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Synergetic activity of a quorum sensing inhibitor and antibiotics to combat oral adhesivity of *Streptococcus mutans*

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ABSTRACT

Streptococcus mutans is the principal pathogen causing human dental caries which can switch between planktonic and biofilm (plaque) forms on oral surfaces. This eventually leads to the development of dental caries and a marked reduction in the effectiveness of antibiotic treatment. The objective of the study is to evaluate the effect of the compounds characterized from the bark ethanolic extract of *Melia dubia* (quorum sensing inhibitor) that could curtail the biofilm formation and subsequent adhesion of *S. mutans* and synergistically bolster the activity of antibiotics by regulation of gene expression. The results reveal that at a concentration of 20µg/ml, the extract has a consistent biofilm inhibitory action that also corresponds to a near-normal cell growth, suggesting the absence of antibiotic activity. This concentration of extract shows a considerable increase in biofilm inhibition and reduction in cell growth when used in combination with penicillin (MIC of 30mg/mL) and gentamycin (MIC of 10mg/ml). This Quorum Sensing Inhibitor (QSI) in the bark extract has a great potential in eliminating the biofilm of *S. mutans*, especially when used in a combinatorial control regime with penicillin and hence is a promising solution to eradicate the complications arising out of dental plaque formation.

KEYWORDS

Melia dubia;
Streptococcus mutans;
Quorum-sensing inhibitor;
Synergistic interaction.

INTRODUCTION

Dental caries and its associated oral problems are prevalent among populations throughout the world. Statistical estimates suggest that over 60% in India and around 50% globally are afflicted by dental caries[1,2]. The oral cavity serves as a thriving habitat for bacterial communities which are varied in structure and composition[3]. Bacterial species in the oral cavity total more than 700, inclusive of pathogenic and non-pathogenic microbes. Among the pathogens, oral *Streptococci* accounts for more than 20% of the population. *Streptococcus mutans*, a gram-positive bacterium, is a major etiological agent of oral disorders[4] and the most cariogenic among invasive oral *Streptococci*[5]. Clarke was the first to isolate *S. mutans* from dental sources, where it prefers a biofilm lifestyle[6]. Such a biofilm form is vital not only for its survival, but also to establish its

pathogenicity[7]. Recently, it was found that biofilm formation along with bacteriocin production and competence development of the *S. mutans* was regulated through the population dependant pathway of Quorum sensing[8]. Quorum sensing is induced by chemical messengers (such as pheromones or autoinducers) that get released into the environment and tend to increase in concentration in proportion to the growing population. The Quorum sensing circuit of *S. mutans* involves six gene products, encoded by the genes *comAB, comCDE,* and *comX*[9]. The genes *comC, comD* and *comE* encode a competence stimulating peptide (CSP) precursor, its histidine kinase sensor protein, and a cognate response regulator respectively. The genes *comAB* encode a CSP-specific secretion system which consist of an ATP-binding cassette (ABC) transporter (comA) and its accessory protein (comB), involved in the processing and secretion of the CSP. The *comX* gene encodes for an alternative sigma factor (comX) that evokes the transcription of late competence genes. Lately *comW* and *comY* were also found to participate in the quorum sensing signaling, though the exact role is obscure.

Biofilms are substratum-attached microbial communities, anchored in the organic polymer matrix. They act as an effective barrier and protect the bacterial populations embedded in them. In *S. mutans* biofilms are found to have enhanced transformation efficiency than the planktonic forms[9]. The biofilm phenotype also exhibits an increased acid tolerance and limits the penetrations of the antimicrobials to the inner cells, making it more resistant[10,11]. Bacteria embedded in biofilms are more than a 1000-fold resistant to the action of existing antimicrobial compounds[12,13]. A quest for suitable natural substances that can combat caries disease has been the focus of researchers[14]. The emergence of multi-drug resistant strains has led us to explore alternate strategies to handle the disease causing microbes. Of late, targeting the quorum sensing system to control the biofilm-mediated infections has gained limelight. The use of quorum sensing blockers specifically attenuates the virulence, by selectively removing the pathogens with little or no side reactions to the host organism[15]. Honey has been known to contain antimicrobial[16] and antibiofilm[17] compounds towards combat of *S. mutans*. The use of medicinal herbs of Indian origin for treating dental caries is common, even though it is not corroborated by scientific evidence[18]. The main benefit is the lower chance of occurrence of hypersensitivity and associated side effects. Essential oils contain bioactive compounds which act as biofilm inhibitors in *S. mutans*[19]. Various parts of neem are used as folk medicines for diverse aliments. Chewing sticks originating from *Azadirachta indica* (Neem) are used as oral hygiene aids throughout Asia and Africa[20]. *Melia dubia*, a plant from Meliaceae family has been used in folk medicine as a bactericidal agent[21].

