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Inhibitory Potential of Mangiferin on Glucansucrase Producing *Streptococcus mutans* Biofilm in Dental Plaque

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Abstract: Glucansucrase secreted by *Streptococcus mutans* and composed of virulence genes alters oral microbiota, creating adherent environment for structural bacteria colony forming dental biofilm. The present investigation studied the inhibitory and binding potentials of mangiferin against *S. mutans* and its enzyme glucansucrase implicated in biofilm formation. Antibacterial activity against planktonic *S. mutans* was carried out. Using reverse transcription PCR, the expression of crucial virulence genes, *gtfB*, *gtfC*, *gtfD*, *gbpB*, and *comDE* were determined. The effect of mangiferin on teeth surfaces biofilm was ascertained by scanning electron microscopy (SEM). Docking analysis of *S. mutans* glucansucrase and mangiferin revealed the binding energy of −7.35 and ten hydrogen interactions. Antibacterial study revealed that mangiferin was not lethal to planktonic *S. mutans,* but a concentration-dependent inhibition of glucansucrase activity was observed. The inhibitory effect of water-insoluble glucan synthesis was apparently more marked relative to water-soluble glucan synthesis attenuation. Mangiferin significantly downregulated the expression of the virulence genes, indicating a mechanism involving glucanotranferases, specifically inhibiting colony formation by attenuating bacterial adherence. SEM images revealed that *S. mutans* biofilm density was scanty in mangiferin treated teeth compared to non-treated control teeth. Our data therefore suggest that mangiferin inhibited *S. mutans* biofilms formation by attenuating glucansucrase activities without affecting bacteria growth.

Keywords: mangiferin; *S. mutans*; glucansucrase; dental plaque; biofilm

1. Introduction

Dental plaque is regarded as one of the most common biofilm-related infectious dental problems reported in public health settings [1]. Plaque formation can cause dental caries, periodontitis, and endodontic infections [2,3], with resultant infections leading to dental decay. The public health problem of dental plaques and disease of dental caries are known to cut across all strata of the entire human population [4]. This has been attributed to the consumption of dietary free sugars (sucrose), either as

additives or preservatives, and they are linked to biofilm induced tooth decay [5,6]. The phenomenon of biofilm formation is complex with its control suggested to be a critical approach in curtailing the challenges faced by healthcare professionals [7]. Generally, dental plaques occur when the oral microbial pH is lowered, thereby creating the presence of increased acid producing and acid tolerant bacteria in a structurally and functionally organized biofilm formation [8]. Subsequently, as plague develops over time, the low pH becomes dominant and demineralizes the enamel and dentin [9]. The resultant plaque develops in such an ordered way that it is of a diverse microbial composition. As plague formation evolves over time, a low pH becomes dominant, demineralizing the enamel and dentin [9]. One of most common bacteria associated with dental plaques and a major contributor to the breaking down of tooth enamel, causing tooth decay, is *Streptococcus mutans* (*S. mutans*) [9]. The ability to cause dental plaque biofilm by this bacterium has been attributed to glucansucrase, an enzyme secreted extracellularly by *S. mutans*. Glucansucrase is reported to use fermentable carbohydrates to form high molecular-weight sticky glucan that is associated with the pathogenesis of biofilm production [10]. Extracellular glucan is a critical virulent element of *S. mutans* and can either be water soluble or insoluble contributing to the development of dental biofilm [11]. The main component of these virulent genes is three glucanotransferase enzymes (*gtftB*, *gtfC*, *gtfD*). They catalyze the synthesis of extracellular glucans in concert with fermentable carbohydrates, all of which are important as virulence factor in biofilm formation. Therefore, the pathogenicity of *S. mutans* is dependent on the virulence elements that enable it to adapt to low oral pH due to its characteristic aciduricity property [12]. The virulence characteristics such as mass, viscosity, and stickiness creating an adherent environment for the bacterium to survive and thrive on teeth surfaces, thus contributing to the stability of structural dental plaque integrity [10–12]. In addition to this, glucans possess binding proteins, an example of which is the glucan binding protein B (*GbpB)* that facilitates the binding of bacteria to secreted glucans [13]. Furthermore, *S. mutans* produces two-component signal transduction system protein, *ComDE* [14], known to regulate the expression of virulent gene elements. As an environmental sensor, downregulation of this gene will result in biofilm defects [15]. Currently, the treatment of dental biofilm involves the mechanical removal of the plaque and the use of antimicrobial mouthwash such as chlorhexidine. However, these agents are non-specific in their action, and hence disrupt the oral microbiome by targeting commensals. Their reported side effects include teeth discoloration and gingival epithelial cells cytotoxicity [16,17]. In view of these treatment limitations, coupled with the emergence of bacterial resistance, alternative molecules of natural origin with potential antibacterial activity that have been used as remedies could be evaluated. A recent study reported a number of natural compounds, such as anthraquinones, apigenin, chitosan, α-mangostin, and myricetin to exhibit anti-biofilm activities. According to the report, most of these products are not selective in their action [18]. Therefore, there is a need for alternative compounds with selective biofilm inhibitory potential targeting *S. mutans* without altering the oral microflora as well as having minimal side effects. One such compound is mangiferin, a major component of *Mangifera indica*, which belongs to the Anacardiaceae family, and has been documented to have a promising pharmacological profile [19,20]. Extracts of *Mangifera indica* have been used in herbal medicine for various indications [21,22]. Studies also reported an anti-adherence effect of the extracts of *Mangifera indica* on early and late plaque bacterial colonizers [23,24]. Periodontitis is caused by organisms, which colonize biofilm, therefore the action of *S. mutans* in attracting various bacteria strains such as *Aggregatibacter actinomycetemcomitans*, could induce inflammatory reactions [25]. Carvalho et al., [21], observed that orally administered mangiferin was effective in the treatment of experimental periodontitis in rats. In addition, the antimicrobial activity of mangiferin against *S. mutans* GS-5, a cariogenic strain, has been documented [26]. In addition, other compounds of natural origin, such as biacalm and palmtoylethanolamide, were found to be effective in the treatment of periodontitis [27]. Based on the previous studies that showed the effectiveness of mangiferin in the treatment of experimental periodontitis in rats and the inhibition of the activity of cariogenic strain *S. mutans*, we hereby further evaluate its antibiofilm potential. We hypothesize that it might be an effective natural agent against dental plaque development by inhibiting

glucansucrase activity. Therefore, the present investigation evaluated the anti *S. mutans* activity of mangiferin and its inhibitory activity against glucansucrase using an enzymatic assay. Also, by using computational and biological assays, we investigated the antibacterial activity of mangiferin against planktonic *S. mutans* and its binding potential to glucansucrase. We also evaluated the inhibitory potential of mangiferin on glucansucrase activity and its ability to form biofilms. Furthermore, the impact of mangiferin on the expression of the virulence genes, namely *gtfB*, *gtfC*, *gtfD*, *gbpB*, and *comDE*, was determined. This was undertaken with a view to ascertaining the anti *S. mutans* biofilm formation by mangiferin, a naturally available and less toxic substance than the current available pharmaceutical products. The null hypothesis to reject is that there is no significant difference between the mangiferin treated group and control group.

