3, 5-Di-tert-butylphenol Combat Against *Streptococcus Mutans* Via Impedes Acidogenicity, Acidurance and Biofilm Formation

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Abstract

Streptococcus mutans is a common pathogen present in oral cavity and it cause dental caries for all aged group of people, in particular, children's. *S. mutans* have several virulence factors such as acidogenecity, aciduricity, adhesion and biofilm formation. These virulence factors are working together and lead to initiate development of caries in tooth surface. The present study aimed to investigate the anticariogenic potential of 3, 5-Di-tert-butylphenol (3, 5-DTBP) against *S. mutans*. 3, 5-DTBP biofilm inhibitory concentration (BIC) was found at 100 µg/ml concentration without any lethal effect on the growth. Moreover, 3, 5- DTBP significantly reduced water soluble and water insoluble glucans production, in concurrence with downregulation of *gtfBC* genes. Moreover, acidogenicity associated virulence factors such as lactate dehydrogenase and enolase enzymatic production was arrested upon 3, 5-DTBP treatment. In addition, 3, 5-DTBP greatly reduced acidtolerance ability through impedes of F_1F_0-ATPase. Gene expression analysis unveiled the downregulation of *gtfB*, *gtfC*, *gtfD*, *vicRK*, *comDE*, *gbpB*, *smu0630* and *relA* upon 3, 5-DTBP treatment. The present study paves the way for exhibiting 3, 5-DTBP as a promising therapeutic agent to control *S. mutans* infections.

Introduction

Dental caries is one of the leading oral infectious diseases caused by *Streptococcus mutans* (Marsh 2004). *Streptococcus mutans* is a primary causative agent since its adeptness to extreme environmental stress conditions while biofilm formation and host interactions (Smith and Spatafora 2012). *S. mutans* have equipped multiple factors which assist them to adhere, interact with nearby microbiota which leads to initiate the progression of infection (Brown et al. 2005). *S. mutans* cariogenic virulence and survival ability were controlled by two component signal transduction systems (TCSTS). VicRK is well defined TCSTS, controlled various virulence factors and physiological progress of *S. mutans* such as biofilm formation, regulate *gtfBCD* expression, acid tolerance, oxidative stress tolerance, genetic competence and cell death (Senadheera et al. 2005; Deng et al. 2007; Mattos-Graner and Duncan 2017). ComDE is another virulence gene, regulates gene competence and biofilm formation (Li et al. 2002). In the co-operation of these genes function, assist *S. mutans* to adhere/colonization on the tooth surface which initiate caries development progress (Martins et al. 2018). *S. mutans* synthesis various organic acids (pH range between 3-5 pH) done by the carbohydrate fermentation process (Matsui and Cvitkovitch 2010). *S. mutans* have survived on a low pH environments; F_1F_0-ATPase is involved to pump the protons from the cell and maintains the inner cell pH level (Lemos and Bume 2008). In addition, biofilm formation keeps control of the antimicrobials penetration, it acts as a primary barrier to protect the bacterial cells during a low pH environment. In particularly mature biofilm cells are high acid tolerance compared to planktonic cells (Welin-Neilands and Svensäter 2007). For their regular usage of antiseptic drugs for oral infections, it could cause severe to mild side effects such as teeth staining and hypersensitivity (Bagis et al. 2011). Hence, the new antimicrobial agents/alternative therapeutic action is needed to control oral infections in clinical settings.

Plants are a promising source for identifying antimicrobial agents with minimal side effects, compared to commercial antibiotics and other sources. This property added additional value for plants sources to produce appropriate antimicrobial agents for clinical applications against infectious diseases (Gyawali and Ibrahim 2014). Several antimicrobial compounds have been discovered from plant sources and many of them are commercially available, with some compounds already have been used for caries prevention (Cardoso et al. 2016; Vijayaumar and Ramanathan 2018; Zhang et al. 2020). The present study is aimed to unveiling the antibiofilm and anticariogenic efficacy of organic compound 3, 5-di-tert-butylphenol (3, 5-DTBP) against a primary etiological pathogen *Streptococcus mutans* and to reveal the mechanism underlying its anticariogenic activity.

Materials And Methods

Bacterial strains and growth conditions

Streptococcus mutans UA159 (reference strain) from American Type Culture Collection (ATCC 700610) was used in the study. It was maintained on tryptone soya agar (TSA) and cultured regularly in tryptone soya broth (TSB) at 37° C for 24 h. The standard cell suspension of *S. mutans* for all *in vitro* experiments was prepared by adjusting the optical density (OD) of overnight culture of 0.4 at 600 nm (1 x 10^8 CFU/ml) using TSB. Note: In all the assays performed in TSB supplemented with 1 % sucrose.

Phytochemicals

Vaccenic acid, glutamic acid, sodium oleate, 4-phenyl butyric acid and 3, 5-di-tert-butylphenol (3, 5-DTBP) were dissolved in methanol (10 mg/ml). The aliquots were filtered (using 0.25 µm pore filter sterilized) and used for antibiofilm assay. All the compounds were purchased from Sigma Aldrich (USA) with maximum purity. Methanol was used as vehicle control in all the assays were 3, 5-DTBP was used.

Screening of phytochemicals antibiofilm efficacy against *S. mutans*

Totally five phytochemicals were screened for their antibiofilm activity using 24 well sterile microtitre plates. Each well contains 1 ml of TSB (supplemented with 1 % sucrose) with 1 % of overnight culture bacterial culture (1 x 10^8 CFU/ml). For screening, 100 µg/ml of each compound was loaded and assay plate was kept at 37° C for 24 h. After incubation, OD at 600 nm was measured. Then planktonic cells were discarded and each well was washed gentle with sterile distilled water to eliminate loosely bound cells and then the plate was allow to air dried. For quantification, biofilm cells were stained with 0.4 % of crystal violet (CV) for 10 min and excess stain was removed by washing plates thrice with distilled water and air dried for 5 min. Biofilms cells were destained using 20 % glacial acetic acid and OD was read at 570 nm (Vijayakumar and Ramanathan 2020a).

Determination Biofilm inhibitory concentration (BIC) of 3, 5-DTBP

To determine the BIC of 3, 5-DTBP, 24 well MTP assay was performed as mentioned above with various concentrations of 3, 5-DTBP ranging from 20, 40, 60, 80, 100 and 120 µg/ml. Well containing TSB (supplemented with 1 % sucrose) + *S. mutans* + methanol were maintained as control. The absorbance of control and treated wells was measured at 570 nm.

Microscopic analysis of biofilm

In microscopic analysis, bacterial cells were allowed to form biofilm on glass slides (1 x 1 cm) in the presence and absence of 3, 5-DTBP for 24 h at 37° C. After incubation, the slides were washed with sterile distilled water and stained as needed. For light microscopic analysis, the washed slides were stained with 0.4 % CV and washed to remove excess stain and allow to air dried. Then, the glass slides were visualized at magnification of 400X under light microscope. For confocal laser scanning microscope (CLSM) analysis, the washed slides were stained with 0.1 % of acridine orange for 10 min at dark followed by washing to remove the excess stain and drying. Finally, the glass slides were visualized under CLSM at magnification of 200X (Vijayakumar et al. 2021).

Effect of 3, 5-DTBP on adherence to glass surface

The effect of 3, 5-DTBP on the sucrose-dependent and sucrose independent adherence of *S. mutans* over the smooth glass surface was performed as described earlier (Hasan et al. 2014). Briefly, *S. mutans* was grown anaerobically at 37° C at an angle of 30° in boiling test tubes containing 10 ml of TSB with or without 5 % sucrose in the presence and absence of 3, 5-DTBP and incubated for 24 h. After incubation, the planktonic cell suspension was removed and the adhering cells were washed by adding 0.5 M of sodium hydroxide followed by agitation. Then, the adhering cells were washed and suspended in PBS saline and the adherence was quantified using a spectrophotometer at OD 600 nm. The percentage of adherence was calculated according to the formula:

Adherence (%) = (OD of adhered cells/OD of total cells) x 100

Effect of 3, 5-DTBP on cell surface hydrophobicity (CSH) of *S. mutans*

To evaluate the effect of 3, 5-DTBP on the CSH of *S. mutans* was performed by microbial adhesion to hydrocarbon (MATH) assay as described earlier with slight modifications (Khan et al. 2010). Briefly, overnight cultures grown in TSB in the presence and absence of 3, 5-DTBP were washed and suspended with sterile saline (0. 85 %) and their optical density (OD) as 0.3 at 600 nm. The cell suspension (3 ml) was taken to the test tubes containing 0.25 ml of toluene. Then, the tubes were agitated uniformly for 2 min and let to equilibrate for 10 min at room temperature. After separation of the toluene phase from the aqueous phase, the OD of the aqueous phase was measured using a spectrophotometer at 600 nm. The percentage of hydrophobicity was calculated according to the formula:

% of hydrophobicity = [Initial OD600nm − Final OD600nm/initial OD600nm] x 100.

