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Naringenins: The Genesis of Novel Defenders –Effect Of Naringenins on the Growth of Streptococcus Mutans

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Abstract
Objective: Dental caries is an infectious bacterial disease that has plagued human beings for centuries. Streptococcus mutans has been recognized to play a major role in causation of dental caries. Various anti-Microbial agents that act against Streptococcus mutans(S.mutans) have been developed but most of them have other side effects. Thus new biomaterials with lower cost and higher biocompatibility should be explored to fight Streptococcus mutans. Naringenins from citrus plants such as grapefruits are found to be effective against pathogenic bacteria such as Listeria monocytogenes, Escherichia coli, and Staphylococcus aureus.The aim of this study was to evaluate the effect of naringenins on the growth of Streptococcus mutans
METHODOLOGY: The Minimum inhibitory concentration (MIC) of naringenin against Streptococcus mutans was determined using standard broth dilution method.A twenty four hour growth curve assay was then performed.
Results: Naringenin showed an inhibitory effect on the growth of Streptococcus mutans and the Minimum inhibitory Concentration was determined to be between 160 and 320 μg/mL of naringenin. One way Anova followed by post hoc test showed that both Group 1(p-value 0.000738<0.05) and Group 2 (p-value 0.000462) showed a statistically significant difference when compared with the Control Group. No statistically significant difference was found between Group 1 and Group 2 (p-value 0.974793>0.05)
Conclusion: The antibacterial effect of Naringenins against Streptococococcus mutans in this study makes it an alternative agent in the management of dental caries.

Keywords: *Naringenin, Streptococcus mutans, antibacterial effect, Dental caries.*

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I. Introduction:

Human beings have been plagued by dental caries for centuries. Dental caries is a multifactorial disease of bacterial origin,that is characterized by the localized destruction of dental hard tissues. *Streptococcus mutans* plays a cardinal role in causation of dental caries[1] .They are strong acid producers and hence cause an acidic environment concocting the risk for cavities. These bacteria are able to rapidly metabolize dietary sugars to acid, creating a low pH locally.

Several anti-microbial agents, such as chlorhexidine[2], metal nanoparticles[3], quaternary ammonium compounds[4] , and anti-microbial peptides[5] , have been developed to fight against dental caries. Various natural products such as Propolis [6] and Chitosan[7] have also been shown to be effective against dental caries. However, these are associated with various side effects. Chlorhexidine has been known to cause alteration in taste, burning sensation, staining of teeth and restoration and rarely causes oral mucosal desquamation and parotid swelling [8]. Quaternary ammonium compounds (QACs) may be a potential key driver in the emergence of antimicrobial resistance [4]. They are generally referred to as 'hard antibacterial agents' because they are poorly metabolised and are excreted primarily in a non-metabolised form [9].

The major disadvantage of metal nano particles, is that they do not link chemically to the matrix made up of polymer and can leach out eventually along with other elutable materials, leading to health risks .The

silver element can be ionized in the presence of water, tissue exudates or body fluids and can readily interact with amino acid residues, proteins, especially those with thiol groups, free anions and receptors on mammalian and eukaryotic cell membranes [10]. AMPs are sensitive to the physiological salt concentration or the ionic strength, pH, and proteolytic activity in body fluids[11].

Thus novel biomaterials with lower costs and higher biocompatibility should be explored to fight against *Streptococcus mutans w*ho is the major cariogenic bacteria[1].More naturally derived plant extracts have recently gained great attention. Naringenins are predominant flavanones from citrus plants such as grapefruits, oranges and tomatoes .They have been found to have a bioactive effect on human health and have been known to exhibit many pharmacological properties such as anti-oxidant[12], anti dyslipedemic[13], anti-diabetic[14] and anti-inflammatory[15] actions. They are also found to be effective against various pathogenic bacteria such as *Listeria monocytogenes*, *Escherichia coli and Staphylococcus aureu*s [16].The inhibitory effect of naringenin on the growth of *Streptococcus mutans* has not been widely studied. Therefore, the aim of this study is to evaluate the antibacterial effect of naringenins on streptococcus mutans at two different concentrations by using growth curve.

