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ANTIBIOFILM AND ANTIADHESION ACTIVITIES OF PHALERIA MACROCARPA AGAINST ORAL STREPTOCOCCUS MUTANS

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Graphical abstract

Fruit (A) and leaves (B) of *Phaleria macrocarpa*

Abstract

Dental caries is a major concern in oral healthcare. Continuous research have been performed extensively in finding new compounds that are capable to solve the problems. *Phaleria macrocarpa* has been identified effective against hypertension, diabetic, cancer, and diuretic acid. In this study, antiadhesion and antibiofilm activities of *Streptococcus mutans* were investigated using crude extracts of fruit, leaf and stem of *P. macrocarpa*. Minimal inhibitory concentration (MIC) assay was conducted to identify the lowest concentration of the extracts required to suppress the activity of *S. mutans*. This assay confirmed that all tested extracts were able to inhibit the bacterial activities with concentration of less than 8 mg/mL and thus can be classified as a natural antimicrobial agents. The extracts were found capable of reducing 50 to 80% of both adhesion and biofilm activity of *S. mutans* at 1.56 mg/mL. Results from this study provide a preliminary data for the effectiveness of *P. macrocarpa* crude extracts as antiadhesion and antibiofilm agent against *S. mutans* and may have potential for antiseptic agent to treat oral dental caries.

Keywords: *Phaleria macrocarpa, Streptococcus mutans*, dental carries, antiadhesion, antibiofilm

Abstrak

Karies gigi merupakan isu utama dalam penjagaan kesihatan mulut. Kajian secara berterusan dan menyeluruh telah dijalankan bagi mencari kompoun baru yang berupaya untuk menangani masalah ini. *Phaleria macrocarpa* telah dikenalpasti berkesan terhadap masalah tekanan darah tinggi, diabetis, kanser dan asid diuretik. Dalam kajian ini, aktiviti antirekatan dan antibiofilem daripada *Streptococcus mutans* telah dikaji dengan menggunakan ekstrak buah, daun dan batang *P. macrocarpa*. Ujian kepekatan perencatan minima (MIC) dijalankan untuk mengenalpasti kepekatan minima ekstrak yang diperlukan untuk menyekat aktiviti *S. mutans*. Ujian ini mengesahkan bahawa semua ekstrak berupaya untuk menghalang aktiviti bakteria pada kepekatan kurang dari 8 mg/mL dan dapat diklasifikasi sebagai bahan antimikrob semulajadi. Kesemua ekstrak diperhatikan berupaya mengurangkan aktiviti perekatan dan biofilem *S. mutans* sebanyak 50-80% pada kepekatan 1.56 mg/mL. Hasil kajian ini diharap dapat memberikan maklumat awal berkenaan keberkesanan ekstrak *P. macrocarpa* sebagai agen antirekatan dan antibiofilem terhadap *S. mutans* dan mungkin berpotensi sebagai agen antiseptik bagi merawat karies gigi.

Kata kunci: *Phaleria macrocarpa, Streptococcus mutans*, karies gigi, antirekatan, antibiofilem

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1.0 INTRODUCTION

Dental caries is the localized destruction of susceptible dental hard tissues by acidic by-products from bacteria fermentation of dietary carbohydrates at sites with a pre-existing natural and diverse microflora in the human oral cavity [1]. It is chronic, without intervention and progressive disease and its prevalence increases as children get older [2]. It causes oral pain and tooth loss and via DNA mapping and other strategies, there is evidence showed that dental caries can be transmitted vertically, mostly from mother to child in womb [2].

Dental caries frequently happen in high intake of dietary sugar, promoting the growth of acidogenic and aciduric bacteria, such as *Streptococcus mutans, Streptococcus sobrinus* and *Lactobacillus acidophilus*. As a result, these bacteria secrete acid to its environment, creating a low pH surrounding and causes demineralization of tooth surface, leading to tooth decay [1].