Estimation of water soluble and water insoluble glucans

The standard phenol/sulfuric acid estimation method was performed to quantify the water soluble and water insoluble glucans as described earlier (Yano et al. 2020). Briefly, *S. mutans* was cultured in TSB (supplemented with 1 % sucrose) in the presence and absence of 3, 5-DTBP and incubated anaerobically for 24 h at 37° C. After incubation, the cell free culture supernatant (CFCS) was harvested by centrifugation. The supernatant was subjected to ethanol precipitation to obtain extracellular water soluble glucans. The remaining cell pellet was resuspended in 1M NaOH and centrifuged to remove the bacterial cell and, the water insoluble (alkali-soluble) glucan was recovered by ethanol precipitation of the harvested supernatant.

Glycolysis pH drop assay

The level of the glycolysis pH drop of *S.mutans* was measured as earlier described method (Ban et al. 2012). The cells of *S.mutans* from the suspension cultures were centrifuged at 15000 g, washed once with salt solution (50 mM KCl and 1 mM $MgCl_2$) and resuspended in a salt solution with the presence and absence of 3, 5-DTBP (100μg/ml). The pH was adjusted to 7.2 to 7.4 with 0.2 M KOH solution. Glucose was then added to obtain a concentration of 1% (w/v) and the decrease in pH was assessed over a period of 60 min.

Terminal pH determination assay

To analyze the effect of 3, 5-DTBP on acid production, terminate pH assay was performed. Briefly, an overnight culture of *S.mutans* was inoculated with TSB (pH 7.4), with presence and absence of 3, 5-DTBP and incubated at 37° C for 24 hours. The pH of bacterial broth was determined at the before and after 24 h incubation. The changes in pH were recorded (Korithoski et al. 2007).

Lactic acid measurement

Briefly, the cells of *S. mutans* were harvested, washed twice with PBS and resuspended to a final concentration of 1 x 10^8 CFU/ml in a 24 well plate with 1.5 ml buffered peptone water (BPW) supplemented with 0.2 % sucrose containing BIC of 3, 5-DTBP in each well and incubated for 120 min at 37° C. After incubation, planktonic cells were removed by centrifugation (8,000g, 5 min, 4° C), the supernatant (50 μl) was added to 2 ml of 0.2 % iron (III) chloride, stirred and absorbance was measured at 390 nm (Borshchevskaya et al. 2016).

Lactate dehydrogenase assay

To evaluate the lactate dehydrogenase assay (LDH) activity, the overnight bacterial culture (OD_{600} = 0.8) was incubated at 37° C with Tris − HCl buffer, pH 7.0 containing 0.5 mg/ml of lysozyme for 1 h. After the incubation, the cell free supernatant (CFS) was collected from centrifugation at 10000g for 20 min at 4° C. Furthermore, the CFS was dialyzed with 10 mM phoaphate buffer saline (PBS) (pH 7.0) at 4° C for overnight. The dialyzed crude LDH protein content was measured by the Bradford method (Bradford 1976). After that, the LDH activity was determined by the previous method described by (Crow and

Pritchard 1977). The crude LDH was pretreated with 3, 5-DTBP (at 100 µg/ml) at 30 min at room temperature, and the LDH activity was estimated by measuring the rate of nicotinamide adenine dinucleotide (NADH) oxidation at 340 nm. The standard reaction mixture (for 200 µg/ml) contained 180 µl of 50 mM PBS pH 7.0 with 0.167 mM NADH and 10 mM sodium pyruvate; 10 µl of 1 mM fructose 1, 6-diphosphate (FDP) and 10 µl of pretreated LDH. The results were expressed as enzymatic activity compared to that of the control.

Enolase activity assay

To determine the enolase activity with permeabilized cells of *S.mutans* were measured by a method described earlier (Belli et al. 1995). Permeabilized cells were pretreated with 100 µg/ml concentration of 3, 5-DTBP for 30 min at room temperature. The enolase activity was observed by the formation of phosphoenolpyruvate (PEP) at 240 nm. The standard reaction mixture (for 200 µl) contained 180 µl of 20 mM KPO_4 buffer, pH 6.5 with 2 mM $MgSO_4$; 10 µl of 17.6 mM D- (+)- 2- phosphoglycerate and 10 µl of permeabilized cells already treated with the test concentration of the compounds. The results were expressed as enzymatic activity compared to that of the control.

Acid tolerance assay

Acid tolerance ability of *S. mutans* in the presence and absence of 3, 5-DTBP, was assessed by acid tolerance assay. *S. mutans* was grown till mid logarithmic phase and pelleted by centrifugation. The pellet was divided into two aliquots, one aliquot was directly resuspended in TSB at a killing pH of 3.5 and incubated at 37° C for 2 h. These cells were considered as unadapted cells. Then, the other aliquot was first suspended in TSB at a pH of 5.5 and incubated at 37° C for 1 h to ease adaptation. The resulting adapted cells were then transferred to TSB at a lethal pH of 3.5 and incubated for 2 h at 37° C. Cell fractions were removed from these unadapted and adapted cultures at regular intervals (at 0, 1 and 2 h) and their viability was tested by TSB agar plates at favorable pH (pH 7.5) for 48 h at 37° C (Viszwapriya et al. 2017).

F_1F_0 – ATPase activity assay

The F_1F_0 – ATPase activity was determined by using permeabilized cells of *S.mutans* by adding the cells to 10 % toluene followed by series of freezing and thawing as described earlier method. The F_1F_0 – ATPase activity was evaluated by the release of inorganic phosphate in the following reaction mixture 75mM of Tris – maleate buffer (pH 7.0) containing 5mM adenosine – 5 – triphosphate (ATP), 10mM of M_gCl_2, permeabilized cells along with BIC concentration of 3, 5-DTBP. The release phosphate was determined as previously described method (Bencini et al. 1983).

Auto aggregation assay

S.mutans was grown in the presence and absence of 3, 5-DTBP (100 µg/ml) and incubated at 37° C for 24 h. The bacterial suspensions were then centrifuged and the pellet was resuspended in PBS (pH 7.4).

The suspensions were kept undisturbed and visually observed for aggregation at the interval of 10 min to 30 min. After incubation, 200µl of the upper layer was read at 600 nm (Sorroche et al. 2012). The percentage of autoaggregation was calculated using the formula

% aggregation = [(OD$_{initial}$ − OD$_{final}$)/OD$_{initial}$)] x 100

H$_2$O$_2$ sensitivity assay

S. mutans survival ability was analyzed using H$_2$O$_2$ sensitivity assay as reported earlier. Briefly, S. mutans control and 3, 5-DTBP treated cells were centrifuged at 8000 rpm for 10 min and pellets were suspended in 1ml of phosphate buffered saline (PBS), subsequently treated with 0.2 % of H$_2$O$_2$ and incubated at 37° C for 3 h and then plated on TSB agar and incubated at overnight, to analyze the viable bacterial cells. After incubation, colonies formed were counted to plot the graph (Van Sorge et al. 2013).

Growth curve and viability of S. mutans

To evaluate the effect of 3, 5-DTBP on growth of S. mutans, 100 ml of TSB was inoculated with 1 % of overnight culture of S. mutans in the presence and absence of BIC of 3, 5-DTBP. The zero hour OD was measured at 600 nm and the culture was incubated at 37° C. The OD values were taken at 3 h interval for 24 h and the growth curve was plotted as OD against time interval (Subramenium et al. 2015).

Further, the cell viability of S. mutans quantified using the XTT (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay. Briefly, the XTT sodium salt and menadione were dissolved in sterile PBS (1 mg/ml) and acetone (1mM) respectively. For each experiment, XTT −menadione solution was prepared freshly at the ratio of 12.5.1 respectively. After incubation, both biofilm and planktonic cells were collectively removed from the 3, 5-DTBP treated and untreated wells. The cells were washed twice with sterile PBS and resuspended in the 175 µl PBS with 25 µl of XTT-menadione solution. This mixture was incubated in dark at 37° C for 8 h. The viability of tested strains was measured spectrophotometrically by the reduction of XTT-menadione into the orange colored formation at 490 nm (Vijayakumar et al. 2020b).

RNA extraction and cDNA preparation

The total RNA of S. mutans grown in the presence and absence of 3, 5-DTBP for 24 h was isolated using a method described earlier (Oh and So 2003). The isolated RNA samples were reversed transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems) as per the manufacturer's instructions. Then cDNA samples were used for subsequent real-time polymerase chain reaction (qRT-PCR) analysis.

Real-Time PCR (qRT- PCR) analysis

The qRT- PCR was performed using 7500 thermal cycler system (Applied Biosystems, USA) with the SYBR Green chemistry kit (Applied Biosystems, USA) following the manufacturer's instructions. The oligonucleotide primers were designed with Primer3 software and are listed in **Table. 1**. The cycling

parameters were as follows: initial denaturation of 5 min at 95° C, followed by 40 cycles of denaturation at 95° C for 30 s, annealing at 56° C for 30 s, extension at 72° C for 30 s and a final extension at 72° C for 1 min. The data was acquired at each cyclic extension step. All the experiments were performed and analyzed in triplicate. The 16S rRNA was used as calibrator to normalize the gene expression. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

Statistics

All the experiments were performed in triplicate at least three times. The data were expressed as mean ± standard deviation (SD). One way ANOVA, 5-DTBP was performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). Results with *P<0.05 and was indicated statistically significant.

