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Efficiency of *Hylocereus undatus* Extracts on Biofilm Formation of *Streptococcus mutans* *In vitro*

Suwonchoochit T[1], Nagasevi N[1], Thamprechavai P[1], Nuntana Aroonrerk[1*]
Department of Stomatology
Faculty of Dentistry, Srinakharinwirot University, Bangkok

***Corresponding Author:**
Nuntana Aroonrerk
[1]Associate professor, Department of Stomatology, Faculty of Dentistry, Srinakharinwirot University, Bangkok, Thailand, 10100

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Abstract

Background: *Hylocereus undatus* or dragon fruit contains high level of essential fatty acid and has been reported in promoting wound healing and antibacterial activity.

Objective: This study was to investigate the biofilm inhibitory activity and antibacterial activity of *Hylocereus undatus* extracts. *Streptococcus mutans* (*S. mutans* ATCC25175) was used in the experiment since it is the primary causative agent of biofilm formation and dental caries.

Materials and methods: The white pulp and pink peel of dragon fruit were extracted using 95% methanol. Antibacterial activity of the extracts was examined by broth microdilution method. Biofilm formation inhibition activity was evaluated by modified microtiter dish assay. *S. mutans* ($2.0x10^6$ CFU/ml) was inoculated in Todd Hewitt broth with 3% sucrose at 37 ºC for 24 h. Biofilm formation was assessed by 0.1% crystal violet, the color was soluble in 30% acetic acid and determined by microplate reader at 570 nm. Positive control was kanamycin and negative control was 0.1% DMSO. Kruskal Wallis test was used to statistically analyze the results and find the differences between group by Dunn test.

Results: The minimum inhibitory concentration (MIC) of dragon fruit pulp and peel extracts were 5 mg/ml and >10 mg/ml, respectively. The minimum biofilm inhibitory concentration (MBIC) of dragon fruit peel extracts was 0.625 mg/ml with 85.39±1.45% of biofilm inhibition and MBIC of flesh extracts was 2.5 mg/ml with completely inhibited.

Conclusion: Dragon fruit extracts had potential in antibiofilm formation activity against *S. mutans*, especially the peel extract had more efficiency than the pulp extract.

Keywords: Dragon fruit, *Streptococcus mutans*, biofilm, MIC, MBIC

INTRODUCTION

Oral diseases are significant global health problem in all countries and populations. Over 3.5 billion cases, more people are affected than by other disease group. The main oral diseases encompass tooth decay of permanent and deciduous teeth, severe periodontal disease, and oral and lip cancer [1]. Dental caries can be found commonly in human at all ages. People sometimes consider this as meaningless pain. However, it can lead to serious health issues and cannot be ignored. The prevalence of dental caries among 151 Thai children aged 9-18 months evaluated was 32.5%, 15.9% had at least one cavity (cavitated caries) and 16.6% had white lesions (non-cavitated caries) [2]. As regards most non-communicable diseases (NCDs), oral conditions are chronic and firmly socially patterned. Children living in poverty, socially criticized groups, and elderly persons are the most influenced by oral diseases and have poor

approach to dental care [3]. Their oral hygiene was usually poor, designated by high levels of dental biofilm and high numbers of mutans streptococci. As a result of the failure to incorporate oral health into general health promotion, millions suffer incurable toothache and poor quality of life and wind up with few teeth [4].

Streptococcus mutans is a cariogenic bacterium that plays a crucial role in the beginning of dental caries, both in fissures and on smooth enamel surfaces [5]. An influential virulence property of the bacterium is its capability to form oral biofilm on tooth surfaces [6, 7]. *S. mutans* also produces glucosyltransferases, multiple glucan-binding proteins, protein antigen c, and collagen-binding protein, surface proteins that related to produce oral biofilm, thus causing dental caries [8].

