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In vitro* antimicrobial and antibiofilm activity of *Saraca indica* against *Streptococcus mutans

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Abstract

Objective: *Streptococcus mutans* (*S. mutans*) primarily inhabits biofilms that form on tooth surfaces, also known as dental plaque. *S. mutans* is a gram positive, non-motile coccus is resistant to most of the conventional antibiotics including rifampicin, ciprofloxacin, and metronidazole. Due to the abundance of various virulence factors found in *S. mutans* it is recognized as a key pathogen in the formation of dental cavities. So, there is a need of a natural compound which have an imprecise therapeutic value all over the world. This research purpose is to investigate the antimicrobial and antibiofilm activity of the plant extract *Saraca indica (S. indica)* against *S. mutans* isolate. **Methods:** Examined the antimicrobial and antibiofilm activity of natural plant extract of *Saraca indica (S. indica)* against *S. mutans* by several in-vitro investigations such as crystal violet biofilm inhibition assay and growth curve. **Results:** At 2.5mg/mL, methanolic extract of *S. indica* inhibited *S. mutans* and in case of biofilm assay, statistical analysis shows 55.22% of biofilm inhibition at a subinhibitory dose of 0.625mg/mL. Altogether, our investigations demonstrate that *S. indica* exhibits both antimicrobial and antibiofilm properties against *S. mutans*. **Conclusion:** Findings from the current study demonstrated that methanol extract derived from *S. indica* exhibit a antibacterial properties and significant reduction in biofilm formation were demonstrated through *in vitro* studies.

Keywords: *Streptococcus mutans, Saraca indica,* biofilm, antibiotic resistance, virulence factors,

INTRODUCTION

Streptococcus mutans (*S. mutans*) recognized as the primary causative agent of dental caries [1]. Dental plaque, or biofilms that develop on the surfaces of teeth, are the primary residence of *S. mutans* [2]. These bacteria utilize glucosyltransferase enzymes (Gtfs) to convert dietary sucrose into both soluble and insoluble glucans. This transformation facilitates extracellular aggregation, leading to the formation of a stable biofilm on the tooth surface [3,4]. The rise and development of antimicrobial resistance among clinical pathogens complicates infectious disease treatment and management [5]. These pathogens are resistant to a variety of antibiotics, posing a significant risk to human beings. Antibiotic-resistant streptococci have been linked to a variety of mechanisms, include the efflux pumps and changes in the antimicrobial target [6].

Due to variety of virulence factors present in *S. mutans* it is consider as the major pathogens connected to the onset of caries [7]. Environmental conditions and genetic factors can both influence the activity of *S. mutans* virulence factors and the formation of biofilms in

the oral cavity [8]. One of the main virulence factors in biofilm that contributes to the development of dental caries is the capacity of *S. mutans* to create organic acids through a variety of carbohydrate metabolism activities (acidogenicity) and to survive in low pH environments. As well as Self-produced extracellular polymeric substances (EPS) matrix consists of protein, polysaccharides, and nucleic acid make up the ecological environment known as a biofilm (plaque), which play a major role in pathogenicity of *S. mutans* [9,10]. Quorum-sensing (QS) signaling molecule called competence-stimulating peptide (CSP) which triggering a chain of events that affect gene expression and various physiological responses leads to different phenotypic outcomes [11].

Various studies have yielded significant insights on mitigating or eliminating *S. mutans* cariogenic potential by compromising its environmental tolerance capacities, such as resistance to oxidative and acidic stress, utilization of carbohydrates, maintenance of intracellular metal ion homeostasis [12]. A study reported the evaluation of dental students knowledge of inheritance patterns, analyzed using SPSS, revealed that 78% of participants were aware of who the father of genetics is, while 22% were not. Among the participants, 25% knew the law of dominance, 10.42% knew the law of independent assortment, and 64.58% preferred all of the above [13].

Natural products have proven the valuable source of novel pharmacological compounds, and there is renewed interest in investigating them as potential drug candidates, particularly in the context of combating antimicrobial resistance [14]. Plant based bioactive compound include flavonoids and chalcones they have an ability to inhibit by disease pathogenesis genes by disrupting QS-associated virulence factors [15]. A study demonstrated that a mouthwash containing green tea, stevia, and fresh coriander showed a zone of inhibition of 32 mm against Streptococcus mutans at a concentration of 100 µg/ml [16]. Additionally, a study demonstrated that gold nanoparticle dental varnish has an effective remineralization effect on demineralized enamel [17].

