Original Article

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Caesalpinia sappan: A promising natural source of antimicrobial agent for inhibition of cariogenic bacteria

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Summary **From the previous findings, the ethanolic fractionated extract of *Caesalpinia sappan* (F-EtOH) has high activity against *Streptococcus mutans*, the most severe cariogenic bacteria. The present study was aimed to isolate and identify the active compound of F-EtOH and compare its inhibitory activity against the biofilm of *S. mutans* as well as the cytotoxicity to oral fibroblast cells with F-EtOH. Compound isolation was done by column chromatography. The active compound was identified using liquid chromatography-mass spectrometry with electrospray ionization and nuclear magnetic resonance spectroscopy. It was found that the major compound of F-EtOH is brazilin. F-EtOH and brazilin were compared for inhibitory potential on the biofilms of three strains of *S. mutans*. The results exhibited that both F-EtOH and brazilin had potential on inhibiting biofilm formation and eradicating the preformed biofilms and their activity was dose dependent. F-EtOH showed significantly less toxic to normal periodontal ligament fibroblast than brazilin. At low concentration of 1- and 2-MBC, F-EtOH showed higher effective than brazilin. The results of our study suggest that the antibacterial activity of F-EtOH is according to the synergistic effects of the existing compounds including brazilin in F-EtOH.**

Keywords: Sappan wood, brazilin, oral pathogens, antibiofilm, cytotoxicity

1. Introduction

Many *Streptococcus* spp. are normal flora microorganisms in oral cavity but some insidious species are found to be oral pathogenic stains. *Streptococcus mutans* is considered to be one of the severe cariogenic bacteria leading to dental caries (*1,2*). They can early colonized on hard tooth surfaces and the epithelial tissues to form a biofilm or known as a dental plaque, which later contains multiple bacterial species (*3*). These biofilms can produce acid that destroys the tooth's enamel layer which leads to periodontitis and dental carries (*4*). Although, the oral pathogens may be controlled by meticulous mechanical oral hygiene but they cannot be completely exterminated from oral cavity. Controlling oral microorganisms and keeping dental plaque at levels compatible with oral health are important. Therefore, many oral health care products are formulated to contain antiplaque or antiseptic agents to achieve good oral health.

Using plant extracts instead of chemical synthetic agents in treatment of certain bacterial infections are of increasing interest for green environment. Various potential plant extracts have been reported on antimicrobial activity against oral pathogens (*5*). Nowadays, only a few of them are considered to be used as ingredients in dental products whereas many of them are not, according to their limited properties. For example curcumin, the high effective secondary metabolite compound from turmeric has been reported to have strong ability against biofilm formation of *S. mutans* (*6*). However, curcumin is not used as ingredient in toothpaste or mouthwash because of its low aqueous solubility and rapid degradation, as well as its yellowish color that affects the physical characteristics of the products (*7*). Therefore, searching for other potential plants is still needed.

Caesalpinia sappan or sappan wood is the plant

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commonly found in many Asian countries. In Thailand, it has been used as traditional medicine since the ancient time to promote blood circulation. The heartwood part of *C. sappan* can produce natural red dye that can be used as coloring agent in food, beverage, and cosmetics. For biological properties, it has been reported to have antioxidant (*8,9*), anti-inflammation (*10*), anti-rheumatoid arthritis (*11*) and antimicrobial activity (*12-14*). Many phenolic compounds, such as xanthone, coumarin, chalcones, flavones, and homoisoflavonoids have been found from *C. sappan* (*15*). Brazilin [(6a S-cis)-7, 11b-dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10(6H)-tetrol] is reported as one of the major constituents present in the heartwood part of *C. sappan* (*16*). The color of brazilin can be changed from amber to red in pH ≥ 7 (*17,18*). The intensity of red color is depends on the amount of brazilin. The extract of *C. sappan* heartwood has been reported to have activity against several kinds of bacteria including *S. mutans* (*19,20*). While brazilin has also been reported to have inhibition activity against many oral pathogens (*14*). From the literature review, there is still lack of deep detail of *C. sappan* extracts against oral pathogens, particularly on inhibition of the biofilms of such severe pathogens. Our previous study presented that the ethanolic fractionated extract of *C. sappan* (F-EtOH) showed strong antibacterial activity on these bacteria (*20*). The current study was aimed to identify the active compound of F-EtOH and compare the antibiofilm activity and cytotoxicity between F-EtOH and its isolated active compound. The minimum bactericidal concentration (MBC) of both F-EtOH and the isolated active compound was determined before antibiofilm activity investigation.

