkaloids | Flavonoids | Phenols |
|---|---|---|---|---|---|---|---|---|---|
| Methanol | ++ | - | ++ | - | ++ | + | + | - | ++ |
| Chloroform | ++ | - | + | + | + | + | + | - | + |
| Diethyl ether | + | - | + | - | - | + | + | - | + |

+: Positive, ++: Strongly positive, -: Negative

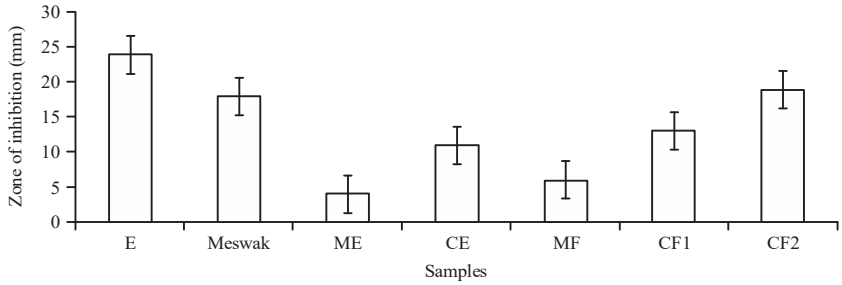


Fig. 3: Antibacterial activity of the sample extracts in disc diffusion assay

All the values were average of triplicates and expressed as Mean±SE, E: Erythromycin, ME: Methanol extract, CE: Chloroform extract, MF: Methanol fraction, CF1: Chloroform fraction 1, CF2: Chloroform fraction 2

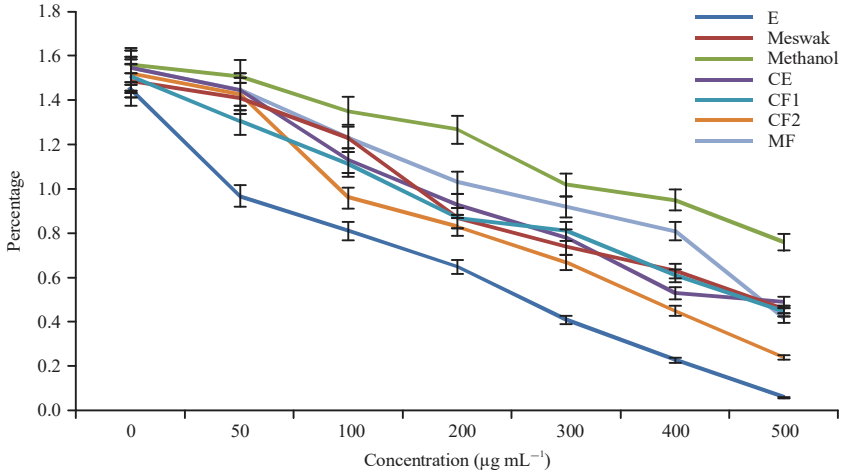


Fig. 4: Inhibition (%) of the *S. mutans* to the plant samples

All the values were average of triplicates and expressed as Mean±SE, E: Erythromycin, CE: Chloroform extract, MF: Methanol fraction, CF1: Chloroform fraction 1, CF2: Chloroform fraction 2

The purified fractions were also tested for the inhibition (%) and CFU $mL^{-1}$ studies. The serial micro-dilution results obtained were analyzed using the one way ANOVA and indicated that there is a significant difference in the sensitivity of the micro-organism to the various extracts ($p<0.05$). In the present study, the mean MIC values of the plant extracts against *S. mutans* were statistically significant (Fig. 5a, b).

**Antibacterial activity kinetics:** In this study, a significant decrease in the bacterial growth was observed within a short period of exposure for all extract concentrations (except for 1/4× MIC which was very less and similar to the control). Bacterial growth remained inhibited or low up to 9-24 h after incubation. After 24 h, the growth was found to be stabilized.

The antibacterial kinetic activity of neem fractions against *S. mutans* (Fig. 6) clearly showed that the control group represented a typical growth curve including lag phase and stabilization phase. The 1/4 MIC values seemed to more or less similar to the control group. The 1/2MIC and MIC values also could slow the lag phase for a small fraction of time and then with an extended log phase (Fig. 6). About 2 MIC values were almost similar to the positive control, where they could prolong the lag phase for exactly 24 h and thereafter with a small log phase. This showed the significant effect of the antibacterial activity of the fractions.

**Biofilm inhibition assay:** Biofilm formation inhibition results were found to be promising for the CF2 fraction when compared to methanol and CF1 fraction.