Saraca indica (S. indica) is a medicinal plant belonging to the family *Leguminosae*. It has drooping branches commonly referred to as Asoka [18]. This tree is significant in Indian cultural practices. The dried bark of the Ashoka tree contains tannins, sterol, catechol, and other organic calcium compounds. The plant's bark was previously utilized to treat piles, fractures and bleeding from ulcers, menstrual bleeding, and indigestion. Flowers have been used in children with the pitta energy degradation, syphilis, hyperdipsia, dysentery inflammation, and scabies [19]. *S. indica* have an anti-inflammatory, antioxidant, and antibacterial properties

because they contain several bioactive compounds flavonoids, terpenoids, alkaloids, phenol, steroids, and saponins [20] These compounds may act individually or synergistically to inhibit the growth and survival of pathogenic bacteria [21]. The primary goal of this study is to investigate the effect of *Saraca indica* against *S. mutans* isolate. To the best of our knowledge, *S. indica* anti-QS properties have not been thoroughly studied in relation to tooth pathogens like *S. mutans*.

MATERIALS AND METHOD

The current investigation was an in *in vitro* trial evaluated in the presence of *Saraca indica* (*S. indica*) compound were obtained from indigenous botanical garden located in Chennai, Tamil Nadu, India. The authenticity of the plant was verified by a qualified botanist. After being extracted from the plant, the leaves cleaned with water and dried naturally almost ten days to treat against *S. mutans*.

Solvent extraction

10 grams of *S. indica* leaves powder mixed in 50mL of methanol distributed across two maceration containers for 48 hours. Following the extraction phase, the resulting suspension was filtered using Whatman No. 1 filter paper which was layered over the funnel housing the filter paper with a white muslin cloth. The filtrate was concentrated in a hot water bath that was precisely kept at 50°C. The desiccated filtrate was quantified, and the dried material was meticulously weighed before being stored at 4°C for later use.

Bacterial Strain and Growth condition

Clinical samples of *Streptococcus mutans* (*S. mutans*) used in the current investigation was obtained Saveetha dental college and hospital, Tamil Nadu, India. Bacteria were habitually grown aerobically in Luria Bertani (LB) (HiMedia, India) broth culture maintained at 37^{0} C with a shaking incubator (100rpm) for 24 hours. Characteristic growth patterns were seen on BHI agar (Brain Heart Infusion Agar), MSB Agar (Mitis Salivarius-bacitracin) and various phenotypic tests were conducted such as gram staining, catalase, oxidase, motility, Hugh-Leifson’s oxidative-fermentative (OF), and citrate were documented. The bacterial cultures were routinely sub-cultured for use in the experiments, and biochemical confirmation was done on all occasions.

Antimicrobial activity of *S. indica.*

The agar well-diffusion method was utilised to ascertain the antibacterial activity of *S. indica.*[22]. The bacterial cultures of with *S. mutans* tested were spread on Mueller Hinton

agar (MHA) (HiMedia, Mumbai, India) using a swab that is sterilised and dampened with the bacterial suspension. A well with a diameter of 8mm was punched with a sterile cork borer into the MHA medium and filled with 40µL (*S. indica*) for a well. Plates were then incubated in for 24 hours at 37°C in an upright position. After incubation, the zone wells were measured using a Vernier calliper in mm scale for the detection of antibacterial activity.

Antibiotic susceptibility testing

The Kirby-Bauer disk diffusion method was tested to determine antimicrobial susceptibility against *S. mutans*, Briefly, the inoculum containing a bacterial culture of *S. mutans* was spread on MHA plate with a sterile swab moistened with the bacterial suspension against classical antibiotics.