2. Materials and Methods

2.1. Chemicals and Bacteria Strain

Streptococcus mutans (*S. mutans*) MTCC 890 obtained lyophilized culture from the Institute of Microbial Technology (IMTECH), Chandigarh, India was used for the investigation. This was sub-cultured in Brain heart infusion (BHI) broth and incubated at 37 °C for 24 h. All the chemicals used in the study were bought from Himedia, India, unless otherwise stated.

2.2. Preparation of Protein Structure, Ligand Structure and Active Site Prediction

The RCSB database (http://www.rcsb.org/pdb/home/home.do) was used to retrieve the three-dimensional structure of *S. mutans* glucansucrase (3AIB). While the mangiferin ligand chemical structure was be retrieved from the compound database of Pubchem, (http://www.ncbi.nlm.nih.gov/search). Ligand structures were retrieved in sdf was converted to pdb format with Pymol software (Schrodinger, LLC. 2010. The PyMOL Molecular Graphics System, Version 2.3.1). Also, using Computed Atlas of surface Topography of proteins (CASTp), the possible binding sites of Glucansucrase were ascertained to include pockets located on protein surfaces as well as voids buried in the protein interior.

2.3. Molecular Docking

Earlier described methods [28] for docking calculations were performed with AutoDock (version 1.5.2 revision 2) and executed with AutoDock version 4.2. The mangiferin that acted as the reference drug for procedure validation was docked in to the active site. In addition, the assignment of a perfect grid to each ligand and the subsequent obtaining of grid box values are as described previously [28]. Based on the binding energy, each resulting docked conformed ligand were ranked into cluster and visually analysed using the PyMOL software version 2.3.1.

2.4. Anti-microbial Activity of Mangiferin and Time-Kill Assay

A disc diffusion assay was used to evaluate the antibacterial effect of varying dilutions of mangiferin on cariogenic *S. mutans*. The following concentrations of mangiferin 125 μm, 250 μm, 500 μm and 1000 μm were used for the screening of its antimicrobial effect while DMSO and ciprofloxacin (20 μg)) were the negative and positive controls respectively. The method described earlier [29] was used for the time kill assay of mangiferin against *S. mutans*. A bacterial suspension made up of 4×10^6 CFU/mL of *S. mutans* was prepared in tubes containing 8 mLs of BHI broth to which mangiferin concentrations that ranged between 62.5–1000 μm were added. A vehicle control was also set up and this consisted of DMSO and bacterial inoculum. All tubes were incubated at a temperature of 37 °C in a shaking incubator for 42 h. At time intervals of 0, 24, 30, 36, and 42 h, 1 mL of the bacterial suspension was drawn out, serially diluted in BHI broth, and then plated out on Muller Hilton agar. All plates were incubated for 24 h at a temperature of 37 °C, after which colony-forming units (CFU) were counted.

2.5. Biofilm Susceptibility Assay

The *S. mutans* biofilm inhibitory potential of mangiferin was performed in a sterile 24 well cell culture plate was used to determine the inhibitory effects of mangiferin on *S. mutans* biofilm as had been *previously described* [30]. *Tubes* of bacterial suspension consisting of 1×106 CFU/mL of *S. mutans* in BHI broth supplemented with 1% (wt/vol) sucrose and varying mangiferin concentrations (62.5–1000 μm) were incubated at 37 °C for a 72 h period. Media with DMSO was employed as vehicle control. Media and mangiferin with respective concentrations were refilled with every 24 h. At 72 h post incubation, the supernatant containing unbound cells and media components from each well was removed and stained with 0.1% (wt/vol) crystal violet solution for 5 min. Plates were then rocked in 95% ethanol at room temperature for 30 min and absorbance recorded at 595 nm. In addition, *S. mutans* biofilms were cultured on glass coverslips with different concentrations of mangiferin (0, 62.5, 125, 250, 500, and 100 μm) for 24 h at 37 °C. The coverslips were washed trice with sterile Phosphate Buffered Saline (PBS) to remove unbound cells and stained for 30 min in the dark with Acridine orange. A fluorescent microscope (Optika, Germany) was used to record image stacks in five random locations at 40× magnification. In each experiment, the light intensity, background level, and contrast were maintained at the same level.

2.6. The Assay of Glucansucrase Enzyme Inhibition

The method used earlier by other researchers [31] was employed in the assay glucansucrase enzyme. To this effect, *S. mutans* was used as the source for the extraction of crude glucansucrase enzyme using the method previously described [31]. The bacterium was inoculated into 50 mL of an enzyme-producing medium made of BHI supplemented with 1% sucrose in a 250 mL flask. This was incubated under a 180-rpm shaking condition at a temperature of 25 °C. To separate the cells, samples were drawn out, centrifuged at 10,000 rpm for 10 min at 4 °C and the cell-free supernatant used for enzyme assay. The method described by Kim et al. [32] was used for enzyme assay with the reaction mixture consisting of different mangiferin concentrations, 100 mm sucrose, 50 mm sodium acetate buffer (pH 5.2), and 20 μL cell free extract. The resultant was incubated for 30 min at a temperature of 30 °C while the end product of sucrose quantified dinitrosalicylic acid (DNS) method. Obtained results were calculated in enzymatic units with one unit defined as the amount of glucansucrase enzyme required to catalyse the release of 1 μm fructose per min at 30 °C.