The biofilm inhibition assay was found to be highly significant in terms of positive control and CF2. Though, it was time dependant the effect was significantly seen in CF2, when compared to MF and CF1 (Fig. 7). The results were in accordance to kinetics study. A two-way ANOVA between the treatments and time period was conducted to compare the effect of biofilm inhibition of the extracts. All effects were statistically significant at the 0.05 significance

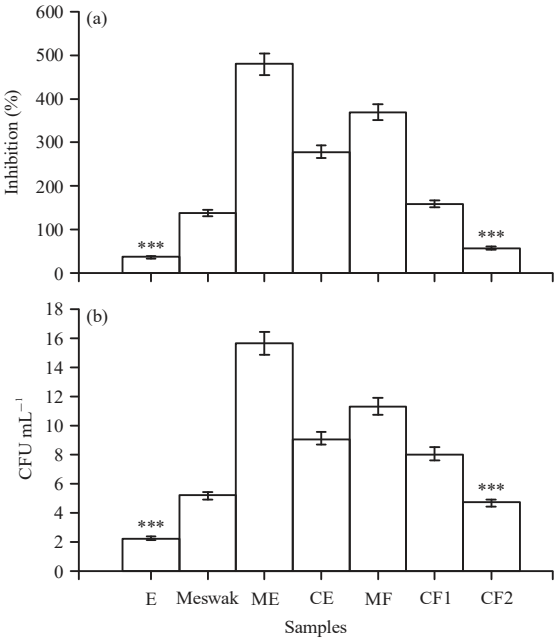


Fig. 5(a-b): Graph showing the (a) MIC and (b) CFU $mL^{-1}$ values of the plant extracts and fractions

All the values were average of triplicates and expressed as Mean±SE, ***Very highly significant, E: Erythromycin, ME: Methanol extract, CE: chloroform extract, MF: Methanol fraction, CF1: Chloroform fraction 1, CF2: Chloroform fraction 2

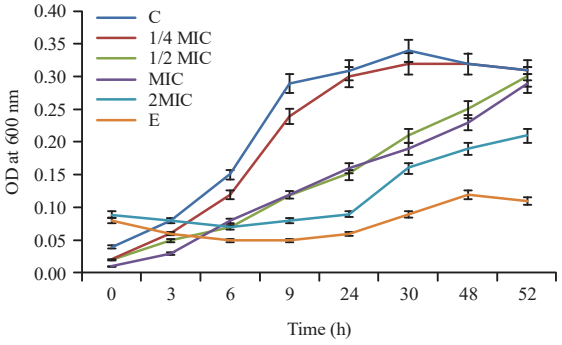


Fig. 6: Kinetics of antibacterial activity obtained from *S. mutans* exposed to the chloroform fraction 2 of neem bark extracts at four different concentrations of MIC values obtained from the MIC curve

All the values were average of triplicates and expressed as OD at 600 nm

level. There was a significant effect between the control and treatments remembered at the $p<0.05$ level [$F(4,8) = 4.20837$, $p = 0.039971$]. However, there was no significant effect on the time period. *Post hoc* comparisons using the Tukey HSD test indicated that the mean score for the control was significantly different from positive control and fraction CF2.

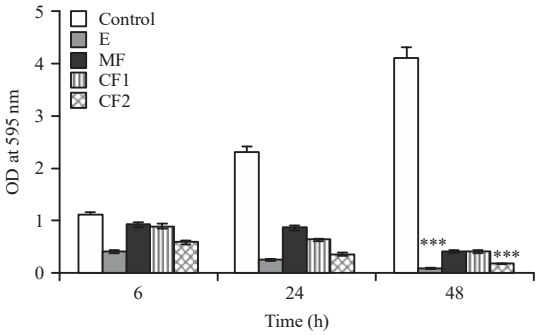


Fig. 7: Biofilm inhibition of the extracts on *S. mutans*

Erythromycin was used as positive control, control was without treatment, all the values were average of triplicates and expressed as Mean±SE, ***Very highly significant

**Validation of the qPCR data:** Real time PCR analysis was performed on the selected genes (*SrtA*, *AgI/II*, *comD*, *recA*). The band length of the PCR products were corresponding to the selected genes and were approximately 470, 490, 217 and 382, respectively. The PCR product for the housekeeping gene, *recA* was found to be 382 bp.

House keeping gene expression seems to be normal and used as normalizer in the qPCR. The relative expression was figured out basing on the band intensity as observed in the gel. On treatment with the experimental sample fraction, Sortase A gene expression was relatively lowered when compared to the control (Fig. 8) and the same was seen in case of Antigen I/II and comD also, but the under expression seems to be almost similar in terms of comD and *SrtA*. The underexpression order was of *SrtA*>comD>AgI/II. The validation was in accordance to the qCR data.