Results

Screening of phytochemicals antibiofilm activity against *S. mutans*

Antibiofilm potential of five different phytochemicals (100 µg/ml) was assessed by 24 well MTP assay along with CV staining. The results obtained from, OD at 570 nm was significantly reduced upon treatment with 3, 5-DTBP (*p<0.05)* whereas remaining compounds slightly inhibited the biofilm formation of *S. mutans* and found to be no statistical significant (**Fig. 1a**)

Determination of biofilm inhibitory concentration (BIC) of 3, 5-DTBP

S. mutans biofilm was treated with various concentrations of 3, 5-DTBP ranging from 20 – 120 µg/ml. Concentration dependent biofilm inhibition was observed upon 3, 5-DTBP treatment with maximum inhibition (91 %) was observed at 100 µg/ml concentration (*p<0.05*), the above concentrations do not show any significant effect hence this concentration was fixed at BIC of 3, 5-DTBP and further experiment was done with this concentration (**Fig. 1b**).

Microscopic analysis

Antibiofilm potential of 3, 5-DTBP was confirmed by light microscopic and CLSM analysis. Light microscopic images showed significant reduction in biofilm upon 3, 5-DTBP treatment in *S. mutans*. In control slide shows a dense biofilm formation covered on the entire surface wherein minimum number of cells was observed in the surface treated with BIC of 3, 5-DTBP (**Fig. 1c**). Further, biofilm architecture arrangements were assessed by CLSM and the results confirmed the antibiofilm activity of 3, 5-DTBP. BIC of 3, 5-DTBP treated slides showed a significant reduction of biofilm matrix thickness and minimum number of cells adhered on to the surface compared to the control (**Fig. 1d**).

Effect of 3, 5-DTBP on adherence of *S. mutans*

Adherence ability plays a crucial role in oral microorganisms; in particular, *S. mutans* has able to attach the tooth surface with the support of sucrose dependent and sucrose independent progress. The present

study showed that 3, 5-DTBP at BIC significantly reduced the sucrose dependent and sucrose independent adherence activity by 65 % ($p<0.05$) and 58 % ($p<0.05$) respectively (**Fig. 2a**). The present finding indicated that the 3, 5-DTBP treatment is more efficient to inhibit sucrose dependent adherence wherein compared than sucrose independent adherence.

Effect of 3, 5-DTBP on CSH of *S. mutans*

Bacterial cell surface hydrophobicity is an important virulence factor responsible for bacterial adherence. The MATH assay results showed that 3, 5-DTBP (100 μg/ml) treatment greatly reduced the CSH activity by 77 % when compared to the untreated control. The CSH inhibition at BIC was found to be no statistical significant (**Fig. 2b**).

Inhibition of water soluble and insoluble glucans

The obtained results showed that 3, 5-DTBP treatment greatly reduced the water soluble and water insoluble glucans production by 48 % and 87 % wherein compare than respective controls (**Fig. 2c**). The decreased level of water insoluble glucan synthesis at BIC was found to be statistically significant ($p<0.05$) and water insoluble glucan inhibition was found to be no statistical significant.

3, 5-DTBP inhibits acidogenicity of *S. mutans*

The present studies observed the effect of 3, 5-DTBP on acidogenicity of *S. mutans* monitoring by glycolytic pH drop assay, terminal pH, lactic acid quantification assay and LDH assay. The results showed that the 3, 5-DTBP treatment at BIC greatly reduced the initial rate of *S. mutans* cells pH drop. In control sample final pH value was recorded at 4.6 whereas the 3, 5-DTBP treated cells final pH value was recorded at 6.7 respectively (**Fig. 3a**). The terminal pH assay results showed that the 3, 5-DTBP treated cells terminal pH was recorded at 5.5 whereas the control sample terminal pH was recorded at 4.4 respectively (**Table 2**). Also, the 3, 5-DTBP treatments significantly reduced the *S. mutans* lactic acid production ($p<0.05$) (**Fig. 3b**) and LDH activity by 80 % ($p<0.05$) respectively (**Fig. 3c**). As well as 3, 5-DTBP treatment greatly reduced the enolase activity by 70 % respectively. The enolase inhibition at BIC was found to be no statistical significant (**Fig. 3d**), which were consistent with the results of glycolytic pH drop assay.

3, 5-DTBP inhibits the *S. mutans* aciduricity

Effect of 3, 5-DTBP on acid tolerance was assessed by acid tolerance assay and F_1F_0-ATPase assay. Results unveiled the survival rate of *S. mutans* at pH 5.0 was greatly reduced in the presence of 3, 5-DTBP (**Table 3**). Consequently, 3, 5-DTBP treatment effectively inhibits the activity of the F1F0-ATPase of *S. mutans*. The F_1F_0-ATPase activity was reduced by 76 % ($p<0.05$) at BIC of 3, 5-DTBP (**Fig. 4**).

Auto-aggregation assay

Aggregation is an important process; the bacterial cells are fused and settled on the bottom and construct the microcolonization. The auto-aggregation process is essential for construct aggressive biofilm formation. Then, the effect of 3, 5-DTBP on the auto-aggregation activity of *S. mutans* was assessed. The results showed that 3, 5-DTBP treated cells aggregation rates are faster than the control cells. This property of 3, 5-DTBP initiates to inhibit the biofilm formation by the new strategy. The treated cells showed 85 % auto-aggregation, even control cells showed 41 % of auto-aggregation, when 30 min of incubation in undisturbed conditions (**Fig. 5a**). The auto-aggregation reduction at BIC was found to be no statistical significant.

Effect of 3, 5-DTBP on H_2O_2 sensitivity of *S. mutans*

H_2O_2 sensitivity assay was performed to test the ability of 3, 5-DTBP to sensitize *S. mutans* cells towards oxidative stress. The results showed that 3, 5-DTBP significantly reduced the number of cells resistant to H_2O_2 when compared to control cells (**Fig. 5b**). The oxidative stress reduction at BIC was found to be no statistical significant.

Effect on 3, 5-DTBP on growth and cell viability of *S. mutans*

The present study confirms the non-bactericidal property of 3, 5-DTBP against *S. mutans*, growth curve analysis was done in the presence and absence of 3, 5-DTBP (100 μg/ml). Results from growth curve assay, it is clear that 3, 5-DTBP do not affect the *S. mutans* growth pattern wherein compare than control (**Fig. 6a**). Further, the non-lethal effect of 3, 5-DTBP was also assessed by using metabolically active cells grown in the presence and absence of 3, 5-DTBP. The results showed no significant variance between the 3, 5-DTBP treated and non-treated metabolic cells. (**Fig. 6b**). These obtained results at BIC were found to be no statistical significant.

3, 5-DTBP inhibits expression of virulence genes of *S. mutans*

To analyze the influence of 3, 5-DTBP at molecular level, gene expression profiles of glucosyltransferase synthesis genes (*gtfB*, *gtfC* and *gtfD*), and other virulence genes (vicR, *gbpB*, *smu0630* and *comDE*) was performed using real-time PCR. 3, 5-DTBP at 100 μg/ml concentration inhibited the expression of *gtfB*, *gtfC*, *gtfD*, *vicR*, *gbpB*, *smu0630*, *comDE* and *relA* genes by 4.3, 8.1, 7.6, 5.5, 4.9, 5.1, 4.6 and 4.2 fold respectively, compared to control (**Fig. 7**). All the genes were found to be statistically significant (*p< 0.05*) at BIC of 3, 5-DTBP.

Discussion

The present study investigates the anticariogenic potential of 3, 5-DTBP against *S. mutans* which has been considered as an etiological pathogen to cause dental caries. 3, 5-DTBP exhibited a concentration dependent antibiofilm (100 μg/ml) efficacy against *S. mutans* (**Fig. 1b**). Hence, 100 μg/ml concentration was considered as BIC of 3, 5-DTBP. Light and CLSM microscopic analysis to confirm the concentration dependent antibiofilm efficacy of 3, 5-DTBP against *S. mutans* (**Fig. 1c, d**). Furthermore, 3, 5-DTBP non-

bactericidal property was confirmed by growth curve assay and XTT assay (**Fig. 6a, b**). The present results were similar to the previous report (Rathna et al. 2016) who has reported that 3, 5-DTBP isolated from *Pleurotus florida* inhibits *C. albicans* biofilm formation without affect fungal growth.

The bacterial biofilms are made up of polysaccharides, proteins, lipids, extracellular DNA (eDNA), lipoproteins and lipooligosaccharides (Flemming and Wingender 2010). Among these extracellular polysaccharides (EPS) was found to be an abundant component in the biofilm matrix (Koo et al. 2010). In *S. mutans* EPS is synthesized by glucosyltransferases (gtf), *gtfB* produces water insoluble glucans, which found the scaffold of the EPS matrix and *gtfC* produces the water insoluble and soluble glucans, which is responsible for providing the binding site and structure of *S. mutans* biofilms (Xiao et al. 2012). The present study, 3, 5-DTBP at 100 μg/ml concentration exhibited a significant inhibition in the synthesis of water soluble and water insoluble glucans (**Fig. 2c**). The phenomenal reduction of adherence and EPS production in 3, 5-DTBP treatment, which signifies the anti-adherence mediated biofilm inhibition proficiency.

