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ORIGINAL ARTICLE

Antibacterial Activity of *Azadirachta indica*, *Pongamia pinnata*, *Psidium guajava*, and *Mangifera indica* and their Mechanism of Action against *Streptococcus mutans*

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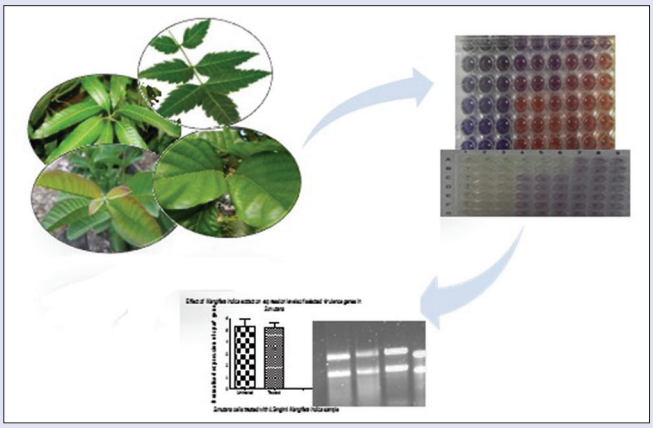
ABSTRACT

Background: Curative plants have reportedly been used to make chewing sticks/toothbrushes intended for the treatment of oral diseases. **Objective:** The *in vitro* antibacterial activities of *Azadirachta indica*, *Pongamia pinnata*, *Psidium guajava*, and *Mangifera indica* were evaluated against *Streptococcus mutans*, along with the cytotoxicity and antioxidant and synergistic potentials. The effect of *M. indica* on the expression of crucial virulence genes *spaP* and *gtfB* of *S. mutans* was determined. **Materials and Methods:** The antibacterial activity was determined using a modified microdilution method. The antioxidant potential was evaluated using diphenyl picrylhydrazyl (DPPH), Griess reagent, and nitroblue tetrazolium calorimetric assays. The synergistic activity was investigated using a modified checkerboard method, while the cytotoxicity was determined according to a cell proliferation 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt assay. Reverse transcription was the chosen method for determining the difference in expression of the *spaP* and *gtfB* genes after treatment with the plant sample. **Results:** *M. indica* and *A. indica* had the highest antibacterial activity at concentrations of 0.3 mg/ml and 6.25 mg/ml, respectively. *A. indica* had the best free radical scavenging of DPPH, exhibiting 50% inhibition at 28.72 μg/ml; while *M. indica* showed better superoxide scavenging potential than the positive control quercetin. Both *M. indica* and *A. indica* had adequate activity against the nitric oxide-free radical (12.87 and 18.89 μg/ml, respectively). *M. indica* selectively reduced the expression of the *gtfB* gene, indicating a mechanism involving Glucotranferases, specifically targeting bacterial attachment. **Conclusion:** The overall good antibacterial activity of *M. indica* correlated with the general good antioxidant capacity, while showing a potentially unique mechanism of bacterial inhibition, targeting virulence gene expression.

Key words: Antibacterial, antioxidant, gene expression, oral pathogens

SUMMARY

- *Mangifera indica* and *Azadirachta indica* had very good antibacterial activity against *Streptococcus mutans* and moderate toxicity against Vero cells
- *M. indica* had the best antioxidant capacity overall
- *M. indica* reduced the expression of *gtfB* gene at 0.5 mg/ml.



Abbreviations used: AA: Ascorbic acid; BHI: Brain–heart infusion; CHX: Chlorhexidine; DPPH: Diphenyl picrylhydrazyl; DMSO: Dimethlysulfoxide; NBT: Nitroblue tetrazolium; NO: Nitric oxide; XTT: 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt.

