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Research Article

Antibacterial activity of Medan Butterfly pea (*Clitoria ternatea* L.) corolla extract against *Streptococcus mutans* ATCC®25175™ and *Staphylococcus aureus* ATCC®6538™

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Abstract

Streptococcus mutans (*S. mutans*) and *Staphylococcus aureus* (*S. aureus*) pathogenicity that alter biofim, has become one of risk factor in orthodontic treatment. The medicinal plant's Butterfly pea efficacy as an antibacterial agent should be confirmed in dentistry. The purpose of this study is to investigate the antibacterial activity of Medan butterfly pea corolla extract (BPCE) against *S. mutans* ATCC®25175™ and *S. aureus* ATCC®6538™. This is a laboratory experiment with Post Test Only Group Design. The minimum inhibitory concentration of BPCE is 6.25 mg/mL. The best concentration of butterfly pea extract to inhibit biofilm formation (antibiofilm) is 100 mg/mL. There was a significant difference ($p < 0.05$) for antibiofilm activity assays and determination of intramembrane cellular leakage. Although Medan BPCE was inadequate enough in forming antibiofilm and caused intramembrane leakage of *S. mutans* and *S. aureus*, further studies in exploring the potential morphological traits of these herbs related to orthodontic products are quite promising.

Keywords

butterfly pea, *Streptococcus mutans*, *Staphylococcus aureus*

Introduction

Colonies of *Streptococcus mutans* (*S. mutans*) as oral bacteria on the tooth surface could decrease the pH of the oral cavity to a critical level which will cause demineralization of enamel and leads to caries. As a result, the teeth will not function optimally and increase the risk of build-up of plaque in the gingival region, which can lead to gingivitis and periodontitis (Forssten et al. 2010; Dani et al. 2016). The role of *Staphylococcus aureus* (*S. aureus*) has also been considered in the differential diagnosis of oral cavity disease and cross-infection sources in a large dataset of retrospective laboratory data (McCormack et al. 2015).

The salivary pellicle is a mediator in which oral bacteria can attach to tooth surfaces and dental restorations. This pellicle acts as the receptor for several bacteria in the oral cavity, which is the iatrogenic effect of malocclusion or fixed orthodontic treatment. Previous studies stated that the saliva pellicle plays a significant role in initiating oral

Streptococcus and *Staphylococcus* bacteria inside the oral cavity, especially in fixed orthodontic components. There is a two-step mechanism of bacteria adhesion in the oral cavity. In the initial stage, the bacteria adhere to the surface of oral cavity through the pellicle and multiply until the pellicle turns into plaque in the second stage. As major oral pathogens, *S. mutans* and *S. aureus* attach to the host through their receptors in the salivary pellicle (Krzyściak et al. 2014; Dani et al. 2016; Taher 2017; Fischer and Aparicio 2021).

Orthodontic patients should follow some instructions in controlling their oral hygiene and diet in order to prevent oral and systemic complications such as plaque accumulation, caries, gingivitis, periodontitis and other systemic problems. Antibacterial mouthwash is one of the daily oral care preventive procedures to minimize those possible complications (Taher 2017). Oral mouthwashes that are prescribed for fixed orthodontic patients can be useful as a coadjuvant in controlling the cariogenic biofilm (Pithon et al. 2015). Especially during pandemic COVID-19, using the preprocedural mouthwashes is recommended before oral procedures to reduce the crossinfection risk and SARS-CoV-2 viral load while treating patients(Vergara-Buenaventura and Castro-Ruiz 2020). Although there is no difference in oral management between chlorhexidine and herbal mouthwashes in gingivitis patients, the herbal mouthwashes are favourable due to minimal or no side effects and are less harmful (Kalkundri and Dinnimath 2018; Cai et al. 2020; Reddy T. and Preethi 2020).