2.7. Scanning Electron Microscopic Analysis

The scanning electron microscope was used to examine the effect of mangiferin on tooth surfaces with *S. mutans* biofilm. Human upper premolar teeth obtained from the reserved bank of Teeth "N" Jaws Center, Chennai (India) was used for the analysis. They formed part of those obtained in patient care therefore needing no informed consent from the patients. An earlier described method [33] was used for the preparation and processing of tooth samples. A bacterial suspension consisting of 1×109 CFU/mL *S. mutans* inoculated into 1 mL of BHI that was supplemented with 1% sucrose was introduced to a 24-well micro titre containing sterile teeth. To another set up containing, the same preparation was added 500 μm of mangiferin. All set up were incubated for a period of 48 h at 37 °C. Obtained sample were fixed in 2.5% glutaraldehyde for one hour followed by dehydration in increasing concentrations (25%, 50%, 75%, 95%) of ethanol for a period of 30 min each. Final dehydration was for 10 min in 100% ethanol before allowing too dry at room temperature for 4 h. Samples coated with platinum/palladium were examined using JEOL JCM-6000 scanning electron microscope (JEOL, Ltd., Tokyo, Japan).

2.8. Analysis of Bacterial Adhesion and Exopolysaccharide (EPS) Synthesis

Viable CFU of *S. mutans* biofilm teeth samples that of untreated and mangiferin treated and their polysaccharide composition were analysed according to methods described previously [34].

In addition, water and insoluble EPS were extracted as previously described [35], while quantification was by colorimetric assays according to earlier described method [36].

2.9. RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

RNA was extracted from *S. mutans* biofilms treated with 0, 500, and 1000 μm mangiferin concentrations using TRIzol reagent (Sigma-Aldrich) according to manufacturer's guidelines. The extracted RNA served as the template for reverse transcription using the cDNA synthesis kit (Takara) according to the guidelines of the manufacturers. The resultant cDNA was stored at −20 °C until needed for real-time PCR and served as the template for qPCR assay. The 16S rRNA primers used in the investigation are shown in Table 1. Quantitative PCR assay was performed using Qiagen (Hilden Germany) Rotor-Gen® Q thermal cycling system. A 20 μL of PCR reaction mixture was made up of 2 μL of template cDNA, SYBRGreen PCR Master Mix (Thermo Scientific), and 0.5 μm appropriate forward and reverse PCR primers each. The programming was as follows: initial incubation was carried out for 2 min at 50 °C this was followed by 40 cycles of denaturation at 95 °C for 15 s, then annealing and extension at 60 °C for 60 s. The resultant cDNA and those of the negative control were amplified using the primers shown in Table 1. The fold changes in gene expression were calculated with the ΔΔCt method and expressed according to recommendation [37].

Table 1. Nucleotide sequences of primers used for relative quantification of expression pattern of genes involved in *S. mutans* biofilm formation.

Gene	Primer Sequence (5′–3′)	Amplicon Size (bp)	Role in Biofilm Formation
gtfB	FP: AGCAATGCAGCCAATCTACAAAT RP: ACGAACTTTGCCGTTATTGTCA	95	Water insoluble glucan production.
gtfC	FP: GTGCGCTACACCAATGACAGAG RP: GCCTACTGGAACCCAAACACCTA	107	Water soluble and insoluble glucan production.
gtfD	FP: TGGCACCGCAATATGTCTCTTC RP: CAATCCGCAATAACCTGAATACCG	183	Water soluble glucan production.
gbpB	FP: ATGGCGGTTATGGACACGTT RP: TTTGGCCACCTTGAACACCT	50	Glucan binding protein.
comDE	FP: ACAATTCCTTGAGTTCCATCCAAG RP: TGGTCTGCTGCCTGTTGC	80	Competence-stimulating peptide involved in quorum sensing mechanism
16S	FP: CCTACGGGAGGCAGCAGTAG RP: CAACAGAGCTTTACGATCCGAAA	100	

2.10. Hemo Toxicity

Freshly obtained EDTA human blood (A, Rh+) from blood bank of A. G. Padmavathi Hospital Blood Bank, Puducherry, India was washed three times by centrifugation (2500 rpm for 10 min) in PBS at room temperature (20–25 °C). Lymphocytes and platelets (Buffy coat) were removed from gradient centrifugation and a 10% red blood cells (RBC) suspension was prepared in PBS (1×), aliquots of 2.5 mL of 10% RBC was mixed with 250 and 500 μm of mangiferin. Chlorhexidine 0.2% was used as positive control. Glutaraldehyde was added to stop the reaction. The supernatant was removed and the liberated hemoglobin in the supernatant was measured spectrophotometrically as absorbance at 541 nm. For negative control 5% DMSO with 10% of RBC suspension making a final concentration of 1% was used. The experiment was performed in triplicate and mean ± standard deviation (SD) was calculated as follows: % Hemolysis = (Absorbance sample/Absorbance positive control) × 100.

2.11. Oral Cell Toxicity

The biocompatibility of mangiferin in human oral epithelial cell line (HOEC) was procured from the Division of Cellular Immunology, Pondicherry Centre for Biological Sciences, India. The cells were routinely maintained in culture flasks (TPP, Switzerland), at 37 °C in 5% CO_2, in complete DMEM media (Gibco, MA).

MTT Assay and Cytotoxicity Analysis

Determination of cytotoxic activity of mangiferin, 80% confluent cell lines were trysinized and seeded on 96-well plates at a density of 5×10^3 cells per well. The cells were incubated overnight and treated with concentrations of 250 and 500 μm mangiferin, while 0.2% Chlorhexidine was used as positive control. Negative control group cells were treated with 5% DMSO at the same concentration with culture media and attain final concentration of 1% DMSO (v/v) in each well. Cell viability was determined by a 3,4,5-dimethylthiazol-2,5 biphenyl tetrazolium bromide (MTT–Life Technologies, USA) assay. The absorbance of cell viability was examined using a spectrophotometer with a microplate reader at a wavelength of 595 nm (Biorad; plate reader, USA).

2.12. Data Analysis

Experiments were performed in independent triplicates and data are presented as the mean ± SD. Statistical analysis was performed using Graphpad Prism (version 8, San Diego California, USA). One-way ANOVA was used to compare statistical difference between control and treated groups followed by Tukey's multiple comparisons test. Significant difference was taken as $p < 0.05$.

3. Results

3.1. Computational Analysis of S. mutans Glucansucrase Binding Potential of Mangiferin

Docking analysis of *S. mutans* glucansucrase and mangiferin revealed binding energy of −7.35 and 10 hydrogen interactions (Table 2). It was noticed that a hydrogen bond between ASPS'593/OD1 of glucansucrase and C7-OH of mangiferin with a bond length of 2.2 Å. Two hydrogen bonds were noticed between ASP'588/OD2 of glucansucrase and C1-OH and C6'-OH group of mangiferin respectively with bond length of 2.0 Å. Similarly, two hydrogen bonds were noticed between ASP'909/OD1 of glucansucrase and C2'-OH and C3'-OH group of mangiferin with bond length of 2.1 Å and 2.5 Å respectively. Amino acid, GLN'592/1HE2 of glucansucrase and C2'-O group of mangiferin revealed a hydrogen interaction with a bond length of 2.4 Å. While both the amino acids HIS'587/HE2 and ARG'475/1HH2 of glucansucrase interacted with C6'-O group of mangiferin with a hydrogen bond length of 2.4 Å and 2.0 Å (Figure 1A,B; Table 2).

