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Interference of naringin, a bioactive compound characterized from the methanolic leaf extract of *Citrus maxima*, with the biofilm-forming property of cariogenic bacterium *Streptococcus mutans*

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Abstract
Microbial adhesion on the tooth surface is the initiating factor for plaque formation. The majority of the early plaque flora is constituted by different *Streptococci sp*. In this study, we have reported the antimicrobial and antibiofilm activity of naringin against the cariogenic bacterium *Streptococcus mutans* MTCC 497. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the identified bioactive compound naringin were determined using a micro-dilution assay. The antibiofilm activity was screened at sublethal concentrations of naringin (75%, 50%, and 25 % of MBC) using the microtiter plate method. Further, the cytotoxicity of naringin and chlorhexidine mouth wash was tested on L132, normal human epithelial cell lines using a phase-contrast inverted microscope and MTT assay. Our results revealed, bioactive compound naringin exhibited a significant antimicrobial activity with MIC 12.5mg/ml, MBC 6.25mg/ml, and IC50 value 4.563mg/ml. There was more than an 85% reduction in the biofilm formation at 75% sub-MBC level Concentration. Naringin-treated cells, showed no signs of deformity even at the highest tested concentration while there was evidence of cell toxicity in the cells treated with the lowest concentration of chlorhexidine mouth wash. LC50 value of naringin was found to be 174.884 mg/ml indicating it to be safe. The findings of this study endorse that naringin has the strong ability to interfere *In vitro* with the biofilm formation of *Streptococcus mutans*, the primary etiological agent of dental caries. It also showed the least toxicity in comparison to chlorhexidine mouth wash, the choice of drug used to control dental plaque.

Keywords: naringin, antimicrobial, antibiofilm, dental plaque, *Streptococcus mutans*

Introduction
The microbes exist in two forms namely planktonic and sessile. Planktonic cells enter the sessile phase by attaching to a biotic or abiotic surface to form a biofilm. A biofilm is a complex habitat composed of not just one, but a divergent range of microorganisms living enclosed in a hostile environment, composed of a matrix of polysaccharides, proteins, lipids, nucleic acids, and other chemical materials [1]. This association is a reversible one where the planktonic cells can get detached to form a new biofilm by attaching themselves to a new surface. Dental plaque is an example of a biofilm. Once the dental plaque is formed, the cells start metabolizing the food particles by producing acids that destruct the tooth enamel leading to dental caries and gingivitis [2]. It is very difficult to combat this army of cells with static and cidal agents due to various factors like the hindrance offered by the thick matrix for the permeability of the chemicals, efficient multidrug efflux pump, horizontal gene transfer, and secretion of antibiotic modifying enzymes, and the quorum sensing defense. Due to this extreme resistance, the dental biofilms are often treated with high doses of antibiotic agents or disrupted by a physical intervention that damages the tooth surface [3]. This concern has forced investigators to find an alternative antibiofilm agent with minimal side effects. The development of novel therapeutic measures to control the biofilm formation rather than killing the pathogen using plant-based products appears to be a promising approach. In our study, we have used naringin separated and identified from the methanolic leaf extract of *Citrus maxima*, [29] to screen its antibiofilm activity against *Streptococcus mutans*, which is the primary etiological agent of dental caries. Since flavonoids are natural agents, they are safer in comparison to chemical agents and also have an upper edge over antibiotics as they have multiple approaches to killing the cell, making it hard for the bacteria to develop resistance against them [4]. Naringin is a flavanone glycoside, found in citrus fruits, grapefruits, cherries, beans, cocoa, oregano, and tomatoes, responsible for their bitter taste in them. It is produced from the flavanone naringenin and disaccharide neohesperidose [5]. Naringin and its derivatives have reported antimicrobial activity against pathogenic bacteria. [6, 7] It also exhibits strong anti-inflammatory and antioxidant activity [8], naringin and naringenin in combination have exhibited anti-cancerous activity [9] and are also known to have anti-quorum and antibiofilm activity [10, 11, 12]. The objective of the present study was to study the antimicrobial activity of flavonoid naringin, to screen its inhibitory influence on biofilm formation of *Streptococcus mutans* MTCC 497, and to evaluate its toxicity in comparison to chlorhexidine mouthwash.

Materials and Methods

Bacterial culture

The bacterium used in this study was *Streptococcus mutans* MTCC 497, which was kindly provided by Maharani Lakshmi Ammanni college for women, Bengaluru, Karnataka, India. It was cultured in brain heart infusion broth (BHB) followed by Brain heart infusion agar (BHA) with 0.1% sucrose at 37 ℃ in a 5% CO_2 enriched condition. All the media components used were purchased from HiMedia Laboratories Pvt Ltd.