Development of innovative and cost-effective herbal-based products by supplying and updating current antibacterial activity information from various plants in some tropical countries is to improve the community efforts to live healthy and independent. Among the various types of medicinal plants available in Indonesia, the evidence based-health of butterfly pea (*Clitoria ternatea* L.), which is well-known as 'kembang telang' has been reported in multi-sectoral studies, especially in the health sector (Muhammad Ezzudin and Rabeta 2018; Marpaung 2020; Purba 2020). This herbal provides antioxidant, antidiabetic, hepatoprotective, antiasthmatic, anti-inflammatory, anticancer, and antimicrobial based on the phytochemical components, such as flavonoids, anthocyanins, flavonol glycosides, kaempferol glycosides, quercetin glycosides, and myricetin glycosides. (Kamilla et al. 2009; Pratap Gowd et al. 2012; Lijon et al. 2017; Chusak et al. 2018; Widyarman et al. 2018; Kumar and More 2019; Lakshan et al. 2019; Haditio et al. 2021). This is an important perennial herbaceous plant that has morphological variations with the genetic richness of some tropical countries in the world, including Indonesia (Oguis et al. 2019; Suarna and Wijaya 2021). The inhibition zone of butterfly pea is more effective against *Staphylococcus aureus* than *S. mutans*. Due to previous studies that have reported about secondary metabolites that have been produced by butterfly pea herb (stem, leaf, flower, seed, and root), the phytochemical screening reported that flavonoids were found in flower, seed, and root (Kamilla et al. 2009; Anthika et al. 2015).

There are some major bioactive compounds that have potential antimicrobial activities and can be isolated from flowers extracts, as follows: phenolics, phenolic acids and quinones, tannins, terpenoids and essential oils, glycosides, and alkaloids (Voon et al. 2012; Siahaan and Aryastami 2018). The importance of the chemical constituents and pharmacological effects of Indonesia Butterfly Pea, have been reported in some local health studies in Indonesia (Angriani 2019; Marpaung 2020; Purba 2020; Haditio et al. 2021). There are several chemical compounds were reported in butterfly pea herb, such as kaempferol, quercetin, myricetin, taxaxerol, tannic acid, 3-monoglucoside, β-sitosterol, delphiniein-3,5-*O*-bisglucoside, malvidin-3-*O*-β-glucoside, p-hydroxycinnamic acid, ethyl-α-D-galactopyranoside, anthoxanthin glucoside, kaempferol-3 neohesperidoside, quercetin 3-neohesperidoside, hexacosanol, myricetin-3-*O*-neohesperidoside, myricetin-3-*O*-rutinoside, kaempferol-3-glucoside (Pendbhaje et al. 2011). The present study of butterfly pea in Bali Island in Indonesia showed that the flower has the most variable organ and is easily recognized with variance in colour and structure of corollas (Suarna and Wijaya 2021). As a nation with the second-largest biodiversity in the world, the Indonesian archipelago enables the development of butterfly pea essential oils as novel and cost-effective herbal-based medicines for treating a variety of ailments, including dentistry that should be considered nowadays.

Based on the previous studies that reported the benefits of Butterfly pea flower, the goal of this study is to analysis the antibacterial activity of Medan butterfly pea corolla extract against *S. mutans* ATCC®25175™ and *S. aureus* ATCC®6538™. The assessment of antibacterial was based on antibiofilm activities and membrane cell leakage of DNA, protein, calcium, and potassium ions.

Methods

Preparation and identification of butterfly pea flower extract (BPCE)

The BPCE is derived from the medicinal plants family that grows in the yard of residents at Medan Polonia district, North Sumatra, Indonesia. Those flowers were originated from pod separation by taking the whole normal dark blue corolla (Fig. 1). The corolla was then cleanly washed using running water and then were dried in a low-temperature oven at under 50 °C and then ground into powder. The powder was blended and dissolved in absolute ethanol until it was possible to obtain butterfly pea corolla extract. Following that, the extract was divided into four concentrations (12.5 mg/mL, 25 mg/mL, 50 mg/mL, and 100 mg/mL) using dimethylsulfoxide (DMSO). (Pratap Gowd et al. 2012).

Figure 1. Photographic image of Medan Butterfly Pea.

Streptococcus mutans and *Staphylococcus aureus* culture

S. mutans ATCC®25175™ was cultured with a liquid media of Tryptone-Yeast-Cysteine-Sucrose-Bacitracin (TYCSB) and *S. aureus* ATCC®6538™ was cultured with a liquid media Nutrient agar at 37 °C for 24 hours in the Universitas Sumatera Utara Pharmacy Faculty's microbiology laboratory.