2. Materials and Methods

2.1. *Chemicals and reagents*

Hexane, ethyl acetate, ethanol, methanol, chloroform, and dimethyl sulfoxide (DMSO) of analytical grade and HPLC grade were from RCI Labscan (Bangkok, Thailand). Difco™ Brain heart infusion (BHI) broth and agar were from Bacton, Dickinson and Company (Sparks, Maryland, USA). Human blood for blood agar preparation was supported by Maharaj Nakorn Chiang Mai Hospital (Chiang Mai, Thailand). Dulbecco's modified eagle medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), and antibiotic-antimycotic solution (AA) were from Gibco™, Life Technologies (Grand Island, New York, USA). Thiazolyl blue tetrazolium bromide was from Sigma-Aldrich (St. Louis, Missouri, USA). The other chemicals and solvents were of the highest grade available.

2.2. *Plant materials and fractionated extracts preparation*

The heartwood of *C. sappan* was collected from the local area in Chiang Mai Province of Thailand and identified by the botanist in the botanical herbarium of Faculty of Pharmacy, Chiang Mai University to obtain the reference voucher numbers (002276). F-EtOH was prepared according to the previous report (*20*). Briefly, the dried powder of *C. sappan* heartwood was subjected to fractionated extraction by maceration method using 3 different solvents; hexane, ethyl acetate, and ethanol, respectively. The filtrate from ethanol extraction was subjected to a rotary evaporator, EYELA rotary evaporation N-1000 (Tokyo, Japan) for removing the solvent and to obtain F-EtOH.

2.3. *Identification of the major compound*

Liquid chromatography–mass spectrometry (LC-MS) with electrospray ionizationc (ESI), Flexar SQ300 MS (Single Quad) (PerkinElmer, Waltham, Massachusetts, USA) was used to determine the molecular mass of the compound. The maximum absorption was determined using UV-2450 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan) with spectrum mode in wavelength range of 200-700 nm. Nuclear magnetic resonance (NMR) spectra of the isolated compound were obtained from Bruker 400 MHz NMR spectrometer, UltraShieldTM (Billerica, Massachusetts, USA). ^{1}H NMR was operated at 400 MHz and ^{13}C NMR was operated at 100 MHz. CD_3OD was used as a solvent.

As brazilin was previously reported to be the major compound in *C. sappan* (*20*), for quantitative determination of the major compound of F-EtOH, the pure brazilin was dissolved in methanol at the final concentration ranges of 0.5-500 µg/mL and used for preparing calibration curve. HPLC analysis was carried out using HPLC Shimadzu CLASS-VP™ model (Kyoto, Japan) and the reversed phase Eurospher 100, *i.d*. 4 mm, C18 column, Knauer (Berlin, Germany). F-EtOH and brazilin were dissolved in methanol to proper concentrations before injection. Aqueous solution of 1% v/v acetic acid in DI water (A) was mixed with methanol (B) at a volume ratio of 3:1. This solution was used as the mobile phase with the flow rate of 1 mL/min, injection volume of 10 µL, running time of 30 min, and detected at 280 nm. Running system was performed at room temperature. A standard curve of brazilin was constructed. The amount of brazilin in F-EtOH was calculated from the linear equation of $y = 8710.9x + 435.18$ ($r^2 = 0.9998$).

2.4. *Bacterial strains culture conditions*

Three strains of *S. mutans* including *S. mutans* DMST9567, *S. mutans* DMST18777, and S. *mutans* DMST41283 were cultured and incubated under anaerobic condition at 37°C in 5% CO_2 using anaerobic chamber BACTRONII-2, SHEL LAB® (Cornelius, Oregon, USA). Blood agar plates were prepared from

5% human blood in BHI agar.

2.5. *MBC determination and antibiofilm susceptibility*

The MBC of the test samples was determined according to the method previously described (*20*). Briefly, the diluted samples were added into the suspension of 1 × 10^6 CFU/mL of the tested bacterial strains in the 96-well plates and incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. Subsequently, the cultures were streaked on blood agar plates and incubated at 37°C in 5% CO_2 anaerobic chamber for 24 h. The lowest concentration in the plates that bacterial growth could not be visible was considered as the MBC.

In antibiofilm susceptibility test, the antibiofilm formation and eradication of the preformed biofilms were investigated. For antibiofilm formation, the samples were prepared to have the final concentrations of 1-, 2-, and 4-fold MBC. The sample solutions of 100 µL were transferred to 96-well plates followed by adding 50 µL of BHI broth and 50 µL of the culture suspensions (1 × 10^6 CFU/mL). Chlorhexidine at 0.12% (CHX) was used as a positive control. The plates were incubated in anaerobic chamber for 24 h. After incubation, nonadherent planktonic cells were removed and the wells were gently rinsed with 200 µL of phosphate-buffered saline (PBS). The adherent biomass was stained with 200 µL of 0.1% (w/v) crystal violet at room temperature for 30 min. The solutions were removed, and the wells were rinsed 3 times with 200 µL of PBS. Then, 100 µL of 30% (v/v) acetic acid was added to dissolve the crystal violet stains and measured at 595 nm using a microtiter plate reader Model 680, BIO RAD (Tokyo, Japan). The percentage of biofilm formation was calculated by the following equation; *% biofilm formation = As × 100/Ac*. Whereas *Ac* is the absorbance of the control culture (untreated cell) and *As* is the absorbance of the culture treated with the sample. The lower percentage of biofilm formation indicates the higher inhibitory activity of the test samples.