Adhesion, acidogenicity (ability to synthesis acid) and aciduricity (ability to tolerate acid) are the major virulence factors in *S. mutans* (Banas 2013). Hence, the present study mainly focused on these virulence productions. Adherence plays a crucial role in oral microorganisms, in particular, *S. mutans* attaching the tooth surface, which leads to cascade of biofilm formation (Matsumoto et al. 1999). Generally, *S. mutans* adherence was occurred in two distinct mechanisms such as sucrose dependent and sucrose independent mechanisms. Several studies are documented that sucrose dependent adherence plays a crucial role in causing dental caries and biofilm formation compared to sucrose independent adherence. Here, 3, 5-DTBP significantly inhibited both sucrose dependent adherence and sucrose independent adherence by 65 % ($p<0.05$) and 58 % ($p<0.05$) respectively (**Fig. 2a**). The present finding was compared with the previous study (Gowrishankar et al. 2014) who has reported that *Bacillus amyloliquefaciens* (MMS-50) bioactive metabolite cyclo (L-leucyl- L-prolyl) inhibits sucrose dependent and sucrose independent adherence by 50 % and 55 % respectively. Cell surface hydrophobicity mainly involved host –pathogen interactions and bacterial cell attachment on tooth surface via hydrophobic interactions. The previous studies reported that restrict of *S. mutans* hydrophobic nature, which inhibiting the biofilm formation and reduces the opportunities of bacterial attachment and caries development (Khan et al. 2012). The CSH assay revealed that 3, 5-DTBP has the potential to modify the cell surface hydrophobic properties of *S. mutans* (**Fig. 2c**).

Acidogenicity is an important virulence factor, which is responsible to produce high pH acid from the dietary carbohydrate (glucose) with the help of glycolysis pathway. In this progress, several enzymes are involved directly to help us synthesis the acid. Lactate dehydrogenase is an important enzyme mainly involved in the conversion of pyruvate into lactate acid in anaerobic condition. As well as the lack of LDH, affect the NAD+ conversion from NADH. NAD+ is required when glyceraldehyde-3-phosphate dehydrogenase oxidizes glyceraldehyde-3-phosphate in glycolysis. Moreover, the increasing level of NADH affects the redox potential of the cell, which is documented to be toxic for *S. mutans* (Postma et al. 1993). This fact has driven the study to evaluate the LDH activity of *S. mutans* in the presence of 3, 5-

DTBP. The results showed the significant reduction of LDH production compared to control sample (**Fig. 3c**). Moreover, the present study found that 3, 5-DTBP treatment significantly inhibited the enolase activity (**Fig. 3d**). Enolase is a glycolytic enzyme responsible for the formation of phosphoenolpyruvate, in bacteria utilized phosphoenolpyruvate as the source of energy through the phosphotransferase system. Then, the lack of enolase activity could be reduced phosphoenolpyruvate production which leads to decrease the acidogenesis (Xu et al. 2011). The reduction of acidogenesis ability was further assessed by glycolyic pH drop assay. The results unveiled that 3, 5-DTBP treatment significantly reduced the initial and final rate of pH drop signifying the inhibition of acid production ability of *S. mutans* (**Fig. 3a**), the lack of enzymatic production could be a possible reason to diminish the bacterial glycolytic pathway. In addition, terminate pH assay to confirm the impairment of acidogenic property, the results showed that 3, 5-DTBP treated cells significantly maintain the pH level compared to control (**Table 2**). Furthermore, the glycolytic pH drop assay result implies impairment of *S. mutans* acid tolerance ability upon 3, 5-DTBP treatment.

F_1F_0-ATPase plays a crucial role in *S. mutans* acid tolerance ability; it regulates the bacterial cell pH gradient across the membrane. F_1F_0-ATPase regulates cytoplasmic alkalization because alkaline pH is essential for regular cell functioning and enzymes activity (Hamilton and Buckley 1991). The previous study suggests that impairment of F_1F_0-ATPase enzymatic activity affects the acid tolerance ability (Hasan et al. 2014). Hence, the insufficiency of F_1F_0-ATPase may be involved in increasing the cytoplasmic acidity and subsequently decreased acid tolerance. The present study revealed that 3, 5-DTBP treatment greatly affects the F_1F_0-ATPase activity (**Fig. 4**). Further, the acid tolerance assay to confirm, 3, 5-DTBP treated cells lost their ability to survive on the acidic conditions (**Table 3**). Hence, the present study confirms the inhibition of *S. mutans* cariogenic property (adherence, acidogenesis and acid tolerance) by targeting the biomolecular mechanisms responsible for the *S. mutans* cariogenesis.

In addition, 3, 5- DTBP anticariogenic potential was confirm by transcriptomic analysis. The results showed the downregulation of gtf genes (*gtfB*, *gtfC* and *gtfD*). It could be a positive sign to eradicate the *S. mutans* virulence production. Gtfs plays a crucial role in synthesis of glucans, which is essential for adherence on tooth surface. Generally, *gtf* enzymatic action was regulated by *gtfC*, *gtfD*, *gtfB* genes. The *gtfB* and *gtfC* genes are working in a similar model and synthesis the end product of water insoluble glucans. On the other way, *gtfD* gene synthesis water soluble glucans (Yamashita et al. 1999). Hence, the downregulation of gtf genes might be a possible reason to impedes the glucans production upon 3, 5-DTBP treatment (**Fig. 7**). The present result was compared to previous study (Ren et al. 2016) who has documented that interfering of glucosyltransferase could reduce the ability of biofilm formation and cariogenic virulence of *S. mutans*. Glucans binding protein plays an important role to regulate the affinity of glucans which increasing the bacterial adherence rate to the tooth surface. The results showed the significant downregulation of *gbpB* could responsible for reduction of bacterial adherence upon 3, 5-DTBP treatment (**Fig. 7**).

The two component signal transduction systems (TCSTS) which responsible to regulate bacterial biofilm formation, adaptation, survival and virulence production. These signal transduction systems act as molecular switches, which modify the gene function to survive in the extreme environment (Senadheera et al. 2005). The TCSTS is accomplished by two regulatory elements *vicK* (histidine kinase) and *vicR* (response regulator). The previous study reports that *vicR* gene controls the biofilm formation and *gtfBCD* genes expression, though downregulation of *vicR* greatly reduced the biofilm formation and glucan synthesis (Senadheera et al. 2005). The present investigation found the *vicR* gene downregulation upon 3, 5-DTBP treatment; it could be a possible reason for inhibition of biofilm formation and suppression of *gtf* expression. The result was parallel to the previous study betulin inhibits *S. mutans* biofilm formation via impedes of *vicR* (Viszwapriya et al. 2017) (**Fig. 7**). Moreover, the current study also found that 3, 5-DTBP treatment modifies the growth nature of *S. mutans* in a liquid medium. The obtained result showed the overnight culture of control cell suspension is evenly formed, in contrast, 3, 5-DTBP treated cells are co-aggregate together and settled at the bottom of the medium (**Fig. 5a**). The previous study has reported a similar form of aggregation observed in *vicK* mutans strains (Senadheera et al. 2005). VicRK genes are arranged in an operon model and their expressions are co-regulated (Tremblay et al. 2009). Then, the downregulation of *vicR* could downregulates the *vicK* expression, which could be a possible reason behind the cell aggregation upon in 3, 5-DTBP treatment. In addition, *vicK* mutant strains formed unstable biofilms. The present findings added one more attention, as 3, 5-DTBP treatment correspondingly inhibits biofilm formation in the existence of sucrose.

relA gene encodes guanosine tetra-phosphate which is responsible to regulate acid tolerance (Liu et al. 2011). The downregulation of *relA* gene could be a possible reason for impairment of acid and stress tolerance. *comDE* gene regulates the *S. mutans* quorum sensing mechanism and mediated virulence productions (Senadheera and Cvitkovitch 2008). Hence, the suppression of *comDE* gene expression directly affects quorum sensing as well as mediated biofilm formation, acid tolerance and acid production. In addition, *smu0630* is a hypothetical protein involved in biofilm formation (Brown et al. 2005). The downregulation of *smu0630* (**Fig. 7**) gene attenuates the biofilm formation, which may is an alternative approach to reduce/control *S. mutans* biofilm formation (Liu et al. 2011).

In the present study demonstrate the antibiofilm potential of 3, 5-DTBP explored antibiofilm and anticaries activity against *S. mutans*. 3, 5-DTBP at 100 µg/ml inhibited the biofilm formation and significantly reduced the adhesion, acidogenesis and aciduricity property through impairment of enzymatic productions and downregulating the *gtf* and *vicR/K* virulence genes. Based on this data, we believed that 3, 5-DTBP as a potential antibiofilm and anticaries agent could control the *S. mutans* cariogenesis.

