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Optimization of *Piper sarmentosum* Extract Concentration and Exposure Time to Inhibit Biofilm Formation of Dental Plaque-Causing Bacteria

Yong Sze Min[a], Nurriza Ab Latif[a], Nurzila Ab Latif[a]

[a] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.

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*Corresponding author
nurzila@utm.my

Abstract

Streptococcus mutans and *Streptococcus sobrinus* are the primary oral pathogens that can lead to the formation of dental plaque. *Piper sarmentosum* is a medicinal plant has the potential alternative solution to solve the problem. The objectives of this project were to optimize the extraction concentration, total phenolic compounds (TPC) and total flavonoid compounds (TFC) in *P. sarmentosum* by using 80% methanol and 95% ethanol as the extraction solvents and the exposure time of the extract of *P. sarmentosum* to inhibit the biofilm formation of both *S. mutans* and *S. sobrinus*. The extract with higher TPC and TFC was screened by using GC-MS analysis. The minimum inhibitory concentration (MIC) of the plant extract against the oral pathogen was determined by using an MTT colourimetric assay. The anti-adherence and biofilm-inhibitory assays were performed when the exposure time of the plant extract towards the oral pathogen reached 1, 3, 6 and 24 hours. The 80% methanolic extract exerts a higher ability to extract more phenolic and flavonoid compounds compared with 95% ethanol as the extraction solvent. The MIC of the 80% methanolic extract towards *S. mutans* and *S. sobrinus* was 50 mg/mL and 100 mg/mL, respectively. A dose-dependent manner was observed in both anti-adherence and biofilm inhibitory assays. The best biofilm-inhibitory activity was observed when the exposure time of the plant extract towards the oral pathogen reached 1 hour. These findings served as preliminary ideas about the potential of *P. sarmentosum* to develop into oral care products.

Keywords Dental plaque-causing bacteria, *P. sarmentosum*, GC-MS, biofilm-inhibition assay

1.0 INTRODUCTION

Dental plaque is one of the most common diseases in the human oral cavity. The formation of dental plaque is due to the aggregation of microbes and food particles in the oral cavity (Albutti et al., 2021). The dental plaques were the most ordinary habitat for the oral pathogen, where the oral pathogen will aggregate in a matrix affixed to a surface known as a biofilm (Jeyaraj et al., 2022). *Streptococcus mutans* (*S. mutans*) and *Streptococcus sobrinus* (*S. sobrinus*) (Marsh, 2018) were the major causes of dental plaques, especially *S. mutans*. Palombo (2011) suggested that a person who likes to consume high-fermentable sugar had a higher percentage of *S. mutans* and *S. sobrinus* in plaque. Gram-positive bacteria with the ability to significantly lower the pH in the oral cavity can cause enamel demineralization, leading to caries development and an increased risk of plaque accumulation in the gingival region (Satria et al., 2022).

The most traditional methods used to remove dental plaques include flossing and toothbrushing. However, an additional approach is needed to assist in removing dental plaque, as both flossing and toothbrushing may not effectively remove the entire dental plaque (Sambunjak et al., 2019). The chlorhexidine mouth rinse products claim antimicrobial activity

and can assist in reducing dental plaque but introduce some adverse effects including alteration of taste, numbness in the mouth and tongue and even swelling of the parotid gland (Deus & Ouanounou, 2022). The most unacceptable adverse effect of chlorhexidine mouth rinse products by consumers is the tooth staining effect that increases tartar on the tooth surface (Deus & Ouanounou, 2022).

These days, more people are concerned about the security of their consumers' purchases. Consumers prefer natural products to synthetic ones as most are worried about the side effects that could present in the future, although the products were approved to be safe to use (Scott et al., 2020). Natural products derived from medicinal plants have gained attention due to their diverse bioactive compounds and potential antimicrobial properties. *Piper sarmentosum*, commonly known as the daun kaduk, is an edible plant native to tropical and subtropical areas belonging to the *Piperceae* family (Sun et al., 2020). It has gained attention from researchers as the properties possessed by the phytochemicals produced by these plants are profitable and beneficial to humans (Sun et al., 2020).

The growth requirement of *P. sarmentosum* is not fastidious and widely distributed in Southeast Asia, especially Thailand and Malaysia (Sun et al., 2020). According to Sun et al., 2020, *P. sarmentosum*'s leaf has the potential to medicate dental carries and toothaches. *P. sarmentosum* also exhibited antimicrobial activity (Thongphichai et al., 2023) towards various bacteria, such as *Pseudomonas aeroginosa* and *Escherichia coli* (Sanusi et al., 2017). Extracts from the leaves of plants contain significant amounts of bioactive compounds such as polyphenols, primarily flavonoids, which have been shown to have antibiofilm and antibacterial properties (Sun et al., 2020).

Consequently, the phytochemical produced by P*. sarmentosum* could become an antimicrobial agent for pathogenic bacteria such as *S. mutans* and *S. sobrinus*. The phytochemicals could be extracted from plants and possess the same characteristics as chlorhexidine, which could indirectly resolve the side effects of chlorhexidine and contribute to healthcare settings.