Table 2. Hydrogen bond interaction of mangiferin and amino acid residues of *S. mutans* glucansucrase.

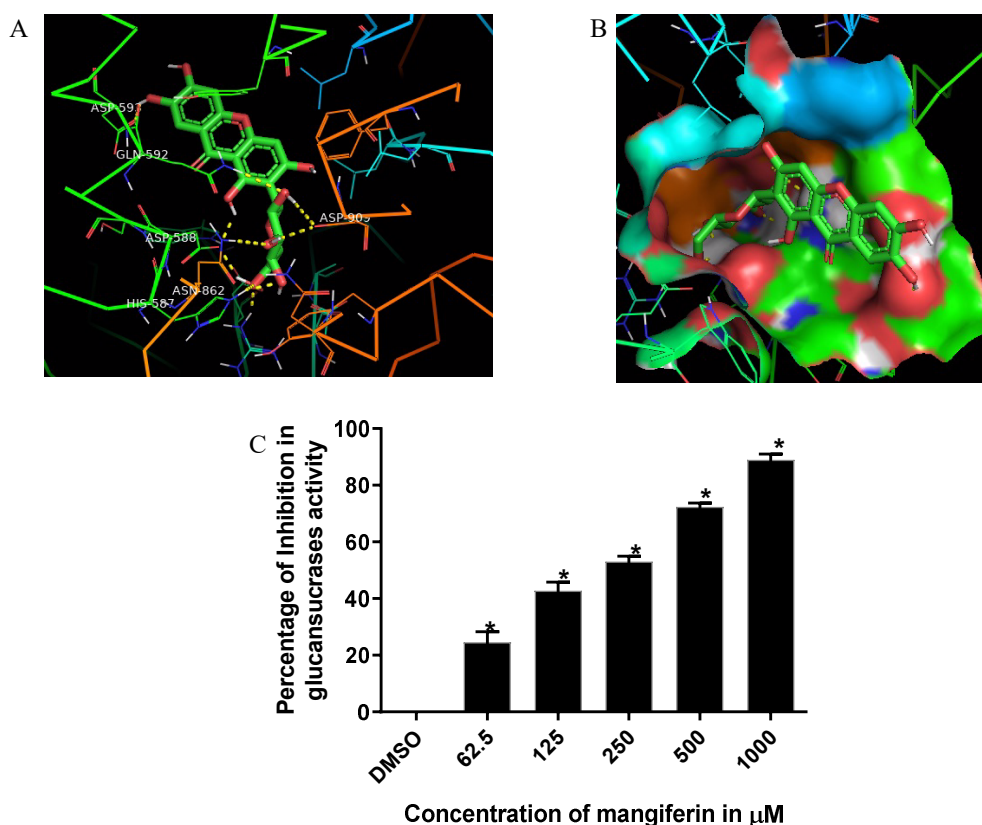| SL.No	Ligand	Binding Energy	Ligand Efficiency	Intermole Energy	Ligand Atoms (Ring)	Docked Amino Acid Residue (Bond Length)
1.	Mangiferin	−7.35	−0.25	−8.66	C7-OH (B)	ASP'593/OD1 (2.2 Å)
					C1-OH (A)	ASP'588/OD2 (2.0 Å)
					C2'-O	GLN'592/1HE2 (2.4 Å)
					C2'-OH	ASP'909/OD1 (2.5 Å)
					C3'-OH	ASP'909/OD1 (2.1 Å)
					C3'-O	ASN'862/1HD2 (2.5 Å)
					C4'-O	HIS'587/HE2 (2.0 Å)
					C6'-O	HIS'587/HE2 (2.4 Å)
					C6'-O	ARG'475/1HH2 (2.0 Å)
					C6'-OH	ASP'588/OD2 (2.0 Å)

Figure 1. (**A**) Docked orientation of mangiferin to *S. mutans* glucansucrase (PDB ID: 3AIB); (**B**) Glucansucrase binding pocket representation for mangiferin in glucansucrase; (**C**) *S. mutans* glucansucrase inhibitory potential of mangiferin. The values are mean of three replicates ± standard deviations (SD). * $p < 0.05$, represents significant difference compared with the control group.

3.2. Mangiferin Inhibition of S. mutans Glucansucrase Activity

The resultant outcome of computational studies motivated us to study the biological effect of mangiferin on *S. mutans* glucansucrase activity. Inclusion of mangiferin in glucansucrase reaction mixture resulted in concentration dependent inhibition in glucansucrase activity (Figure 1C). Mangiferin revealed glucansucrase inhibition starting from the lowest test concentration (62.5 µm). Mangiferin at the higher concentrations of 500 µm and 1000 µm inhibited more than 75% of glucansucrase activity as compared with those of the DMSO control.

3.3. Antibacterial Activity of Mangiferin against Planktonic S. mutans

Results of the antibacterial investigation using disc diffusion assay revealed that mangiferin was not lethal to *S. mutans* in the test concentrations ranging from 62.5 µm to 1000 µm (Figure 2A). Compared to the DMSO control, a significant decrease was initially observed with concentrations of 250 µm after 30 h but did not affect the overall growth curve. Even then, the growth curve assay with an extended incubation period of up to 42 h also indicated that mangiferin was not lethal to planktonic *S. mutans* culture (Figure 2B).