**Real time PCR:** The expression of the selected genes *SrtA, comD, AgI/II* and the house keeping gene *recA* were quantified using real-time RT-PCR (Fig. 9). As shown in Fig. 9, the pattern of expression of the genes from the Ct values depicted the down and upregulation of the selected genes. The *SrtA* gene after treatment was down-regulated by -15.72 fold when compared to the control 1.68. *AgI/II* gene after treatment was also down-regulated by about -11.23 fold when compared to the control 6.62. The *comD* gene was also shown to be down regulated during treatment by -18.01 when compared to control 1.78. All the data were normalized to the reference gene *recA* and kept at 1 (Fig. 10). The expression patterns obtained were in accordance to the conventional PCR results.

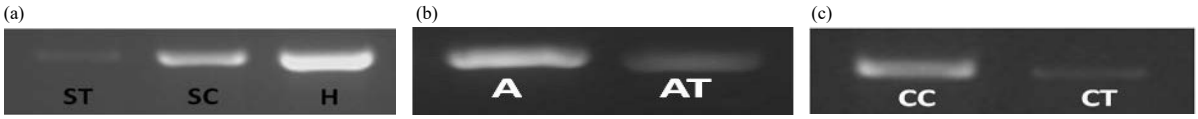


Fig. 8(a-c): 1.2% gel images showing the band length of the selected genes
ST: *SrtA* treatment, SC: *SrtA* control, H: House keeping gene, A: AgI/II control, AT: AgI/II treatment, CC: comD control, CT: comD treatment

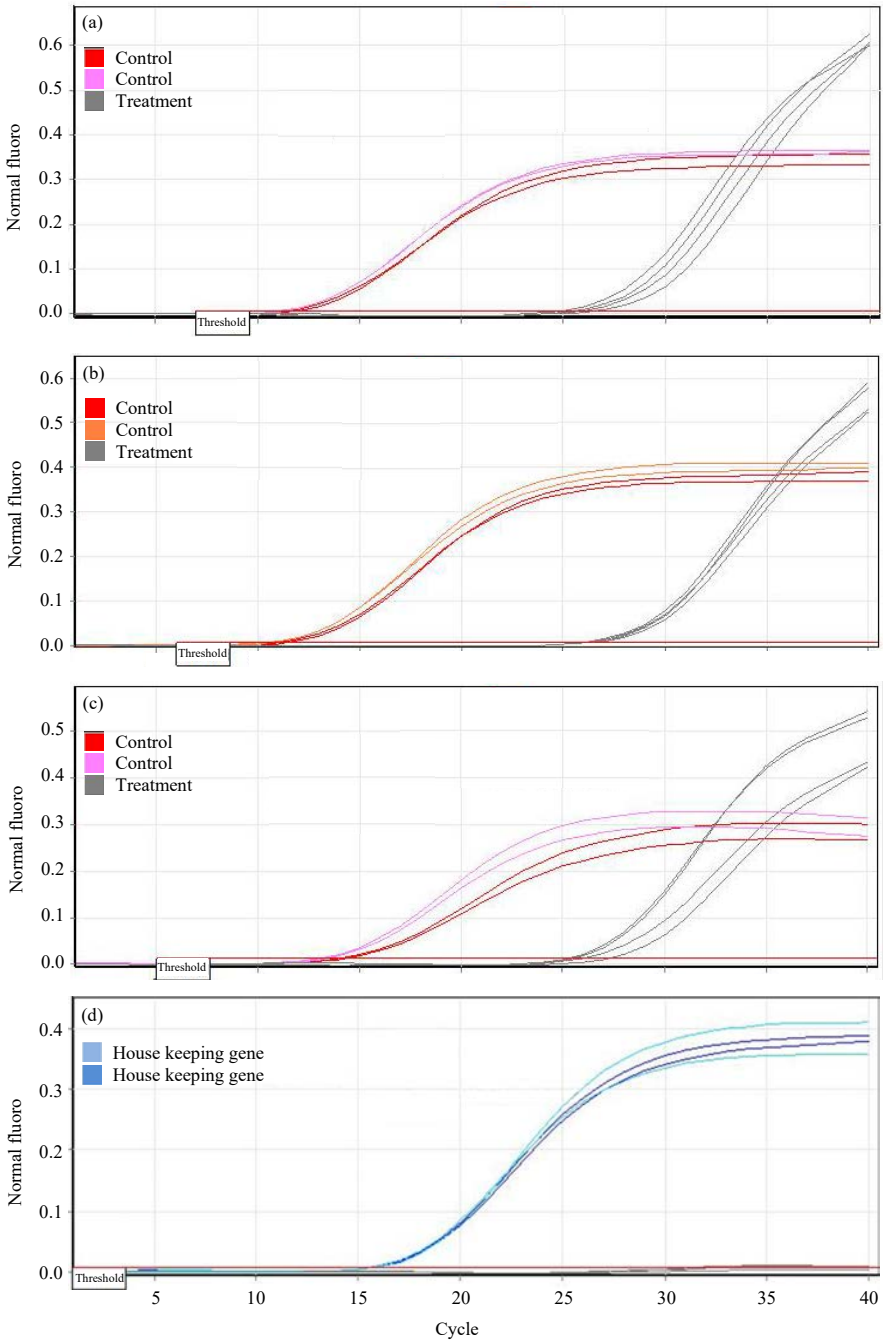


Fig. 9(a-d): Real time quantitative curves of the selected genes (*SrtA*, *comD*, *AgI/II*)
*recA* acts as house keeping gene and used as normalizer, all the experiments were done in quadruplicates