Dentists prescribe antibiotics such as gentamycin and penicillin against the infection of *S. mutans*, in the backdrop of *Streptococci* exhibiting a trend of increasing resistance to action of penicillin. The main objective of this study is to understand the synergistic action of a quorum sensing blocker (*M. dubia* bark extract) and antibiotics (gentamycin and penicillin) on the adhesivity of *S. mutans*.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The strain of *Streptococcus mutans* [Microbial Type Culture Collection (MTCC) #890] was procured from the MTCC, Institute of Microbial Technology, India. Biofilm assay and protein analysis were conducted by *S. mutans* in Brain Heart Infusion (BHI) medium supplemented with 5% sucrose.

Preparation of *Melia dubia* bark extracts

Processing of the material

Bark samples of *Melia dubia* was collected. The authentication of the bark samples was done by Dr.M.Jagadeesan. Voucher herbarium (TUH 285) specimens of the plant were deposited in the Department of Environmental and Herbal science, Tamil University, Tanjore, India[22]. The cleaned patches of bark were shade dried in a sterile environment and powdered.

Preparation of crude extracts

Bark extract was prepared using ethanol as the solvent of preference[23]. Dry bark powder (100g) was soaked in 70% ethanol (1L) for 8-10 days and stirred every 10th hour using a sterile glass rod. After the ex-

traction process, it was filtered and the alcoholic filtrate was lyophilized and stored at -80ºC[22]. The crude extract was formulated by dissolving a known amount of the dry extract in PBS (Phosphate buffer saline) to have a stock concentration of 10mg/mL.

Protein quantification

The culture samples grown in the presence of the plant extract were collected every six hours and the intracellular and extracellular protein was quantified using Hartree-Lowry protein estimation[24]. To 1mL of sample, 0.9mL of reagent A (7mM sodium potassium tartrate, 0.81M sodium carbonate, 0.5N NaOH final concentration) is added and kept for ten minutes of incubation in a 50ºC bath. Then it is cooled to room temperature and 0.1mL of reagent B (70mM sodium potassium tartrate, 40mM copper sulfate) is added, and then incubated at room temperature for 10 minutes. 3mL of reagent C (Folin-Ciocalteau reagent 1:15 (v/v)) is rapidly added and finally incubated for ten minutes in a 50ºC bath. Absorbance is read at 655nm.

Assessment of anti-biofilm effect of bark ethanolic extract of *Melia dubia*

Biofilms were developed in 96-well polystyrene microtitre plates. The growth of biofilm was commenced by inoculating 1mL of *S. mutans* overnight culture into 150mL of BHI broth with 5% sucrose and containing varying concentrations (10µg/mL, 20µg/mL, 30µg/mL, 40µg/mL, 50µg/mL, and 100µg/mL) of bark ethanolic extract. The microtitre plates were then incubated at 37 ^{0}C without agitation for 36 hours, analysis done every 6th hour of growth. After the required period of incubation, media and non-adhered cells were decanted from the wells of the microtitre plates. The planktonic cells that remained were removed by rinsing. The adhered biofilm in the wells was fixed with 37% formalin and 2% sodium acetate (in 1:10) and the cells constituting the biofilm were stained with 0.1% crystal violet for 15 minutes at room temperature. The cells were cleansed of the crystal violet by using sterile water, following which bound dye was eliminated from the cells using 95% ethanol. Full extrusion of the dye was facilitated by setting the plates on a shaker for 5 minutes. Optical density was measured at 600nm using a BioRad iMark™ Microplate reader[25].