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INTRODUCTION

The mouth is often viewed as a separate entity to the rest of the body, as such, many people tend to neglect their oral health and often do not take the necessary steps required to prevent diseases such as dental caries.[1,2] These oral diseases have overwhelming consequences on the general quality of life, interfering with basic actions such as chewing and communicating.[2] The pain associated with toothaches has been linked to the loss in the working hours of many countries and was found to contribute to absenteeism in the workplace and schools.[3] This has led to many people turning to the roots and bark of curative plants in order to make chewing sticks/toothbrushes, primarily for maintaining good oral health and preventing the onset of oral diseases such as dental caries.[4,1,2]

Plants belonging to the families Meliaceae, Fabaceae, Myrtaceae, and Anacardiaceae have reported applications in the treatment of oral ailments such as toothache and oral thrush. The plants considered for the present study were *Azadirachta indica*, *Pongamia pinnata*, *Psidium guajava*, and *Mangifera indica*, commonly known as Neem, Karanj, Mango, and Guava, respectively. *M. indica* has been traditionally used

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as an ayurvedic component in the treatment of a variety of symptoms caused by oral diseases, such as healing wounds in the mouth that result from oral thrush as well as the treatment of toothache associated with dental caries. The reported traditional use of *P. guajava* in various parts of the world has been for the treatment for toothache and against caries, where the leaves, shoots, roots, and bark are used to make concoctions. The roots of *P. pinnata* have been traditionally used to clean gums and teeth, a preventative measure for the development of caries.[5] The stems, roots, and twigs of *A. indica* have been used to make chewing sticks and toothbrushes for the removal of plaque.[6]

With the development of high-throughput screening methods, it has become possible to conduct scientific validations of plants which are traditionally used for oral care.[7-9] Those plants that are able to specifically inhibit the activity of bacteria such as *S. mutans,* Gram-positive cocci, and the main causative agents of dental caries are of particular interest. In addition to their antibacterial activity, plants often possess natural antioxidants in the form of small secondary compounds, for instance, polyphenols, flavonoids, and other associated compounds.[10,11] These particular compounds are important in the prevention of disease development by reducing the oxidative damage that certain microorganisms such as *S. mutans* can cause, often as part of their own important cellular processes.[12-15] Medicinal plants can therefore be investigated for their radical scavenging potential.

Bacteria such as *S. mutans* possess important virulence factors that are important for the development and spread of disease in hosts.[16] *S. mutans* has three main virulence factors, namely, direct attachment, through unique enzymes on the surface of their membrane such as AntigenI/II and Glucotranferases (GTF); biofilm formation; and acid production.[17] Biofilm formation and acid production are directly dependent on successful attachment; therefore, a plant sample that can target attachment will effectively prevent disease development.[18] The *spaP* and *gtfB* genes encode for the enzymes AntigenI/II and GTF, respectively, and give *S. mutans* the unique ability to produce extracellular polysaccharides that allow it to bind onto the tooth surface.[19-21]

The antibacterial activities of *A. indica*, *P. pinnata*, *P. guajava,* and *M. indica* were tested against *S. mutans* (American Type Culture Centre [ATCC] 25175) using a calorimetric microdilution method. The radical scavenging potential of the samples was measured using the diphenyl picrylhydrazyl (DPPH), nitric oxide (NO), and nitroblue tetrazolium (NBT) scavenging assays. In addition to determining the antibacterial activity of the samples, it was assessed whether the inhibition of bacterial cells was due to a decrease in expression of the *spaP* and *gftB* genes in cells treated with plant extract. RNA was isolated from treated and untreated cells in order to compare the expression of the particular genes at mid-log phase. The main aim of this study was to determine the antibacterial and antioxidant activities as well as the possible mechanism of action of traditional medicinal plants used on a large scale from many decades ago.

MATERIALS AND METHODS

Materials

The leaves of the plants considered for the current study were gathered at the Hatia region of Ranchi district, Jharkhand, India. The plants were then identified at the Department of Botany, St. Xavier's College, Ranchi, Jharkhand, India, by Dr. Ajay Srivastava (HOD, Department of Botany, St. Xavierme College, Ranchi, Jharkhand, India). A herbarium specimen voucher for each plant was kept at the herbarium of the department of Botany at St. Xavier's College.

DPPH and PrestoBlue® were procured from Sigma Aldrich, South Africa. Brain–heart infusion (BHI) broth and agar, Anaerocult®, and McFarland standard 1 were purchased from Merck Chemicals (Pty) Ltd. (Wadeville, South Africa). Chlorhexidine (CHX) gluconate was bought from Dental Warehouse (Sandton, South Africa) and *S. mutans* were bought from the ATCC 25175.

Plant extract preparation

The leaves of each of the selected plants were dried out at room temperature and then subsequently ground to a fine powder. The powdered material was then placed on a shaker for 48 h, using ethanol as an extractant. After filtering the plant material, the ethanol was evaporated using a rotary evaporator.