For antibiofilm activity that the preformed biofilms were eradicated, 50 µL of the culture suspensions (1 × 10^6 CFU/mL) and 150 µL of BHI broth were transferred to 96-well plates and incubated in an anaerobic chamber for 24 h. After incubation, nonadherent planktonic cells were removed and the wells were gently rinsed with 200 µL of PBS. Next, 100 µL of BHI broth was added in each well and the adherent biomass was treated with 100 µL of the test sample at the concentrations described above. The plates were further incubated in an anaerobic chamber for 24 h. After incubation, the nonadherent planktonic cells were removed by gently rinsing the wells with 200 µL of PBS. The viability biomass was stained with 200 µL of 0.1% w/v crystal violet at room temperature for 30 min. The solutions were removed, and the wells were rinsed 3 times with 200 µL of PBS. Then, 100 µL of 30% v/v acetic acid was added to dissolve crystal violet stains and measured at 595 nm using a microtiter plate reader. The biofilm eradication activity of the samples was evaluated from the percentage biofilm left which was calculated by the following equation; *% preformed biofilm = As × 100/Ac*. Whereas *As* is the absorbance of the culture treated with the samples and *Ac* is the absorbance of the control culture (untreated cells). The lower percentage found indicates the higher eradication activity of the test samples.

2.6. *Cytotoxicity*

The cytotoxicity of F-EtOH and its major component against normal cells was evaluated. Periodontal ligament (PDL) fibroblast cells were collected from the healthy human subjects. This experiment was under ethical clearance No. 02/2015, approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University, Thailand. Cell viability was determined by MTT assay. The PDL cells were cultured in completed DMEM (supplemented with 10% v/v FBS and 1% v/v AA) and incubated in humidified atmosphere, 5% CO_2 at 37°C. For the test, the cell suspension at a density of 1 × 10^4 cells/well was cultured in 96-well plates and then incubated under the same condition for 24 h. After that, the medium was removed and replaced with 100 µL of completed DMEM and 100 µL of the samples (final concentration ranged from 3.9-2,000 µg/mL in 0.4% v/v DMSO). The plates were further incubated for 24 h. Then, 100 µL of the medium was removed from each well and 100 µL of MTT solution (0.5 mg/mL in PBS) was added and further incubated for 4 h. Next, the medium was removed, and the formed formazan crystals were dissolved by mixing with 100 µL of DMSO for 10 min. The absorbance was measured at 540 nm and 690 nm as a reference wavelength using a microtiter plate reader. The cell viability was compared with the untreated culture or vehicle control culture. The percentage of cell viability was calculated using the following equation; *% cell viability = OD × 100/* OD_0. Whereas *OD* is the optical density of the well containing cells treated with the samples and OD_0 is the optical density of the well containing cells treated with 0.4% v/v DMSO (a negative control). The higher percentage of cell viability indicates the lower cytotoxicity of the samples.

2.7. *Statistical analysis*

The results of all experiments were conducted in triplicate and expressed as mean ± SD and statistically analyzed *via* SPSS statistic 17.0 software. ANOVA and Turkey's Multiple test have been determined the significant at $p < 0.05$.

3. Results

3.1. *Sappan wood extract and the major compound*

F-EtOH obtained had the same outer appearance as the previous report (*20*). Isolation of F-EtOH using column chromatography yielded many fractions but only fraction-6 (F6) was identify since our previous work found that F6 has the highest activity. It was found that F6 was a relatively pure compound. The HPLC chromatograms of F-EtOH and F6 were compared as shown in Figure 1. It is found that F6 demonstrated a major single peak at a retention time of 6.83 min (Figure 1A). Meanwhile, F-EtOH demonstrated 2 obvious peaks and 2 tiny peaks exhibited at obviously distinct retention times (Figure 1B). The peak of F-EtOH at the same retention time as F6 is the largest one. The results of identification of the single compound of F6 using ^{1}H NMR spectra and ^{13}C-NMR spectra are as follows and shown in Figure 2.