3.4. Mangiferin Suppressed S. mutans Biofilm

Similar to the inhibition of glucansucrase activity, mangiferin was also seen to have inhibited *S. mutans* biofilm formation in a concentration dependent manner (Figure 3). Also, biofilm inhibitory activity of mangiferin was noticed for the least test concentration of 62.5 µm (Figure 3A). Although the biofilm inhibitory potential was less in lower test concentrations, this inhibitory ability by mangiferin

was seen to have increased in concentration-dependent manner. The test concentration of 1000 μm inhibited more than 90% of the *S. mutans* biofilm formation on plastic culture plates. Furthermore, microscopic examination of biofilm in plastic culture plate revealed concentration dependent decrease in bacterial biofilm on the surfaces (Figure 3B). Also, treatment with mangiferin treated (62.5 μm to 1000 μm) biofilm stained with acridine orange, observed under fluorescent microscope at 40× magnification, showed a concentration-dependent reduction (Figure 3C). This significant reduction ($p < 0.05$) of *S. mutans* biofilm formation ranged from 11% to 94%, evidently indicating that mangiferin has the potential to inhibit dental plaque biofilm formation.
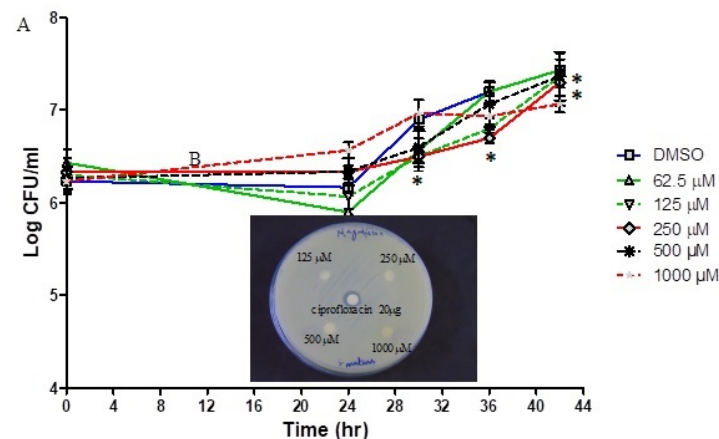

Figure 2. (**A**) Effect of mangiferin on growth pattern of *S. mutans* planktonic cell cultures using time kill assay. The concentrations of mangiferin ranged from 62.5 μm to 1000 μm. (**B**) Antibacterial effect of mangiferin against *S. mutans* using disc diffusion method. The DMSO treated *S. mutans* cells grown in BHI broth was used as control. Data are means ± standard deviation (SD) (n = 3). Significance difference was set at * $p < 0.05$, from the control group.
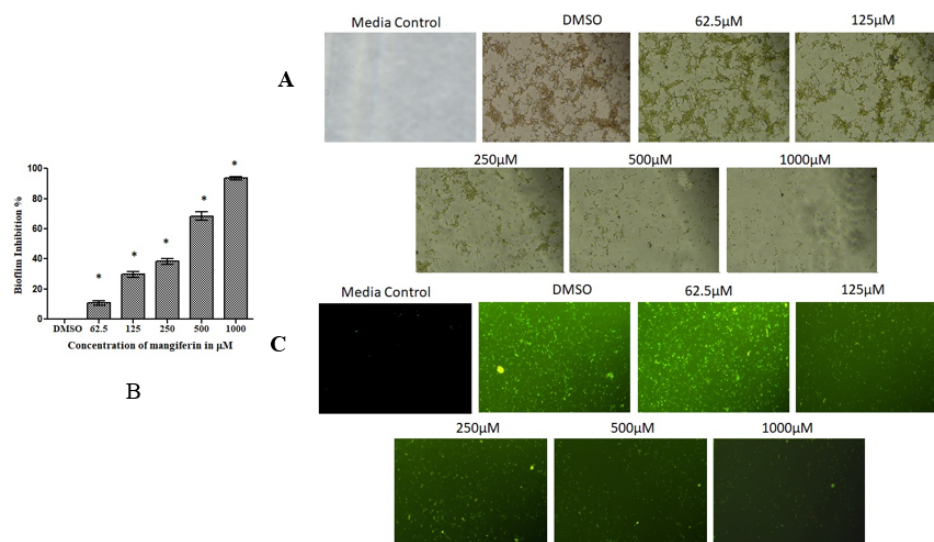

Figure 3. Mangiferin inhibitory potential against *S. mutans* biofilm formation. (**A**) Biofilm inhibition of mangiferin in concentrations of 62.5, 125, 250, 500 and 1000 μm compared to DMSO control. The values are mean of three replicates ± SE. * $p < 0.05$, represents significant difference compared with the control group. (**B**) Microscopic observation of *S. mutans* biofilm under the influence of mangiferin at 40× magnification; (**C**) Control and mangiferin treated (62.5 μm to 1000 μm) biofilm stained with Acridine orange observed under fluorescent microscope at 40× magnification.

3.5. Mangiferin Suppressed S. mutans Biofilm on Dental Surface

On confirmation of biofilm inhibitory potential of mangiferin on plastic culture plates, we analyzed its biofilm inhibitory potential on dental surfaces. Results from our study revealed that mangiferin reduced colony-formatting units of biofilm on dental surface in a concentration dependent manner. Unlike plastic surfaces, mangiferin at low concentration (62.5 μm) did not inhibited *S. mutans* biofilm on dental surface (Figure 4). In contrast, the biofilm formation on dental surfaces started to decrease when the tooth surfaces were treated with the concentrations ranging from 125 μm. Treating dental surfaces with higher concentrations of mangiferin (500 μm and 1000 μm) revealed more than a 75% reduction in *S. mutans* biofilm.
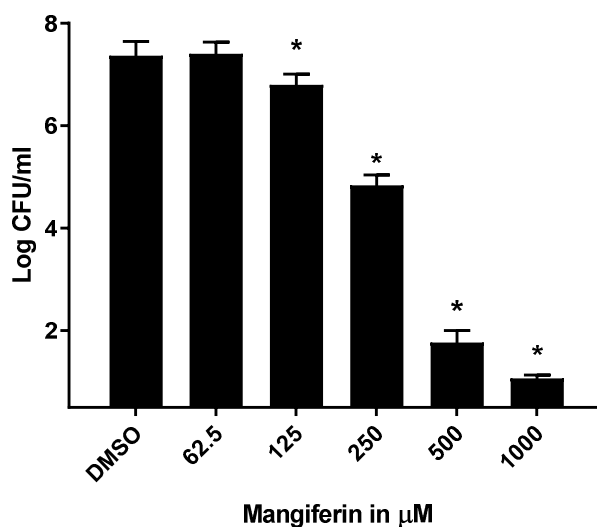

Figure 4. Effect of mangiferin on *S. mutans* biofilm in teeth analyzed as viable colony forming units. Data are means ± standard deviations (SD) (n = 3). * $p < 0.05$ represents statistical significant difference from the control group.

3.6. Mangiferin Inhibition of Glucan Synthesis in S. mutans Biofilm

Similar to glucansucrase activity and biofilm formation, glucan synthesis in *S. mutans* was reduced by mangiferin. Mangiferin concentrations ranging from 125 to 1000 μm reduced water-soluble glucan synthesis by *S. mutans* (Figure 5A). Similar to its effect on water-soluble glucan synthesis, mangiferin also decreased the synthesis of water insoluble glucan in concentration dependent manner (Figure 5B). Both water soluble/insoluble glucan syntheses in *S. mutans* biofilm were not affected when it was treated with a lower concentration of mangiferin (62.5 μm).