Plant materials and preparation of the naringin

The bioactive compound naringin was isolated, purified, and characterized from the methanolic leaf extract of *Citrus maxima* from our previous study as described by Apoorva *et al.,* (2020) [30], The compound was dissolved in 1% Dimethyl sulfoxide at a concentration of 25mg/ml.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration and minimum bactericidal concentration of the natural compound naringin were evaluated against the test organism *S. mutans* MTCC 476 using broth microdilution assay, following the procedure described by Oliveira *et al.,* (2006) and Lima *et al.,* (2012). 25mg/ml of naringin was dissolved in 1% DMSO, which was serially diluted in twofold dilution. 20µl of BHIB inoculated with the test bacteria approximately containing 1×10^8 CFC/ml were added to the wells containing antimicrobial agent at a final volume of 100µl/ well ranging from 25- 0.04mg/ml. The microplate was incubated at 37 ℃ and 5% CO_2 for 24h. The bacterial growth was measured by absorbance at OD_{570} nm using, Thermo scientific MULTISCAN GO Micro ELISA auto reader. Gentamycin was used as a positive control at 25mg/ml and 1% DMSO as the negative control. The lowest concentration of the naringin which restricted the visible growth of the test bacteria was considered as the MIC. The samples were assayed in triplicates and repeated twice The MBC of the naringin was determined by streaking a loopful of each concentration ≥ MIC on the BHA medium. The plate was incubated at 37℃ and 5% CO_2 for 24 to 48 h. MBC was defined as the lowest concentration of the bioactive compound showing no bacterial growth.

Antibiofilm assay

Inhibitory activity of naringin on the biofilm-forming property of *S. mutans* MTCC 497 was screened at sublethal concentrations of 75%, 50%, and 25 % of MBC, using 96 - well microtiter plate method by following the methodology used by Antunes *et al.,* (2010). 300µl of trypticase soya yeast broth (TSY) inoculated with the test organism at 10^6 CFU/ml concentration was aliquoted into each well of the microplate. Chlorohexidine mouthwash at 0.2% concentration was used as a positive control and inoculated medium without the bioactive compound was used as the negative control. The plate was incubated at 37℃ and 5% CO_2 for 24 to 48 h. At the end of incubation, the supernatant was carefully removed and each well was washed thoroughly with sterile distilled water to remove free-floating cells. The plate was then air-dried for 30min, the adhering biofilm was stained using a 0.1% aqueous solution of crystal violet for 15 min at room temperature. Following incubation, the excess stain was removed by washing the plate gently with sterile distilled water. The bound dye to the cells was solubilized by the addition of 250µl of 95% ethanol. After 15min of incubation, absorbance was measured at 570nm using a Thermo scientific MULTISCAN GO Micro ELISA auto reader. The percentage of biofilm inhibition was calculated using the formula

$$\text{Percentage of inhibition } \% = \frac{\text{Control } OD_{570\,nm} - \text{Treated } OD_{570\,nm}}{\text{Control } OD_{570nm}} \times 100$$

Cytotoxicity ASSAY

Cell lines and culture condition

The Normal, Human epithelial cell line, L132 was initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained in Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in a 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and an antibiotic solution containing: Penicillin (100µg/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator.

Cytotoxicity evaluation

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in a 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator. After 24 hours the growth medium was removed, freshly prepared naringin and chlorhexidine mouthwash in DMEM were five times serially diluted by two-fold dilution (100µL, 50µL, 25µL, 12.5µL, 6.25µL in 500µl of DMEM) and each concentration of 100µl was added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non-treated control cells were also maintained.

Cytotoxicity assay by direct microscopic observation

The entire plate was observed after 24 hours of treatment in an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. [13] Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity assay by MTT method

15mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of the incubation period, the sample content in wells was removed and 30µl of reconstituted MTT solution was added to all test and control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After incubation, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide: DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down to solubilize the formazan crystals. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm [14].

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} \times 100$$

Statistical analysis

All the results were represented as mean ± standard deviation of at least two independent experiments performed in triplicates. Statistical analysis was performed by using student t-test, ANOVA models, differences were accepted as statistically significant at $P < 0.05$.