Biofilms consist of microbial communities embedded in a 3D extracellular matrix. The matrix is composed of a complex form of extracellular polymeric substances (EPS) which provide to the unique feature of biofilm behavior and virulence [9]. Biofilm-grown microbes are also prominent for their resistance to a range of antimicrobial agents including sanitizers and antibiotics [10]. The microtiter dish assay is a decisive tool for the study of the initial phase in biofilm formation and has been used for the research study of bacterial biofilms [11, 12]. Additionally, many researches display that biofilms grown in microtiter dishes do develop some features of mature biofilms, such as antibiotic tolerance and resistance to immune system effectors [11].

Varieties of antibacterial compounds can be derived from natural sources or synthetic chemical compounds [13] Recently, scientists have acknowledged to traditional folk medicines or natural products to discover the scientific basis of therapeutic effects such as antibacterial agents [14]. Apart from plants, fruits also have turned into the principal item for scientists to be investigated since their bioactive compounds close related with herbs, frequently commonly attributed as phytochemicals such as carotenoids, polyphenols, xanthones and anthocyanins that are abundantly present in fruits such as grapes, plum, bergamot and mangosteen [15-18].

Hylocereus species or dragon fruit is commonly found in Asian food markets, have a unique taste, shape, and the hot pink color. Many researchers have found betalains, the major bioactive compounds from red dragon fruit peels of *Helocereus* spp. [19, 20]. Betalains possess strong antioxidant, anti-inflammatory, antiangiogenic, and glutathione S-transferase-inducing activity [20]. Furthermore, *H. undatus* have been reported in promoting wound healing on diabetic rats [21]. However, the examinations on antibacterial and antibiofilm formation activities of *H. undatus* are missing as less popular in the markets.

Based on the explanation above, dental caries should be prevented and treated with caution. Therefore, the present study was aimed to evaluate the antibiofilm formation and antibacterial properties against *S. mutans* using the methanol extracts of *H. undatus*.

MATERIALS AND METHODS

We used quantitative methods to gather data regarding the amount of biofilm formation and the number of bacteria in wells of each solution, both with and without the methanol dragon fruit extracts. Our experiments were divided into three parts, crude extracts preparation, biofilm formation assay and antibacterial method. After undergoing all processes of the experiment, the amount of biofilm and the number of bacteria in each well were measured as optical density (OD) at 570 nm and 600 nm by using a microplate reader.

Crude extracts preparation

Dragon fruit (*H. undatus*) was harvested in Thailand in 2020 and purchased at the local market (Bangkok). Immediately the fruits were peeled, and the pulp were separated. Then the pulp and the peel were dehydrated in the oven at 70℃ up to dryness. Then the samples are powdered by electric mill. A certain weight of the dried powder was soaked in 95% methanol (with ratio of 1:10 (w/v) at room temperature for 72 h with agitated in the dark. The liquid samples were filtered with Whatman filter paper, freeze-dried, and then extracted. The supernatants were concentrated using rotary evaporator (Heidolph Rotary Evaporator, Schwabach, Germany) at 40℃, and the extract was stored at -20℃ [22]. The dry residues were dissolved in absolute dimethyl sulfoxide (DMSO, Merck, Germany) to a concentration of 100 mg/ml and used as stock solution.

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Bacterial stain

Laboratory control strain *S. mutans* (ATCC25175) was purchased from American Type Culture Collection (Rockville, Md., USA). Active cultures for experiments were prepared by transferring a loopful of bacterial cells from the stock cultures to Mueller-Hinton broth (MHB, Oxoid, Basingstoke, UK) that were incubated under 5% CO_2 for 48 h at 37°C. The bacterial cultures were diluted with the media to achieve optical densities corresponding to 2.0 x 10^6 CFU/ml. Todd Hewitt broth (THB, Oxoid) with 3% sucrose (w/v) was used for antibiofilm formation test but MHB was used for antibacterial activity test.

