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Original Article

Efficacy of tannic acid and tannic acid-fluoride on *Streptococcus mutans* and gingival fibroblast cell viability

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Abstract

The objective of this study was to investigate the antibacterial activity of tannic acid (TA) and tannic acid-fluoride (TF) against *Streptococcus mutans (S. mutans)* and gingival fibroblast viability. Activity of TA and TF on minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) against *S. mutans*, glucosyltransferase enzymes (GTFs), acid production, biofilm formation and cell viability were determined. TA and TF MIC values against *S. mutans* were 0.05% TA and 75 ppm fluoride + 0.025% TA, respectively. At 1/2 MIC of TA and 1/4 MIC of TF, there was significant inhibition of both biofilm formation and GTFs activity on *S. mutans* without any toxicity to gingival fibroblasts. However, neither TA nor TF had any effect either on *S. mutans* growth or acid production. Therefore, the combination of TA and F acted synergistically to inhibit biofilm formation and GTFs activity of *S. mutans* without any toxicity to gingival fibroblasts.

Keywords: tannic acid, tannic acid-fluoride, fluoride, *Streptococcus mutans*, gingival fibroblasts

1. Introduction

Dental plaque, also known as dental biofilm, is a group of bacteria held together by a gel-like intermicrobial matrix on the tooth's surface. Plaque contains a large number of different types of bacteria (Zijnge *et al.*,2010). The major causative bacteria for dental caries are *Streptococcus mutans (S. mutans), S. sobrinus* and *L. casei* based on the enzyme glucosyltransferase (GTFs). This GTFs plays an important role in the process of breaking down the bond between glucose and fructose molecules of sucrose (Wang, Wang, Lei, Hao, & Jiang, 2022). The glucose, which is broken down from sucrose, can combine into two types of polymers: soluble and insoluble glucans. Water soluble glucans such as dextrans are composed of alpha-1,6 bonds as the core, and less often alpha-1, 2, alpha-1, 3 and alpha-1, 4 branches are found in colonies of *S. mutans*, as dextran is readily degraded by oral bacterial dextranase. Therefore, it is believed that dextrans act as a reservoir for bacteria rather than playing a role in adhesion. The insoluble glucans such as mutan, which consists of the alpha-1, 3 bonds, are axial and branched. The formed alpha-1, 4 and alpha-1, 6 mutans play an important role in the process of bacterial adhesion to enamel and biofilm development (Ham *et al.*, 2022).

Numerous studies have been conducted on several food compounds that can inhibit the enzyme GTFs. One of these previous studies showed that nutrients in beverages and some fruits, for example, tea, cocoa, grapes and cherries, have polyphenol compounds that can inhibit GTFs of the *S. mutans* strain 6715 (Kashket, Zhang, & Van Houte, 1996). The effects of polyphenols on insoluble glucan formation, *S. mutans* JC-2 adhesion to saliva–coated hydroxyapatite discs (S-HA), and caries reduction, were reported in rats that were infected with *S. mutans* JC-2 (Komori *et al.*, 1993). Moreover, green tea polyphenols can inhibit the formation of insoluble glucans, *S. mutans* JC-12 adhesion on S-HA discs, and reduce caries incidence in a S-infected animal study (Otake, Makimura, Kuroki, Nishihara, & Hirasawa, 1991). Normally, proline-rich

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proteins in saliva are involved in bacterial adhesion to the salivary pellicle, hence it is possible that green tea polyphenols, mainly tannic acid (TA), can bind to the salivary glands, resulting in an inhibitory effect on S-HA adhesion (Lei *et al.*, 2021).