Antibacterial assays

Oral pathogen

S. mutans (ATCC 25175) cells were proliferated on BHI agar (Merck Chemicals (Pty) Ltd., Wadeville, South Africa) placed in an anaerobic jar containing Anaerocult® A (Merck KGaA Darmstadt, Germany) which provided the anaerobic condition required for growth. This was done for 48 h at 37°C, with subculturing done weekly. The inocula were obtained by adding *S. mutans* colonies to BHI broth, until the concentration of the bacterial cells aligned with McFarland standard 1 (3×10^8 cfu/ml), determined spectrophotometrically.

The bacterial inoculum was prepared by suspending *S. mutans* colonies in BHI broth until turbidity was compatible with McFarland Standard 1 (3×10^8 cfu/ml) (Merck Chemicals [Pty] Ltd., Wadeville, South Africa).[22]

PrestoBlue® colorimetric assay to determine the minimum inhibitory concentration of each plant extract

The microplate method as described by Eloff[23] and Seukep *et al*.[24] was adapted and used to determine the inhibitory potential of the plant samples by calculating the concentration at which the plant samples inhibited the growth of the tested bacteria,; minimum inhibitory concentration (MIC). PrestoBlue® was used as the colorimetric indicator reagent in the assay. The plant extracts were dissolved in 100% acetone (Merck Chemicals [Pty] Ltd), which was shown to be nontoxic to the bacterial cells and diluted down in a 96-well plate enriched with BHI broth (Merck Chemicals [Pty] Ltd) to concentrations varying from 12.5 to 0.1 mg/ml. Five percent CHX gluconate was used as a positive control with concentrations ranging from 3.8×10^4 to 12.5 mg/ml, after which 3×10^8 cfu/ml of 24 h-old inocula were added to the plates and incubated at 37°C for a further 24 h before adding the color indicator PrestoBlue®.[25,23] The MIC was taken as the concentration at which there was no color change from blue to pink, indicating bacterial growth inhibition.

Synergistic antibacterial activity

The synergy between the different plants was determined by combining the checkerboard and ratio methods as described by Lall *et al*., Eloff, and Orhan *et al*.[25,23,26] In order to determine the combined antimicrobial interaction between *M. indica* and *A. indica, P. pinnata,* and *P. guajava,* the samples were prepared to concentrations ranging from 0.10 to 12.5 mg/ml. In a sterile 96-well plate, 100 µl of *M. indica* was then added in a ratio from 9:1 (90 µl *M. indica*: 10 µl *A. indica*) to 1:9 (10 µl *M. indica*: 90 µl *A. indica*); 9:1 (90 µl *P. pinnata*: 10 µl *P. guajava*) to 1:9 (10 µl *P. pinnata*: 90 µl *P. guajava*). The sample combinations in their ratios were then serially diluted down in 2-fold dilutions. A 100 µl solution of *S. mutans* inocula (3 × 108 cfu/ml) was added to the plates and incubated at 37°C, under anaerobic conditions for 24 h before adding the color indicator PrestoBlue®.[25,27]

Analysis

The fractional inhibitory concentration (FIC) was used to determine possible synergy, where the sum of FIC gave a Fractional Inhibitory Index (nFIC), determined using the following formula:

The ∑ FIC was interpreted in several ways; if the ∑ FIC was ≤0.50, the combination was synergistic; >0.5–1, the combination was additive; >1–≤4, the combination was noninteractive; or >4, the combination was antagonistic.

$$\sum \text{FIC} = \frac{\text{MIC}(\text{Plant A} + \text{Plant B})}{\text{MIC}\left(\text{Plant A}\right)} + \frac{\text{MIC}(\text{Plant A} + \text{Plant B})}{\text{MIC}(\text{Plant B})}$$

In vitro antioxidant activity

2,2-Diphenyl picrylhydrazyl assay

The degree of scavenging capacity for each plant sample was determined using the method published in the study by Henley-Smith *et al.*[28] with slight modifications. All reactions were carried out in 96-well plates. The plant samples and ascorbic acid (positive control) were prepared to stock solutions and then diluted down to concentrations varying from 500 to 3.9 μg/ml and 100 to 0.781 μg/ml, respectively. A 90-μl solution of 0.04 M DPPH was added to all the wells and the plates were incubated in a dark room for half an hour. In the presence of an antioxidant, the solution was reduced from purple to yellow/clear.