^{1}H NMR data (400 MHz, CD_3OD): δ 2.77 (1H, d, J = 15.6 Hz, H-7), 3.02 (1H, d, J = 15.6 Hz, H-7), 3.69 (1H, d, J = 11.2 Hz, H-6), 3.92 (1H, d, J = 11.2 Hz, H-6), 3.96 (1H, s, H-12), 6.29 (1H, d, J = 2.4 Hz, H-4), 6.46 (1H, dd, J = 10.5, 2.4 Hz, H-2), 6.60 (1H, s, H-11), 6.70 (1H, s, H-8), 7.19 (1H, d, J = 8.1 Hz, H-1). ^{13}C NMR data (100 MHz, CD_3OD): δ 43.0 (C-7), 51.2 (C-12), 71.0 (C-6), 78.2 (C-6a), 104.4 (C-4), 110.1 (C-2), 112.6 (C-11), 113.0 (C-8), 115.7 (C-1a), 131.5 (C-7a), 132.4 (C-1), 137.6 (C-11a), 145.5 (C-10), 145.8 (C-9), 155.9 (C-3), 158.0 (C-4a).

The UV-visible spectrum of F6 showed maximum absorption at 224.5 and 289 nm. The mass spectrum of the compound showed a molecular weight at *m/z* 286. Using the NMR spectra as well as UV absorption and mass spectrum to compare with the data reported previously (*21,22*), we considered that the isolated compound of F6 was identical to brazilin ($C_{16}H_{14}O_5$), which the chemical structure is shown in Figure 3.

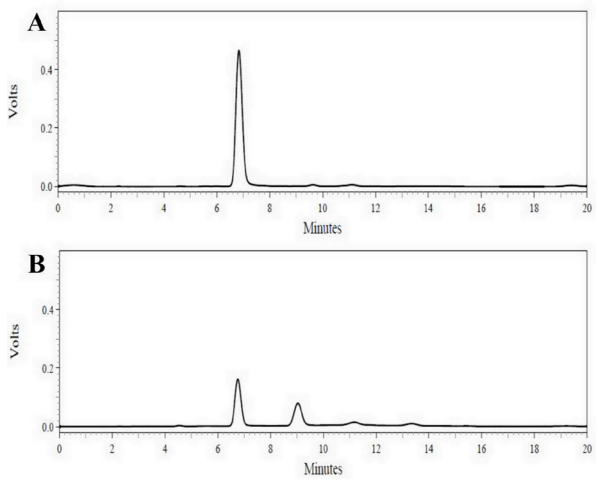

Figure 1. HPLC chromatograms of F6 (A) and F-EtOH (B) detected at 280 nm.

The physical appearance of F6 is orange red powder. An amber color solution was observed when it was solubilized in ethanol, methanol, DMSO or in the solutions of pH less than 7. The color of the solution changed to red or pink when the pH was increased to 7 or higher. These physical characteristics as well as the identified NMR spectra confirmed that the pure compound of F6 was brazilin (*17,21,22*). The physical appearance of F-EtOH was red brown powder. HPLC analysis indicated that the content of brazilin in F-EtOH was 325.14 ± 25.91 µg/mg of F-EtOH. It was also

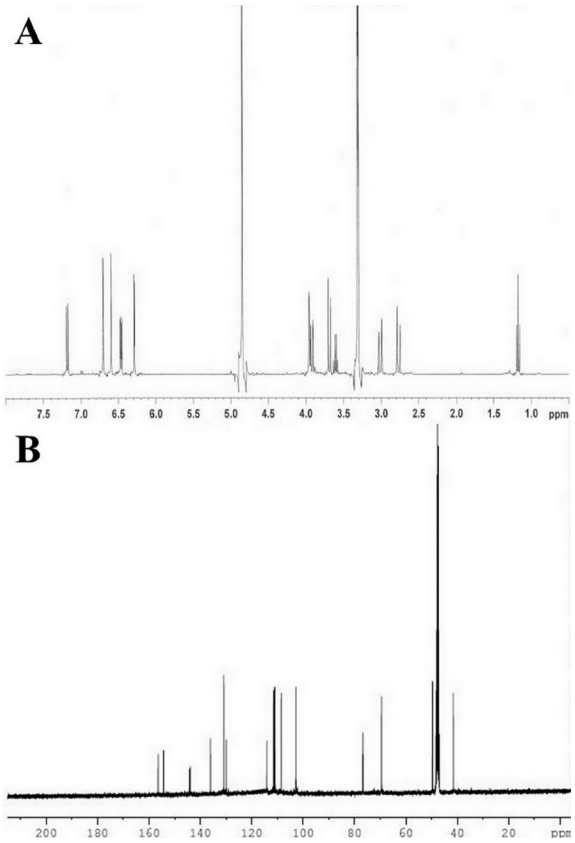

Figure 2. Nuclear magnetic resonance (NMR) spectra of brazilin from *C. sappan*, ^{1}H NMR (A) and ^{13}C NMR (B).