Declarations

Conflict of interest

The authors declare that they have no conflict of interest.

Research Involving Human Participants and Animals: This study does not contain any experiments with human participants or animals performed by any of the authors.

Authors Contribution list:

KV;; Conceptualization of whole work, performed the experiments, data analyzed and co-wrote the manuscript

SM,: Co-wrote the manuscript, performed the experiments and data analyzed.

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References

1. Bagis B, Baltacioglu E, Oezcan M, Ustaomer S (2011) Evaluation of chlorhexidine gluconate mouthrinse-induced staining using a digital colorimeter: an in vivo study. Quintessence Int 42.
2. Ban SH, Kim JE, Pandit S, Jeon JG (2012) Influences of *Dryopteris crassirhizoma* extract on the viability, growth and virulence properties of *Streptococcus mutans*. Molecules 17:9231-44.
3. Banas JA (2013) Delaying *Streptococcus mutans* Colonization in Children Leads to Reduced Caries Experience. J Evid Based Dent Pract 13:67-9.
4. Belli WA, Buckley DH, Marquis RE (1995) Weak acid effects and fluoride inhibition of glycolysis by *Streptococcus mutans* GS-5. Can J Microbiol 41:785-91.
5. Bencini DA, Shanley MS, Wild JR, O'Donovan GA (1983) New assay for enzymatic phosphate release: application to aspartate transcarbamylase and other enzymes. Anal Biochem 132:259-64.
6. Borshchevskaya LN, Gordeeva TL, Kalinina AN, Sineokii SP (2016) Spectrophotometric determination of lactic acid. J Anal Chem 71:755-8.
7. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analy Biochem 72:248-54.

8. Brown Jr TA. Ahn SJ, Frank RN, Chen YYM, Lemos JA, Burne RA (2005) A hypothetical protein of *Streptococcus mutans* is critical for biofilm formation. Infect Immun 73:3147-3151.

9. Brown TA, Ahn SJ, Frank RN, Chen YY, Lemos JA, Burne RA (2005) A hypothetical protein of *Streptococcus mutans* is critical for biofilm formation. Infect Immun 73:3147-51.

10. Cardoso JG, Iorio NL, Rodrigues LF, Couri ML, Farah A, Maia LC, Antonio AG (2016) Influence of a Brazilian wild green propolis on the enamel mineral loss and *Streptococcus mutans'* count in dental biofilm. Arch Oral Biol 65:77-81.

11. Crow VL, Pritchard GG (1977) Fructose 1, 6-diphosphate-activated L-lactate dehydrogenase from *Streptococcus lactis*: kinetic properties and factors affecting activation. J Bacteriol 131:82-91.

12. Deng DM, Liu MJ, Ten Cate JM, Crielaard W (2007) The VicRK system of *Streptococcus mutans* responds to oxidative stress. J Dent Res 86:606-610.

13. Flemming HC, Wingender J (2010) The biofilm matrix. Nat Rev Microbiol 8:623-33.

14. Gowrishankar S, Poornima B, Pandian SK (2014) Inhibitory efficacy of cyclo (l-leucyl-l-prolyl) from mangrove rhizosphere bacterium– *Bacillus amyloliquefaciens* (MMS-50) toward cariogenic properties of *Streptococcus mutans*. Res Microbiol 165:278-89.

15. Gyawali R, Ibrahim SA (2014) Natural products as antimicrobial agents. Food control 46:412-29.

16. Hamilton IR, Buckley ND (1991) Adaptation by *Streptococcus mutans* to acid tolerance. Oral Microbiol Immunol 6:65-71.

17. Hasan S, Singh K, Danisuddin M, Verma PK, Khan AU (2014) Inhibition of major virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a synergistic approach of infection control. PLoS One 9:e91736.

18. Hasan S, Singh K, Danisuddin M, Verma PK. Khan AU (2014). Inhibition of major virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a synergistic approach of infection control. PLoS one, 9: 91736.

19. Karuppiah V, Thirunanasambandham R, Thangaraj G (2021) Anti-quorum sensing and antibiofilm potential of 1, 8-cineole derived from *Musa paradisiaca* against *Pseudomonas aeruginosa* strain PAO1. World J Microbiol Biotechnol 37:1-12.

20. Khan R, Adil M, Danishuddin M, Verma PK, Khan AU (2012) In vitro and in vivo inhibition of *Streptococcus mutans* biofilm by *Trachyspermum ammi* seeds: an approach of alternative medicine. Phytomedicine 19:747-55.

21. Khan R, Zakir M, Khanam Z, Shakil S, Khan AU (2010) Novel compound from *Trachyspermum ammi* (Ajowan caraway) seeds with antibiofilm and anti-adherence activities against *Streptococcus mutans*: a potential chemotherapeutic agent against dental caries. J Appl Microbiol 109(6):2151-9.

22. Koo H, Xiao J, Klein MI, Jeon JG (2010) Exopolysaccharides produced by *Streptococcus mutans* glucosyltransferases modulate the establishment of microcolonies within multispecies biofilms. J Bacteriol 192:3024-32.

23. Korithoski B, Lévesque CM, Cvitkovitch DG (2007) Involvement of the detoxifying enzyme lactoylglutathione lyase in *Streptococcus mutans* aciduricity. J Bacteriol 189:7586-92.

24. Lemos JA, Burne RA (2008) A model of efficiency: stress tolerance by *Streptococcus mutans*. Microbiology 154:3247.

25. Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG (2002) A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. J Bacteriol 184:2699-2708.

26. Liu C, Worthington RJ, Melander C, Wu H (2011) A new small molecule specifically inhibits the cariogenic bacterium *Streptococcus mutans* in multispecies biofilms. Antimicrob Agents Chemother 55:2679-87.

27. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25:402-8.

28. Marsh PD (2004) Dental plaque as a microbial biofilm. Caries Res 38:204-11.

29. Martins ML, de França Leite KL, Pacheco-Filho EF, de Miranda Pereira AF, Romanos MTV, Maia LC, Fonseca-Gonçalves A, Padilha WWN, Cavalcanti YW (2018) Efficacy of red propolis hydro-alcoholic extract in controlling *Streptococcus mutans* biofilm build-up and dental enamel demineralization. Archiv Oral Biol 93:56-65.

30. Matsui R, Cvitkovitch D (2010) Acid tolerance mechanisms utilized by *Streptococcus mutans*. Future Microbiol 5:403-417.

31. Matsumoto M, Minami T, Sasaki H, Sobue S, Hamada S, Ooshima T (1999) Inhibitory effects of oolong tea extract on caries−inducing properties of Mutans streptococci. Caries Res 33:441-5.

32. Mattos-Graner RO, Duncan MJ (2017) Two-component signal transduction systems in oral bacteria. J Oral Microbiol 9:1400858.

33. Oh ET, So JS (2003) A rapid method for RNA preparation from Gram-positive bacteria. J Microbiol Methods 52:395-8.

34. Postma PW, Lengeler JW, Jacobson GR (1993) Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol Mol Biol Rev 57:543-94.

35. Rathna J, Bakkiyaraj D, Pandian SK (2016) Anti-biofilm mechanisms of 3, 5-di-tert-butylphenol against clinically relevant fungal pathogens. Biofouling 32:979-993.

36. Ren Z, Cui T, Zeng J, Chen L, Zhang W, Xu X, Cheng L, Li M, Li J, Zhou X, Li Y (2016) Molecule targeting glucosyltransferase inhibits *Streptococcus mutans* biofilm formation and virulence. Antimicrob Agents Chemother 60:126-135.

37. Senadheera D, Cvitkovitch DG (2008) Quorum sensing and biofilm formation by *Streptococcus mutans*. In Bacterial signal transduction: networks and drug targets. Springer, New York, pp. 178-188.

38. Senadheera MD, Guggenheim B, Spatafora GA, Huang YC, Choi J, Hung DC, Treglown JS, Goodman SD, Ellen RP, Cvitkovitch DG (2005) A VicRK signal transduction system in *Streptococcus mutans*

affects gtfBCD, gbpB, and ftf expression, biofilm formation, and genetic competence development. J Bacteriol 187:4064-76.

39. Smith EG, Spatafora GA (2012) Gene regulation in *S. mutans*: complex control in a complex environment. J Dent Res 91:133-141.

40. Subramenium GA, Vijayakumar K, Pandian SK (2015) Limonene inhibits streptococcal biofilm formation by targeting surface-associated virulence factors. J Med Microbiol 64:879-90.

41. Sorroche FG, Spesia MB, Zorreguieta Á, Giordano W (2012) A positive correlation between bacterial autoaggregation and biofilm formation in native *Sinorhizobium meliloti* isolates from Argentina. Appl Environ Microbiol 78:4092-101.

42. Tremblay YD, Lo H, Li YH, Halperin SA, Lee SF (2009) Expression of the *Streptococcus mutans* essential two-component regulatory system VicRK is pH and growth-phase dependent and controlled by the LiaFSR three-component regulatory system. Microbiology 155:2856-65.