Nitric oxide radical inhibition activity

The method published in the study by Orhan *et al.*[26] with slight modifications was used to calculate the plant samplest ability to scavenge NO. The plant samples and ascorbic acid (positive control) were prepared to stock solutions of 10 mg/ml and serially diluted to final concentrations of 1000 μg/ml to 7.8 μg/m. After which 50 μl of sodium nitroprusside was added to each well and the plates were kept in light at 25°C for 1 h 30 min. Once the incubation period was complete, a 50 μl solution of Griess reagent was then added to each well barring the negative control, where dH_2O was added in its place. For the NO scavenging assay, the presence of an antioxidant reduced the pink solution to a yellow color.

Nitroblue tetrazolium scavenging by alkaline dimethlysulfoxide method

The alkaline dimethlysulfoxide (DMSO) method was used to produce superoxide-free radicals (in this case, NBT), the reduction of which was determined in the presence and absence of a plant sample. All reactions were carried out in 96-well plates. The plant samples were prepared to stock solutions of 1000 to 7.8 μg/ml. The positive control, quercetin, was also prepared in the same way. A solution of 100 μl 100% DMSO was added to each well of the plate. After which, 10 μl of NBT was added. The presence of a reducing agent turned the solution from blue to blue-green, with the increase in the intensity of color change, indicating the increase in reducing power of the sample.

Statistical analysis

The ability of the plant samples to scavenge the free radicals in each antioxidant assay was determined using a BIO TEK Power-Wave XS multi-well reader (A.D.P., Weltevreden Park, South Africa) at a wavelength of 515 nm for DPPH, 546 nm for NO, and 560 for NBT, using KC Junior software (Highland Park, Winooski, Vermont, USA). The IC_{50} values of the samples were calculated using GraphPad Prism version 4 software (Graph Pad software version 4, San Diego, CA, USA) together with Windows Excel 2013.

Cytotoxicity

The potential toxicity of the plant samples on normal cells was determined using a cell proliferation kit II, where the effect that the samples had on Vero (monkey kidney) cells was measured using the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) method.[15] A solution of 100 μl of cells (1×10^5) was added to Mico plates, which were then incubated for 24 h allowing for the seeded cells to attach to the bottom of the plates. A stock solution of each sample was used to make dilutions ranging from 100 to 31 μg/ml, which were then added to the microplates and incubated for 48 h. The XTT reagent (0.3 mg/ml) was added and the plates were incubated for a further 1–2 h. Actinomycin D was used as a positive control. After incubation, the absorbance of the color was spectrophotometrically quantified using an ELISA plate reader, which measured the optical density at 490 nm with a reference wavelength of 690 nm.

RNA extraction, reverse transcription (cDNA synthesis), and reverse transcription-polymerase chain reactions

Having obtained good antibacterial and antioxidant activities by the *M. indica* plant extract, it was selected further for mechanistic studies. The change in the expression of virulence genes *spaP* and *gtfB* caused by treatment with this plant extract was determined using reverse transcription-polymerase chain reactions. Approximately 1×10^6 bacterial cells were treated with 500 μg/ml concentration of the *M. indica* sample in triplicate after which the treatments were incubated for 24 h at 37°C in an anaerobic environment. After incubation, intact RNA from untreated and cells treated with the plant extract was extracted using a Qiagen RNeasy® mini kit, with slight modifications.[29] A nanodrop spectrophotometer calculated the $OD_{260/280}$ and $OD_{260/230}$ ratios of each sample after which gel electrophoresis was carried out in order to scrutinize the quality of RNA. Five hundred nanograms of synthesized RNA was used to prepare a cDNA pool using the cDNA synthesis kit (Thermo scientific, SA). The cDNA was then used to conduct semi-quantitative Polymerase chain reaction (PCR), with Image J (NIH image, Biocompare, USA) and GraphPad Prism used to estimate the mRNA levels in treated and untreated cells.