Antibiofilm formation

The biofilm inhibition of the extracts followed a described method [12]. Briefly, the peel and the pulp extracts were subsequent two-fold dilution was performed in DMSO. 20 ml of a two-fold concentration of extract (undiluted, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640) was pipetted to the prepared 96-well microtiter test plates. Then each well was incubated with 180 ml of suspended *S. mutans* culture in THB with 3% sucrose (w/v). The 96-well plate was incubated under 5% CO_2 for 24 h at 37°C without agitation. The biofilm was washed with phosphate buffer saline pH 7.4 (PBS) and then stained with 200 ml of 0.1% crystal violet for 15 minutes, then rinsed 3 times with PBS. After that, a decolorizer, 200 ml of 30% acetic acid was added and agitated. All experiments were set up in triplicate. The amount of biofilm formation of each well was determined by assessment of the crystal violet color by optical density readings at 570 nm with a Biochrom Asts Expert Plus Microplate Reader. Kanamycin was used as a positive control. Wells containing bacteria (0.1% DMSO) were used as the negative control. The amount of biofilm in each well was compared with that in the negative biofilm control. The minimum biofilm inhibitory concentration (MBIC) recorded the lowest concentration of the substance that its OD was significantly three times lower than that in the next lower concentration.

Antibacterial Activity

The minimum inhibitory concentrations (MIC) of mouthwashes were determined using broth microdilution method which was modified from Wiegand I, et.al. [23]. Briefly, initial candidate extract was prepared in DMSO, and subsequent 2-fold dilution was performed with DMSO. 20 µl of a two-fold concentration of extract (undiluted, 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640) was pipetted to the prepared 96-well microtiter test plates. Then each well was incubated with 180 ml of suspended *S. mutans* culture in MHB. The 96-well plate was incubated under 5% CO_2 for 48 h at 37°C. All experiments were set up in triplicate. The resulting turbidity was observed. MIC was determined by assessment of turbidity by optical density readings at 600 nm with a Biochrom Asts Expert Plus Microplate Reader. Kanamycin was used as positive control. 0.1% DMSO was used as a negative control. The amount of growth in each well was compared with that in the negative growth control. The MIC recorded the lowest concentration of the substance that can inhibit visible growth of the organism.

Statistical analysis

All experiments except were set up in triplicate. Data are presented as means $\pm$ SEM. Data were analyzed and processed using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA). Kruskal Wallis test was used to compared amount of biofilm formation. The Dunn test was used to analyze the results and find the differences between groups. The level of statistical significance was set at P value of < 0.05.

RESULTS

Antibiofilm Formation Activity

Figure 1: Biofilm formation of H. undatus extracts and kanamycin with different dilutions

Data could be read from Figure 1.

The pulp and peel dragon fruit extracts were assayed for their antibiofilm formation activity against *S. mutans* ATCC25175. Kanamycin as a positive control showed the highest antibiofilm formation activity (MBIC value of lower than 0.0782 mg/ml) compared to both the pulp and peel extract. More interestingly, the antibiofilm formation activity of the peel extract (MBIC value of 0.625 mg/ml) was higher than the pulp extract (MBIC value of 2.5 mg/ml), significant at $p < 0.05$ (Figure 1 and Table 1).

Antibacterial Activity

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In the experiment of antibacterial activity of *H. undatus* extracts against *S. mutans*, we found that pulp and peel of *H. undatus* extracts had MIC value of 5 mg/ml and >10 mg/ml, respectively. While positive control (kanamycin) had MIC value of 0.625 mg/ml. The pulp exhibited higher antibacterial activity compared to that of the peel extract. However, the higher concentration that possessed higher than 10 mg/ml of peel extract was not examined.

Data could be read from Table 1.