Assessment of synergistic interaction of bark ethanolic extract and antibiotics

Synergistic interaction between the bark ethanolic extract and the antibiotics (gentamycin and penicillin) was observed by agar well diffusion method. Onto Mueller Hinton agar plates, an inoculum of 1mL was spread. Care was taken to punch holes such that the inhibitory zone of one compound does not coincide with that of another. The wells were inoculated with 200µL of bark extract, antibiotic and the antibiotic supplemented with the bark extract. Phosphate buffered saline (PBS) was used as blank. The plates were incubated overnight at 37ºC. The influence of this synergistic interaction on biofilm formation of *S. mutans* was also studied by following the biofilm assay described above.

Autoaggregation assay

For calculating the percentage autoaggregation, overnight grown culture of *S. mutans* was inoculated in the freshly prepared BHI broth containing the antibiotics (gentamycin and penicillin) along with the bark ethanolic extract. The optical density of the samples was measured initially (OD_0) at 660nm and thereupon after sixty minutes, the cell samples were centrifuged briefly at 2000rpm for 2 minutes. The optical density of the supernatant was measured (OD_{60}). The degree of auto aggregation was calculated by using the equation

$$\% \text{ Autoaggregation} = \frac{OD_0 - OD_{60}}{OD_0} \times 100 \quad (1)$$

Statistical analysis

The experimental results were given as Arithmetic mean ± Standard error. Differences were considered statistically significant at the value of probability less than 5% ($P<0.05$)[22].

RESULTS

Influence of bark ethanolic extract of *M. dubia* on biofilm

The Quorum sensing inhibitory activity of the *M. dubia* ethanolic bark extract was analyzed under dif-

ferent concentrations and time intervals. The maximum biofilm inhibition (95.7%) was recorded at the lag phase when exposed to 10µg/ml concentration (results not shown), and consistently decreased in other phases without any remarkable change in the growth pattern. For a concentration of 20µg/ml of the bark ethanolic extract, the biofilm inhibitory results are significantly consistent at every phase of growth, with consistent biofilm inhibition of 40% to 60% (Figure 1). Interestingly, the cell growth increases in a time dependent manner. The 30µg/ml of bark ethanolic extract shows that the biofilm inhibition decreases gradually from the beginning, having values less than zero percentage. The cell growth pattern remains unchanged (results not shown). On exposure to higher doses of the extract (40µg/ml, 50µg/ml and 100µg/ml), no consistency in the biofilm inhibitory activity was noted in the extract (results not shown). At higher concentrations too, the growth is over 80% of that of the control. The collected data suggests that the minimum biofilm inhibitory concentration (MBIC) of the extract on *S. mutans* is 20µg/ml.

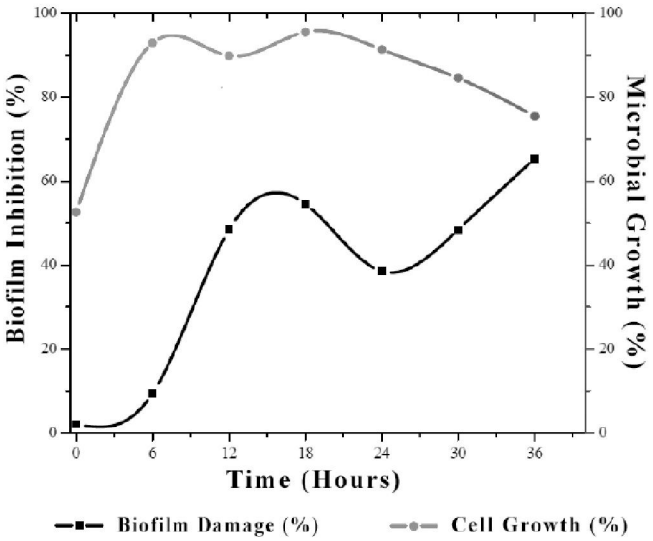

Figure 1 : Influence of 20µg/ml concen FigureraFigureion of Figurehe bark eFigurehanolic exFigureracFigure of *M. dubia* on Figurehe biofilm of *S. muFigureans*

Influence of the extract on intracellular protein (ICP) and extracellular protein expression (ECP)

The drug dose (10µg/ml) increased the concentration of ECP gradually and reached maximum in the late stationary phase. The ICP profile had a fluctuating response and no significant conclusions could be drawn at this concentration. The drug dose (20µg/ml), effective on inhibiting biofilm, showed an ICP and ECP expression of the range 60% to 140%. The maximum expression was observed at the late exponential phase for ICP (139.5%) and mid-exponential phase for ECP (141.7%) (Figure 2). The drug dose of 30µg/ml shows a fluctuating response for ECP and the ICP expression levels were highest (47.2%) at the mid exponential phase of growth. Similar patterns of ICP and ECP levels were seen in the concentrations of 40µg/ml and 50µg/ml of the extract. But in an extract concentration of 100µg/ml, the effect on protein expression levels are inconsistent for ECP and the ICP expression profile was seen to gradually increase and reach maximum at the mid exponential phase.