3.7. Mangiferin Down-Regulated the Expression of Genes Involved in Biofilm Formation

To study the effect of mangiferin on biofilm-related gene expression, we performed quantitative real time PCR to quantify the effect on the biofilms formed by *S. mutans* using test concentrations of 500 μm and 1000 μm. Among the genes examined, the expression of *gtfB* involved in water-soluble extracellular polysaccharide synthesis was found to be down regulated by the treatment with mangiferin (500 and 1000 μm). Similarly, the expression of genes involved in water-soluble and insoluble glucan production (*gtfC*) was also down regulated by mangiferin in dose dependent manner. In addition, the expression of *gtfD*, involved in synthesis of water-soluble glucan was down regulated by mangiferin treatment. In addition, mangiferin also down regulated the expression of *gbpB* gene encoding glucan binding protein. The gene encoding *ComDE*, involved in an intraspecific cell–cell communication quorum sensing system in *S. mutans,* was also down regulated by mangiferin (Figure 6).
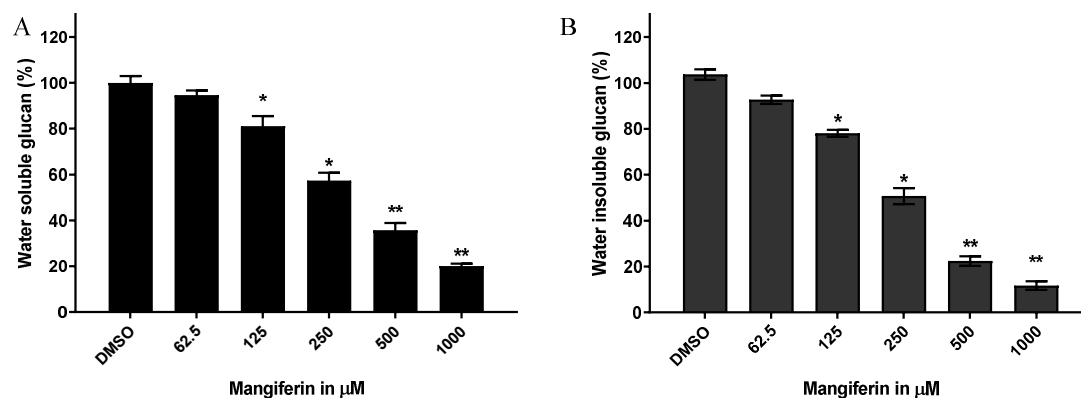
Figure 5. Effect of mangiferin on glucan synthesis in *S. mutans*. (**A**) Water soluble glucan; (**B**) Water insoluble glucan. Data are means ± standard deviations (SD) (n = 3). * $p < 0.05$, and ** $p < 0.001$ represent statistical significant differences from the control group.
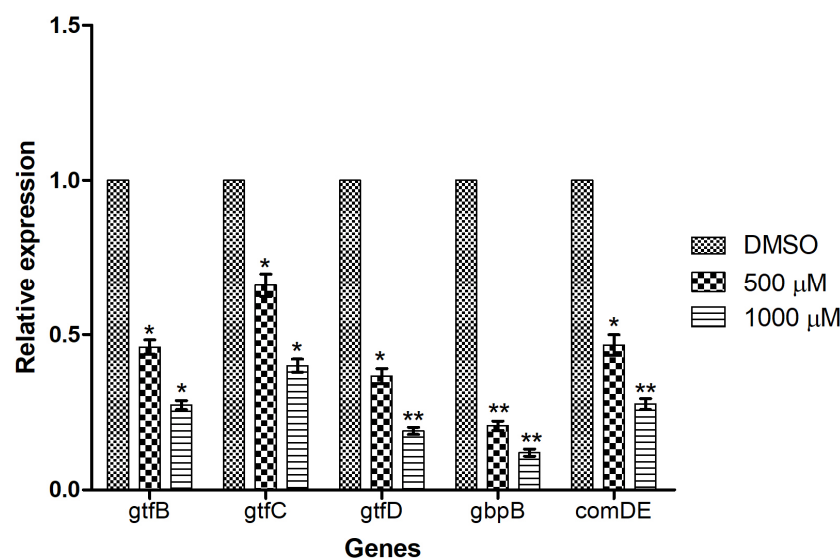

Figure 6. Effect of mangiferin on expression of genes involved in *S. mutans* biofilm formation. Data means ± standard deviations (SD) (n = 3). * $p < 0.05$, ** $p < 0.01$, represents significantly statistical difference from the control group.

3.8. Scanning Electron Microscopic Observation of Biofilm

In addition to the biofilm inhibitory potential of mangiferin on plastic culture plates, we also analyzed the SEM data of control and mangiferin treated biofilms. The SEM images revealed attached of *S. mutans* biofilm in both the control and mangiferin treated tooth surfaces (Figure 7A,B). In contrast, mangiferin induced biofilm disruption (or inhibition) and associated microbial cell morphology was examined using scanning electron microscope of mangiferin treated tooth surface (Figure 7B). As shown in Figure 7B, the *S. mutans* biofilm density was scanty in mangiferin treated tooth on comparison to non-treated control tooth (Figure 7A). In addition, both the pattern of biofilm and cells distribution was also different in the control and the mangiferin treated tooth surface, showing scanty and distorted colonies.
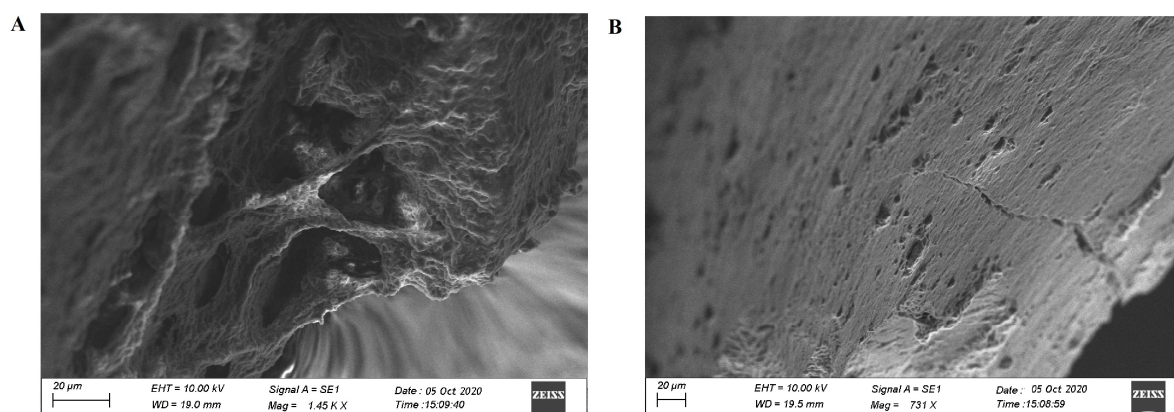
Figure 7. SEM images showing biofilm of *S. mutans* on control and mangiferin treated tooth. (**A**) The biofilm formation on vehicle control (DMSO) treated tooth surface; (**B**) Biofilm formation on mangiferin (500 μm) treated tooth surface.