2.0 EXPERIMENTAL

2.1 Preparation of *P. sarmentosum* extract

The *P. sarmentosum* samples were obtained from Taman Senai Utama, it appeared node-by-node rooting accompanied by a unique and recognizable scent, which aligned with the study of Sun et al. (2020). The leaves of *P. sarmentosum* appeared in a heart shape with a waxy surface. *P. sarmentosum* was found in a tropical area and under a shady tree, which was consistent with the findings of Rahman (2016). The leaves collected were separated from the barks before proceeding to the next procedure. The leaves of *P. sarmentosum* were cleaned under running water and dried at 50 ℃ for 24 hours in an oven (Protech, Malaysia) (Rahman et al., 2021). The leaves were then ground into fine powder with a grinder (Waring Commercial, USA). The remaining fine powder of *P. sarmentosum* was stored in an airtight container for further use. The fine powder of *P. sarmentosum* was mixed with 80% methanol and 95% ethanol (Merck, USA) with a solid-liquid ratio of 1:10 placed in an Erlenmeyer flask, and shaken with 100 rpm. The extract of *P. sarmentosum* was filtered with Whatman No.101 filter paper and proceeded to the concentration process using rotary vapour (IKA/RV10, Germany) under a vacuum at 40° C (Syed-Ab-Rahman et al., 2014). The extraction product was freeze-dried (Labogene, Denmark) to obtain powdery form products. The percentage of yield was determined by employing the following formula:

$$\text{Percentage of yield (\%)} = \frac{Weight\ of\ extract\ (in\ g)}{Weight\ of\ samples\ (in\ g)}\ x100\%$$

2.2 Total Phenolic Compound (TPC), Total Flavonoid Compound (TFC) and GC-MS Analysis

The total phenolic compound was determined using the Folin-Ciocalteu reagent method (Yun & Pa'ee, 2022). 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent (Supelco, USA) was added to 0.5 mL of *P. sarmentosum* extract and incubated under room temperature for 8 minutes, followed by adding 2 mL of 7.5%(w/v) sodium carbonate (Na_2CO_3) (Sigma-Alrich, USA). The mixture was vortexed and incubated at room temperature for 1 hour. The solutions were poured into cuvettes after an hour, and a UV-vis spectrophotometer (Beckman Coulter, US) was used to measure the absorbance at 765 nm. Standard solutions with concentrations ranging from 20 µg/mL to 100 µg/mL were constructed using a simple dilution process utilizing gallic acid (Chemiz, Malaysia) as the standard stock solution. All of the procedures were done in triplicate and the results were expressed in mgGAE/ gDE.

The aluminium chloride colourimetric method was used to determine the total flavonoid content (TFC) of the *P. sarmentosum* extracts. The total flavonoid content was measured using a UV-vis spectrophotometer (Yun & Pa'ee, 2022). 4 mL sterile distilled water and 0.3 mL 5% (w/v) sodium nitrite ($NaNO_2$) (Sigma-Alrich, USA) were added to 1 mL of *P. sarmentosum* extract and incubated for 5 minutes under room temperature followed by adding 0.3 mL of 10% (w/v) aluminium chloride ($AlCl_3$) (Sigma-Alrich, USA) to the mixture and incubated for 6 minutes. 2 mL of 1M sodium hydroxide (NaOH) (Merck, USA) was added to the mixture and followed by 2.4 mL of sterile distilled water, making the final volume of the mixture 10 mL. The mixture was thoroughly mixed before being incubated for 15 minutes. The solution was placed into a cuvette after 15 minutes and a UV-Vis spectrophotometer (Beckman Coulter, US) was used to detect the absorbance at 510 nm. Standard

stock solutions for quercetin were prepared using a simple dilution process. Standard solutions with concentrations ranging from 20 µg/mL to 100 µg/mL were prepared for the standard curve and the results were expressed in mgQE/gDE.

In advance of GC-MS analysis, Derivatization by silylation using N, O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was performed to enhance the detection sensitivity. 1 mg of *P.sarmentosum* leaves methanolic extract was prepared in an Eppendorf tube. Pyridine was used to prepare 1 ml of methoxyamine hydrochloride with a concentration of 15 mg/ml. Then, 50 µL of methoxyamine hydrochloride was added to the plant extract and vortexed for 30 seconds followed by incubation at 37 ˚C for 90 minutes. After incubation, 80 µL of derivatizing agent, BSTFA with 1% trimethylchlorosilane (TMCS) and 20 µL of n-hexane were added to the mixture. The mixture was then vortexed for 2 minutes and further incubated at 70 ˚C for 60 minutes. After the incubation period, the mixture was subjected to centrifugation at 13,000 x g for 15 minutes. The supernatant was collected and transferred with extra care into a GC-MS glass vial for GC-MS analysis by using the GC-MS QP-2010 system in T03, UTM (Sobrinho et al., 2022).

2.3 Biofilm-Inhibitory Assay

The minimum inhibitory concentration (MIC) of *P. sarmentosum* extract was determined using an MTT assay (Noor et al., 2013) using 96 well plates. A series of serial dilutions were performed on the *P. sarmentosum* extract (from 0.78 mg/mL to 100 mg/mL). As preparation for the assay, the overnight culture of *S. mutans* was aliquoted into a falcon tube and centrifuged at 3000 rpm for 15 minutes. The supernatant of the bacterial suspension after centrifugation was discarded and resuspended with 0.9% saline solution. The bacterial culture of *S. mutans* was diluted to approximately 10^5 cells/with the aid of 0.5 McFarland standard, and 100 µL of the bacterial suspension was added to each well of the 96 well-plate according to the designated layout followed by 100 µL of plant extract with designated concentration, the 96-well plate was incubated for 37 ℃ for 24 hours. 50 µL of 3-[4,5-dimethylthiazol2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Elabscience, USA) was added to each of the wells to observe the viable cells. The purple formazan indicates the growth of bacteria, and the pale-yellow appearance indicates no bacterial growth. The positive control was constructed by replacing the plant extract with 0.2% chlorhexidine, while the blank was constructed by replacing the plant extract with 10% DMSO. After the addition of MTT solution, the 96-well plate was covered with aluminium foil and incubated for 3 hours. 5% DMSO was used to solubilize the crystal formation in each well. The minimum concentration of the *P. sarmentosum* extract to inhibit bacterial growth was considered the MIC value, which was observed based on the colour change basis. A similar procedure was repeated by replacing *S. mutans* with *S. sobrinus.*