Total phenol concentration (TPC) determination

A folin reagent was used to assess total phenol concentration (TPC) of sample. Briefly, 100 mL of BPCE (500 µg/mL) was mixed with 7.9 mL of distilled water and 0.5 mL of folin-reagent ciocalteu's (1:10 v/v) and vortexed for 1 min. After mixing, 1.5 mL of 20% aqueous sodium bicarbonate was added, and the mixture was left to stand for 90 min while being shaken intermittently. A UV/Vis spectrophotometer was used to measure the absorbance at 775 nm. Total phenolic content was measured in milligrams of gallic acid equivalent per gram of extract. The methanol solution served as a control. All experiments were performed in triplicate (Satria et al. 2017). The formula for calculating total phenolic concentration:

$$C(GAE) = \frac{c \times V}{M} \times F$$

Abbreviations:

C (GAE)	concentration determined from a standard curve (µg/mL)
c	concentration of phenolic as gallic acid equivalent
V	the volume utilized in the assay (mL)
M	mass of the sample used in the experiment (g)
F	dilution factor

Total flavonoid concentration (TFC) determination

As previously reported, the total flavonoids in the extracts were measured spectrophotometrically. Concisely, 2 mL of BPCE in methanol was combined with 0.10 mL of 10% aluminium chloride ($AlCl_3.6H_2O$), 0.10 mL of 1 M sodium acetate ($NaC_2H_3O_2.3H_2O$), and 2.80 mL of distilled water. A UV/Vis spectrophotometer was used to detect absorbance at 432 nm after 40 min of incubation. We created a calibration curve with quercetin as the standard to calculate the content of flavonoids. The flavonoid concentration was given in milligrams per gram of extract as quercetin equivalents. All experiments were performed in triplicate (Jamuna et al. 2012; Satria et al. 2017).The following equation was used to calculate total flavonoid concentration:

$$C(QE) = \frac{c \times V}{M} \times F$$

Abbreviations:

C (QE)	Flavonoid concentration as a quercetin equivalent
c	concentration measured from a standard curve (µg/mL)
V	assay volume (mL)
M	mass of the sample used in the experiment (g)
F	dilution factor

The procedure of antibacterial activities assays

Antibacterial activity of BPCE was tested using the MIC (Minimum Inhibitory Concentration) test with the diffusion method. A series concentration of BPCE (12.5, 25, 50, and 100 mg/mL) was used with 0.2% chlorhexidine as positive control and DMSO as a negative control. The minimum inhibitory concentration (MIC) was the lowest concentration of BPCE that could still inhibit the activity of *S. mutans* and *S. aureus* bacterium. The MIC itself could be observed after 24 hours from the inhibitory zone and measured with a digital calliper. (Abd-El-Aziz and Sallam 2020).

Antibiofilm activities assays

Antibiofilm assay was performed using six-well plates. The test was carried out using the various BPCE concentrations ranging from 12.5, 25, 50 and 100 mg/mL. A total of 0.1 mL of bacterial suspension solution and 5 mL of TYCSB (Tryptone-Yeast-Cysteine-Sucrose-Bacitracin) liquid medium and *S. aureus* bacterial suspension in Nutrient Broth were put into well plates and then incubated for 24 hours. After washing the well plates with distilled water, the crystal violet solution was added to the wells. The well plates were then washed using distilled water, and 96% ethanol was added to the well plates. The absorbance was measured with UV/Vis spectrophotometer at

600 nm could be used to quantify absorbance or optical density (OD). Biofilm percentage was calculated using the formula as mentioned below (Alvita et al. 2017):

$$\%\text{biofilm:}\frac{\text{bacterial absorbance value - treatment absorbance}}{\text{bacterial absorbance value}}\times 100\%$$

Determination of DNA and protein leakage

The leakage of DNA and protein was confirmed by determining the integrity of the cell membrane. The experimental was performed based on previous methods (Miksusanti et al. 2008; Budiman and Lia Aulifa 2020). Ten mL of *S. mutans* suspension for 24 h was taken and centrifuged for 15 min at 3500 rpm. The pellets were washed with phosphate buffer pH 7 followed by adding 10 mL of buffer and shaken for 24 h. BPCE was added into the solution with various concentrations (12.5, 25, 50, and 100 mg/mL) then centrifuged at 3500 rpm for 15 min. The supernatant was kept and analyzed spectrophotometrically at 260 nm and 280 nm. This measurement was done at the Industrial Chemical Technology Polytechnic Medan, Indonesia.