Furthermore, a previous study by Lei *et al.* reported that oolong tea extract can inhibit the GTFs enzyme from *S. sobrinus* 6715 (Lei *et al.*, 2021). Most of that tea extract was polymeric polyphenols, which were able to inhibit GTFs from *S. sobrinus* 6715, resulting in a statistically significant decrease in glucan production, and the GTFs were inhibited by oolong tea extract and green tea extract (G. F. Ferrazzano, Amato, Ingenito, De Natale, & Pollio, 2009). A comparative study was made of extracts from Chinese black tea (*Camellia sinensis*) and herbs in Thailand, namely *Andrographis paniculata*, *Cassia alata*, *guava*, *mane*, and *stingray* on the inhibition of *S. mutans* ATCC 25175 adhesion (Limsong, Benjavongkulchai, & Kuvatanasuchati, 2004; Yabuta *et al.*, 2018).

A study on the effects of tea constituents on the dissolution of human tooth enamel when exposed to acids in the laboratory (H. Yu, Oho, & Xu, 1995) found that the components of tea substances such as TA, catechin, and caffeine reduced the dissolution of enamel when exposed to acids, and the acid resistance performance was enhanced when these agents were combined with fluoride. The effect of promoting acid resistance may be due to their chemical structures (Zhang, Ma, Wang, Wu, & Zou, 2021). Thus, in the dental erosion resistance process, it is possible that the tea's organic compounds act as bridge bonds to calcium, phosphate and organic substances in the enamel. When used in combination with fluoride, it results in the formation of an insoluble substance on the enamel, which provides acid resistance. However, there is no evidence about TA combined with fluoride (TF) influencing dental caries pathogenesis.

From the above information, TA are the key compounds in plant extracts that have antibacterial activity. Therefore, we are interested in studying the effects of TA and TF on *S. mutans* in terms of the bactericidal, growth, acid production, and GTFs; and also effects on gingival fibroblast viability.

2. Materials and Methods

2.1 Bacterial strain and culture medium

S. mutans ATCC 25175 was maintained with weekly sub-culturing in Tryptic soy (TS) broth (BD, Sparks, MD, USA) containing 1% glucose (TSG broth) and long-term storage at -80ºC in TSG broth containing 10% v/v glycerol. *S. mutans* ATCC 25175 was resuscitated on mitis salivarius bacitracin agar for 48 hrs at 37ºC. Typical colonies were reinoculated in TSG broth (Charu *et al.*, 2022).

2.2 Preparation of samples

Three samples were freshly prepared in this experiment including 0.25-10% w/v of TA in distilled water, 250-20,000 ppm of fluoride (F) and 0.25-10% w/v of TA combined with 250-20,000 ppm of F (TF).

2.3 Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

Using a microdilution technique, the MIC and MBC of TA, TF and F samples against *S. mutans* ATCC 25175 were determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Janet A. Hindler, 2022). Chlorhexidine 0.05% v/v was used as a positive control. 100 µL of the diluted sample and 100 µL of 1×10^5 CFU/mL bacterial suspension were combined in a 96 well-plate and cultured at 37°C. After 24 h, the lowest concentration which displayed no visible growth was recorded as MIC. The MBC was the lowest extract concentration killing 99.9% of the bacteria inoculum after 24 h incubation at 37 °C. The experiments were done in triplicate (Zeng, Nikitkova, Abdelsalam, Li, & Xiao, 2019).

2.4 The effects of TA, TF and F samples on *S. mutans* ATCC 25175 growth

TA, TF, and F samples were adjusted in 2MIC, MIC, 1/2MIC, 1/4MIC, and 1/8MIC and combined with 10^6 CFU/ml of *S. mutans* ATCC 25175 in 2xTSD+ 2% glucose. Deionized water was the negative control. The growth rates at 2, 4, 6, 8, 10, and 24 h were determined by counting the *S. mutans* on the TSA agar plate. All assays were performed in triplicate (Yapong *et al.*, 2022).