For the anti-adherence assay, the overnight culture of *S. mutans* was aliquoted into a falcon tube and centrifuged at 3000 rpm for 15 minutes. The supernatant of the bacterial suspension after centrifugation was discarded and resuspended with BHIB and calibrated to OD_{600} =1.0. The anti-adherence assay was performed as represented by Bhadoria et al. (2019). 100µL of *P. sarmentosum* extract with the designated concentration was added to the universal bottle, followed by 100µL of the bacterial suspension. The chlorhexidine (SolarBio Lifescience, China) acts as the positive control, while the universal bottle that only consists of bacterial suspension and DMSO acts as the blank. The universal bottle was inclined at 30 degrees and incubated at 37 ℃ in an anaerobic condition for 24 hours. After the incubation period, The solution in the universal bottle was removed, and the cells that were not tightly attached to the surface were removed by the addition of 0.5M of NaOH (Merck, US). The adhered cells were resuspended with 0.9% saline solution (Fisher Scientific, Canada) and proceeded to the spectrophotometric analysis by using a microplate reader (BMG Labtech, Germany). The percentage of inhibition of adherence was calculated using the formula:

$$\%\ of\ adhered\ cells = \frac{OD600\ of\ adherent\ cells}{OD600\ of\ blank}\ x\ 100$$

The whole process was repeated by replacing the *S. mutans* with *S. sobrinus.*

For the biofilm-inhibitory assay, the overnight culture of *S. mutans* was aliquoted into a falcon tube and centrifuged at 3000 rpm for 15 minutes. The supernatant of the bacterial suspension after centrifugation was discarded and resuspended with BHIB and calibrated to OD_{600} =1.0. A biofilm inhibitory assay was used to determine the percentage of biofilm inhibition contributed by the *P. sarmentosum* extract (Satria et al., 2022). The assay was performed using a 96-well plate, where 100 µL of *S. mutans* bacterial suspension was added to the well, followed by 100 µL of plant extract with the designated concentration and 50 µL of 250 mg/mL sucrose solution. The 96-well plate was incubated at 37 ℃ for 1,3,6 and 24 hours. The 0.2% chlorhexidine served as the positive control, while the 10% DMSO without plant extract served as the blank. After the incubation period, the solution on the 96-well plate was discarded, and each of the wells was washed twice using sterile distilled water. The wells were then stained with 1% crystal violet solution (Chemiz, Malaysia) for 15 minutes to allow the staining of biofilm in the well, and the biofilm was extracted by using 99% absolute ethanol (Merck, USA) and transferred to a new 96 well-plate. The 96 well-plate proceeded to the spectrophotometry analysis by using a microplate reader (BMG Labtech, Germany) at 600 nm (Alvita et al., 2017). The biofilm percentage was calculated by using the formula:

$$\%\ \text{of biofilm inhibition} = \frac{OD600\ of\ blank - OD600\ of\ treated\ group}{OD600\ of\ blank}\ x\ 100$$

The whole process will be repeated by replacing the *S. mutans* with *S. sobrinus.*

All statistical analyses were performed using GraphPad Prism version 9 for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Values were expressed as the means ± standard error mean (SEM) of at least three individual assays. Analysis of variance was performed using the One-Way ANOVA test. A P value < 0.05 was considered statistically significant.

3.0 RESULTS AND DISCUSSION

3.1 Percentage of extraction yield

The selection of 80% methanol and 95% ethanol as solvents in this study was guided by their high polarity, which is particularly effective for extracting a broad spectrum of bioactive compounds (Syed-Ab-Rahman et al., 2014). Methanol and ethanol, at these concentrations, are commonly used in natural product research because they dissolve polar compounds effectively and are less likely to denature sensitive bioactive compounds (Thongphichai et al., 2023). However, the information about the extraction yield, total flavonoid compound and the identity of the bioactive compounds in the *P. sarmentosum* extracted using 80% methanol and 95% ethanol were not stated clearly in the previous study.

The percentage of extraction yield was calculated and presented in Table 1. From the percentage of extraction yield, the 80% methanolic extract and 95% ethanol yielded 11.41% of extract and 9.91% plant extract, respectively. The results obtained coincided with the results obtained by Akmal et al. (2023) for methanolic extract, which is around 11%, and (Me et al., 2020) for ethanolic extract, which is 9.84%. Kraikrathok et al. (2013) suggested that the extraction yield was related to the polarity of the solvent, which means that a solvent with higher polarity could obtain a higher extraction yield.

Table 1: Percentage of extraction yield for both methanolic and ethanolic extract of *P. sarmentosum.*

Types of extract	Percentage of extraction yield (%)
80% methanolic extract	11.41
95% ethanolic extract	9.91

3.2 Total Phenolic Compound (TPC), Total Flavonoid Compound (TFC) and GC-MS Analysis

Figure 1 shows the (a) total phenolic content (TPC) and (b) total flavonoid content (TFC) for both 80% methanolic extract and 95% ethanolic extract and the total phenolic content of the 80% methanolic extract was higher than 95% ethanolic extract. The total phenolic content of 95% methanolic extract obtained was 9.8707 ±0.679 mgDAE/ gDE, which is much lower than the previous result obtained by Thongphichai et al. (2023) which obtained 28.58 mgDAE/ gDE. The total phenolic content for 80% methanolic extract was 20.4984 ±2.878 mgDAE/ gDE, slightly lower than Chan et al. (2014), which obtained a TPC value of 37.9 mgDAE/ gDE. Several factors may affect the TPC of the *P. sarmentosum* extract, as the extraction temperature and solid-liquid ratio were not clearly stated by the authors.