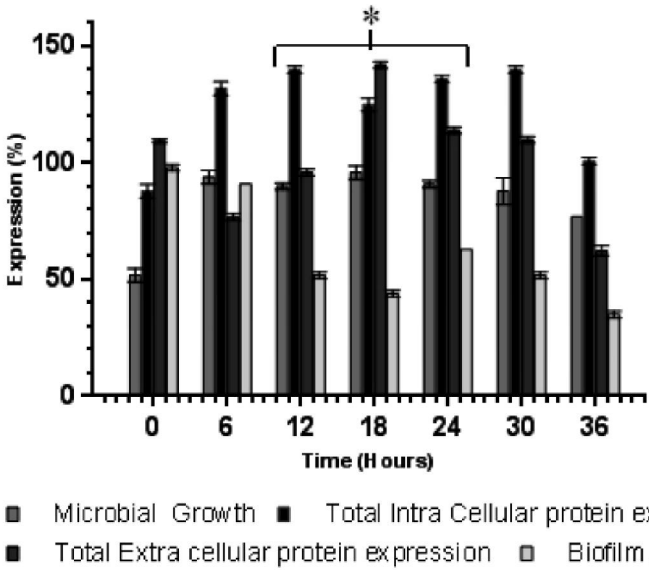

Figure 2 : Influence of 20µg/ml concenFigureraFigureion of exFigureracFigure on Figurehe microbial growFigureh, biofilm adhesion and FigureoFigureal proFigureein expression (inFigureracellular and exFigureracellular).

Figure 2. Influence of 20µg/ml concentration of extract on the microbial growth, biofilm adhesion and total protein expression (intracellular and extracellular).

Influence of time and varying concentration of ethanolic bark extract of *M. dubia* on the synergistic action with the antibiotics

The study utilized two antibiotics (gentamycin and penicillin) that are applied under current clinical practices. Since the above data shows that 20µg/ml extract concentration proved to be the minimum inhibitory concentration for the quorum sensing inhibitor (QSI), its

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significance was further utilized to understand the synergistic action with antibiotics in either increasing or decreasing the activity of antibiotics in a time dependent manner.

Figure 3 shows that the biofilm inhibiting efficacy of gentamycin was considerably reduced in the presence of extract. The highest inhibition was recorded at the late stationary phase for gentamycin (98.9%) and gentamycin along with the extract (96.27%). The biofilm inhibiting efficacy of penicillin was enhanced by the addition of extract initially (till early exponential phase) and gradually it was reduced. When compared to the action of gentamycin, the gentamycin along with the extract showed little variation in the cell growth. The cell growth pattern is not affected greatly by the addition of the extract. In the case of penicillin, the cell growth pattern in both cases showed a marginal difference. There was slight enhancement in the presence of the extract. This may be due to the down-regulation of the genes by the extract.