2.5 The effects of TA, TF and F samples on the adhesion of *S. mutans* ATCC 25175

Briefly, approximately 1 x 10^6 CFU/ml overnight cultures of *S. mutans* ATCC 25175 were diluted in 2xTSG containing 1% w/v sucrose. 100 µL of two-fold serial dilutions of the TA, TF and F samples were prepared in 96-well plates, followed by the addition of 100 µl of diluted bacterial suspension. The 1/2MIC, 1/4MIC, and 1/8MIC of TA, TF and final inoculums of 5 x 10^5 CFU were placed in each well. TSG containing 1% w/v sucrose without any antibacterial agent was used as the negative control and 0.2% w/v NaF was used as the positive control. After 24 h culturing, the biofilms were stained with 100 µl of 0.1% v/v crystal violet for 15 min and rinsed thoroughly with sterile water twice. The bound dye was released from the cells with 200 µl of 95% ethanol. The absorbance of the solution was measured at 560 nm using a microplate reader (Anthos Zenyth 200rt, Biochrom, UK). The relative amount of biofilm formed was calculated by comparing the optical density (OD) at 560 nm of the test samples with the controls. The experiment was repeated three times (Yamanaka, Kimizuka, Kato, & Okuda, 2004; Zeng *et al.*, 2019).

2.6 The effects of TA, TF and F samples on the GTFs of *S. mutans* ATCC 25175

GTFs were prepared from 1-liter of cultured *S. mutans* ATCC 25175. The supernatant was collected by centrifugation and the GTFs were precipitated by adding 45% ammonium sulfate. After being placed at 4ºC for 48 h, the precipitate was centrifuged at 8,500 x g at 4 ºC for 30 mins.

The precipitate was dissolved in PBS with pH 7.4, dialyzed in PBS with pH 6.8 at 4ºC for 48 h, and then lyophilized. The reaction mixture contained 50 µl of 0.6 M acetate buffer (pH 5.5) + 50 µl crude enzymes + 200 µl of tested TA, TF of F sample. Distilled water was used to replace the extract as a negative control. The reaction was set at 37 °C for 3 h, then heated at 100 °C for 5 min and centrifuged at 13,000 x g for 6 min. The supernatant was removed and the pellet was washed twice with distilled water. The glucan was determined by the phenol-sulfuric method (H. H. Yu, Lee, Lee, Kim, & You, 2007) by adding 150 µl of 5% phenol + 750 µl of 99% sulfuric solution and then heating at 110 °C for 15 min. The OD was read at 490 nm. The assays were done in triplicate (Yamanaka *et al.*, 2004; Zeng *et al.*, 2019).

2.7 The effects of TA, TF and F samples on the acid production of *S. mutans* ATCC 25175

The effects of TA, TF and F samples on the acid production by *S. mutans* ATCC 25175 were examined according to (Nattapon Rotpenpian, 2022) with a slight modification. After 18 h culturing, cells were collected by centrifugation at 6,500 x *g* for 20 min at 4 ºC and washed twice with PBS at pH 7.4. The cell pellet was resuspended at 40 mg cells/ml in 135 mM KCl and incubated at 37 ºC for 3 h before use. The reactions were set up by adding 1 ml of *S. mutans*, 3 ml of 135 mM KCl, 0.5 ml of 1% glucose, and 0.5 ml of TA and TF samples at 1/2MIC or 1/4MIC.

Sterile water was used as a negative control and chlorhexidine at final concentration of 0.05% v/v as the positive control. The initial pH of each reaction was adjusted to 7.2-7.4 with 0.2 M KOH. The glycolytic pH-drop was monitored using a pH meter at 30, 60, 90, 120 and 150 mins. The experiments were set up 3 times (Xiao *et al.*, 2006).