Currently, there are few methods used to control oral biofilm. This includes physical methods, which usually involve heat and mechanical scrubbing, brushing, scraping and high pressure spraying. These physical methods may cause pain and discomfort to patients.

Apart from that, some anti-microbial agents in the market for instance Chlorhexidine gluconate, have been widely used as an antiseptic mouthwash to maintain oral hygiene. However, unexpected side effects had been reported including brown staining of tooth, taste change, shortness of breath, desquamation and soreness in oral mucosa [3]. Thus, there is a continuous need to search for an alternative medicine for the treatment of dental carries. In recent years, there has been increasing interest in the use of traditional medicinal plants due to the fact that some of active compounds isolated from medicinal plants exhibit potent antimicrobial and anticancer activities.

In this study, the fruit, leaf and stem extracts of *Phaleria macrocarpa* (also known as Mahkota Dewa) were used to investigate its potential effectiveness in inhibiting biofilm formation by *S. mutans*. Previously, this plant was used by the natives of Indonesia as traditional medicine to treat hypertension, diabetic, cancer and diuretic disease [4]. Scientific studies have proven that this plant exhibit anticancer, antihyperglycemic, antihyperlipidemic, vasorelaxant, antioxidant, anti-inflammatory, antimicrobial and antifungi activities [5, 6].

2.0 EXPERIMENTAL

2.1 Plant Material

Fruits, leaf and stem bark of *P. macrocarpa* were collected from Pontian, Johor, Malaysia, in May 2010, and were identified by Dr. Shamsul Kamis, Plant Taxonomist, Biodiversity Unit, Universiti Putra Malaysia (UPM), Serdang, Selangor, Malaysia. A voucher specimen (SK 2248/13) representing this collection has been deposited in the Biodiversity Unit's Herbarium, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia.

2.2 Extraction

The dried fruits (500 g) were extracted with ethanol at room temperature for 48 hours. The residue was extracted twice and the extract was filtered. The filtrate was then evaporated to dryness using vacuum distillation and rotary evaporator at 50 °C to give ethanol crude extract (24.41 g, 4.80%). The ethanol crude extract was partitioned separately with ethyl acetate and evaporated to afford the crude extract (2.40 g, 9.83%). Meanwhile, the dried and ground leaf (580 g) and stem (500 g) were extracted sequentially using (2 L) each of *n*-hexane followed by ethyl acetate and methanol at room temperature for 48 h each. Evaporation of the ethyl acetate and methanol extracts of the leaf afforded 8.06 g (1.61%) and 15.66 g (3.13%) of respective extracts while the stem part gave ethyl acetate extract (2.63 g, 0.53%) and methanol extract (17.15 g, 3.43%), respectively.

2.3 Minimum Inhibitory Concentration

One hundred microliter crude extracts prepared were transferred into 96-well microplate. Meanwhile, the bacteria inoculum cultured overnight in brain heart infusion (BHI) was centrifuged at 3000 rpm for 10 minutes at 4 °C to obtain the cell pellet. The cell pellet was then resuspended in 0.9 % of saline solution and the bacteria cell density was adjusted to about 10^8 CFU/mL by comparing with 0.5 Mcfarland standard solution. Subsequently, 1 mL of the bacteria in saline was transferred into 9 mL of sterile saline solution for further diluting it to the standard concentration of 10^7 CFU/mL for MIC determination. Next, 100 µL of the bacteria solution prepared was added into the microwell containing 100 µL of the plant extract. Then, the 96-well microplate was incubated at 37 °C for 24 hours. The amount of living cells in the plate was then quantified using an MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay.