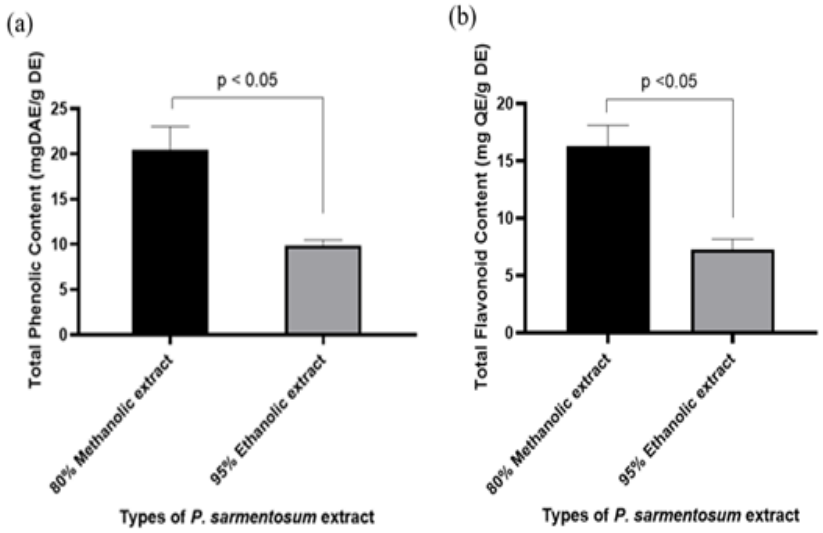

Figure 1(a) Total phenolic content (TPC) and (b) Total flavonoid content (TFC) of 80% methanolic extract and 95% ethanolic extract of *P. sarmentosum*. Results are expressed as means ± SEM, where p < 0.05**.**

The total flavonoid value for both the 80% methanolic and 95% ethanolic extracts was generally lower than the total phenolic content of the respective extracts. Sulaiman and Balachandran (2012) suggested that the value of flavonoid content will be lower than the total phenolic content as the flavonoid was considered a subset of phenolic compounds. Previous results performed by Wairata et al. (2022) also showed that the flavonoid content extracted by 80% methanolic extract will be higher than that of 95% ethanolic extract, as the flavonoid will more likely accumulate in a polar solvent. In general, the phenolic content and the flavonoid content in the *P. sarmentosum* extracted by using 80% methanol will be higher than 95% ethanol. Sultana et al. (2019) demonstrated that the addition of water facilitates the phytochemical extraction process by assisting the solvent penetration into the plant tissues. Therefore, the 80% methanol which consists of a higher water level than 95% ethanol, can yield a higher content of phenolic and flavonoid compounds.

Besides factors such as extraction temperature, extraction duration and solid-liquid ratio, the age of the plant also affects the phenolic content of the plant, The phytochemicals content of the plant decreased when it reached the age of 1 year and the action of repeatedly harvesting also caused the reduction of phytochemical content of the plant (Samat et al., 2020). Other than that, Ibrahim et al. (2022) also supported that methanol was more suitable for extracting the phenolic content of a medicinal plant based on the concept of like-dissolve-like and had a higher affinity to extract phenolic compound which is often polar in nature.

Figure 2 shows the gas chromatogram of *P. sarmentosum* extracted with 80% methanol. Table 2 shows the result from the analysis of GC-MS where 8 compounds detected from the analysis had proven to have antibiofilm and antimicrobial abilities towards *S. mutans* and *S. sobrinus*. The fatty acids detected from the samples were in TMS derivatives form.

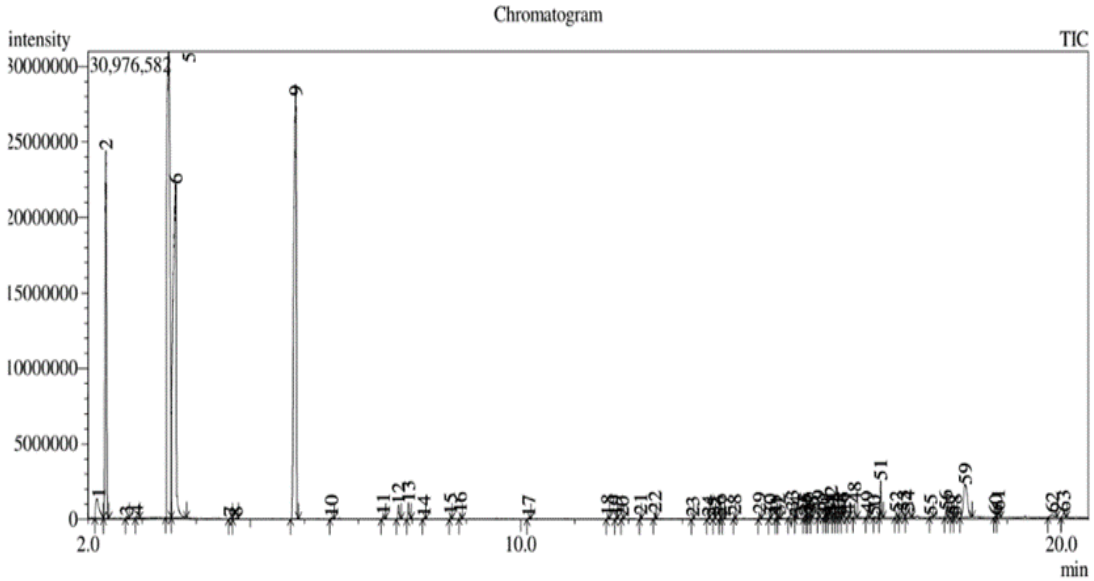

Figure 2 Gas chromatogram of 80% methanolic extract of *Piper sarmentosum* that was derivatized by using BSTFA.