Table 1: MBIC and MIC of H. undatus extracts and kanamycin

DISCUSSION

In the present study, pulp and peel methanol extracts from dragon fruit (*H. undatus*) were used to determine the antibiofilm formation. The effect of various concentrations of extracts on the biofilm formation of *S. mutans* were examined. A significant different between the dragon fruit extracts was observed for the biofilm formation (Figure 1 and Table 1). From Figure 1, no significant different in optical density with a decrease concentration of the peel extract ranged from 20 to 0.625 mg/ml, then the optical density was rising five-to-six-times at the next concentration (0.3125 mg/ml). Therefore, the minimum biofilm inhibitory concentration (MBIC) of dragon fruit peel extracts was 0.625 mg/ml with 85.39±1.45% of biofilm inhibition. But the MBIC of the pulp extract was 2.5 mg/ml with completely inhibited biofilm formation. The numbers of the microorganisms attached to polystyrene were also reduced without affecting their cell viability, this is consistent with the biofilm inhibiting activity of betacyanin from red pitahaya (*H. polyrhizus*) extract [24].

In the experiment, the MIC values of the pulp and peel extracts of *H. undatus* by broth microdilution method were 5 mg/mL and >10 mg/ml, respectively. The results in our study were contradict evidence from the previous research, which have suggested that the peel extract had higher antibacterial activity than the pulp extracts [25]. This may be the different in the fruit species (*H. polyrhizus*) and the extraction method.

Many researchers found that the plant *Hylocereus* had antioxidant activity. Antioxidants such as betacyanin, betalain, flavonoid, phenolic compound, and ascorbic acid, etc. [19, 26, 27]. These natural antioxidants have antibacterial and anti-biofilm effects; especially action against gram positive bacteria [28]. A large amount of antioxidant content such as betacyanin, polyphenol, flavonoid was found in the pulp and peel of *H. undatus*. The antioxidants were significantly higher in the peel than in the pulp [22, 29]. Due to the amount of antioxidant, the peel was more capable of inhibiting the biofilm than the pulp extract of the dragon fruit.

Betacyanin is a main component of the red dragon methanol extract. It has antibacterial and antibiofilm formation activities against gram-negative and gram-negative bacteria. It was reported to have anti-biofilm activity against *Staphylococcus aureus* (MBIC value of 2.5-0.313 mg/ml) and *Pseudomonas aeruginosa* (MBIC value of 0.313-0.625 mg/ml). Betacyanin significantly reduced hydrophobicity of *S. aureus* and *P. aeruginosa* [24]. Yong et al. (2019) suggested that betacyanin can function as anti-biofilm tool against the primary step of biofilm formation, especially on a hydrophobic surface as polystyrene [24]. In addition, *S. mutans* have ability to bind or adhesion to the surface both with nonspecific interaction using hydrophobic property and specific interaction by antigen I/II protein on the substrate [26].

In addition, flavonoid is a group of polyphenols found in the pulp of the dragon fruit [22, 26]. Flavonoid can inhibit the synthesis of bacterial DNA and RNA. It has ability to interfere the activity of the transpeptidase, a bacterial enzyme that cross-links the peptidoglycan chain to form rigid cell wall. Then the cell wall formation is disturbed and finally destroyed [30]. Myricetin and quercetin are members of the flavonoid class of polyphenolic compounds which also found in dragon fruits [31]. Myricetin significantly reduced exopolysaccharides in the extracellular matrix, which is a major role in biofilm formed by *S. mutans* and *Candida albicans* [32]. The anti-biofilm activities of quercetin in biofilm formation of *S. mutans* was also examined and results suggested the potential of quercetin as an alternative anti-caries therapeutic agent [33].

The results of several studies indicated that a large amount of antioxidant content such as betacyanin, polyphenol, flavonoid was found in the pulp and peel of *H. undatus*. The antioxidants were significantly higher in the peel than in the pulp [22, 29]. Due to the

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number of antioxidants, the peel was more capable of inhibiting the biofilm than the pulp extract of the dragon fruit.

CONCLUSION

From this study, it can conclude that *H. undatus* can be used for dental caries prevention as it showed antibiofilm formation activity. Both pulp and peel methanol extracts have antibiofilm activity against cariogenic bacteria *S. mutans*. Thus, these extracts can be used for production of antibiofilm dental preparations to be applied for therapeutic motives or in oral hygiene products. The peel extract proved to have antibiofilm activity at lower concentrations than that of pulp extract demonstrating the extraction of more compounds with antibiofilm activity.