Results

The alarming rate of increased resistance against conventional antibiotic treatment, across the globe, has forced our researchers to find substitutes for antibiotics, to avoid the dissemination of deadly resistant microbes. Hence in this study, we are focusing on using phytochemicals as one of the alternative natural sources to control dental plaque formation. Naringin isolated and identified by us, from the methanolic leaf extract of *Citrus maxima* [29] was used to screen its antimicrobial and antibiofilm activity against *S. mutans* MTCC 497.

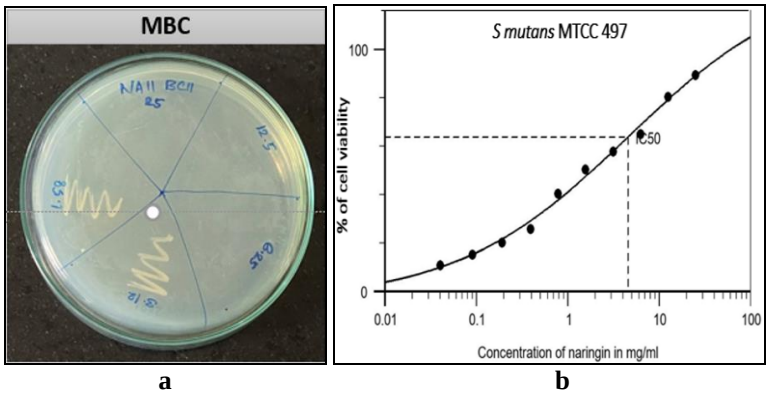
Minimum inhibitory concentration and minimum bactericidal concentration

The mean absorbance value recorded at 570nm is as tabulated in Table no. 1. Naringin was observed to inhibit the growth of the test organism from 6.25mg/ml hence, the minimum inhibitory concentration of naringin that restricted the visible growth of the tested organism was found to be 12mg/ml. From Fig.1, the lowest dilution that showed no visible growth when streaked onto BHA agar plates, post-incubation was 6.25mg/ml and is considered as the MBC of naringin against *S. mutans* MTCC 497. To calculate the IC50 value the percentage of cell viability was calculated and plotted against the concentration of naringin using an online IC50 calculator AAT Bioquest. The concentration of naringin that inhibits the growth of half of an inoculum (IC50) of the tested organism was calculated to be 4.563mg/ml.

Table 1: MIC of naringin against *S. mutans* MTCC 497.

Organism tested	Mean OD at 570nm											
	25 mg/ml	**12.5 mg/ml**	**6.25 mg/ml**	**3.12 mg/ml**	**1.56 mg/ml**	**0.78 mg/ml**	**0.39 mg/ml**	**0.19 mg/ml**	**0.09 mg/ml**	**0.04 mg/ml**	**+ control**	**- control**
S. mutans MTCC497	0.0476 ± 0.006	0.0777± 0.004	0.1806± 0.014	0.3053± 0.004	0.315± 0.004	1.3545± 0.006	2.5024± 0.070	2.604 ± 0.008	2.814± 0.067	2.9131± 0.087	0.025 ± 0.004	2.9688 ± 0.136

Mean ± Standard deviation



a **b**

Fig 1: a- BHA agar plate streaked with different dilutions of naringin. b- Graph depicting the IC50 value of naringin for *S. mutans* MTCC 497.

Antibiofilm assay

The effect of naringin on the plaque-forming ability of *S. mutans* MTCC 497 was evaluated at sub-MBC concentrations of 75%, 50%, and 25%, and its effect is shown in Fig 2. At 75 % of MBC value, naringin was efficiently inhibiting the adhesive property of the tested organism.