Evaluation of minimum inhibitory concentration

The **minimum inhibitory concentration** (MIC) for *S. indica* against *S. mutans* was determined using the broth micro dilution method, which evaluated at different concentrations between 10 mg/mL-0.019mg/mL. MIC for the methanol extract was determined using established protocols [23]. Briefly, Eppendorf tubes containing LB broth were filled with 10µL of broth culture with 0.5 McFarland turbidity standard units (1.5 × 108 CFU/mL). After successive dilution with*, S. indica* all of the tubes were incubated at 37°C for a day. After 24 hours 40µL of 2,3,5-triphenyl tetrazolium chloride (TTC) added to the tubes and watching for a change in colour, the results were confirmed. If there is no growth (no colour) is observed at the lowest concentration is recorded as MIC. According to the findings, sub-MIC antibiofilm studies were carried out.

Crystal violet biofilm inhibition assay

The crystal violet staining assay was used to determine the effect of *S. indica* extract on biofilm formation of *S. mutans*. Overnight culture of *S. mutans* (20µL) was loaded into a microtiter plate containing 180µL fresh BHI medium and the extract added in a dose-dependent manner (1.25mg/mL-0.002mg/mL) and this was incubated for 48 hours at 37ºC. After that surface adherent biofilm was stained with 0.1% crystal violet (CV) solution, the planktonic cells had been eliminated by washing with sterile distilled water. Ten minutes later, the adherent biofilm bounded CV was eluted in 200µL of 70% ethanol, its concentration was determined using a UV-Vis spectrophotometer to measure crystal violet intensity at 520 nm. The experiments conducted in triplicate and the subsequent equation was used to determine the percentage of inhibition.

$$Control\ OD\ 520nm - Treated\ OD\ 520nm/Control\ OD\ 520nm \times 100$$

Bacterial growth curve

S. mutans bacterial growth was examined in both the presence and absence of *S. indica* at a concentration of 0.625mg/mL. For a maximum of 24 hours, the bacterial culture was incubated at 37°C, and the cell density was assessed at OD 600nm every 1 hour.

Estimation of Exopolysaccharide (EPS) in *S. mutans*

The EPS extraction process adhered to a previously defined technique. *S. mutans* cultures were first cultivated aerobically in BHI broth that had been enhanced with 1% sucrose. After adding a control and varying amounts of *S. indica* extract (1.25mg/mL-0.002mg/mL), the cultures were cultured for 24 hours at 37°C in a shaking incubator (100 rpm). The cultures were centrifuged for 15 minutes at 10,000 rpm after the incubation period. The resultant bacterial pellets were centrifuged again for 30 minutes at 10,000 rpm after being reconstituted in 50 mL of high-salt buffer containing 10 mM $KPO4$, 7.5 mM $NaCl$, and 2.5 mM $MgSO4$. The supernatant was mixed with methanol and centrifuged again for 30 minutes at 10,000 rpm. After that, Milli-Q water was used to dissolve the separated EPS. A crimson coloration was obtained by mixing 1 millilitre of precipitated EPS with 1 millilitre of cold 5% phenol and 5 millilitres of concentrated sulfuric acid for EPS analysis. Using a UV-Vis spectrophotometer (Biobase BK-D 590 Double beam scanning UV/Vis China), the intensity of this colour was measured at OD 490 nm in both the control and treated EPS samples. Using OD measurements at 600 nm, the percentage of growth inhibition for the treated strains was compared to the untreated control.

Statistical evaluation

Every experiment was carried out in three duplicates. Statistical significance for the quantification of biofilm (crystal violet assay), growth curve analysis and EPS quantification was analysed. All the experiments were statistically evaluated using Microsoft Corporation,2018. Microsoft Excel.

RESULTS

Biochemical Characterization and Antimicrobial Susceptibility

On a standard culture medium, morphological profiling of the isolates and discrete morphotype observation were conducted. The *S. mutans* appeared as Gram-positive coccus shaped on gram staining, catalase negative, non-motile and oxidase negative.

Antimicrobial susceptibility: The zone of inhibition was measured using *S. indica* extract recorded diameter of 10mm, our findings suggest that *S. indica* has antimicrobial activity against *S. mutans*

Antibiotic sensitivity testing (ABST): ABST was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) Guidelines 2022 [24]. In our finding Metronidazole,

ciprofloxacin, and rifampicin showed no zone of inhibition, indicating that *S. mutans* are resistant to these drugs.