43. Vijayakumar K, Ramanathan T (2018) Antiquorum sensing and biofilm potential of 5-Hydroxymethylfurfural against Gram positive pathogens. Microb Pathog 125:48-50.

44. Vijayakumar K, Ramanathan T (2020) *Musa acuminata* and its bioactive metabolite 5-Hydroxymethylfurfural mitigates quorum sensing (las and rhl) mediated biofilm and virulence production of nosocomial pathogen *Pseudomonas aeruginosa* in vitro. J Ethnopharmacol 246:112242.

45. Vijayakumar K., Bharathidasan V, Manigandan V, Jeyapragash D (2020) Quebrachitol inhibits biofilm formation and virulence production against methicillin-resistant *Staphylococcus aureus*. Microb Pathog 149:104286.

46. Viszwapriya D, Subramenium GA, Radhika S, Pandian SK (2017) Betulin inhibits cariogenic properties of *Streptococcus mutans* by targeting *vicRK* and *gtf* genes. Antonie van Leeuwenhoek 110:153-65.

47. Welin-Neilands J, Svensater G (2007) Acid tolerance of biofilm cells of *Streptococcus mutans*. Appl Environ Microbiol 73:5633-5638.

48. Xiao J, Klein MI, Falsetta ML, Lu B, Delahunty CM, Yates III JR, Heydorn A, Koo H (2012) The exopolysaccharide matrix modulates the interaction between 3D architecture and virulence of a mixed-species oral biofilm. PLoS Pathog 8:e1002623.

49. Xu X, Zhou XD, Wu CD (2011) The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. Antimicrob Agents Chemother 55:1229-36.

50. Yamashita Y, Tomihisa K, Nakano Y, Shimazaki Y, Oho T, Koga T (1999) Recombination between gtfB and gtfC is required for survival of a dTDP-rhamnose synthesis-deficient mutant of *Streptococcus mutans* in the presence of sucrose. Infect Immun 67:3693-3697.

51. Yano A, Kikuchi S, Yamashita Y, Sakamoto Y, Nakagawa Y, Yoshida Y (2010) The inhibitory effects of mushroom extracts on sucrose-dependent oral biofilm formation. Appl Microbiol Biotechnol 86:615-23.

52. Zhang Z, Liu Y, Lu M, Lyu X, Gong T, Tang B, Wang L, Zeng J, Li Y (2020) Rhodiola rosea extract inhibits the biofilm formation and the expression of virulence genes of cariogenic oral pathogen *Streptococcus mutans*. Arch Oral Biol 19:104762.

Tables

Table 1 List of primers used for the real-timePCR analysis

Gene	Primer sequence (5'-3')		References
	Forward	Reverse	
gtfB	ATGGCGGTTATGGACACGTT	TTTGGCCACCTTGAACACCT	Viszwapriya et al. 2017
gtfC	GGTTTAACGTCAAAATTAGCT	CTCAACCAACCGCCACTGTT	Viszwapriya et al. 2017
gtfD	GAAGTATGGCGGTGCTTTCC	ATAACCAACACCACGGCCTA	Viszwapriya et al. 2017
vicR	TGACACGATTACAGCCTTTGATG	CGTCTAGTTCTGGTAACATTAAGT	Viszwapriya et al. 2017
smu0630	GTTAGTTCTGGTTTTGACCGCAA	CCCTCAACAACAACATCAAAGGT	Viszwapriya et al. 2017
comDE	ACAATTCCTTGAGTTCCATCCAA	TGGTCTGCTGCCTGTTGC	Viszwapriya et al. 2017
gbpB	ATGGCGGTTATGGACACGTT	TTTGGCCACCTTGAACACCT	Viszwapriya et al. 2017
relA	ACAAAAAGGGTATCGTCCGTA	AATCACGCTTGGTATTGCTAATTG	Hasan et al. 2014
16S rRNA	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	Viszwapriya et al. 2017

Table 2 Terminal pH determination assay

BIC of 3, 5-DTBP (µg/ml)	Terminal pH determination	
	Before inoculation	After inoculation
Control	7.4 ± 0.3	4.2 ± 0.2
100	7.4 ± 0.3	5.5 ± 0.2

Table 3 Inhibitory effect of 3, 5-DTBP on the acid tolerance of *S. mutans*

Time (h)	Adapted cells (CFU/ml)		Unadapted cells (CFU/ml)	
	Control	Treated	Control	Treated
0	$4.3 \pm 0.3 \times 10^4$	$4.3 \pm 0.3 \times 10^4$	$3.4 \pm 0.4 \times 10^4$	$3.4 \pm 0.4 \times 10^4$
1	$2.7 \pm 0.3 \times 10^4$	$1.2 \pm 0.1 \times 10^4$	$1.3 \pm 0.3 \times 10^4$	$4.4 \pm 0.2 \times 10^4$
2	$1.4 \pm 0.5 \times 10^4$	$3.7 \pm 0.3 \times 10^4$	$7.1 \pm 0.2 \times 10^4$	$7.1 \pm 0.4 \times 10^4$