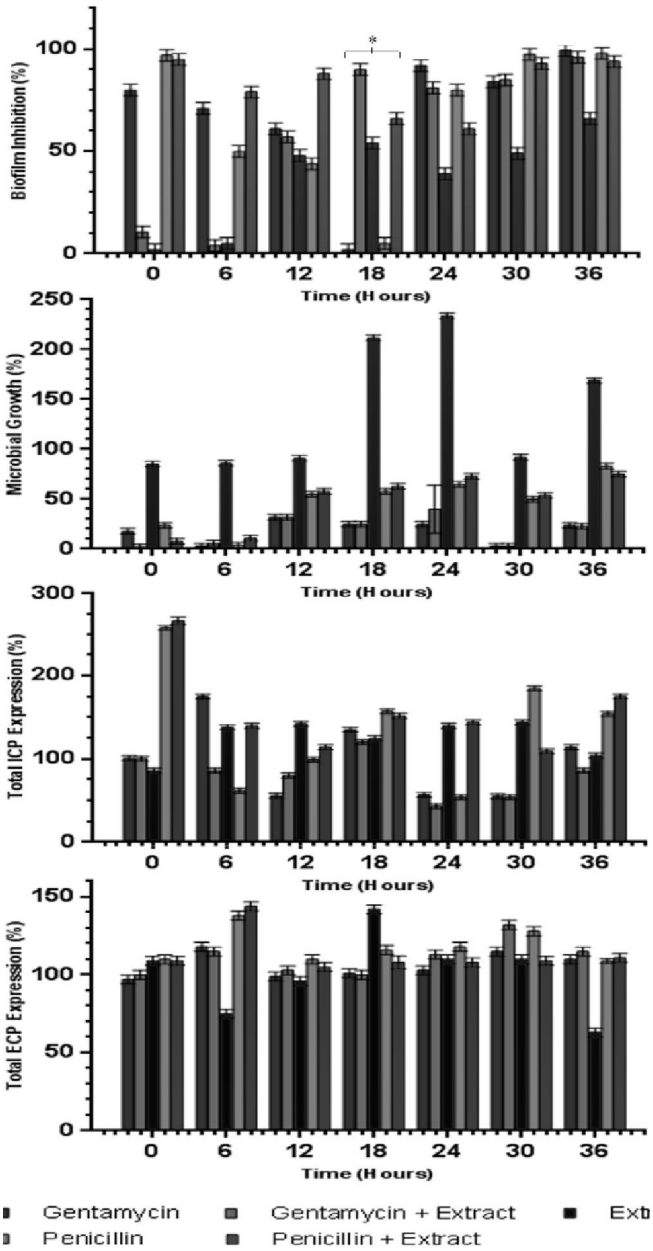

Figure 3 : SynergeFigureic acFigureiviFigurey of exFigureracFigure (MBIC of 20µg/ml) wiFigureh anFigureibioFigureics on biofilm adhesion, cell growFigureh, FigureoFigureal in Figureracellular proFigureein expression and FigureoFigureal exFigureracellular proFigureein expression.

In the Intracellular protein profile there is no significant difference in the activity of gentamycin. The ICP was enhanced to an extent for penicillin in the presence of extract. For gentamycin, there is no significant change in the extracellular protein profile in both the cases. There was a slight reduction in the expression of ECP for the penicillin containing the extract. The above results conclude that the synergistic action of gentamycin and the bark ethanolic extract is of little significance.

In contrast, the synergistic interaction shown between penicillin and the ethanolic bark extract shows that the genes associated with the biofilm formation are initially down-regulated until the early exponential phase. The difference in the protein expression pattern also confirms the same.

The auto aggregation studies conclude that the degree of auto aggregation of the control gradually increases till the exponential phase (24th hour) and then it decreases. Auto aggregation results of the antibiotics follows the same pattern as that of the control, with the values less than the control. There is a gradual increase till the early exponential phase and it reaches maximum at the mid exponential phase (18th hour) for all the cases.

DISCUSSION

Results show that 20µg/ml concentration of extracts proves to be the MBIC. The 20µg/ml concentration of extracts exhibits highest cell growth in all hours, which clearly denotes the absence of antimicrobial activity and is thus a mere quorum-quenching compound. This 20µg/ml extract concentration has the highest ICP expression levels, which shows that it has up regulated

some of the genes coding for ICP. The 12th hour and the 18th hours where biofilm establishment and its inhibition can be visualized; the 20µg/ml shows high level of biofilm inhibition at these hours. The ECP expression is high for this 20µg/ml concentration at 12th and 18th hour which states that it has some effect on adhesion and biofilm formation which is the major use of ECP at these hours. The study involved two compounds, one which is capable of reducing cell growth (antibiotic), and one natural compound (plant extracts) which inhibits biofilm formation. The extracts have some effect on cell membrane permeability, making the bacterial cell more susceptible to antibiotics. During the log phase when virulence and biofilm formation takes place, the biofilm is inhibited in both the cases (18th hour). In the 6th and 12th hour when biofilm formation starts, a greater ICP concentration and increased biofilm inhibition in penicillin (to which *S. mutans* are resistant) is observed. In the stationary phase, the culture containing penicillin has high cell growth and so it is concluded that *S. mutans* is resistant to penicillin after biofilm establishment.

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CONFLICT OF INTEREST

The authors thus declare that there is no financial/ commercial conflict of interest.

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