At the lowest concentration 2.5mg/ml, *S. indica* inhibited *S. mutans*

A two-fold serial dilution method (ranging from 10 mg/ml to 0.019mg/ml) was done to analyse the antibacterial activity of the *S. indica.* We found that *S. mutans* growth was inhibited at the end-point concentration of 2.5mg/ml (Fig 1). Hence, sub-MIC concentration of the *S. indica* was used to determine the anti-biofilm and anti-virulence activities (Table 1).

Table 1: Minimum inhibitory concentration of *S.indica* against *S.mutans*

S.no	Two-fold dilution concentration (mg/mL)	Growth
1	10	-
2	5	-
3	2.5	-
4	1.25	+
5	0.625	+
6	0.312	+
7	0.156	+
8	0.078	+
9	0.039	+
10	0.019	+

Note: “- inhibited,” +” “Growth Growth

***S. indica* inhibited QS-dependent virulence factors and biofilm in *S. mutans* at the sub-MIC level**

By applying 0.1% crystal violet dye to a static microtiter plate, the inhibitory effect of *S. indica* on *S. mutans* ability to produce biofilms was investigated. A maximum of 55% inhibition was found by spectrophotometric measurement with *S. mutans* at a dose of 0.625 mg/mL (Fig 2).

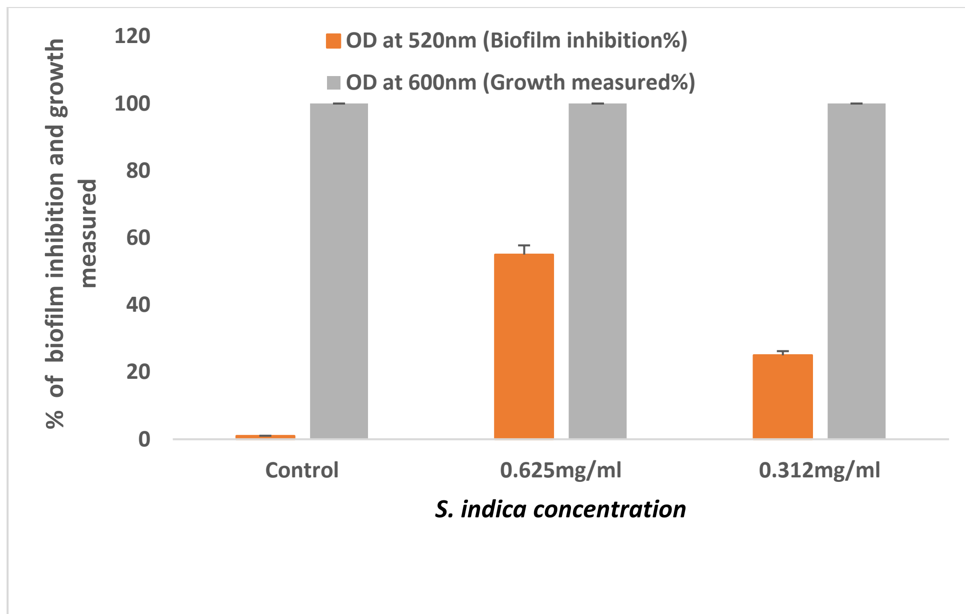

Fig. 2. A) Effect of *S. indica* at sub-inhibitory concentration of 0.625mg/mL and 0.312mg/mL. B) Inhibition of biofilm formation in *S.mutans* treated with 0.625mg/mL and 0.312mg/mL of *S. indica* extract.

Bacterial growth curve analysis

The growth curve was performed in the presence and absence of *S. indica*. The results revealed that *S. indica* doesn't inhibit bacterial growth at 0.625mg/mL (Fig 3). The spectrophotometric analysis shows that there is no difference between the control and treated bacterial cells at 600nm.