Figures
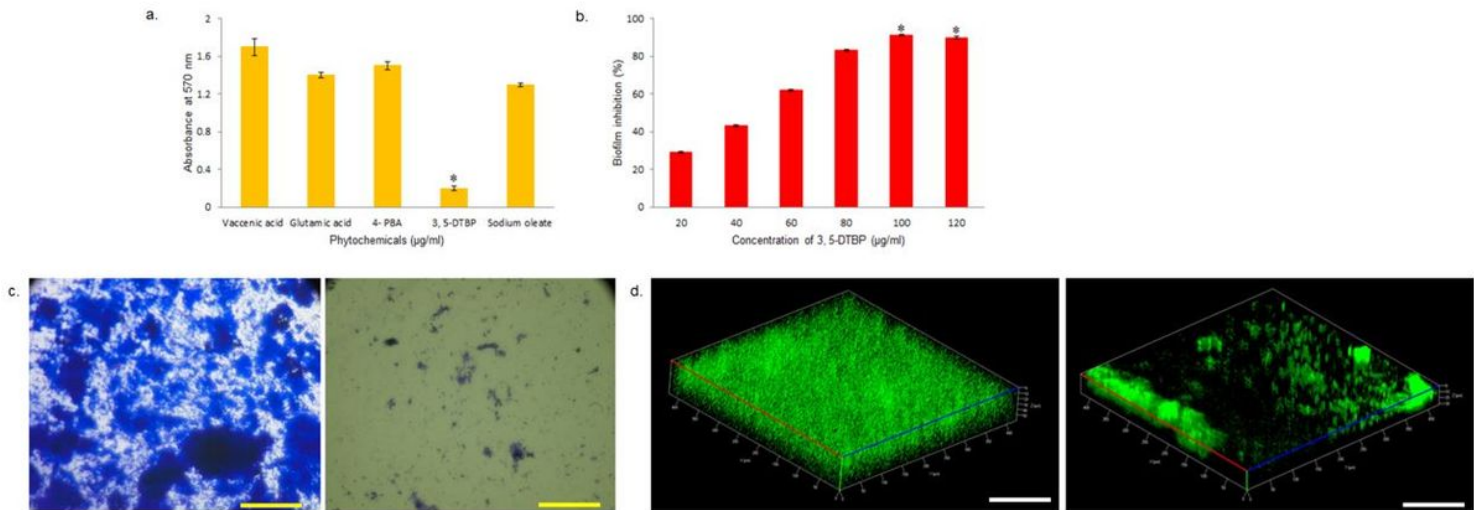
Figure 1

(a) Screening of various phytochemicals for antibiofilm activity against S. mutans. Antibiofilm activity of 3, 5-DTBP against S. mutans. (b) Evaluation of BIC. Graph showing inhibition of S. mutans biofilm by 3, 5-DTBP in a concentration dependent manner. The concentration of 3, 5-DTBP 100 μg/ml showing 91 % biofilm inhibition was considered as BIC. Microscopic analysis to evaluate the 3, 5-DTBP antibiofilm efficacy (c) Light microscopic images (400X) (d) Confocal laser scanning microscope (200X) images showing the antibiofilm potential of 3, 5-DTBP against S. mutans. Error bars indicate standard deviations. * indicates statistical significance (p< 0.05) Scale bars 100 μm.
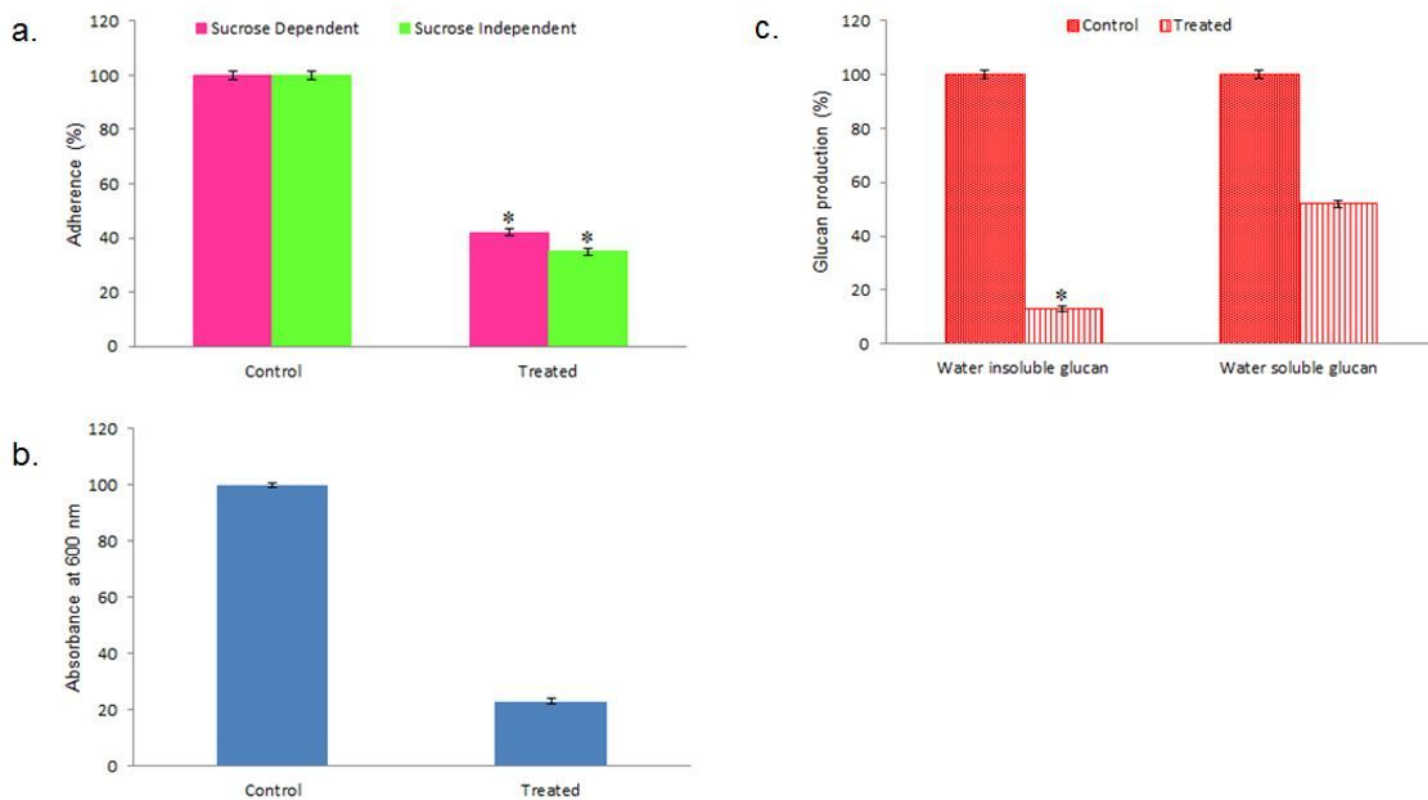
Figure 2

Inhibitory effect of 3, 5-DTBP on adherence of S. mutans. (a) Glass dependent adherence both in the presence of 1 % sucrose (sucrose dependent) and absence of sucrose (sucrose independent). (b) Cell surface hydrophobicity of S. mutans. (c) Effect of 3, 5-DTBP on water soluble and water insoluble glucans production of S. mutans at BIC. Error bars indicate standard deviations. * indicates statistical significance (p< 0.05).
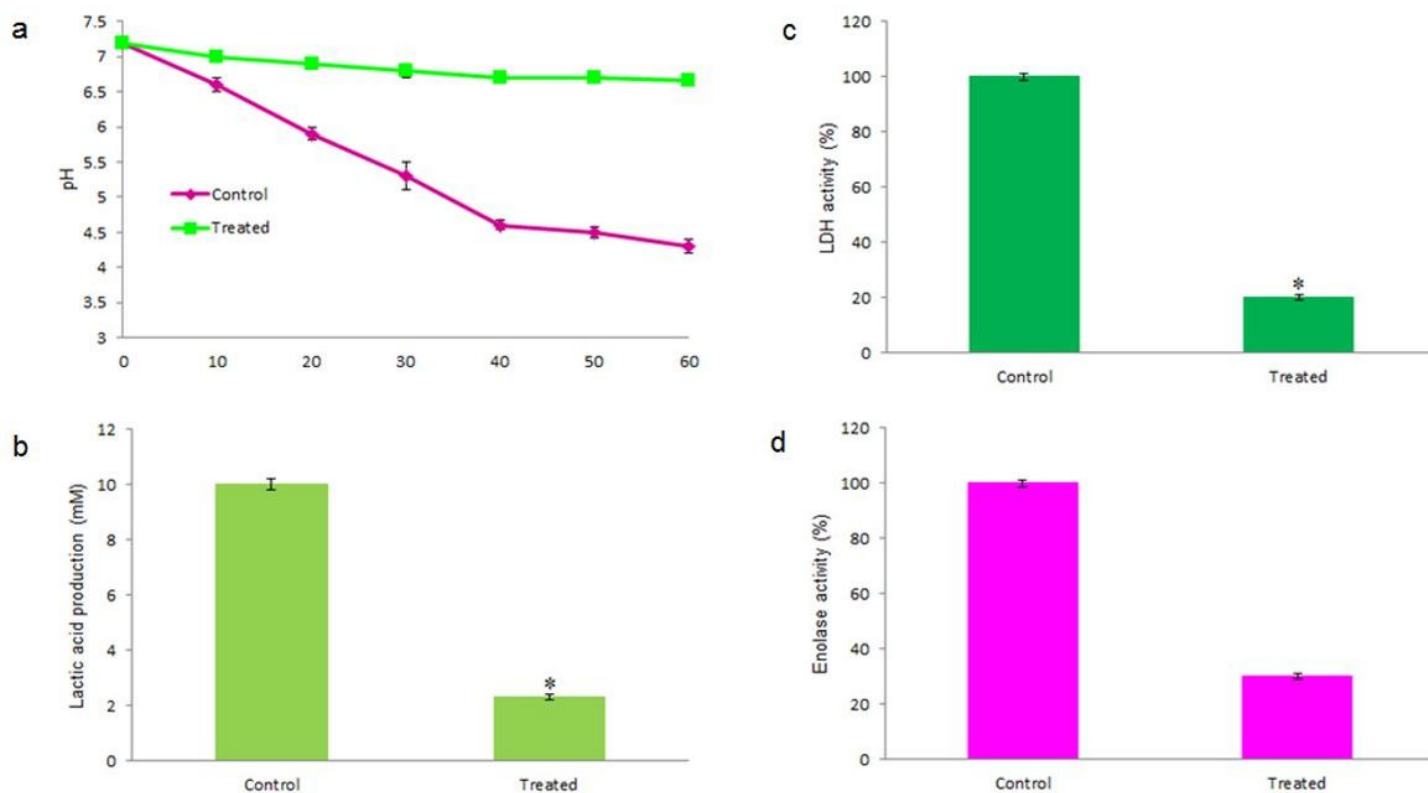
Figure 3

Effect of 3, 5-DTBP on acidogenesis virulence of S. mutans. (a) Glycolytic pH drop (b) Lactic acid production (c) Lactate dehydrogenase activity (d) Enolase activity of S. mutans. Error bars indicate standard deviations. * indicates statistical significance (p< 0.05).
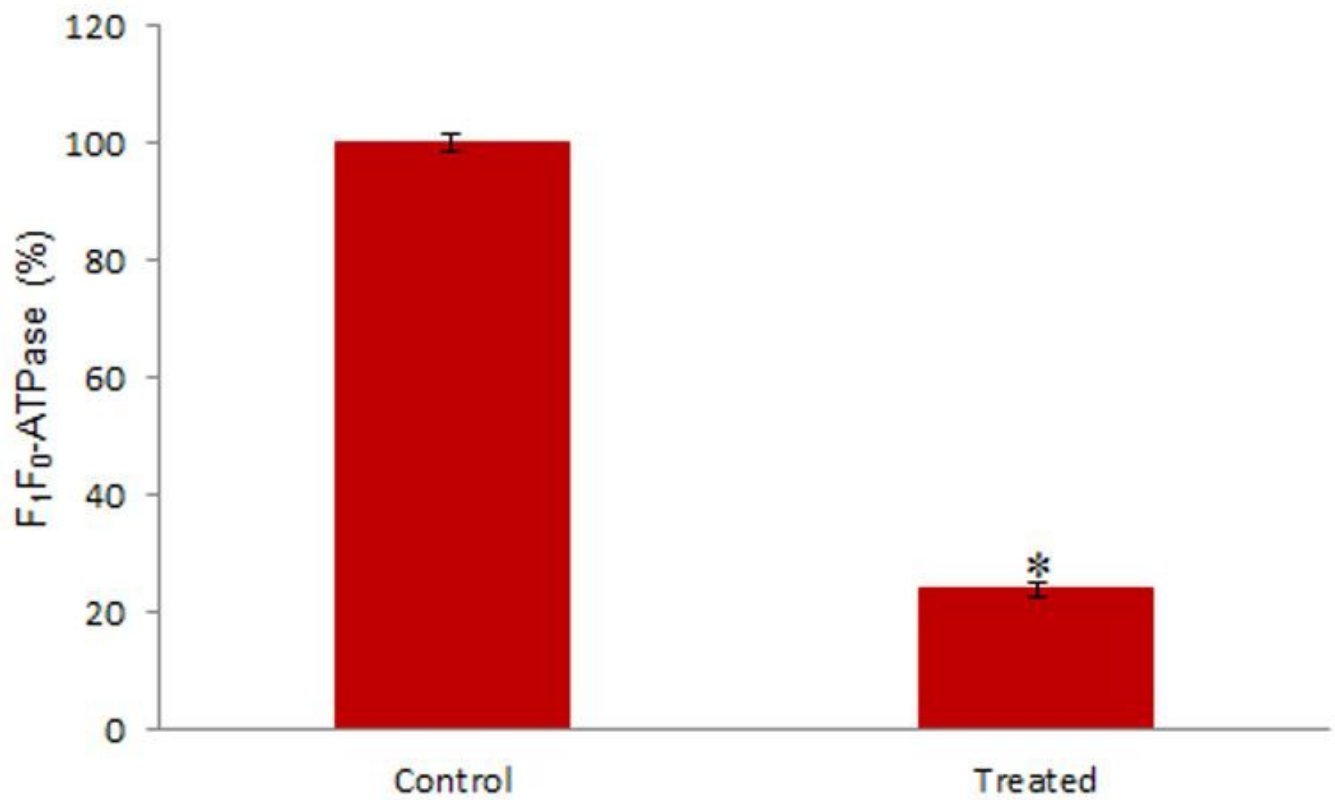
Figure 4