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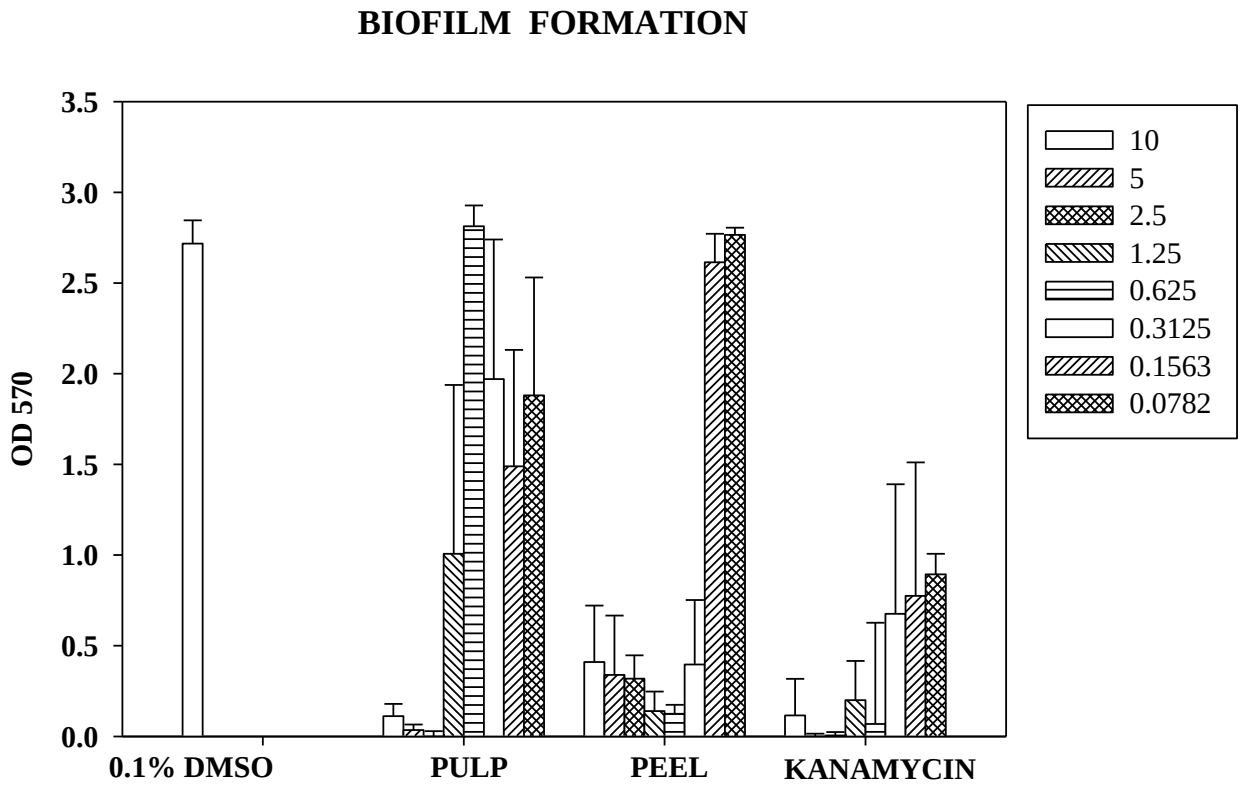

Figure 1: Biofilm formation of H. undatus extracts and kanamycin with different dilutions

Table 1: MBIC and MIC of H. undatus extracts and kanamycin

	MIC (mg/ml)	MBIC (mg/ml)
Pulp extract	5	2.5
Peel extract	> 10	0.625
Kanamycin	0.625	< 0.0782

MBIC or Minimum biofilm inhibitory concentration is the lowest concentration of an agent that can inhibit biofilm formation.

MIC or Minimum inhibitory concentration is the lowest concentration of an agent that can inhibit visible growth of the microorganism.

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