Table 2: Compound detected via GC-MS analysis.

Retention Time (tR)	Peak Area (%)	Compound	Molecular Formula
13.577	0.02	Myristicin	$C_{11}H_{11}O_3$
13.955	0.05	Elemicin	$C_{12}H_{16}O_3$
13.954	0.19	Asarone	$C_{12}H_{16}O_3$
17.870	0.14	Oleic acid	$C_{21}H_{42}O_2Si$
13.181	0.65	Palmitic acid	$C_{19}H_{40}O_2Si$
12.485	0.29	Octanoic acid	$C_{11}H_{24}O_2Si$
16.660	0.74	Stearic acid	$C_{21}H_{44}O_2Si$
12.485	0.29	Lauric acid	$C_{15}H_{32}O_2Si$

Myristicin and Elemicin have been proven to have antimicrobial and antibiofilm ability towards *S. mutans*, where this bioactive compound can also be found in the plant *Myristica fragrans* (Shafiei et al., 2012). Besides, Kalbassi et al. (2022) stated that *E. caryophyllata*, which contains a significant amount of Asarone, reduced biofilm activity in oral bacteria, such as *S. mutans* and *S. sobrinus.* Huang et al. (2011) also proved that fatty acids such as palmitic and octanoic acid can effectively inhibit the growth of *S. mutans*. The other fatty acid, oleic acid, also reduces the EPS production of *Streptococcus* oral bacteria, promoting an antibiofilm effect (Abdel-Aziz et al., 2020). Giacaman et al. (2014) also proposed that the fatty acid chain with the carbon atom between 8 and 15, such as stearic acid, effectively reduced the biofilm development of *S. mutans.*

Lauric acid is also suggested to have antimicrobial activity towards *S. mutans*, where the fatty acid takes part in membrane disruptive behaviour and inhibits the growth of *S. mutans* (Casillas-Vargas et al., 2021).

3.3 Biofilm-inhibitory Assay

Table 3 shows the MIC value for both *S. mutans* and *S. sobrinus* in response to the *P. sarmentosum* extract, which represents 50 mg/mL and 100 mg/mL, respectively. The relatively high MIC values suggest that *P. sarmentosum* extract may have limited efficacy at lower concentrations, highlighting the need for higher doses to achieve antimicrobial effects against these bacteria.

Table 3: Minimum inhibitory concentration (MIC) of *P. sarmentosum* extract towards *S. mutans* and *S. sobrinus.*

Oral pathogen	MIC value (mg/mL)
S. mutans	50
S. sobrinus	100

According to Okwu et al. (2019), an antimicrobial agent with a MIC value below 8 mg/mL was considered suitable with acceptable antimicrobial ability. However, the MIC values for both *S. mutans* and *S. sobrinus* are greater than 8 mg/mL, which indicates that the ability of *P. sarmentosum* extract was insufficient to inhibit the growth of oral pathogens such as *S. mutans* and *S. sobrinus*. This can be related to the bioactive compounds present in the 80% methanolic extract of *P. sarmentosum.* Several fatty acids, such as palmitic acid and octanoic acid, possess the ability to inhibit the growth of *S. mutans*, which was detected from the GC-MS analysis. However, the amounts of that fatty acid were very small, which are 0.05% and 0.29%. When the concentration of the extract increases, the amount of bioactive components in the extract will also increase. This statement can also explain the high MIC values in *S. mutans* and *S. sobrinus* (Haghgoo et al., 2017). The plant *Myristica fragrans* showed a MIC value of 20 mg/mL towards *S. mutans*, which is lower than the *P. sarmentosum* extract used in this experiment. The difference in the amount of bioactive compound in both plant extracts can explain this. The myristicin, which can inhibit the growth of *S. mutans* and *S. sobrinus,* was found in both extracts. However, the myristicin in *P. sarmentosum* only exists at 0.02%, but it represents the main constituent in the bioactive compound of *Myristica fragrans* (Shafiei et al., 2012).

Figure 3 shows the graph of the percentage of adhered cells (%) for (a) *S. mutans* and (b) *S. sobrinus* in the presence of 80% methanolic *P. sarmentosum* extract. This experiment displayed that the depletion of oral pathogen adherence ability on the glass surface by *P. sarmentosum* leaf extract was found to be the dose-dependent effect, in which the oral pathogen adherence activity was negatively correlated with plant extract concentration. The highest anti-adherence activity for both *S. mutans* and *S. sobrinus* was demonstrated when 100 mg/mL of *P. sarmentosum* extract was added to both the oral pathogen, which showed only 56.707 ±0.502% and 47.091±0.956% adhered cells respectively. The results showed that the 80% methanolic extract of *P. sarmentosum* exerted more of an anti-adherence effect on *S. sobrinus* than on *S. mutans*.