2.4 Antiadhesion Assay

Antiadhesion assay was performed according to protocol described in Khan *et al.* [7]. *S. mutans* was grown overnight in BHI medium and centrifuged at 3000 rpm, 4 °C for 10 minutes. The cell pellet was collected, resuspended in PBS and adjusted the $OD_{600\ nm}$ to 1.0. One hundred microliter of the cell suspension was then inoculated into 10 mL of sterile BHI broth containing 0.25 % (w/v) of sucrose in a universal bottle. Then, plant extracts at different concentrations, ranging from 0.78 mg/mL to 6.25 mg/mL were added into the respective universal bottle. Subsequently, the universal bottles were inclined at about 30° and incubated anaerobically at 37 °C for 24 hours. The suspension in universal bottle was discarded after 24

hours incubation. Next, 10 mL of 0.5 M NaOH was added and gently swirled to remove the none or weak-adhering bacteria cells on the glass surface. The mixture was discarded. Then, 2 mL of 0.90 % sterile saline solution was added into the universal bottle. The cell density measured with spectrophotometer at 600 nm. The antiadhesion activity of the bacteria was determined using the formula as shown below:

$$\text{Antiadhesion activity (\%)} = \frac{\text{OD of adhering cells}}{\text{OD of growth cells}}$$

2.5 Antibiofilm Assay

S. mutans grown overnight in BHI medium was centrifuged at 3000 rpm, 4 °C for 10 minutes. Then, the cell density was adjust to $OD_{600\ nm}$=1.0 with BHI broth and were then further diluted with sterile BHI broth containing 0.25 % of sucrose at ratio of 1:100. The 96-well plate was first coated with saliva. After coating the well with saliva, 100 µL of BHI broth was added into each well. Then, 100 µL of 12.50 mg/mL plant extract was added to the first well to make the initial concentration of 6.25 mg/mL of plant extract. In determining the biofilm formation activity of the bacteria in different concentration of crude extract from *P. macrocarpa*, 2-fold dilution was performed until the final concentration of 0.22 mg/mL. Subsequently, 100 µl of pre-adjusted *S. mutans* was added into the wells. The positive and negative controls used were bacteria culture with penicillin and without extract respectively. The plate was then incubated anaerobically at 37 °C for 24 hours. After 24 hours incubation, the plate was washed gently with 200 µL of sterile distilled water for twice. Fifty microliter of 0.1 % crystal violet was then added to stain the biofilm-forming bacteria for 15 min. The wells were then rinsed with 200 µL of sterile distilled water for another 2 times, and 200 µL of 99 % ethanol was added into the wells to solubilise the bound dye in the stained living cells. The mixture was then transferred to a new plate before quantifying the biofilm formed by *S. mutans*. The quantity of the biofilm-forming bacteria was measured using microplate reader at 600 nm.

2.6 Statistical Analysis

All the experiments were carried out in triplicates. For each outcome, data were summarized as mean ± standard error of the mean (SEM). The significance differences between the two set of data was calculated by using Student's t-test. All statistical analysis were carried out using GraphPad Prism (GraphPad Software, Inc.).

3.0 RESULTS AND DISCUSSION

3.1 MIC Determination

The MIC of the *P. macrocarpa* crude extracts was identified positively inhibiting the activity of *S. mutans* at different concentration, ranging from 0.40 mg/mL to 4 mg/mL. As shown in Table 1, at 0.41 mg/mL, both leaf and stem ethyl acetate crude extracts were able to inhibit bacterial activity. In contrast, the ethanolic fruit extract was able to inhibit *S. mutans* activity at 3.91 mg/mL, almost ten times higher than the MIC of leaf and stem ethyl acetate.

Table 1 Minimal inhibitory concentration (MIC) of *P. macrocarpa* extracts against *S. mutans*

Part	Solvent used	MIC value (mg/ml)
Fruit	Ethyl acetate	2.60 ± 1.13
	Ethanol	3.91 ± 0
Leaf	Ethyl acetate	0.41 ± 0.14
Stem	Ethyl acetate	0.41 ± 0.14
	Methanol	0.82 ± 0.28

All the extracts tested have been proven possessing antimicrobial activities against *S. mutans*. According to Van Vuuren [8], natural products with MIC value below 8 mg/mL is considered possessing antimicrobial activity and MIC value below 1 mg/mL is considered noteworthy. Table 1 shows that the ethyl acetate crude extracts of both leaf and stem as well as the stem methanolic crude extracts are highly potential to be natural antimicrobial compounds.