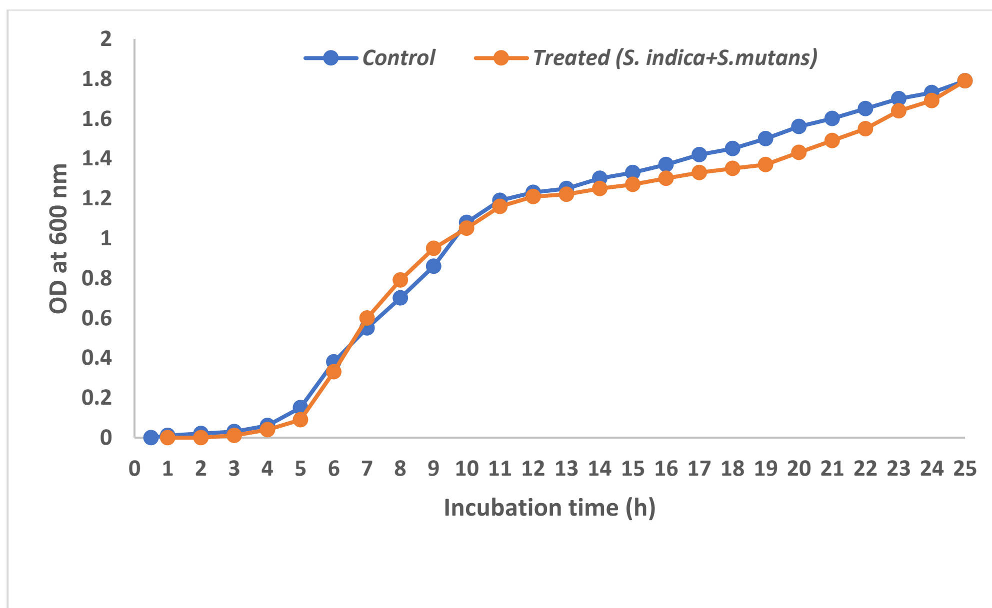

Fig. 3 Growth curve analysis. *S. mutans* grown without (control) and in the presence of *S.indica* extract at the concentration of 0.625mg/mL.

Effects of *S. indica* in *S. mutans*

The purpose of the study was to investigate how *S. indica* affects the production of extracellular polymeric substances (EPS), which are essential to preserving the structural integrity of biofilms. The findings showed that *S. mutans's* generation of EPS was reduced by 10.75%, 18.8%, and 30.25%, respectively, at doses of 0.625 mg/mL and 0.312 mg/mL of *S. indica* (Figure 4). These results highlight the effectiveness of tea tree oil in reducing EPS formation in *S. mutans*, which in turn affects the biofilm architecture.