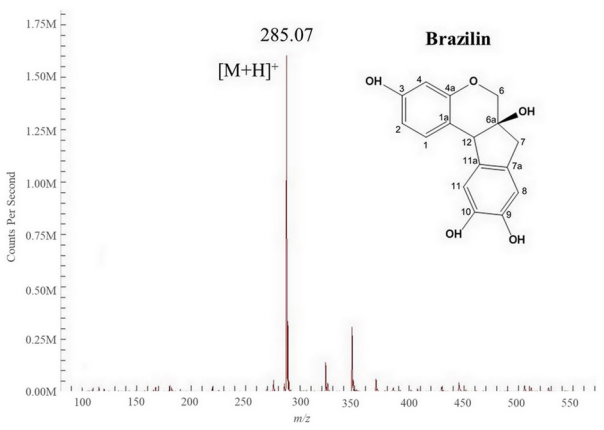

Figure 3. Fragmentation pattern from LC-MS by ESI technique (negative ion mode) of brazilin (*m/z* 286) isolated from F-EtOH.

found that brazilin played a role on the color of F-EtOH solution.

3.2. *MBC and antibiofilm susceptibility*

The MBC of F-EtOH and brazilin against the three strains of *S. mutans* was found to be the same value of 125 µg/mL. To evaluate the potential of F-EtOH as an antibiofilm agent, we examined the inhibition of biofilm formation and the ability to eradicate the preformed biofilms. We also compared these activities of F-EtOH with its major compound brazilin. The results are shown in Figure 4. It was found that all *S. mutans* strains were able to form biofilms completely (100%) after treated with the negative control but significant reduction after treated with F-EtOH and brazilin. Both F-EtOH and brazilin demonstrated dose dependent curves. Biofilm formation of all tested strains of *S. mutans* could be inhibited by F-EtOH and brazilin. After contacting with F-EtOH at 1-fold MBC, only 6.97 ± 0.79, 8.97 ± 1.60, and 9.17 ± 0.97% of biofilm of *S. mutans* DMST9567, *S. mutans* DMST18777, and *S. mutans* DMST41283 could be detected, respectively. After contacting with brazilin at the same concentration, 9.55 ± 1.08, 10.79 ± 0.33, and 14.29 ± 2.32% of biofilm of the three strains, respectively, could be detected. At the higher concentrations as 2-fold MBC, only 2.56 ± 0.21, 4.20 ± 0.13, and 5.25 ± 0.97% of the biofilm of the three strains, respectively, could be detected after contacting with F-EtOH. While, after contacting with brazilin, 9.55± 1.08, 5.59 ± 1.68, and 5.08 ± 1.17%, respectively, could be found. At 4-fold MBC, the biofilm formation of the three strains was almost completely inhibited and not significantly different from CHX. At this concentration, both F-EtOH and brazilin could inhibit more than 85% biofilm formation of all strains. At lower concentrations as 1- and 2-fold MBC, F-EtOH showed significantly higher effective on inhibition of biofilm formation of the three strains than brazilin. The results indicated that F-EtOH and brazilin even at low concentration as 1-fold MBC was sufficient to inhibit biofilm formation of all three strains of *S. mutans*.

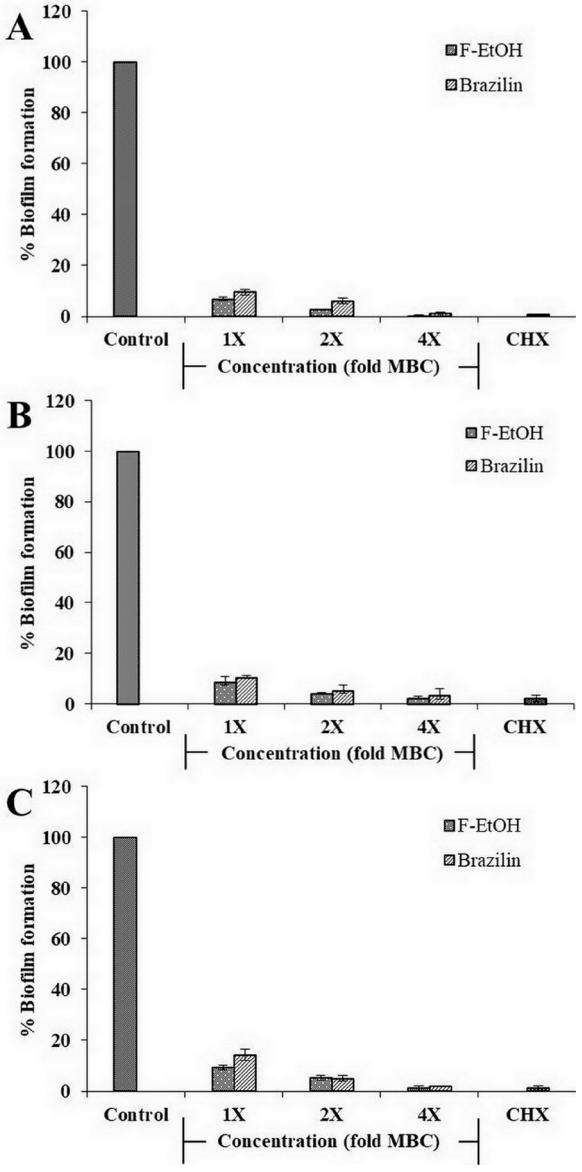

Figure 4. Biofilm formations of *S. mutans* DMST9567 (A), *S. mutans* DMST18777 (B), and *S. mutans* DMST41283 (C) after treating with F-EtOH, brazilin, and CHX in comparison with the untreated control.