II. Materials And Methods

S. mutans(MTCC NO :497) was purchased from Microbial Type Culture Collection and Gene Bank (MTCC) CSIR,Chandigarh and was cultured for 24 h at 37 °C in Brain Heart Infusion (BHI) broth. Naringenin (N5893) was purchased (Sigma-Aldrich) (Figure 1) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL.

The Minimum inhibitory concentration (MIC) was determined according to the instructions of a standard susceptibility broth dilution technique (National Committee of Clinical Laboratory Standards 1993). Briefly, a recovered culture of *S. mutans* was diluted into a 96-well plate using fresh BHI at 10^4 colony forming units(CFU)/mL (Figure 2). In order to obtain the MIC of naringenin for potential clinical guidance, a series of gradient concentration (3.1, 6.2, 12.5, 25, 50, 100, 200, 400, and 800 μg/mL) of naringenin solution were then prepared. Bacterial suspension with BHI served as control**.** After anaerobic incubation at 37 °C for 24 h, a suspension from each well was inoculated on BHI-agar plates.

MIC was defined as the lowest concentration of naringenin that totally inhibited visible bacterial growth but did not completely kill the bacteria. MIC was determined in triplicate, and each concentration was tested in three wells.

For carrying out the growth curve assay, bacterial culture was prepared as described above. Bacterial suspension with BHI served as control, naringenin was added to make final concentrations of 160 and 320μg/mL.

Group 1 – 160 μg/mL of naringenin added to Bacterial suspension
Group 2 – 320 μg/mL of naringenin added to Bacterial suspension
Group 3(Control Group) – Bacterial suspension in BHI broth.

Then the cultures were incubated at 37 ^{o} C for 24 hours. Absorbance value was read at 600nm every 4 hours starting at 0 hour using Microplate Reader (Thermo Scientific Varioskan LUX) (Figure 3). Finally, Growth Curve assay were performed in triplicate and each tested on 3 wells.

STATISTICAL ANALYSIS

The statistical analysis was performed using SPSS v 24.A confidence level of 95% was set. One way ANOVA ,followed by post hoc Tukey's test was performed. A *P*-value of <0.05 was considered as statistically significant.

III. Results

The antibacterial effect of naringenin against *Streptococcus mutans* was assessed using broth dilution method. Naringenin showed an inhibitory effect on the growth of *Streptococcus mutans* and the Minimum inhibitory Concentration was determined to be between 160 and 320 μg/mL of naringenin (Figure 4). The *S. mutans* growth curves showed that both 160 and 320μg/mL of naringenin could obviously inhibit growth of *S. mutans* (Figure 5).

One way Anova followed by post hoc test showed that both Group 1 and Group 2 had a statistically significant difference when compared with the Control Group. No statistically significant difference was found between Group 1 and Group 2 (Table 1).

IV. Discussion

In recent years, the anti-bacterial activities of plant extracts have gained great attention. Natural grapefruit juice (Citrus paradisi) contains several flavonoid glycosides. Flavonoids are a group of pigments contained in plants which are responsible for flower and fruit colouration. According to their molecular structures, they are divided into six classes: flavones, flavanones, flavonols, isoflavones, anthocyanidins and flavanols (or catechins). More than 60 types of flavonoids can be identified in Citrus fruits belonging to five different classes. Citrus flavanones may be present in glycoside or aglycone forms. Naringin (4', 5, 7-trihydroxyflavanone 7-rhamno-glucoside), the so-called 'bitter principal' of grapefruit, is the most abundant of these compounds and is known to have a bioactive effect on human health. Naringenin and hesperetin are the most important flavanones among the aglycone forms. Flavanones are frequently present in their diglycoside form, which confers the typical taste to Citrus fruits.

Naringenins posses a skeleton structure of flavanone having three hydroxy groups attached to the 4', 5, and 7 carbons. It may be found either in its aglycol form which is the naringenin, or in its glycosidic form that is naringin, which has an additional disaccharide neohesperidose attached by means of a glycosidic linkage at carbon 7.