2.8 The effects of TA, TF and F samples on gingival fibroblast cells and cell migration

The protocol was approved by the institutional ethics committee board of the Faculty of Dentistry, Prince of Songkla University (EC6602-007). The protocol was modified from (Charu, 2022). Human gingival fibroblasts (HGFs) were obtained from healthy individuals undergoing surgical crown lengthening at the Dental hospital. HGFs were secluded by explant techniques and cultured in complete media, which was Dubbacco's Modified Eagles (DMEM; GIPCO, Life Technologies, Grand Island, NY, USA) media supplemented with 10% Fetal Bovine Serum (FBS; Biochrom AG, Berlin, Germany) and antibiotics (1% penicillin-streptomycin and 0.1% Amphotericin B 250 µg/ml (GIPCO, Life Technologies, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. HGFs were sampled from third to fifth passages; all experiments were done in triplicate (Charu *et al.*, 2022).

HGFs were seeded in a 96 well-plate at 1×10^4 cells/well and cultured in complete media at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2 for 24 hrs. The cells were then treated with serially diluted TA and TF for 24 h, while 2.5% v/v DMSO was also investigated for its effect on HGFs. After overnight culturing, the test solutions were replaced with complete media and incubated for another 24 hrs. Then the complete media was replaced with medium containing 10 mM HEPES and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) solution and incubated for 3 h at 37 °C. To perform the MTT assay, an MTT solution was first prepared at a concentration of 5 mg/mL in PBS or culture medium. After treatment, the culture medium in each well was carefully removed and replaced with 100 µL of fresh medium containing 10–20 µL of the MTT solution. The plate was then incubated at 37°C for 3–4 h, allowing mitochondrial dehydrogenases in viable cells to reduce the MTT reagent into insoluble formazan crystals. Following incubation, the MTT solution was gently removed, and 100–200 µL of DMSO (or acidified isopropanol) was added to each well to dissolve the formazan crystals. The plate was then incubated at room temperature for 10–15 min with gentle shaking to ensure complete solubilization of the formazan before absorbance measurement. The OD were measured for whole solution by using a microplate reader (Biochrom Ltd., Cambridge, England) at 570 nm. The relative cell viability (%) determination was performed in triplicate and calculated according to the following equation:

$$\% \text{ Cell viability} = [A]_{test} / [A]_{control} \times 100$$

where $[A]_{test}$ is the absorbance of the test sample and $[A]_{control}$ is the absorbance of the control.

2.9 Statistical analysis

All variables are presented as mean ± SD. To call significant differences, the Kruskal-Wallis H test was applied, followed by Dunnett T3 for multiple comparison. The level of significance was set at $p < 0.05$.

3. Results

3.1 The MIC and MBC of TA, TF and F samples against S. *mutans* ATCC 25175

The MIC values of TA, TF and F samples against *S. mutans* were 5, 2.575 and 0.15 mg/ml, respectively. The MBC values of TA and TF against *S. mutans* were 50 and 11.5 mg/ml, respectively. However, the MBC of F was more than 5 mg/ml.

3.2 The effects of TA, TF and F samples on *S. mutans* ATCC 25175 growth, biofilm formation, GTFs activity, and acid production

The concentrations of TA, TF and F samples at MIC, 1/2MIC, 1/4MIC, and 1/8MIC were investigated. None of the TA, TF and F samples could inhibit *S. mutans* growth during 0-24 h, and could not inhibit the glycolytic acid production by *S. mutans* compared to the control; data are not shown.

MIC, 1/2 MIC and 1/4 MIC of TA significantly inhibited *S. mutans* adhesion by 93.45%, 91.35%, and 85.65%, respectively, compared to the negative control. Moreover, at MIC and 1/2 MIC, TF significantly inhibited *S. mutans* adhesion by 92.85% and 92.25%, respectively, compared to the negative control. Furthermore, the MIC and 1/2 MIC of F significantly inhibited *S. mutans* adhesion by

85.85% and 82.75%, respectively, compared to the negative control as shown in Figure 1. Moreover, the MIC and 1/2 MIC of TA and F, as well as the MIC, 1/2 MIC and 1/4MIC of TF significantly inhibited GTFs activity compared to the control as shown in Figure 2.