For the ability to eradicate the preformed biofilm, the results are shown in Figure 5. A dose dependent reduction in cell viability of *S. mutans* was observed. Treating with 1-fold MBC of F-EtOH and brazilin was not much effective on eradication of the preformed biofilm of *S. mutans* DMST41283. More than 50% (52.07 ± 2.65 and 59.59 ± 1.84) of viable cells could be found after treating with F-EtOH and brazilin, respectively. However, at this concentration, 38.54 ± 1.50 and 46.52 ± 0.77% of viable cells of DMST9567 and *S. mutans* DMST18777, respectively were detected after contacting with F-EtOH. Whereas, after contacting with brazilin at this concentration, higher amount of *S. mutans* DMST9567 (49.42 ± 0.91) and *S. mutans* DMST18777 (54.79 ± 3.11%) could be detected. Increasing the extract concentration, a higher reduction in cell viability of the three strains was observed. Treatment with 4-fold MBC of F-EtOH, only 2.34 ± 1.63, 2.34 ± 0.47, and 5.49 ± 1.13% of viable cells of *S. mutans* DMST9567, *S. mutans* DMST18777, and *S. mutans* DMST41283 could be detected, respectively. At the same concentration of brazilin, 2.46 ± 0.82, and 3.79 ± 1.04, and 9.86 ± 2.62% of viable cell could be detected, respectively. This viability reduction indicated that more than 90% of the pathogens could be killed. From these results, it is shown that F-EtOH presented the significantly higher effective than brazilin. F-EtOH and brazilin at concentration of 4-fold MBC could killed *S. mutans* DMST9567 and *S. mutans* DMST18777 as much as CHX. Meanwhile, the reduction of viable *S. mutans* DMST41283 from F-EtOH and brazilin was lower than CHX. It was

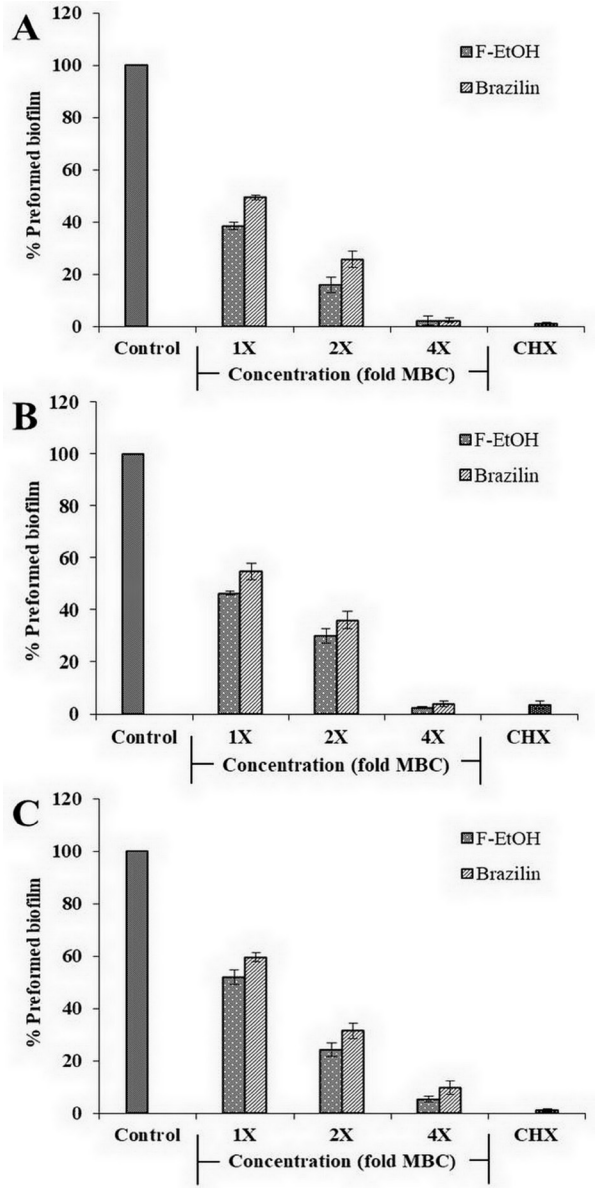

Figure 5. Percentage of preformed biofilms of *S. mutans* DMST9567 (A), *S. mutans* DMST18777 (B), and *S. mutans* DMST41283 (C) after treating with F-EtOH, brazilin, and CHX in comparison with the untreated control.

considered that the biofilm communication of this strain might be tolerant to F-EtOH and brazilin.