3.9. Hemo Toxicity

The hemotoxicity of mangiferin was studied by examining haemolytic activity against human red blood cells (RBCs) using Chlorhexidine 0.2% as positive control. There were no differences between 1% DMSO and mangiferin concentrations used. This indicated that mangiferin did not exhibit any toxicity on RBC. However, 0.2% chlorhexidine showed about 3–4-fold increased haemolysis compared to mangiferin and DMSO (Figure 8A).
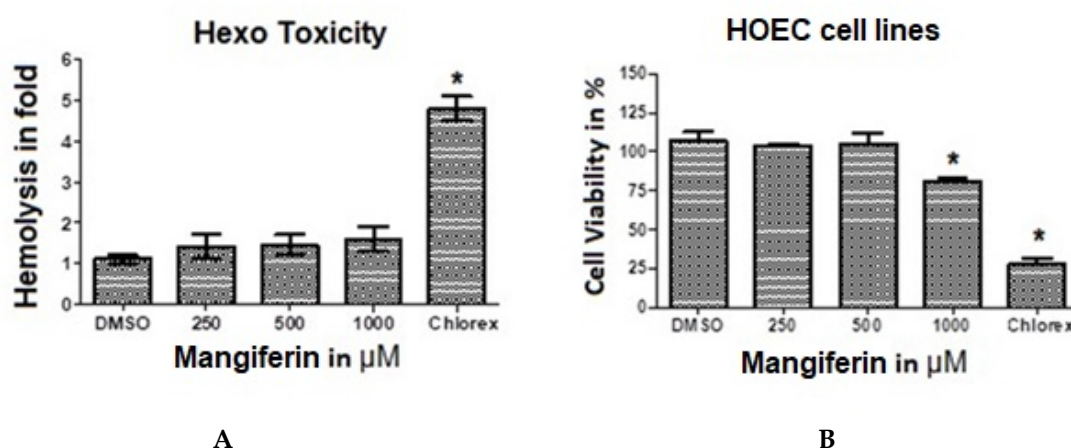

Figure 8. Hemolysis and Cytotoxicity of mangiferin. Hemolytic activity of mangiferin in concentrations of 250 and 500 μm against human RBC cells and HOEC cells. After 24 h of incubation with the mangiferin, (**A**) Hemolytic activity of the mangiferin against red blood cells. The release of hemoglobin was measured using a microplate reader at 414 nm. Hemolytic level was noted in fold. (**B**) Cytotoxicity was measured using an MTT (3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide) assay. * $p < 0.05$ represents significant difference between the control and. mangiferin treated groups.

3.10. MTT Assay and Cytotoxicity Analysis

Results of the effects of mangiferin on the cell viability of human oral epithelial cell line (HOEC) were studied as presented in Figure 8B. There were no effects on cell viability by concentrations of 250 and 500 μm mangiferin. The result obtained is comparable to that observed for the negative control, 1% DMSO. However, 1000 μm of mangiferin gave 75% cell viability compared to 0.2% chlorhexidine, which significantly ($p < 0.05$) decreased cell viability. This indicates that mangiferin does not suppress cell viability and potentially has no deleterious effects when applied on oral mucosa cells.

4. Discussion

Biofilm production by *S. mutans* is critical in the pathogenesis of dental plague associated oral diseases [38,39]. Several natural products have been evaluated for their anti-plaque properties in an effort to attenuate oral bacteria biofilm formation [28,38,39]. However, most of them have shown detrimental effects on the oral microbiome. Numerous studies have shown that inhibiting glucansucrase synthesis will affect the ability of *S. mutans* to produce extracellular polysaccharide-like glucans implicated in the pathogenicity of dental plaque [16,40]. Glucansucrase provides the binding elements that create the enabling environment for biofilm formation. In the present study, we report the inhibitory potentials of mangiferin, a naturally synthesized product from *Mangifera indica*, on glucansucrase's glucan synthesis. Using a computational approach, we demonstrated a significant binding interaction of mangiferin with glucansucrase at domains A and B amino acid sequence. Documented evidence indicates that these domains harbor the enzyme's catalytic sites [41,42]. The binding interactions as revealed by docking analysis in this study occurred at 8 regions of the amino acid components of glucansucrase. Interestingly, ARG 475, ASP 588, and ASP 593, which were involved in this binding interaction, are considered as the enzyme's catalytic active sites [43,44]. ARG 475 was identified as a key stabilizer of the biofilm structure, whereas ASP 593 is reported by Ito et al. [43] to have a critical influence on transglycosylation. This phenomenon is responsible for the production of either soluble or insoluble glucan. Therefore, the binding interaction of mangiferin with glucansucrase provided the impetus to explore its potential to inhibit biofilm formation. Abundant evidence shows that the production of glucan by glucansucrase secreted by *S. mutans* is the main pathogenic feature implicated in dental biofilm formation [45]. Hence, suppressing the ability of glucansucrase to synthesize glucan will in turn reduce the generation of polysaccharide production and disrupt bacteria dental plaque formation. Ren et al. [45] reported that a glucan synthesized polysaccharide matrix enhances early phase bacteria adherence to tooth surfaces. It also acts as a crucial carbohydrate reserve, shielding the microbes from antibacterial action [46]. The present study indicates that mangiferin inhibited glucansucrase activity in a concentration-dependent manner by 75%. However, the observation, that all concentrations of mangiferin did not affect the growth of planktonic *S. mutans* is contrary to earlier reports by Quang et al., [26] and Banavar et al., [47], who documented antibacterial activity of extracts of *Mangifera indica* leaves. A number of molecules of natural origins are reported to inhibit *S. mutans* activity, particularly, essential oils as documented by Le et al. [48] and Ács et al. [49]. This attribute places essential oils as a likely alternative to antimicrobials, particularly with the emergence of multi drug resistance. It could also, reduce their ability to be pathogenic [49]. Ashraf et al. [50], reported antibiofilm activity of *Mentha piperita* essential oils against *S. mutans*. The present work showed a mangiferin-induced suppressing effect on the biofilm formation in concentration-dependent fashion. This effect is consequent to the inhibition of glucansucrase as aforementioned. Colony-forming units (CFU) were reduced as well, indicating that mangiferin could be a potential candidate in the control of dental plaque formation. Our findings are in agreement with the work of Quang et al. [26]. The inability of *S. mutans* to form colonies in the presence of mangiferin decreased the bacteria's propensity to form biofilm in the absence of adherent glucan. This affected the ability of *S. mutans* to create an enabling microbial environment that is appropriate for colonization and subsequently plaque formation [12]. Documented evidence shows that the presence of biofilm is a risk factor for periodontitis, which generates inflammatory reactions [51]. Furthermore, the presence of periodontitis and the attendant inflammation has been associated with diseases like endothelial dysfunction and coronary heart disease [52]. Therefore, the treatment of biofilm associated dental diseases is an important health approach. Moreover, a recent report confirms the link between pathogenic oral bacteria and the progression of periodontal-induced biofilm [25]. Visualization with confocal imaging confirmed concentration-dependent dispersion and loose colonies with mangiferin treatment in this study. These findings indicate that mangiferin potentially disturbed the process of adherence in early *S. mutans* colonizers, thereby interrupting biofilm formation. The studies of Shafiei et al. [38] and Quang et al. [26] thus support this outcome and this could mean an anti-adherent