3.3 The effects of TA, TF and F samples on gingival fibroblast cells

To determine the cell viability in TA, TF and F samples' cytotoxicity evaluation, HGFs were exposed to various concentrations of TA, TF and F. We found that most MIC levels of TA, TF and F as well as 1/2 MIC of F were toxic to HGFs. Other concentrations of TA, TF and F samples were not toxic to HGFs (Figure 3).

3.4 Synergistic effect of TA, TF and F samples

As shown in Table 1, at 1/2 MIC, TA can inhibit biofilm formation and GTFs activity of *S. mutans*, and is also not toxic to HGFs, whereas 1/4 MIC of TF can inhibit biofilm formation and GTFs activity on *S. mutans*, and also not be toxic to HGFs.

4. Discussion

Fluoride is the most common approach used to prevent dental caries formation. CaF_2, or CaF_2-like materials, precipitate on tooth surfaces when compounds containing fluorides are applied, which might serve as a local reservoir of fluoride and induce remineralization (Amissah, Andey, & Ahlschwede, 2021). However, some bacterial strains, including *S. mutans*, have been reported to be resistant to fluoride (Amissah *et al.*, 2021). The presence of sodium fluoride has decreased the overall gene expression level in *S. mutans*. However, its major effect seems to be inducing the expression of genes and other metabolic transporters, which imply specific cellular internalization of sugars (Damé-Teixeira, Deng, & Do, 2019). Our result agree with another study where fluoride from a sustained-release composite resin significantly inhibited biofilm formation, adhesion, and acid production by *S. Mutans*, but did not influence the growth and ATPase activity of the bacterial strain (Hayashi, Matsuura, & Yamamoto, 2022). Moreover, the fluoride-sustained-release composite resin inhibited adhesion, biofilm formation, and acid production by *S. mutans* through the suppression of GTFs expression as well as the expression and activity of enolase (Hayashi *et al.*, 2022).

In recent years, extensive research has been conducted on anti-caries agents, including fluoride, in various formulations (Jafari *et al.*, 2022). Synergetic combinations of fluoride promote such activity, allowing decreased doses of these combinations to have elevated effectiveness. In support of this, our results found that increased doses of TA can cause toxicity on gingival fibroblasts, while decreased doses of TA combined with lower doses of fluoride were effective in antimicrobial activity against *S. mutans*. This agrees with another study, in which the treatment with fluoridated milk or combinations of tannin-fluoride and milk-fluoride provided additional protective effects against enamel erosion more than did tannin-fluoride alone (Boonviriya, Tannukit, & Jitpukdeebodintra, 2017).

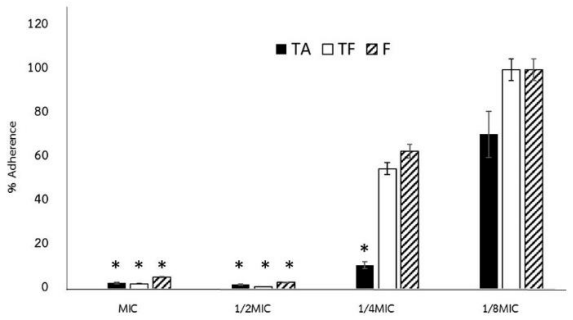

Figure 1. The effects of TA, TF and F samples on *S. mutans* ATCC 25175 adhesion (mean ± SD, $p < 0.05$). * indicates a statistically significant difference ($p < 0.05$) compared to the negative control group.

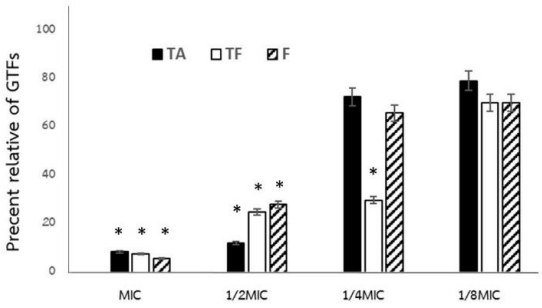

Figure 2. The effects of TA, TF and F samples on GTFs of *S. mutans* ATCC 25175 (mean ± SD, $p < 0.05$). * indicates a statistically significant difference ($p < 0.05$) compared to the negative control group.