Naringenin has been reported to have many pharmacological properties, including anti-dyslipidemic, anti-obesity [17] and anti-diabetic and antifibrotic[18]. It is also found to be a free radical scavenger [19] and an immune system modulator [20]. These low molecular weight substances have high pharmacological potency and low cytotoxicity which make them viable alternatives to conventional therapeutic drugs. They have also shown to possess antibacterial properties against *Staphylococcus aureus, Listeria monocytogenes and Escherichia coli.* Tsuchiya et al. studied the antibacterial activity of phytochemical flavanones against 18 methicillin-resistant *S. aureus* strains and found that Naringenins had antimicrobial activity at concentrations ranging between 0.75 and 1.5 mmol/L. In addition, aliphatic groups at the 6- or 8-position (A flavonoid ring) enhanced the flavanone antibacterial activity [21].

Thus the antibacterial activity of naringenin against *Streptococcus mutans* which is the cariogenic bacteria was assessed in this study. Naringenin showed a satisfactory anti-microbial effect and the MIC was found to be between 160 and 320 g/mL . The S. mutans growth curves showed that both 160 and 320 μg/mL of naringenin could obviously inhibit S. mutans growth. The results of this study are similar to those obtained in study conducted by Yue J et al, in which naringenins inhibited *Streptococuus mutans* growth with MIC ranging between 100 and 200 μg/mL[22].

Generally, antibacterial material exerts their effects by targeting the cytoplasmic membrane and intracellular biomacromolecules such as enzymes and nucleic acids of susceptible bacteria. The interaction of antimicrobial agents with DNA may affect cell function by interfering gene expression and protein synthesis, thereby playing an important role in antibacterial action. On the other hand, because of the interfacial position between the extra-cellular medium and the cytosol, cell membrane is often the primary or first target for antibacterial substances.

Previous studies have shown that the antibacterial activity of naringenin may be due to bacterial cytoplasmic membrane damage [23]. Wang et al investigated the antimicrobial mechanism of naringenin, against food borne *Staphylococcus aureus* American Type Culture Collection (ATCC) 6538. Gas chromatography−mass spectrometry (GC−MS) and fluorescence analysis showed that relatively low concentrations of naringenin cause changes in membrane fatty acid composition and conformation of membrane proteins.Exposure to naringenin at higher levels increased the membrane permeability significantly and even evokes the leakage of intracellular substances with a loss of membrane integrity and significant cell morphological changes of *S. aureus* cells [23].

Mandalari et al compared antimicrobial activity of Naringenin, hesperetin and eriodictyol and their Naringin, neohesperidin and neoeriocitrin di-glycosides against Listeria innocua and *S. aureus* FI10139, among the other strains. Their results suggest that if there exists a difference between aglycones and di-glycosides, aglycones would be more active [24]. Han and You found that the aglycone Naringenin was more effective than Naringin at inhibiting Gram positive bacteria. This would indicate that the attachment of hydrophilic substituents to the 7-O-aglycone would diminish the interaction between the aglycone and the target strain [25]. This is almost certainly true because of the lack of affinity for the phospholipid bi-layer or specific receptors on the cell membrane to initiate cell damage.

Additional evidence has shown that naringenin might target directly to nucleic acids and change the functions of DNA [23]. In the study conducted by Wang et al, along with Multivariate Curve Resolution-Alternating Least (MCR−ALS) analysis, the genomic DNA-binding of naringenin was also quantitatively monitored using Uv-vis spectra and the concentration and pure spectra profiles for the three reaction species (DNA, naringenin and DNA−naringenin) were obtained. In addition, thermal behavior of DNA and docking studies were also performed which discovered that naringenin preferentially bound to the A−T base pairs region

of genomic DNA via groove mode. Mild secondary structural and obvious morphological variations of this biomacromolecule were induced by naringenin as shown by atomic force microscopy and circular dichroism studies. According to these results, it may be suggested that naringenin exerts its antibacterial effects through disruption of cytoplasmic membrane and its DNA targeting effects against *Staphylococcus aureus*[23].