Determination of calcium and potassium ions leakage

Samples of bacterial pellets were prepared using a similar method in DNA and protein leakage analysis. Bacterial pellets were treated with various concentrations of BPCE (12.5, 25, 50, and 100 mg/mL) with 0.2% chlorhexidine and DMSO as positive control and negative control, respectively. The amount of calcium and potassium ions leaked were measured by. The measurement used Z-2000 (Hitachi®) of atomic absorption spectroscopy (AAS) 422.7 nm and 766.5 nm. This experiment was performed at Industrial Chemical Technology Polytechnic Medan, Indonesia (Budiman and Lia Aulifa 2020).

Statistical analysis

All experiments were carried out in triplicate and values were expressed as means ± SD (standard deviation). Statistical tests were performed by SPSS version 20.0 with one-way ANOVA and post hoc test. *P* values of less than 0.05 were considered to be statistically significant.

Results

Total phenolic and total flavonoid contents

The Folin Ciocalteau method was used to determine total phenolic content (TPC), which is based on the reduction of phosphomolybdic-phosphotungstic acid (Folin) reagent to a blue-coloured complex in an alkaline solution (Blainski et al. 2013). The phenolic content of the BPCE was found to be high (338.81 0.85 mg GAE/g). When it comes to total flavonoid content (TFC), the BPCE had flavonoid content of 27.9 0.15 mg QE/g. Flavonoids are a class of polyphenolic compounds that have a variety of biological effects.

Antibacterial activities assays

The antibacterial potential of BPCE was tested against susceptible bacterial isolates and shown in Table 1. The

Table 1. Minimum inhibitory zones and biofilm activity assay of Medan BPCE against *S. mutans* and *S. aureus*.

Bacteria	Concentration (mg/mL)	Inhibitory zone (mm)	*P*	Biofilm Activity (%)	*P*
S. mutans	12.5	0.00±0.00	0.001*	48.44±1.37	0.001*
	25	8.43±0.59		58.47±3.46	
	50	9.27±0.48		62.47±5.84	
	100	9.57±0.76		62.69±8.49	
	Negative control	0.00±0.00		0.00±0.00	
	Chlorhexidine 0.2%	16.80±0.79		80.84±2.16	
S. aureus	12.5	6.90±0.27	0.001*	56.16±0.04	0.005*
	25	7.27±0.32		69.13±0.03	
	50	7.90±0.80		74.51±0.02	
	100	8.83±0.38		76.89±0.02	
	Negative control	6.00±0.00		0.00±0.00	
	Chlorhexidine 0.2%	13.77±0.26		75.62±0.02	

*$p < 0.05$: significant difference.

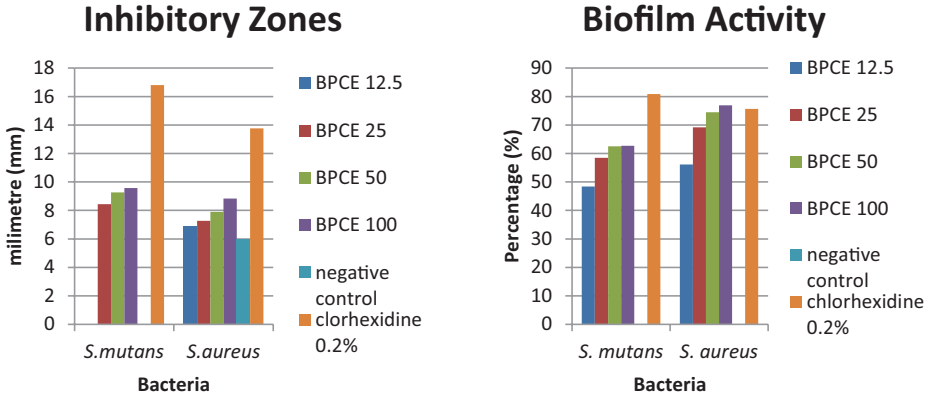

Figure 2. Minimum inhibitory zones and biofilm activity of BPCE against *S. mutans* and *S. aureus* at different concentrations. Each colour represents the millimetre and percentage from different concentrations of BPCE (mg/mL) in each bacteria.