Recently, Altaf *et al.* [6] reported that the fruit and stem of *P. macrocarpa* contain high concentration of saporins, alkaloids, polyphenolics, phenols, flavonoids, lignans and tannins while the leaf is enriched with phalerin. According to Ayaz *et al.* [9] and Rahman and Moon [10], the phenolic and flavonoid compounds are components primarily dominate to antimicrobial activity but study on the constituents of the plant is still very limited. However, Hendra *et al.* [5] has found out that the flavonoid compounds, such as kaempferol, myricetin, naringin, quercetin and rutin, which are part of polyphenol molecules are present in *P. macrocarpa* fruit. All of these components were reported to have positive antimicrobial activity against human pathogenic microorganisms, like *S. mutans* [11].

Meanwhile Lay *et al.* [4] has analysed the ethyl acetated crude extract of the *P. macrocarpa* fruit using gas chromatography mass spectrum. It was identified that there were components such as phenol, 2,6-dimethoxy phenol, 2-methoxy phenol, □-sitosterol, stigmast-4-en-3-one, flamenol, palmitic acid, methyl palmitate, oleic acid, and some other unidentified components found presence in the ethyl acetate fruit extract of the plant. Although crude extracts were being used in this study, it is expected that components suggested are present and some of it possibly contribute to the antimicrobial activity. The low MIC values suggest that there are potential natural antimicrobial compounds in the plant and may become the alternatives to antibiotics in near future.

3.2 Antiadhesion Assay

Bacteria adheres onto the tooth surface prior to dental plaque formation [12]. As discussed by Krol [2], *S. mutans* critically initiates dental caries. Once this bacteria grows in the oral cavity, it produces glucan, which aids its adhesion onto the tooth surface. The bacteria will then start producing acid and polysaccharides by utilising the surrounding. Hence, dental plaque biofilm develops. The critically increasing number of patients suffering oral cavity problems reflects the urge of alternative treatments.

In this study, the antiadhesion effect of 5 crude extracts of *P. macrocarpa* was investigated and results obtained are shown in the Figure 1. In the presence of the crude extract (6.50 mg/mL) from the fruit, there was only 9.72 ± 2.00 % and 14.78 ± 4.28 % of cells attached onto the glass surface containing ethanol and ethyl acetate extracts respectively. Meanwhile, there was 25.71 ± 6.06 % and 11.86 ± 6.06 % of bacteria attached onto the glass surface when treated with stem ethyl acetate and methanolic extract respectively. However, the leave extract extracted using ethyl acetate failed to inhibit the adhesion activities in *S. mutans*.

Study from Ferrazzano *et al.* [13] identified polyphenol components can affect cell permeability and alter the cell charges through interaction with proteins, enzymes and lipid on the microbial membrane. Vegetative organs, such as flowers and fruit of flowering plants contain abundant of polyphenol. Hence, it is believed that the high antiadhesion activity of *S. mutans* could be attributed by the polyphenols in the fruits of *P. macrocarpa*.

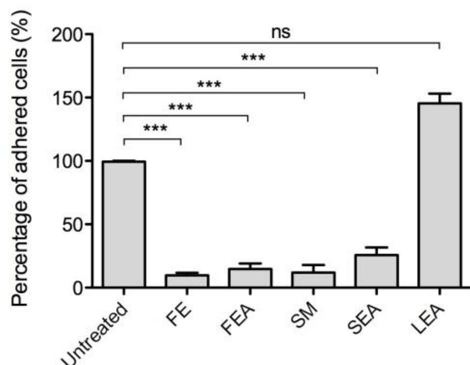

Figure 1 The percentage of *S. mutans* adhering to the glass surface in the presence of 6.5 mg/ml *P. macrocarpa* crude extracts (FE; ethanolic fruit extract, FEA; ethyl acetate fruit extract, SM; methanolic stem extract, SEA; ethyl acetate fruit extract and LEA; ethyl acetate leave extract). Data were expressed as mean ± standard deviation from three independent experiments. ***$P < 0.0001$, ns; non-significant (*t* test).