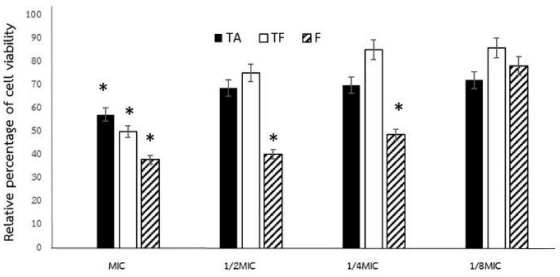

Figure 3. The effects of TA, TF and F samples on gingival fibroblast viability (mean ± SD, $p < 0.05$). * indicates a statistically significant difference ($p < 0.05$) compared to the negative control group.

The possible mechanism of TF effects on *S. mutans* could be that antibacterial activity of TF may lead to the suppression of bacterial colonization caused by the death of its planktonic cells, thereby causing leaks in bacterial cell walls. Therefore, the destruction of bacterial cells occurs due to them losing vital molecules and ions from the cytoplasm (Tanizaki & Inoue, 1979). The ability of the *S. mutans* virulence factor to exert biofilm formation and insoluble glucans via GTFs activity through the breakdown of nutrients permits the bacteria to adhere to surfaces and proliferate dental plaque (Ooshima *et al.*, 2000). Reportedly, plant-derived

Table 1. Synergistic effect of TA and F in TF combination

	1/2 MIC of TA	1/4 MIC of TF	1/2 MIC of F
S.mutan growth	-	-	-
Bioflim formation	+	+	+
Acid production	-	-	-
GTFs	+	+	+
Gingival fibroblast viability	-	-	-

+: significant inhibition
- : not significant

polyphenols, including TA, suppress acid-producing bacteria by decreasing the growth of *S. mutans* and GTFs (Gianmaria F Ferrazzano *et al.*, 2011; Furiga *et al.*, 2008). According to the hypothesized mechanisms, the phenolic molecule may alter the hydrophobic characteristics of the infected cell surface, which then binds to the active site of the insoluble enzyme leading to an inhibition of caries progress (Gregoire, Singh, Vorsa, & Koo, 2007; Jagtap & Karkera, 2000).

Furthermore, TA prevents demineralization by interacting with the organic enamel matrix. The diffusion channel within the tooth structures may change as a result of changes to the organic matrix network in the inter- and intra-prismatic spaces, which could impact the de-mineralization of the enamel (Huang, Liu, Li, Zhou, & ten Cate, 2012). Our results agree with a previous study's findings in that TF promoted enamel remineralization (Boonviriya *et al.*, 2017). Another investigation found that TA inhibited enamel demineralization (Chunmuang, Jitpukdeebodintra, Chuenarrom, & Benjakul, 2007). One possible mechanism is that TF may create bonds between organic molecules and calcium phosphorus, leading to the production of insoluble compounds on the surface of the enamel. Insoluble materials, in contrast to CaF_2, are extremely resistant to acid assault and water cleaning (Boonviriya *et al.*, 2017).

However, this current research was performed *in vitro*, which means that the results may not be directly applicable to the real oral environment, as the latter includes a diversity of oral bacteria, flow of saliva, and the appearance of tooth surfaces. Also, exact mechanisms for caries prevention by TF should be further investigated.

5. Conclusions

The combination of TA and F acted synergistically to inhibit biofilm formation and GTFs activity of *S. mutans* without any toxicity to gingival fibroblasts. Therefore, it is possible that TF has anti-demineralization activity in dental caries pathogenesis.

Acknowledgements

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