Table 2. Membrane intracellular (DNA and Protein) leakage of Medan BPCE against *S. mutans* and *S. aureus*.

Bacteria	Concentration (mg/mL)	DNA leakage	*P*	Protein leakage	*p*
S. mutans	**12.5**	0.13±0.02	0.001*	0.18±0.02	0.001*
	25	0.27±0.01		0.33±0.01	
	50	0.58±0.01		0.65±0.01	
	100	1.05±0.00		1.17±0.02	
	Negative control	0.00±0.00		0.00±0.00	
	Chlorhexidine 0.2%	0.79±0.02		0.73±0.01	
S. aureus	**12.5**	0.09±0.01	0.001*	0.09±0.01	0.001*
	25	0.25±0.02		0.25±0.01	
	50	0.39±0.03		0.43±0.02	
	100	1.09±0.18		1.12±0.09	
	Negative control	0.01±0.01		0.00±0.00	
	Chlorhexidine 0.2%	0.46±0.02		0.53±0.03	

*$p < 0.05$: significant difference.

Table 3. Calcium and potassium ions leakage of Medan BPCE against *S. mutans* and *S. aureus*.

Bacteria	Concentration (mg/mL)	Calcium leakage	*P*	Potassium leakage	*p*
S. mutans	**12.5**	20.81±2.76	0.001*	9.89±1.31	0.001*
	25	23.15±0.83		11.00±0.40	
	50	72.30±0.03		34.34±0.02	
	100	96.13±0.07		45.66±0.04	
	Negative control	25.92±1.60		12.31±0.76	
	Chlorhexidine 0.2%	67.98±0.03		32.29±0.02	
S. aureus	**12.5**	0.03±0.00	0.001*	0.02±0.00	0.001*
	25	0.05±0.01		0.05±0.00	
	50	0.06±0.00		0.05±0.01	
	100	0.08±0.01		0.07±0.01	
	Negative control	0.01±0.00		0.01±0.00	
	Chlorhexidine 0.2%	0.06±0.00		0.06±0.00	

*$p < 0.05$: significant difference.

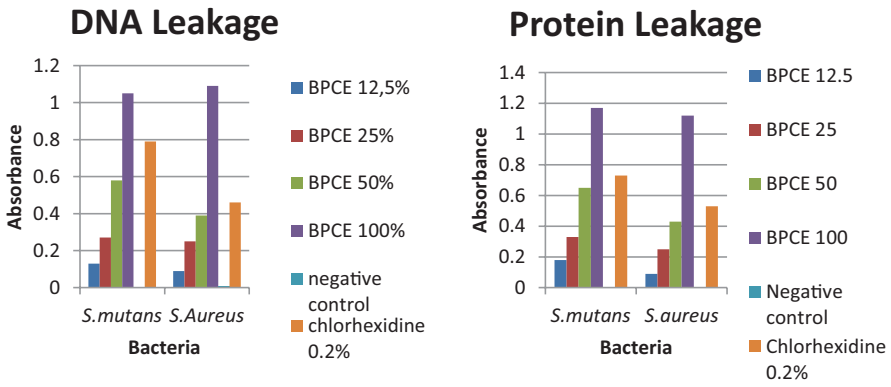

Figure 3. The effect of BPCE on membrane intracellular (DNA and protein) leakage from *S. mutans* and *S. aureus* at different concentrations. Each colour represents the absorbance from different concentrations of BPCE (mg/mL) in each bacteria.