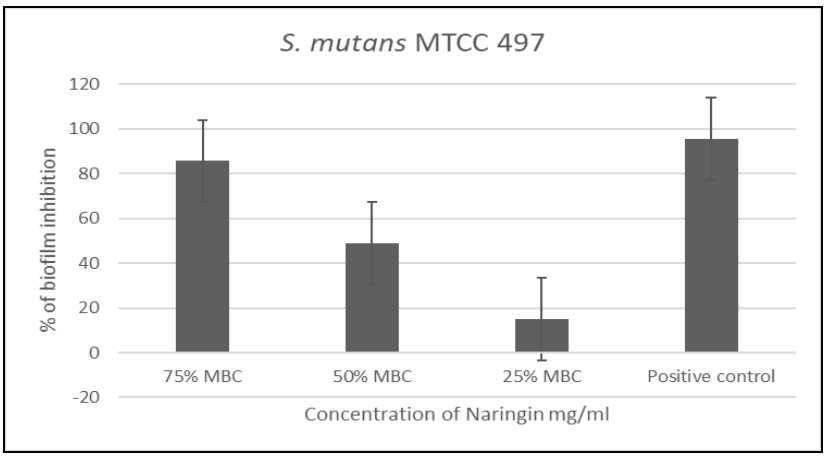
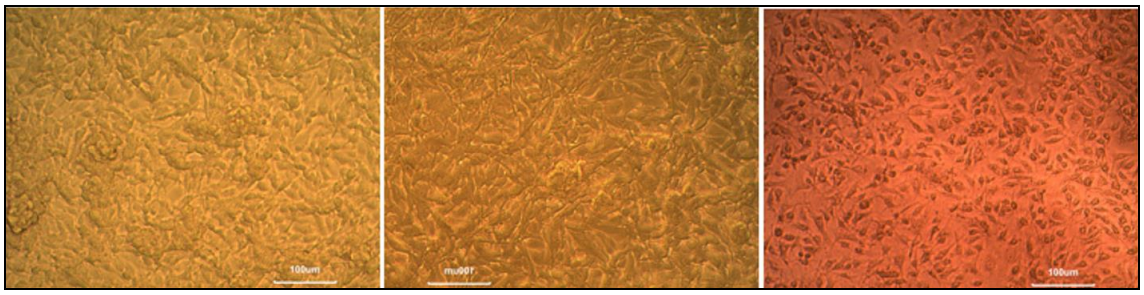


Fig 2: Percentage of biofilm inhibition by chlorohexidine mouthwash used as positive control and naringin at sub-lethal MBC concentrations of 75%, 50%, 25%.

Cytotoxicity assay by direct microscopic observation



a b c

Fig 3: Morphological changes observed in cells after 24 hours of exposure, under phase contrast inverted microscope of a. Naringin at 100mg/ml concentration b. Untreated control c. Chlorohexidine mouthwash at 6.25mg/ml concentration.

Cytotoxicity assay by MTT method

The cytotoxic effect of naringin and chlorhexidine mouth wash in a concentration ranging from 6.25 to 100 mg/ml was studied on the human embryonic lung epithelial cell line, L132 using an MTT assay. The cytotoxic effect was observed on the cells after 24 hours of exposure to the testing agents. The percentage of cell viability was calculated by measuring the absorbance of pink color formazan formed from the reduction of MTT solution. From Fig. 4, at the lowest concentration, naringin showed more than 90% of cell viability while chlorohexidine mouth wash showed just 31.32 %. The LC_{50} for both the testing agents was calculated after 24 hours of exposure using ED50 PLUS V1.0 Software. LC_{50} indicates the lowest concentration of plant extracts that inhibits 50% of cells. Naringin showed a high LC50 value of 174.884mg/ml while chlorohexidine mouthwash's, LC50 was well below the value of the lowest concentration tested for its toxicity.

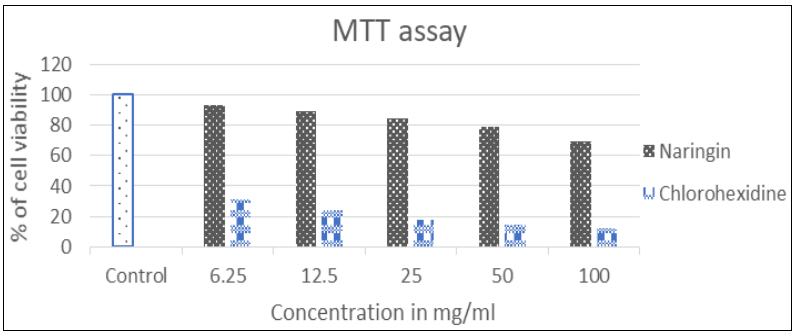


Fig 4: Cytotoxic effect of naringin and chlorohexidine mouthwash against L132 cell line after 24 hours of exposure using MTTC assay, at the concentration ranging from 6.25- 100mg/ml.