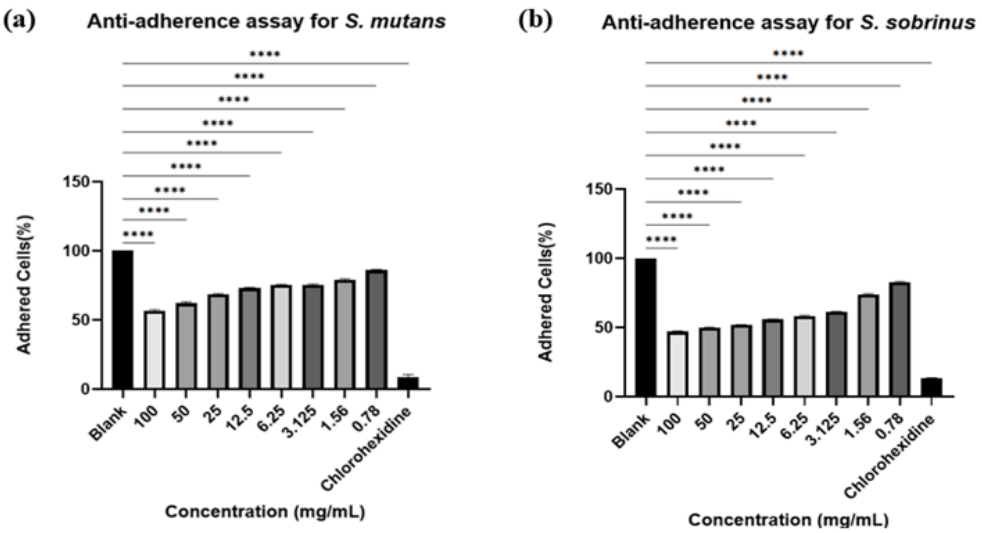

Figure 3 The percentage of adhered cells (%) for (a) *S. mutans* and (b) *S. sobrinus* in the presence of 80% methanolic *P. sarmentosum* extract. Results were expressed as mean ±SEM, **** indicates the significant differences between the treated group and blank when $P < 0.001$ in the One-way ANOVA test.

This can be explained by adding 0.25% sucrose in performing the assay; sucrose was added to activate the glucosyltransferase to synthesize the glucan, which promotes tight cell cohesion on the plaque and adhering abilities (Waldman et al., 2023). According to Saravia et al. (2011), *S. sobrinus* relies more on the presence of sucrose as the

compound to be metabolized and activate the glucosyltransferase for the production of sticky glucan to allow the adherence of the cells compared with *S. mutans*. However, fatty acids such as palmitic acid, stearic acid and oleic acid can down-regulate the activity of glucosyltransferase and decrease the synthesis of glucans that are needed by the oral pathogen (Pakpahan & Fadilah, 2011). Since palmitic acid, stearic acid and oleic acid are present in the *P. sarmentosum* extract, therefore, it can reduce the synthesis of glucan and affecting more on *S. sobrinus*, which mainly relies on the sucrose-dependent mechanism in the glucan synthesis.

Figures 4 and 5 show the graph of the percentage of biofilm inhibition for *S. sobrinus* and *S. mutans*, respectively, when it is exposed to different concentrations of *P. sarmentosum* at 1, 3, 6 and 24 hours. It showed that the highest biofilm-inhibitory activity was achieved during the exposure time at 1 hour, where the percentage of biofilm-inhibitory activity achieved 68.293±0.625% and 67.316±0.625% for *S. sobrinus* and *S. mutans,* respectively. The biofilm-inhibitory activity of the *P. sarmentosum* to both the *S. sobrinus* and *S. mutans* can be achieved due to the presence of bioactive compounds that are able to exert a biofilm-inhibitory effect towards the oral pathogen. Abdel-Aziz et al. (2020) proved that the presence of oleic acid can hinder the primary attachment of the oral pathogen to the tooth surface to form the biofilm. Besides, oleic acid can also reduce the production of EPS and contribute to the biofilm-inhibitory effect (Abdel-Aziz et al., 2020). Besides, palmitic acid present in the *P. sarmentosum* extract can down-regulate glucosyltransferase (*gtf*), which is important in the development of biofilm of oral pathogens. Indirectly, it also promotes biofilm-inhibitory activities in oral pathogens such as *S. sobrinus* and *S. mutans* (Zhang et al., 2021).