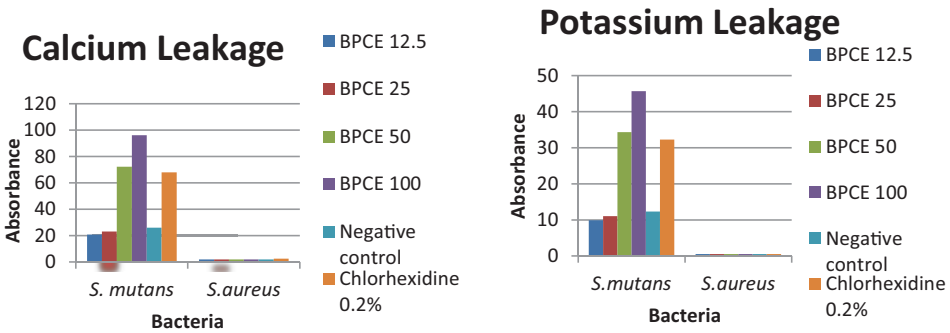

Figure 4. The effect of BPCE on calcium and potassium ions leakage from *S. mutans* and *S. aureus* at different concentrations. Each colour represents the absorbance from different concentrations of BPCE (mg/mL) in each bacteria.

inhibitory zones of BPCE against *S. mutans* presented from the 25 mg/mL MIC and the inhibition zone increased with increasing concentration.

Discussions

The function of butterfly pea as ornamental plants can adapt to all types of soil, from sandy soil to clay and calcareous soils. This plant is quite friendly to tolerate salinity. The saponin, alkaloids, glycosides, phytosterols, and carbohydrates have been reported as phytochemical constituents in butterfly pea flowers (Lijon et al. 2017; Kumar and More 2019). This in-vitro study explored the antibacterial potent of Medan butterfly pea used against *S. mutans* ATCC®25175™ and *S. aureus* ATCC®6538.

Based on Table 1, there was a significant difference ($p < 0.05$) for both minimum inhibition zone and

biofilm activity assay of Medan BPCE against *S. mutans* and *S. aureus*. The inhibition zone of 50 mg/mL BPCE (Medan BPCE) against *S. mutans* was 9.27±0.48 mm, meanwhile for Indian BPCE, the inhibition zone was 7 mm. In addition, compared to Indian BPCE (10 mm) (Pratap Gowd et al. 2012), the inhibition zone of Medan BPCE for *S. aureus* was 7.90±0.80 mm which was smaller and more active than Indian BPCE. Other literature also reported that antibacterial activity of gram-positive was found with an inhibition zone of 13±1 mm at 100 mg/mL, higher than 0.2% chlorhexidine (Kamilla et al. 2009). Although there was a significant difference between Medan BPCE and chlorhexidine, the biofilm activity of Medan BPCE against *S. aureus* with the concentration of 100 mg/mL is higher than chlorhexidine as a reference. This might be due to genes encoding enzymes involved in glycolysis or fermentation, such as phosphoglycerate mutase, triosephosphate isomerase, and alcohol dehydrogenase in *S. aureus*, causing oxygen restriction that leads to disruption of genes during biofilm development. The presence of flavonoid compounds in butterfly pea flower inhibited biofilm formation by inactivation of glucosyltransferase enzymes, which play an important role in forming the biofilm (Krzyściak et al. 2014). Besides, a methicillin-sensitive *S. aureus* (MSSA) strain biofilm production could be inhibited by quercetin since it can exposed the significant anti-inflammatory potential in different cell types (Yang et al. 2020). Thus, this Medan BPCE itself is not so effective as an antibiofilm for gram-positive bacteria whilst the leaf of butterfly pea that contained quercetin is more effective (Lijon et al. 2017).