Discussion

Considering the constantly increasing ineffectiveness of antibiotics, the interest in natural products has increased [16]. We are in the current marketing situation where the products with any plant-based formulation are more widely accepted than purely chemical ones. In this study, we are focused on screening natural products which can be a great alternative to chemical-based oral hygiene products. As oral health is the reflection of our total body health, there is certainly an urgent need to promote preventive measures as the treatment of any dental-oriented problem is very expensive and is an unrealistic option especially post covid. [17] Microbial pathogenesis depends on several factors which are responsible to initiate and continue the infection in the host. In the case of dental plaque, the primary important factor to initiate infection is to adhere to the tooth surface. In our study, we are interested in preventing the adhesion of the cells to the tooth surface using the natural plant bioactive compound, rather than killing and disturbing the flora of the oral cavity and digestive tract. The flavonoid, naringin, used in the current study was separated and identified from the methanolic leaf extract of *Citrus maxima* [29] Naringin was screened for its antimicrobial, antibiofilm property against the cariogenic bacteria *S. mutans* MTCC 497. *S. mutans* is an acidogenic bacterium that adheres to the tooth surface and grows to release various acids such as lactic acid, formic acid, butyric acid during carbohydrate metabolism. These organic acids demineralize the tooth surface and cause dental caries. [18] In the present investigation, naringin was reported to be an antimicrobial agent with a MIC of 12mg/ml and MBC at 6.25mg/ml against *S. mutans.* Naringin has reported a wide range of therapeutic properties ranging from antioxidant, anti-inflammatory, anti-apoptotic, anti-ulcer, anti-osteoporotic, and anti-carcinogenic properties in an extensive review by Rui *et al.,* (2016). It also enhances the bioavailability of diltiazem and paclitaxel during their oral exposure. [20] Celiz *et al.,* (2011), has reported antimicrobial activity of the flavonoid naringin against pathogenic bacteria such *as L. monocytogenes, E. coli,* and *S. aureus* thus proving its inhibitory effect against both gram-positive and gram-negative bacteria. Micro-titre plate assay was employed to detect the antibiofilm property of naringin at sub-lethal concentrations of 75%, 50%, and 25%. Chlorohexidine mouthwash was used as a positive control in this test as it is the prime component of current mouthwashes used for controlling and preventing dental plaque. At a concentration of 75% of the MBC value, naringin was found to prevent the test organism from adhering by 85.63% which is a significant reduction in the biofilm formation in comparison to the chlorohexidine mouthwash standard which inhibited the biofilm formation by 90.53%. This suggests that naringin may interfere with the proteins involved in adhesion. [25] Naringenin the precursor of naringin has previously reported antibiofilm activity against *E. coli, V. harveyi, S. aureus,* and *P. aeruginosa* [21, 22]. Melanoidins [23], embelin and piperine [24], guaijaverin [25], are some of the reported plant bioactive compounds with anti-biofilm forming properties against *S. mutans*. Not all the bioactive compounds from plants are safe to consume or for topical application. This concern for safety makes it critical in the development of novel drugs [26]. In line with the above statement, the cytotoxicity of naringin and chlorhexidine mouthwash was tested on normal human embryonic lung epithelial cell line, L132, using a phase-contrast inverted microscope and by MTT assay. Fig. 2 a reveals that naringin showed no structural changes in the morphology of the cells after 24 hours in comparison to the untreated control cells while the antiplaque chemical agent chlorohexidine mouthwash, treated cells showed severe structural deformities like shrinking and membrane blebbing even at the lowest concentration of 6.25mg/ml. The MTT assay also revealed the high toxicity of chlorhexidine mouthwash with just 31.32% of cell viability post 24 hours of exposure at the lowest concentration while naringin showed more than 90 % cell viability, naringin also proved less toxic at the highest concentration of 100mg/ml with more than 69 % cell viability. In reference to the research article by Nemudzivhadi *et al.,* (2014) [28], low LC_{50} values represent high toxicity, extracts with high LC_{50} are preferable to work with, because of their lower toxicity effects on the host cells. Naringin was reported with LD50 of 174.884mg/ml while chlorohexidine mouthwash's LC50 was well below the value of the lowest concentration tested for its toxicity. Thus, the findings of our study strongly suggest that chlorhexidine mouthwash is toxic even at the lowest concentrations than that used in clinical practices. Coelho *et al.,* (2020) [29], also reports the cytotoxic effects of chlorhexidine on human gingival fibroblasts and suggest limiting its use as an antiseptic and in post-operative situations. Chlorhexidine mouthwashes have also reported several side effects like staining the teeth, tongue, alteration in taste, and an increase in tartar formation on teeth. Severe effects like swollen glands near the neck and mouth irritations are also reported [27]. The results presented herein illustrate the potential of the secondary metabolite, naringin as an antibacterial agent, and its ability to selectively inhibit the biofilm formation in *S. mutans.* The cytotoxicity studies further stress the toxic effect of chlorhexidine mouthwash and the need for better treatment to treat and prevent dental plaque.

Conclusion

The present findings show that naringin can interfere *in vitro* in the crucial steps of dental plaque formation. With further research on the mechanism of action and *in vivo* studies on suitable models, naringin can be considered as an alternative to the toxic chlorohexidine mouthwash in preventing dental plaque.

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