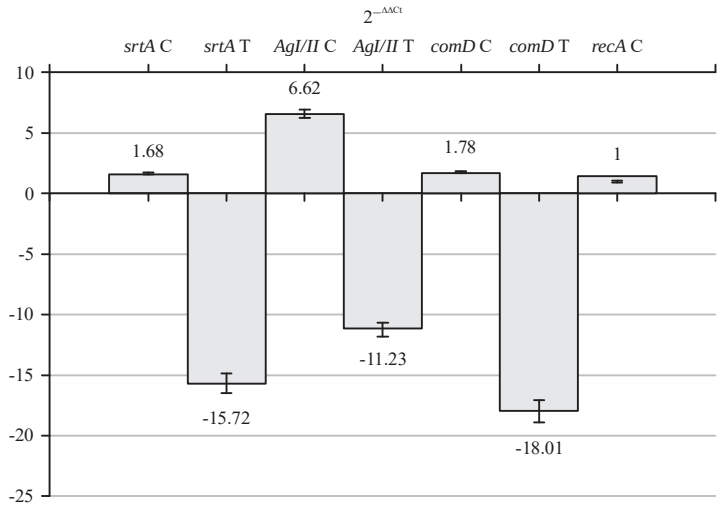


Fig. 10: Validation of real time RT-PCR. The Ct values were used for the calculation of gene expression using the $2^{-\Delta\Delta Ct}$ formula
*recA* acts as house keeping gene and used as normalizer, All the experiments were done in quadruplicates

## DISCUSSION

In the present context the findings showed that neem extract significantly reduced the expression of sortase A by about -15.72 fold. Baker and Thornsberry[31] reported that *S. mutans* was the least sensitive to the erythromycin with MIC value of <2 mg $mL^{-1}$. The study done by Ahn *et al.*[32] also confirmed of the possible role of erythromycin against the *S. mutans* species.

Neem extract was used earlier in the oral studies and our study also confirmed of the same findings. Even studies confirmed that neem extracts were also effective against other oral pathogens like *Streptococcus salivarius*, *Streptococcus mitis* and *Streptococcus sanguis* are also inhibited by neem extracts[33].

Similar findings were reported in *SrtA*-mutants where *S. mutans* showed decreased ability to colonize the oral cavity and teeth[34]. The tannin fractions purified were screened in the study was found to be significantly effective in biofilm assay and disc diffusion assay. Similar studies were done by Akiyama *et al.*[35] in *S. aureus* and found tannic acid to be 10 times more effective than the positive control antibiotics.

This antibacterial activity might be due to mechanisms which were proposed so far and could be inhibition of extracellular microbial enzymes or direct influence on the microbial metabolism[36].

The tannin fraction obtained also confirmed of the inhibition in the biofilm studies. The biofilm formation was found to be significantly reduced ($p<0.05$) with the tannin fractions when compared to positive control[37]. The GCMS analysis obtained fractions were further tested against the *S. mutans* and similar inhibition findings were found.

## CONCLUSION

The neem plant extracts were extracted and the tannin fractions purified used in the present study showed antibacterial activity and inhibition in the formation of biofilm. Yet, our results confirmed in pre-clinical settings to be able to recommend in preventing oral infections.

In summary, these studies demonstrated that neem tannin fractions can inhibit the activity of *SrtA* by inhibiting the biofilm formation of *S. mutans*. Thus, tannin fractions could be a promising lead compounds to develop novel products which can be incorporated into oral care products like toothpastes, to increase their efficiency as anticaries properties.

## SIGNIFICANCE STATEMENT

The present study concluded the potent antibacterial and antibiofilm activity of the tannin fractions against the *S. mutans*. The study also significantly showed negative regulation of the biofilm inducing genes *comD* and *AgI/II* towards the purified fraction. Further, the study was aimed to purify and identify the respective tannins and confirm the validation by both *in silico* and *in vitro* assays. The study could confirm the usage of specific chemical moieties in the tooth pastes which could be very beneficial to stop the dental caries and other oral infections caused by *Streptococcus* species.

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