The flavonoids in the root and flower of the butterfly pea could harm the permeability of the gram-positive bacteria's cell wall, microsomes, and lysosomes since it contains a variety of health-promoting advantages and colouring agents (Panche et al. 2016). The interaction between Medan BPCE and DNA of bacteria (Table 2) related to flavonoids that prevent bacterial cell division and able to harm the bacterial cell walls and cytoplasm by reducing membrane fluidity of bacterial cells (Sankari et al. 2014). Flavonoids could also inhibit the bacterial respiratory electron transport chain, causing bacteria to lack energy in producing macromolecules (Kumar and Pandey 2013). In the previous research, flavonoids showed highly inhibitory activity toward *S. mutans* in prevent oral biofilm formation (Salmanli 2021). Plaque formation by sucrose-dependent mechanism was based on Glucosyltransferase (GTF), produced by combination of *S. mutans* and glucan-binding proteins (GBP). GTF played an important role in the development of virulent dental plaque and responsible for formation of glucans. Moreover, Protein antigen c is the main surface protein of *S. mutans* which related to the development of dental caries caused by its interaction with salivary pellicle on the tooth surface. The reaction of GTF and GBP produced dental plaque which led to dental caries (Krzyściak et al. 2014; Dani et al. 2016; Abbas et al. 2017; Fischer and Aparicio 2021).

Based on Table 3, the concentration of calcium ions was 3.10 mg/g and potassium ions was 1.25 mg/g in butterfly pea extract. The leakage of calcium and potassium ions of *S. mutans* in BPCE is greater than chlorhexidine in MIC 100 mg/mL since calcium and potassium were also contained in butterfly pea. The metal leakage of calcium and potassium of *S. mutans* in BPCE is greater than chlorhexidine in MIC 100 mg/mL because calcium and potassium are also contained in butterfly pea (Muhammad Ezzudin and Rabeta 2018). This finding suggests that flavonoids can affect the bacterial cell wall which calcium ions as a component of cell wall and obstructs the cell membrane permeability that contain potassium ions and causing damage to the cell wall and membrane (Muhammad Ezzudin and Rabeta 2018). This process causes the leakage of the cytoplasm, that contains DNA, proteins, calcium and potassium ions. The leakage of DNA, protein, calcium and potassium ions from cells causes the death of *S. mutans* bacteria that were produced by tannin metabolites (Budiman and Lia Aulifa 2020).

Even though the in-vitro study of antibacterial activity of Medan BPCE against *S. mutans* ATCC®25175™ and *S. aureus* ATCC®6538™ are not so effective, , evaluation of biofilm colonization in any dental material used during treatment, may prevent some avoidable orthodontic complications. Since butterfly pea has become a regional natural dye in the food industry due to it's stability in room temperature (Angriani 2019). Then, high anthocyanin can be considered as an alternative disclosing solution in oral prophylaxis management. Another implementation from the clinical point of view, the BPCE with its high antioxidant property, can be considered as the dye of elastomeric orthodontics in order to obtain the colour stability (da Silva et al. 2016) or additive in candies used for fixed orthodontic patients.

The extract which was obtained from the corolla, was exposed with high flavonoids as the antioxidants. Then the absence of tannins as secondary metabolites was sufficient to block the biofilm activities of gram-positive bacteria such as *S. mutans* and *S. aureus* (Homenta 2016). The maintenance of oral hygiene can prevent complications due to poor oral hygiene during orthodontic treatment. This study also suggests that the high antioxidants which obtained from corolla butterfly pea, is predicted to be able to reduce the stress oxidative from nickel ion that are frequently released from orthodontic components.

The limitation of this study showed that the value of the MIC and antibiofilm does not exceed the value of the positive control test (0.2% chlorhexidine), since there is a main compound of bisbiguanide in 0.2% chlorhexidine. However, flavonoid compounds might be unstable because they can be affected by temperature (Kumar and Pandey 2013). The nature factors such as climate and soil characteristics, the presence of antimicrobial agents, and mode of action will affect the pharmacological activities from different parts of the butterfly pea. According to butterfly pea morphological variations in Indonesia, to identify the optimum extraction condition, high amount of bioactive chemicals and antioxidants, numerous crit-

ical aspects such as the solvent, extraction duration, and temperature should be considered as main factors (Jaafar et al. 2020). Another suggestion, combination with other herbs that favour as traditional medicine in Indonesia, can achieve a safe and effective oral hygiene aid with orthodontics herbal-based products to anticipate the susceptible to reactive oxygen species.

Conclusions

The antibacterial activity of Medan BPCE was insignificant in antibiofilm formation and caused intramembrane leakage of the *S. mutans* and *S. aureus*. Thus, further studies need to be carried out for investigating the morphological traits of these herbs that is quite promising for orthodontic products.

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