V. Conclusion

The antibacterial effect of Naringenins against *Streptococococcus mutans* in this study makes it an alternative agent in the management of dental caries. Further studies are however required for future use in clinical application.

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ABBREVIATIONS

Minimum inhibitory concentration –MIC
Streptococcus mutans – S.mutans
Microbial Type Culture Collection and Gene Bank - MTCC
Brain Heart Infusion - BHI
Dimethyl sulfoxide - DMSO
Colony forming units – CFU
Quaternary ammonium compounds - QACs
American Type Culture Collection - ATCC

Gas chromatography−mass spectrometry - GC−MS

Table 1: Post Hoc Tukey's Test

(I) Group		Mean Difference (I-J)	Std. Error	Sig.
GROUP 1	320µg/ml	0.023429	0.10879	0.974793
	Control	-0.49171	0.10879	0.000738
GROUP 2	160µg/ml	-0.02343	0.10879	0.974793
	Control	-0.51514	0.10879	0.000462
GROUP 3	160µg/ml	0.491714	0.10879	0.000738
	320µg/ml	0.515143	0.10879	0.000462

*The mean difference is significant at the 0.05 level.

Figure legends:
Figure 1: Naringenin (N5893) purchased from Sigma-Aldrich)
Figure 2: Broth Microdilution Method.
Figure 3: Absorbance value read at 600nm using Microplate Reader (Thermo Scientific Varioskan LUX)
Figure 4: Minimum Inhibitory concentration of Naringenin
Figure 5: *S. mutans* growth curves of the control and naringenin (160 and 320 µg/mL) groups.

Figure 1: Naringenin (N5893) purchased from Sigma-Aldrich)

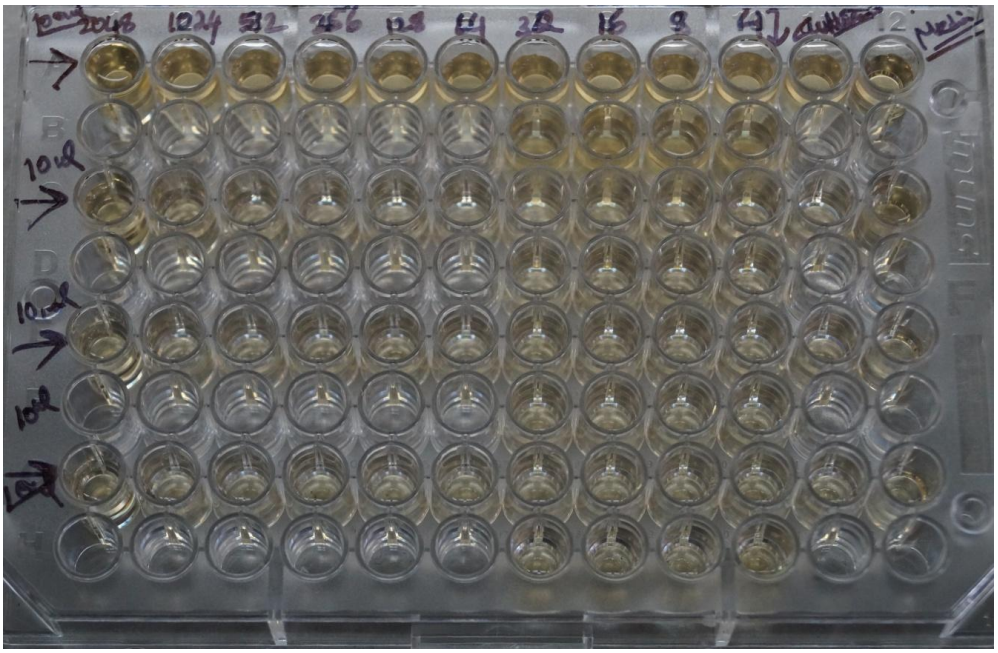

Figure 2: Broth Microdilution Method.