3.4. *Cytotoxicity*

The cytotoxicity of F-EtOH and brazilin on normal PDL cells of healthy volunteers was evaluated by MTT assay. DMSO at the final concentration of 0.4% v/v and its series of 2-fold dilution were also tested as it was used as a solvent for the test samples. The results showed that 85.01 ± 4.34% of cell viability could be detected when DMSO at 0.4% v/v was used indicating that DMSO at this concentration was not toxic to the cells. Comparison between F-EtOH and brazilin, the results are shown in Figure 6. Both F-EtOH and brazilin demonstrated the dose-response curves, however it was

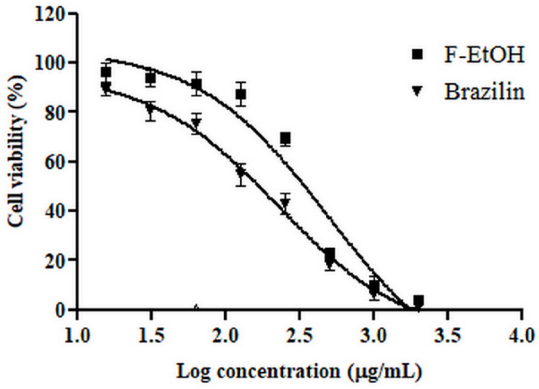

Figure 6. Viability of PDL cells after treating with F-EtOH (■) and Brazilin (▼).

obviously different in levels of toxicity. Cell viability higher than 80% demonstrates safety to normal cell. Monitoring at log concentration of 2.1 which referred to the concentration of 125 µg/mL, cell viability after treating with F-EtOH was 87.36 ± 4.83% whereas that treating with brazilin at the same concentration was obviously reduced to 54.73 ± 4.23%. Decreasing the concentration to 31.25 µg/mL which eqivalent to log 1.49, cell viability after treating with brazilin was 80.59 ± 3.77%. Therefore, brazilin at this concentration is safety to PDL.

4. Discussion

The oral cavity is the hub of an extremely diverse microflora consisting of about 500 species of microorganisms. The surface of mucosal membrane, teeth and tongue as well as gingival pocket can be the suitable habitats for microbial growth. These areas therefore can promote the formation of distinct oral microflora communities. Oral biofilm found on a tooth surface and define as a dental plaque is the important microbial community and identified by the initial adhesion of the early colonizers. The dental plaques commonly found in oral cavity are supragingival, subgingival, buccal mucosa, and tongue coating plaques. The metabolic activities of these normal flora communities can change the properties of the oral environment and to a suitable condition for promoting the growth of oral pathogens. The over growth of these oral pathogens can lead to several oral diseases such as dental caries, gingivitis, and periodontitis (*23,24*). Oral streptococci especially *S. mutans* is significantly role lead to dental caries and gingivitis (*2*). CHX is widely used in oral health care products for the prevention and treatment of oral diseases due to these pathogenic bacteria. However, CHX is cytotoxic to human periodontal cells, inhibits protein synthesis, affects mitochondrial activity, and thus, has adverse effects on vital tissues (*25*). Therefore, it is a need to find new agents that could be used as biological alternatives in management of these oral diseases.

Many bioactive compounds from various plants

have been reported their antimicrobial efficacy on a wide range of microorganisms including oral pathogens. Among phenolic compounds, tannins and the members of flavonoid group, especially flavan-3-ols and flavonols possessed broad spectrum, high antimicrobial activity and show synergism effect with antibiotics. Moreover, they are able to suppress the factors influence to microbial virulence, such as inhibition of biofilm formation, reduction of host ligands adhesion, and neutralization of bacterial toxins (*26*). Although, the specific mode of action of phenolic compounds is not completely understood (*27*). According to the numerous of report, phenolic compound and aromatics are disrupted at the cytoplasmic membrane of microbial cells by changing their structure and function influence to cell contents leak out and the microorganisms die (*28*).