Effect of 3, 5-DTBP on aciduricity of S. mutans. Relative activity of F1F0-ATPase of S. mutans. Error bars indicate standard deviations. * indicates statistical significance ($p < 0.05$).
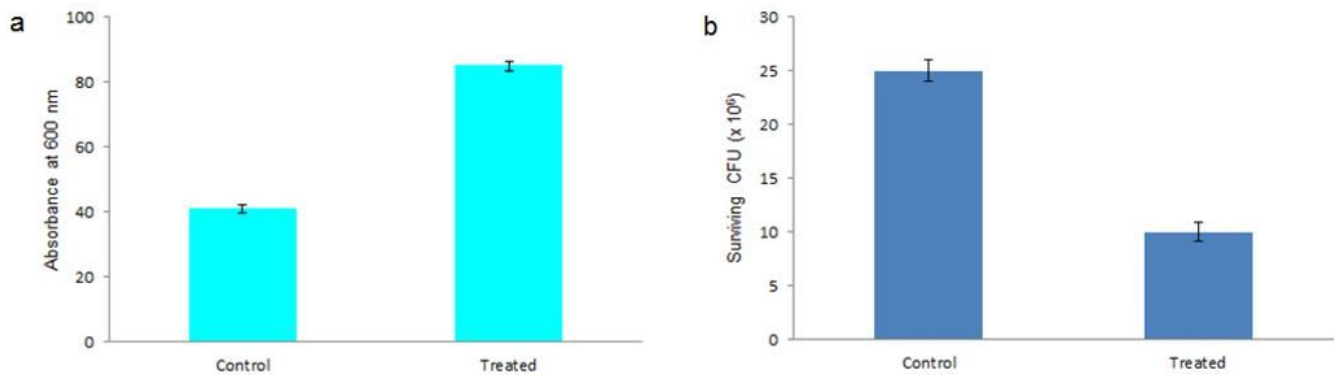

Figure 5

(a) Suspension of S. mutans grown in the presence and absence of 3, 5-DTBP. Auto-aggregation. (b) S. mutans cells surviaval rate observed in the presence and absence of 3, 5-DTBP in H2O2.
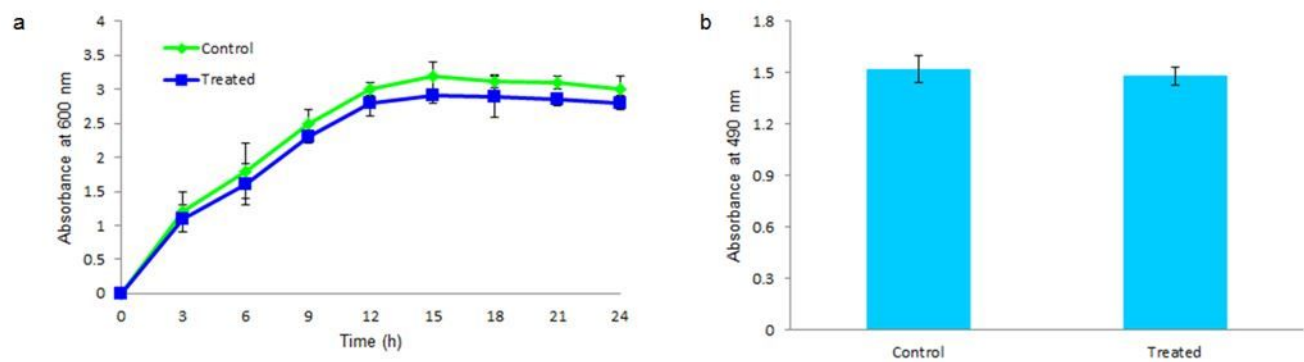

Figure 6

Effect of 3, 5-DTBP on growth and viability of S. mutans at BIC (100 µg/ml) (a) Growth curve analysis. (b) 3, 5-DTBP treated S. mutans cells growth viability was comparable with control cells which was confirmed by XTT assay. Error bars indicate standard deviations.
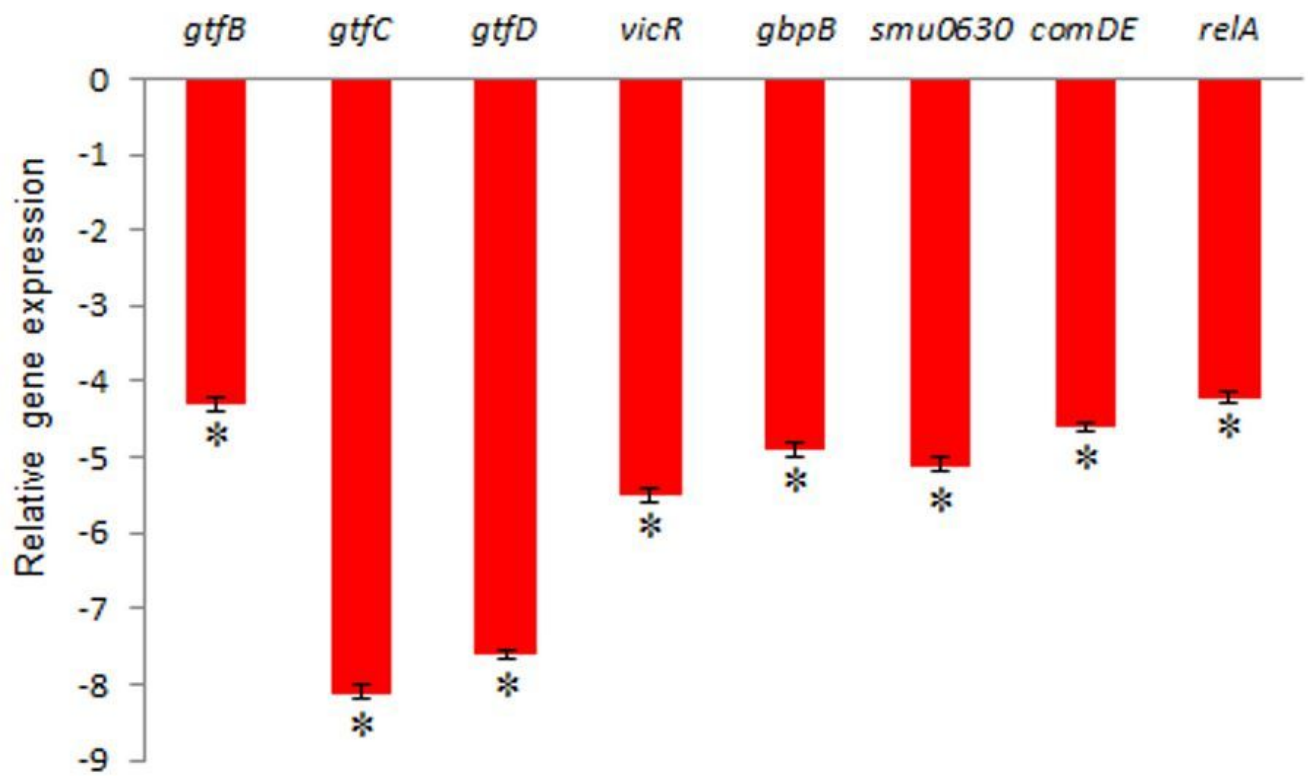
Figure 7

Gene expression profile of candidate genes involved in biofilm formation and cariogenic virulence of S. mutans upon 3, 5-DTBP treatment. Error bars indicate standard deviations. * indicates statistical significance (p< 0.05).