Figure 3: Absorbance value read at 600nm using Microplate Reader (Thermo Scientific Varioskan LUX)

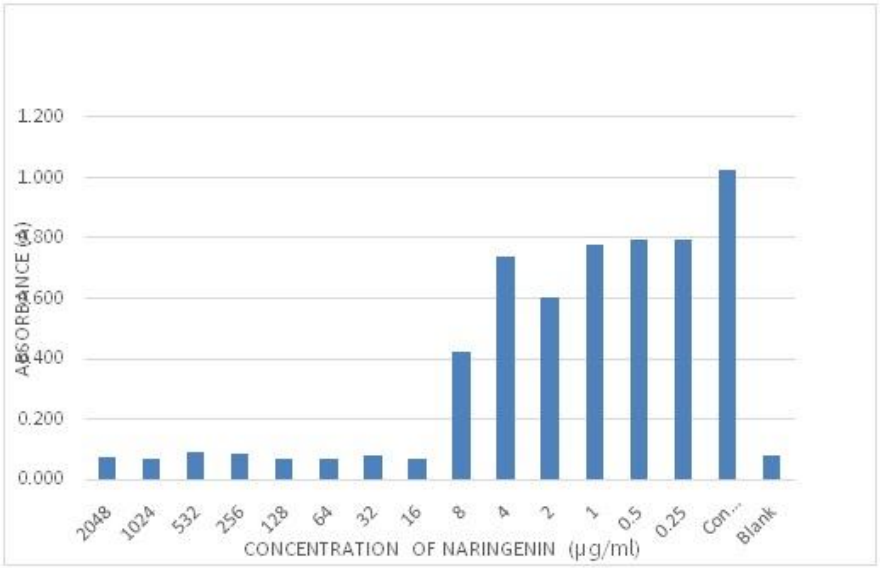

Figure 4: Minimum Inhibitory concentration of Naringenin

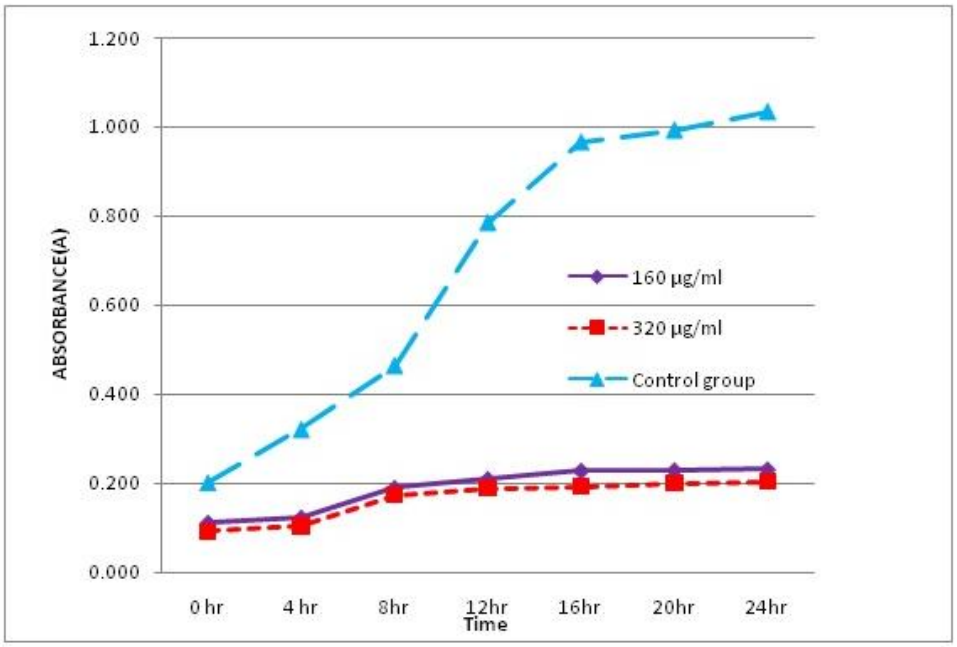

Figure 5: ***S. mutans*** **growth curves of the control and naringenin (160 and 320 µg/mL) groups.**

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