In Thailand, it is well familiar that the most natural health care products contain the extracts of green tea. Its bioactive constituents are catechins and derivatives; epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG), and epicatechin (EC) have the strong oral protection by against oral pathogens such as streptococci and lactobacilli (*29,30*). *C. sappan* has been reported its antimicrobial activity against oral pathogens including gram positive and negative strains lead to oral diseases as dental caries and periodontitis. However, research related to this activity is still needed according to the lack of important detail such as antibiofilm activity which is directly related with severe dental diseases, particularly dental caries, gingivitis, and periodontitis. The current study provides the intensive data from our previous works (*20*) which reported the antibacterial activity of F-EtOH which is the ethanolic fractionated extracts of *C. sappan* possesses significantly higher antimicrobial activity against *S. mutans* and *S. intermedius* than the other extracts. In the present study, we therefore worked deeply on F-EtOH to identify the major active compound of this fractionated extract and compare their antibiofilm activities. The inhibitory activity test was performed against three strains of *S. mutans* because this pathogen is the most severe cariogenic bacteria that can cause dental carries and other important oral disease like periodontitis. Isolation of the major compound from F-EtOH was done by using silica gel column chromatography. The obtained isolated compound was confirmed to be brazilin by comparing the obtained ^{1}H NMR and ^{13}C NMR spectra as well as the supporting data of MS and UV with the data previously reported in the literatures (*11,15,21,22*). Considering the HPLC chromatogram of F-EtOH, it confirmed that F-EtOH contained not only brazilin but also one obvious minor peak and the other two tiny peaks. The calculation based on the HPLC chromatograms of F-EtOH and brazilin resulted that F-EtOH contained brazilin of approximately 300 µg/mg extract. According to the eradication of the preformed biofilm, the result clearly showed that the percentage of preformed biofilms of all strains after treating with F-EtOH at the concentration of 125 µg/mL were significantly less than after treating with brazilin at the same concentration indicating that F-EtOH had stronger inhibitory activity than brazilin. Using the above calculation, it is indicated that F-EtOH at the concentration of 125 µg/mL contains brazilin only 37.5 µg/mL. This result emphasizes that F-EtOH which consists brazilin at approximately 3 folds less than the pure brazilin has stronger activity than the isolated brazilin. The antibacterial potential of F-EtOH, therefore, is considered to be due to the synergistic effect of brazilin and the other minor compounds existed in the extract.

Biofilm formation of *S. mutans* is a severe etiological factor for dental caries. In the current study, we used two methods to investigate the antibiofilm activity of the test samples. One is the test on inhibition of biofilm formation and the other is the inhibitory activity test on eradication of the preformed biofilms of the test pathogenic bacteria. Both methods are different in the time that the samples are exposed to the test bacterial strains. For antibiofilm formation, the bacterial strains were mixed together with the samples at the first time before forming biofilms. This method indicates the ability of the sample to prevent the adsorption or adhesion of microorganisms to surfaces that is the initial stage of biofilm formation by killing the bacterial strains (*31*). Regarding to the inhibitory activity on eradication of the preformed biofilms, the bacterial strains were incubated for 24 h, that their biofilms were completely formed, before mixing with the test samples. F-EtOH showed the higher potential than brazilin on inhibition of biofilms of all strains by both methods. Our study demonstrated that the antibiofilm activity of F-EtOH and brazilin was dose dependent. At the low concentrations, such as at 1- and 2-fold MBC corresponding to 125 and 250 µg/mL, respectively, different potential of inhibition between F-EtOH and brazilin was obviously detected. F-EtOH showed significantly higher effective than brazilin. The concentration of 125 µg/mL was sufficient to inhibit the biofilms from *S. mutans*. Meanwhile, at the high concentration (4-fold MBC) corresponding to 500 µg/mL, brazilin and F-EtOH showed similar inhibitory power to almost complete inhibition (about 98%), and same as the positive control CHX.

The cytotoxicity test on normal cell is necessary for the candidates to be used in human. PDL is the connective tissue consists of fibroblasts cells in the periodontal area (*32,33*). These PDL cells were used in the present study to test for the toxicity of F-EtOH and brazilin to human normal cells. Our results show that PDL could tolerate to F-EtOH higher than brazilin. F-EtOH did not induce nonspecific toxicity to PDL cells when the concentration of 125 µg/mL was used, whereas brazilin at this concentration showed severe

toxic to the cells. This result confirmed the significant difference on higher safety of F-EtOH than brazilin.

In conclusion, this study reveals that the extract of *C. sappan* heartwood prepared from ethanolic fractionated extract F-EtOH and its isolated major compound brazilin possess the effective inhibition against three strains of cariogenic bacteria (*S. mutans*). Their inhibitory activity on pathogenic biofilms is along with the inhibition of biofilm formation and the eradication of the preformed biofilms of these pathogens. The antibacterial potential of F-EtOH at its MBC and 2-fold MBC is stronger than its major compound brazilin and it has significantly less toxicity to normal cells than brazilin. F-EtOH at 125 μg/mL is nontoxic to human normal cells. The antibacterial activity of F-EtOH is the results of synergism of the existed compounds including brazilin. From these results, F-EtOH of *C. sappan* is a promising natural antibacterial candidate suitable for further study on development of oral health care product for treatment and prevention of oral infection and related disorders like dental carries.

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