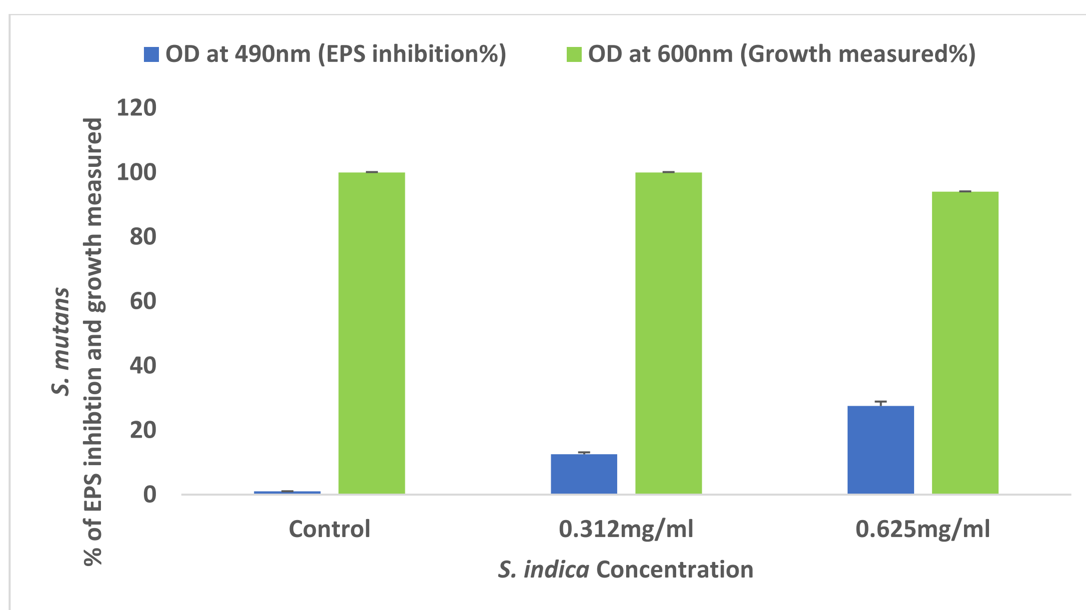

Fig. 4 shows how the growth and generation of EPS are inhibited. The EPS pigment of *S. mutans* was inhibited by *S. mutans*, reaching sub-inhibitory concentrations of 0.312 mg/mL and 0.625 mg/mL, respectively, resulting in levels of 12.5% and 27.5%.

DISCUSSION

Several research groups are currently investigating different strategies to developing new drugs with antimicrobial and anti-biofilm properties required for treating dental infections. Treating infections caused by biofilm-forming *S. mutans* remains a significant challenge due to bacterial resistance to several drugs includes antibiotics [6]. In this study we determine the potency of *Saraca indica* (*S. indica)* a ayurvedic herbs was evaluated by its ability to inhibit QS dependent factors production in *S. mutans.* In our study when the *S. mutans* treated with *S. indica* inhibit cell wall of bacteria. A recent study by Kamarehei et al. [25] explain the role of natural compound curcumin (CUR) have antibacterial and antibiofilm activity against *S. mutans* by downregulating glucosyltransferase enzymes and quorum-sensing genes. In another

study Cui et al [26] reported that marine natural product such as oxazole derivatives, 2-Aminoimidazole (2-AI) derivative also have antibacterial activity against *S. mutans.* Recently Deepika et al [27] reported that *Ocimum sanctum* plant extract illustrates antimicrobial efficacy against anaerobic oral microbes.

We performed the antibiofilm activities of *S. indica* extract against *S. mutans*. In primary evaluation *S. indica* inhibited bactericidal activity at lowest concentration of 2.5mg/ml (Fig 1). The current data slightly support the findings of He et al [28]. In which they reported that an organic compound cinnamaldehyde inhibit the *S. mutans* at MIC concentration of 2 mg/mL. Whereas Salles Branco-de-Almeida et al [29] found out that a compound 7-Epiclusianone derived from *Rheedia brasiliensis* plant which inhibit the *S. mutans* at a concentration of 1mg/mL. A recent study found that graphene had the strongest antimicrobial activity against all oral pathogens [30]. Additionally, a study shown that green synthesis of CaOHAgNPs produced an effective nanoparticle formulation that could be used against common oral pathogens [31].

Afterall, we found that at sub-MIC *S. indica* reduced *S. mutans* QS-dependent biofilm formation in a dose-dependent manner. As per crystal violet assay, big dose of *S. indica* extract significantly reduced biofilm formation without influencing planktonic cell proliferation (Fig 2). Additionally, our investigation indicated that *S. indica*, at concentrations of 0.312 mg/mL and 0.625 mg/mL, significantly reduced EPS production by *S. mutans* by 12.5% and 27.5%, respectively (see Figure 4). The gathered data is camparable with earlier studies [25,32]. Previously it was found out that a plant *Withania somnifera*, also known as Ashwagandha, a member of *Solanaceae* family which reduces the biofilm at higher concentration [33]. One of the primary components of propolis is propolis essential oil (PEO) which reduces the total biomass of biofilms and damages the biofilm structure of *S. mutans* [34]. Recently Rudin et al [35] reported that natural flavonoid phloretin significantly inhibit biofilm formation at sub-MIC level of 200 µg/ml. Altogether *S. indica* important in suppressing the QS system in *S. mutans*. Nonetheless, more research is needed to isolate active components that are likely to have anti-QS and anti-biofilm-producing properties.

CONCLUSION

Our research findings indicate that the methanol extract derived from *S. indica* exhibit an antibacterial properties and significant reduction in biofilm formation which demonstrated through rigorous *in vitro* studies. The findings presented here in not only enhance our understanding of *S. mutans* behaviour but also open avenues for targeted interventions in combating oral infections. This research contributes to the broader field of antimicrobial

resistance and oral health, emphasizing the importance of further exploration to develop effective strategies for managing microbial populations in the oral environment.

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