RESULTS AND DISCUSSION

Potential antibacterial activity against *Streptococcus* mutans (*in vitro*)

Of the four samples tested, *P. pinnata* and *P. guajava* showed no significant antibacterial activity, with only occurring at the highest concentration tested, i.e. 12.5 mg/ml. *M. indica* had very good activity at 0.39 mg/ml, with *A. indica* showing moderate activity at 6.25 mg/ml [Table 1]. The positive control CHX had an MIC of 4.8×10^3 mg/ml, which corresponded to that reported in literature.[28]

A plant extract is regarded as having antimicrobial activity if it has an MIC value of 8 mg/ml or lower.[30,23] A specific interpretation according to the study by Kuete[31] was that the activity of a plant sample was regarded noteworthy when its MIC fell below a concentration of 0.1 mg/ml and adequate when MIC values fell between 0.1 and 0.625 mg/ml, with samples having activity above 0.625 mg/ml indicating low activity. Of the four samples present, the extract of *M. indica* exhibited the best antibacterial activity, with its activity showing better activity than that reported for the leaf extracts of *Eucalyptus spathulata* (which has been known to be added in toothpastes and mouthwash), which had an MIC of 50 mg/ml when tested against *S. mutans*.[32]

Antioxidant capacity

The radical scavenging activities of the different plant samples using different antioxidant assays are summarized in Table 1.

The antioxidant activities of the samples were expressed by an IC_{50} value, which was used to define the concentration of the tested sample at which

50% of the free radical was scavenged. A low IC_{50} value indicated good activity of that sample against that particular free radical.[33]

A. indica had the best activity against DPPH (IC_{50} value of 28 µg/ml), with *P. guajava* exhibiting little-to-no DPPH scavenging activity with an IC_{50} value of 283 µg/ml. The positive control ascorbic acid had an IC_{50} value of 20 µg/ml. From the samples tested, *M. indica* had the best scavenging capacity against NO, with an IC_{50} value of 12 µg/ml, which was similar to that of the positive control ascorbic acid (IC_{50} value of 4 µg/ml). In general, most of the plant samples showed good activity against the NBT-free radical, with *M. indica* and *P. guajava* having the lowest IC_{50} value of 7 µg/ml, which was even better than that of quercetin, the positive control which had an IC_{50} value of 10 µg/ml. *P. pinnata* was the only sample that did not exhibit any NBT scavenging activity.

Many bacterial species use oxidation as a part of their pathogenesis, where many of them attack important molecules such as DNA and other molecules in order to use them as building blocks for bacterial cellular components.[10,34] The production of superoxide has been reported in diseases such as dental caries, where these free radicals are important for converting the environment in the oral cavity to one that is conducive to the establishment of other bacteria. Therefore, a sample with good antioxidant activity may directly interfere with the successful establishment of bacteria such as *S. mutans* in the oral cavity.[35]

All the plant samples in the present study generally exhibited some antioxidant activity across the board. The antioxidant activity that was of particular interest was that of *M. indica,* particularly against the NO radical. This antioxidant activity is of particular importance because the production of NO is an important part of the pathogenesis of certain oral pathogens, as it forms part of the inflammation process (acts as an activation agent). The good NO activity exhibited by *M. indica* also correlated with its good antibacterial activity. *M. indica* had the overall best antioxidant activity of all the plant samples tested. The reducing properties of the samples may be due to the presence of reductones (molecules that break free radical chains by donating hydrogen atoms). In particular, *M. indica* contains the pharmacologically and medicinally important chemical mangiferin, which is a polyphenolic antioxidant.[36]

Table 1: Summary of the concentrations of the plant samples at which 50% of the free radical were scavenged (IC_{50}) in *in vitro* antioxidant assays

Samples	Antioxidant activity IC_{50} (µg/ml)			
	Herbarium specimen	DPPH	NBT	NO
Azadirachta indica (J)	PD-3768	28	9	18
Mangifera indica (L)	PD-951	94	7	12
Millettia pinnata (P)	Rs-187	112	400	53
Psidium guajava (L)	Rs-172	283	7	27
Vitamin C	-	20	-	27
Quercetin	-	-	10	-

NBT: Nitroblue tetrazolium; NO: Nitric oxide; DPPH: Diphenyl picrylhydrazyl

Synergistic assay

The MIC value of the combination of *M. indica* and *A. indica* was 6.25 mg/ml and for *P. pinnata* and *P. guajava* was 6.25 mg/ml. These values were used to calculate the ΣFIC indices. The Σ hei for *M. indica* and *A. indica* was 16 and for *P. guajava* and *P. pinnata* was 1, indicating that a combination of *M. indica* and *A. indica* was antagonistic and that of *P. guajava* and *P. pinnata* was additive.