3.3 Antibiofilm Assay

We next investigated the antibiofilm activity of *P. macrocarpa* extracts on *S. mutans*. Results from this study demonstrated that *S. mutans* treated with 6.50 mg/mL *P. macrocarpa* crude extracts exhibited significant antibiofilm effect in microwells coated with salivary pellicle when compared to untreated cells (Figure 2). The least amount of biofilm formation was recorded in bacteria grown in the presence of fruit ethyl acetate extract, which was 16.98 ± 1.44 %, while the highest amount of biofilm formation was observed in bacteria grown in the presence of stem ethyl acetate extract, which was 43.50 ± 2.00 %.

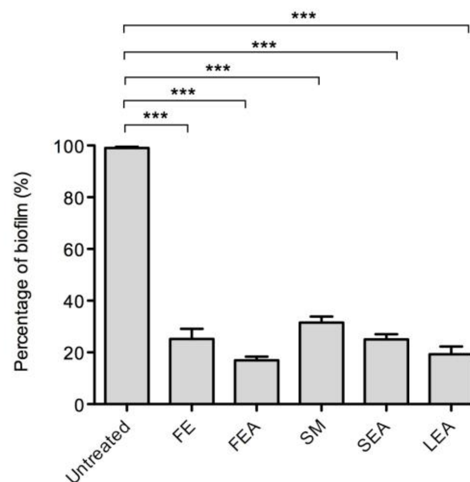

Figure 2 The percentage of biofilm formed by *S. mutans* in the presence of 6.5 mg/ml crude extracts of *P. macrocarpa* (FE; ethanolic fruit extract, FEA; ethyl acetate fruit extract, SM; methanolic stem extract, SEA; ethyl acetate fruit extract and LEA; ethyl acetate leave extract). Data expressed as mean ± standard deviation from three independent experiments. ***$P < 0.0001$, (*t* test)

In this experiment, 0.25 % of sucrose was supplied to improve the biofilm-forming capacity of *S. mutans* [14]. Recently, researchers found that both the sucrose and non-sucrose dependent mechanism in the bacteria contribute to the formation of biofilm [14]. In the presence of sucrose, the acidogenic mutans and aciduric bacteria grow better in the oral cavity can actively synthesize glucosyltransferase (Gtf) enzyme, promoting the formation of water insoluble glucans and activating the glucan binding protein, leading to activation of sucrose-dependent mechanism in the bacteria [14]. Schilling and Bowen [15] reported that the enzyme provides avid binding sites for enhancing the bacteria attachment, particularly in the presence of saliva. Biofilm matrix will then develop onto the tooth surface [16]. In the presence of both sucrose and saliva pellicle, the microwells used provide environment imitating the oral cavity. This promotes *S. mutans* to grow and synthesize Gtf enzyme. The increasing formation of Gtf enzyme by the bacteria subsequently stimulates the *S. mutans* to adhere and form dental plaque biofilm [16]. The overall experimental results obtained from the antibiofilm formation of *S. mutans* in the presence of crude extracts extracted from different parts of *P. macrocarpa* are statistically significant when compared to the non-treated cells.

4.0 CONCLUSION

This study demonstrated the potential of *P. macrocarpa* crude extracts as anticariogenic compound against biofilm formation by *S. mutans*. The result obtained show promising antibiofilm efficacy of the plant extracts in antibiofilm activity. Results from this study may have an implication for future researches in natural antimicrobial products, especially in oral healthcare.

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