property. The behavior of *S. mutans* to aggregate and adhere contributes to the pathogenesis of dental plaque. If not controlled, will eventually lead to dental caries and associated diseases of the oral cavity [10]. In addition, biofilm analysis on the tooth showed that viable CFU were significantly reduced. Meaning that microbial milieu favourable to colonization has been altered by mangiferin. Evidently, from this present study, the synthesis of water-soluble and water insoluble glucans were reduced in the presence of mangiferin. These effects were only achieved in the concentration of 125–1000 μm of mangiferin. Reported studies show that *S. mutans* utilizes water soluble glucan to interact with salivary proteins in the oral cavity, whereas the synthesized water insoluble glucan is used to form stable dental plaques with increased adherence that attract other microbes [39,53]. Other natural products like EGCC and Ethyl gallate have similarly been reported to suppress the propensity of *S. mutans* to synthesize both water-soluble and water-insoluble glucans [39,53]. In our study, mangiferin suppressed biofilm formation on the tooth surface by attenuating the very element that enables adhesion. Furthermore, to evaluate the effect of mangiferin on gene expression of glucansucrase virulence elements used by *S. mutans* to form and maintain functional biofilms. The elucidation of these genes and their presence provided more information about the mode of action of mangiferin. Here, we demonstrated that mangiferin significantly downregulated the expression of *gtfB*, *gtfC*, and *gtfD* virulence genes involved in biofilm formation. The inhibition compromised their ability to express their full virulence attributes. Evidence indicates that reduced expressions of these genes proactively decreased the production of extracellular polysaccharide, bacterial aggregation, and consequently biofilm formation [39]. In addition, mangiferin was found to reduce the expression of glucan binding protein (*gbpB*). It is noted to be responsible for mediating microbial cell adhesion and plays an active role in the maturation of biofilm [54]. Our finding is consistent with the report of Ashraf et al. [50] who documented the reduction of the *gbpB* virulent gene involved in biofilm formation using a modified natural compound. Another essential element critical for dental plaque development, used by *S. mutans* to establish persistent biofilm, is the competence stimulating peptide (CSP) gene *comDE* [55]. The *comDE* gene is involved in the quorum-sensing system, regulating the expressions and competence of glucanotransferase enzymes. It also modulates the oral microbial environmental adaptation of bacteria involved in biofilm formation [56]. In our present investigation, we report that mangiferin suppressed *comDE* gene expression. Hasan et al. [10] demonstrated that the suppression of *comDE* expression is critical for plaque formation, affecting cell wall integrity and the loss of adherence. Reports indicate that *S. mutans* utilizes this gene to respond to changes within the oral environment by membrane signal transduction [55]. Our results are in agreement with other studies that showed the downregulation of this gene and a consequent disruption of biofilm formation [12,57]. Moreover, SEM results analysis revealed that exposure of *S. mutans* to mangiferin on the teeth surfaces showed disruption of plaque formation with scanty biofilm density. The scanty appearance of aggregated cells in the treated teeth sample can be interpreted to mean glucan synthesis inhibition. Unlike the treated teeth, the untreated teeth appeared clustered with a sticky glucan surface. Therefore, the SEM analysis further validates both the glucan synthesis and virulence gene elements inhibitions. Finally, the overall effect of mangiferin is demonstrated by this study to be antibiofilm formation. The fact that mangiferin did not alter the growth of planktonic *S. mutans* appear to set it apart as a potential therapeutic agent. This is suggested to be an advantage in that its use will therefore not alter the oral microbiome. The use of current antibiotics in the treatment of dental plaques affects not only *S. mutans* but also oral bacterial flora. The safety profile of mangiferin was also evaluated on human oral epithelial cell line (HOEC) and it was demonstrated not to be cytotoxic and to have no effect on cell viability. This observation was also demonstrated on human RBC and was seen to produce no haemolysis. Our finding confirms the report of other studies that stated that mangiferin is without any side effects or toxicity [58].

5. Conclusions

The present study has demonstrated that mangiferin has the potential to bind *S. mutans* glucansucrase and inhibit its activity. Given that mangiferin did not affect planktonic *S. mutans*

growth, the oral microbiome will not be altered. The study also showed that mangiferin antibiofilm activity occurs via the inhibition of the virulence gene elements of *S. mutans*. By so doing, adherence attributes are attenuated, colonization reduced, and aggregation diminished, thereby providing an apparent insight into its antibiofilm propensity. Further investigations are needed to expand the scope of mangiferin's antibiofilm potential with a view towards future clinical studies. Particularly as an anti-inflammatory agent, mangiferin has established potential to attenuate inflammatory related diseases caused by periodontal disease.

6. Study Limitations

In this study, we compared the effects of oral mouthwash containing the antibiotic chlorhexidine to show the safety profile of mangiferin as an antibiofilm agent. Therefore, this study has opened a new study perspective, that is, to compare mangiferin with other agents that are currently used in the treatment of dental biofilm. Therefore, the limitation was that we compared it with chlorhexidine alone.

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