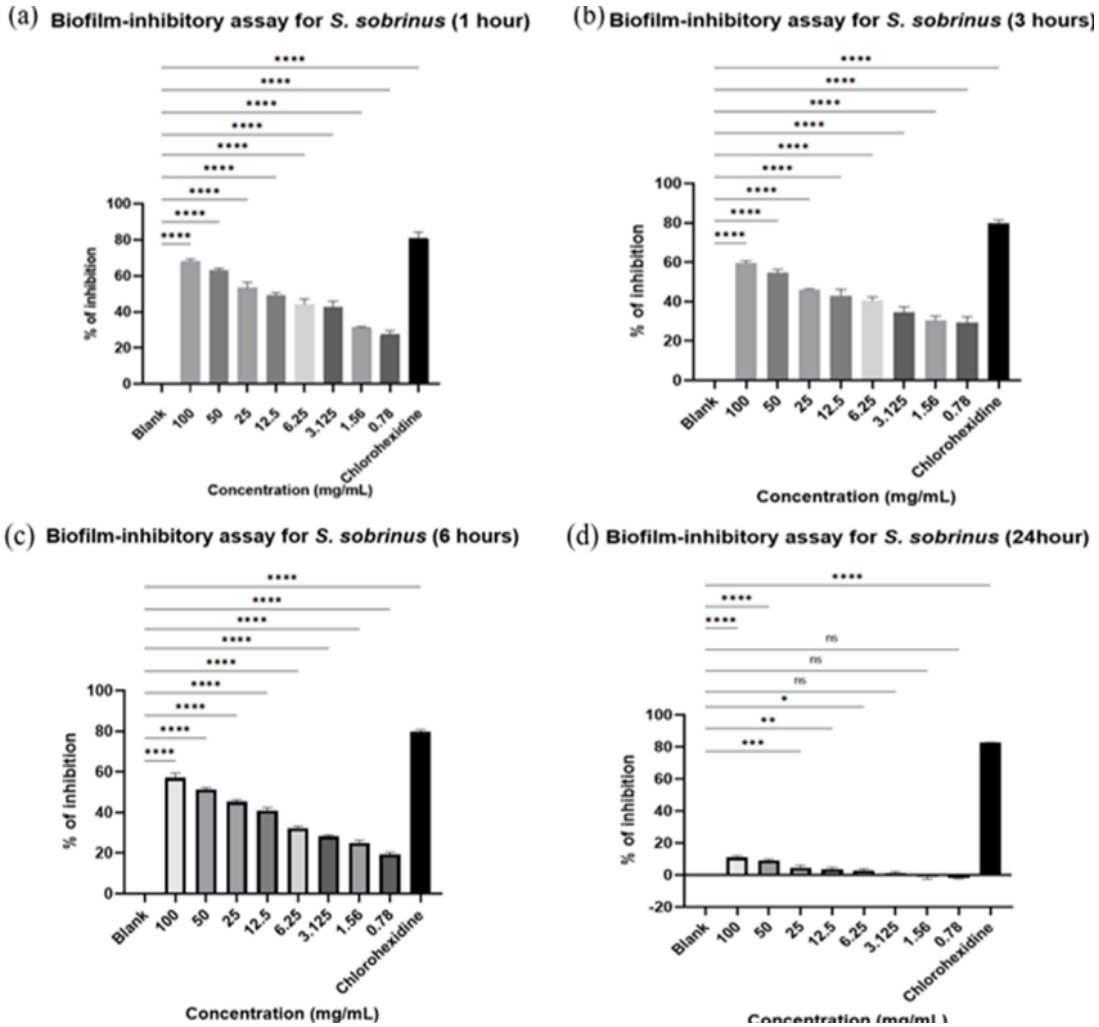

Figure 4 The percentage of biofilm inhibition for *S. sobrinus* when it is exposed to different concentrations of *P. sarmentosum* at (a)1 hour, (b) 3 hours, (c) 6 hours and (d) 24 hours. Results were expressed as mean ±SEM, **** indicates the significant differences between the treated group and blank when P < 0.0001, *** indicates the significant difference between the treated group and blank when0.001< P<0.005, ** indicates the significant difference between the treated group and blank when P<0.01, * indicated the significant differences between the treated group and blank when P<0.05 whereas non-significant (ns) indicates no significant difference between treated medium and untreated bacteria when P<0.05 in One-way ANOVA test.