Reverse transcription-polymerase chain reaction

The degree of expression of the *spaP* and *gtfB* mRNAs in bacterial cells treated with the *M. indica* sample was evaluated by reverse transcription-polymerase chain reactions. The gene for glutamine (*glnA*) was used as the housekeeping gene. The levels of mRNA expressed for the *gtfB* gene were expressed at a lower rate in the presence of the plant extract ($P < 0.01$) while the levels of mRNAs expressed for *spaP* were found to be the same in treated and untreated cells ($P > 0.01$) [Figures 1 and 2]. This suggested that the plant sample did not inhibit the production of the Antigen I/II, expressed for by the *spaP* gene. It may have instead targeted the production of the GTF enzyme, encoded for by the *gtfB* gene at the transcription level.

Cytotoxicity

The plant samples were tested for toxicity against Vero cells, and of all the samples, *P. guajava* showed acute toxicity to the cells, with an IC_{59} value of 23.63 µg/ml. *M. indica,* which was chosen for further investigation in the study, showed moderate toxicity (IC_{50} of 64.72 µg/ml). The other samples also showed moderate toxicity at concentrations ranging from 35.88 to 58.69 µg/ml. Actinomycin D had an MIC of 2×10^3 µg/ml, which was comparable to that reported in literature.

CONCLUSION

Of all the samples tested, *M. indica* (Mango) showed significant results for antibacterial activity. The results provided some scientific validation

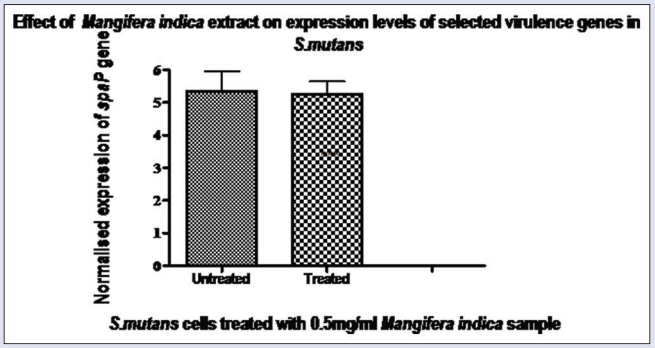

Figure 1: Expression of the *spaP* gene in treated and untreated cells. Expressions of the *spaP* gene were normalized to that of the *glnA* reference gene. Data are expressed as mean ± standard deviation, $n = 6$, *t*-test, $P > 0.05$, not statistically significant. This indicated that there was no difference in expression of the *spaP* gene in untreated and treated cells. This indicated that the sample had no effect on the synthesis of AntigenI/II at the transcription level

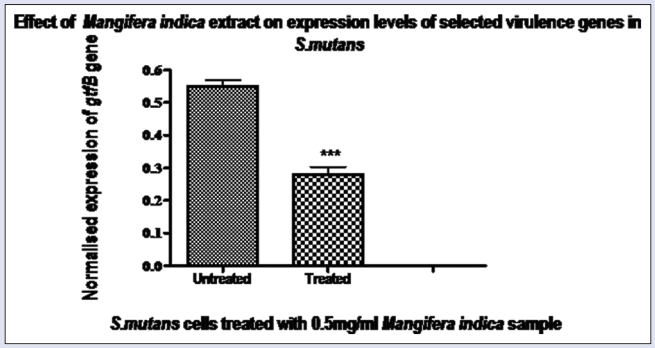

Figure 2: Expression of the mRNA levels of the *gtfB* gene in treated and untreated cells. Expressions of the *gtfB* gene were normalized to that of the *glnA* reference gene. Data are expressed as mean ± standard deviation, $n = 6$, *t*-test, $P < 0.05$, represents a statistical significance when compared to the untreated. The plant sample caused a reduction in mRNA levels for this gene, indicating that the sample may have an effect on the synthesis of the glucotransferase enzyme at transcription level

for the traditional use of *M. indica* leaf extract which inhibited some characteristics of *S. mutans* and thus may be considered beneficial for oral care.[37] Therefore, this study provides a significant contributor to antibacterial and antioxidant activities. Of interest would be *M. indica*, which had the best antibacterial activity as well as generally the best antioxidant activity. More importantly, this plant sample may possess a unique mechanism of action, which involves targeting the expression of the *gftB* gene, thereby targeting bacterial attachment.

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Conflicts of interest

There are no conflicts of interest.

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