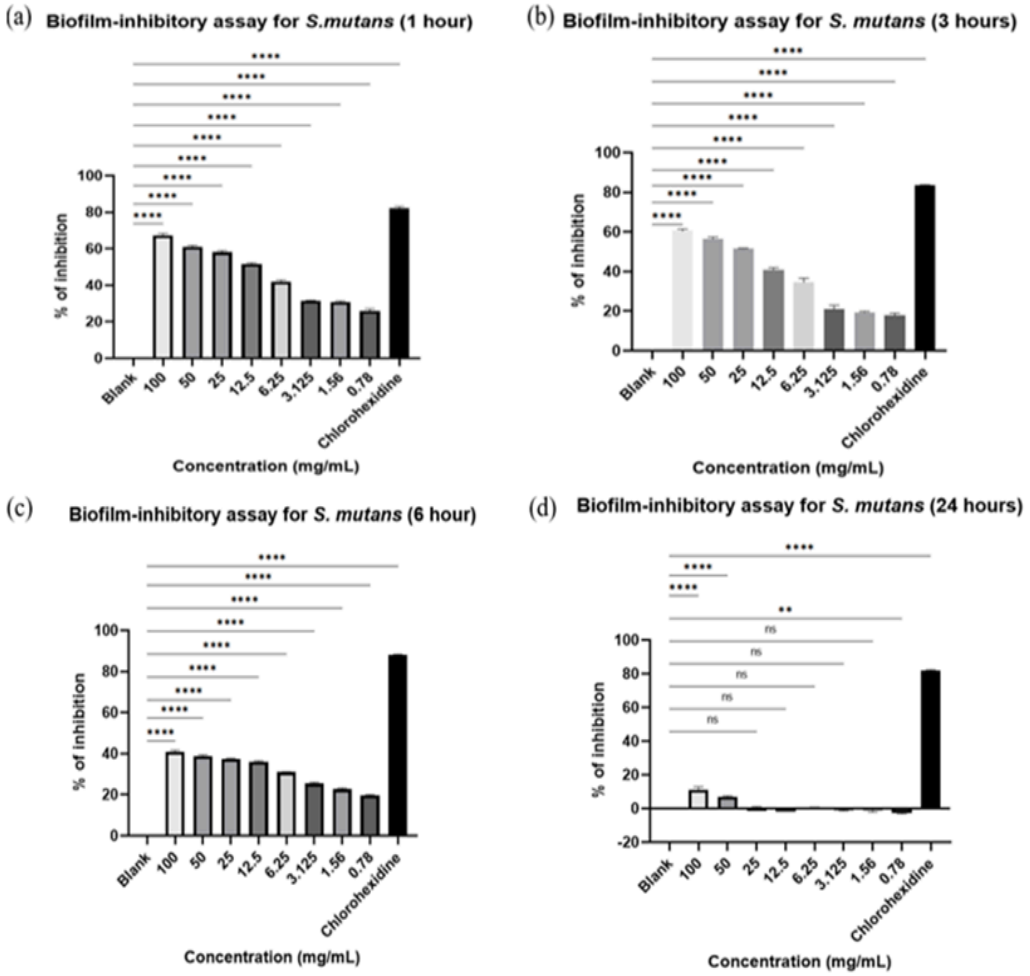

Figure 5 The percentage of biofilm inhibition for *S. mutans* when it is exposed to different concentrations of *P. sarmentosum* at (a)1 hour, (b) 3 hours, (c) 6 hours and (d) 24 hours. Results were expressed as mean ±SEM, **** indicates the significant differences between the treated group and blank when P < 0.001, * indicates the significant difference between the treated group and blank when P<0.005 whereas non-significant (ns) indicates the significant difference between treated medium and untreated bacteria when P<0.05 in One-way ANOVA test.

Other than that, it was investigated that the essential oil of *C. longa,* which also consists of palmitic acid, is able to inhibit the biofilm formation by 80% of *S. mutans* at lower concentrations, which is 2 mg/mL (Lee et al., 2011) compared with *P. sarmentosum* extract. The possible reason for this was the essential oil of C. longa consists of palmitic acid in abundant amounts compared with *P. sarmentosum* extract, which represents 2.05 % and 0.65%, respectively. Moreover, the essential oil of *C. longum* also yielded a significant amount of linoleic acid that also plays a role in the biofilm-inhibitory effect. However, the linoleic acid was absent in the *P. sarmentosum* extract.

The development of biofilm by oral pathogen was the most significant stage of the development of dental plaques. The formation of biofilm by oral pathogen begins when the planktonic bacteria start to settle down on the acquired pellicle. During this stage, the attachment of the bacteria is still considered reversible, meaning that it can be eliminated effortlessly (Rath et al., 2021). This phenomenon can elucidate the biofilm-inhibitory activity of the *S. sobrinus* and *S. mutans* when exposed to *P. sarmentosum* extract for 1 hour. After the reversible attachment stage, the oral pathogen will coaggregate together and further develop into an adhesive slimy layer whose composition includes extracellular polysaccharides, cell debris, and nucleic acid, which is also known as EPS.

The formation of EPS also indicates the beginning of irreversible attachment of the oral pathogen, which is more difficult to eliminate (Rath et al., 2021). After the development of biofilm, the characteristic of resistance to environmental stress and drugs will be developed (Sintim & Gürsoy, 2016). Moreover, Høiby et al. (2010) also stated that biofilm-growing bacteria are easy to mutate due to an increase in horizontal gene transmission in the biofilm. Thus, when the genes that have been passed to other cells are antibiotic-resistant genes, the bacteria are able to produce drug-degrading enzymes and adapt to the environmental conditions quickly (Høiby et al., 2010). This phenomenon can explain the fact that the biofilm-inhibitory effect decreased over the exposure time as the drug-resistant behaviour developed within the oral pathogen.

It is important to identify the optimum doses of the *P. sarmentosum* extract onto the oral pathogen, including ensuring that the *P. sarmentosum* extract can perform its intended function to the best of its ability. Besides, it also provides a baseline for future studies to explore variations, combinations with other compounds, or new formulations, allowing for the

advancement of knowledge about the *P. sarmentosum* extract's potential treatment strategies. By determining the optimum exposure time of the *P. sarmentosum* extract onto the oral pathogen, provided the information that the *P. sarmentosum* extract was effective during the early stage of biofilm development. The *P. sarmentosum* extracts were not effective in inhibiting the biofilm formation when the oral pathogen had reached the biofilm-maturation stage. From the findings, *P. sarmentosum* extract could be recommended as a preventive solution, potentially in products that are applied regularly to inhibit early-stage biofilm formation. However, additional strategies or combined treatments may be required to target and disrupt mature biofilms effectively.

4.0 CONCLUSION

This study primarily used to identify the optimal extraction solvent used to extract the phytochemicals of *P. sarmentosum* and screen the phytochemicals extracted from P*. sarmentosum* that had antimicrobial and antibiofilm activity towards dental-plaque causing bacteria, especially *S. mutans* and *S. sobrinus* by GC-MS. In this study, 80% methanol was identified as the better extraction solvent compared with 95% ethanol as it displayed a higher value of extraction yield, total phenolic compound (TPC) and total flavonoid compound (TFC). From the results of GC-MS, several phytochemicals had been detected to affect the antimicrobial and antibiofilm activity towards *S. mutans* and *S. sobrinus* which includes, myristicin, elemicin, palmitic acid, oleic acid, stearic acid and lauric acid. From the results of the MIC assay, show that the MIC value of *P. sarmentosum* extract against *S. mutans* and *S. sobrinus* was 50 mg/mL and 100 mg/mL respectively. For the anti-adherence assay, the highest anti-adherence activity had been recorded for 56.707 ± 0.502% and 47.091 ± 0.956% of cells that remained adhered to *S. mutans* and *S. sobrinus*, respectively. For the biofilm-inhibitory activity, the highest inhibitory activity was recorded as 67.316±0.625% and 68.293±0.625% for *S. mutans* and *S. sobrinus*, respectively when exposed to the *P. sarmentosum* extract for 1 hour. In a nutshell, this study provides preliminary data on *P. sarmentosum* leaf extract which potentially implies future research in natural antimicrobial products, especially in dental healthcare.

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