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ANTIMICROBIAL ACTIVITY OF PROPOLIS FROM *Trigona thoracica* TOWARDS CARIOGENIC BACTERIA

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ABSTRACT

Propolis is an important bee product which consists of resinous mixture produce by the honeybees from various plant sources. Propolis produced by stingless bee (*Trigona thoracica*), commonly known as 'Kelulut' in Malaysia, is also known to have medicinal values. The *Trigona thoracica* bees are widely distributed throughout Malaysia. The properties of propolis from *Trigona thoracica* have been investigated *in vitro* and *in vivo*. It is renowned to have various biological activities as the antimicrobial, antiproliferative, antiinflammatory and anticancer. Currently, there is limited scientific studies that show antimicrobial activities of propolis against the oral pathogens. Thus, this study is carried out to evaluate the antimicrobial activities of ethanol extracts of propolis (EEP) from *Trigona thoracica* against cariogenic bacteria (*S.mutans* & *S.sobrinus*). This study is performed using the agar well diffusion assay to screen the antimicrobial activity of EEP from *Trigona thoracica* expressed as mean of inhibition diameter and minimum inhibitory concentration (MIC) of EEP will be determined by the broth microdilution method. The mean of inhibition diameter and MIC between EEP and standard antibiotic (metronidazole) against *S.mutans* and *S.sobrinus* is not statistically different. In conclusion, EEP from *Trigona thoracica* has antimicrobial properties against cariogenic bacteria.

Key words: Propolis, *Trigona thoracica*, *Streptococcus mutans*, *Streptococcus sobrinus*, minimum inhibitory concentration

INTRODUCTION

One of the most prevalent oral diseases is dental caries or tooth decay (Peres *et al*., 2019). Dental caries is a progressive destructive condition of the tooth induced by the interaction of sugary diet, saliva, and the bacteria to form dental plaque. Caries can be prevented by reducing the dental biofilms. Dental biofilm is an aggregation of microorganisms to the teeth. Saliva has 10^8 to 10^9 bacteria per milliliter, some of which bind to the teeth and initiate the development of a dental biofilm, formerly known as dental plaque (Larsen & Fiehn, 2017). With access to excess carbohydrates, the dental biofilm will be dominated by mainly gram-positive carbohydrate-fermenting bacteria causing demineralization of teeth, dental caries. The cariogenic bacteria are the culprit of producing acids from the fermentation of sugar that causes a decrease in pH, starting the demineralization process (Bowen & Koo, 2011). Dental caries is caused mainly by mutations in streptococci. These species are known to produce acid as the byproduct of sugar fermentation and hence demineralize the enamel. It includes *Streptococcus sobrinus* and *Streptococcus mutans* (Yadav & Prakash, 2017).

There are many oral hygiene products produced as measures in controlling the development of caries. The products include fluoride toothpaste which has a role in remineralizing action, and triclosan, the antibacterial agent, added in the toothpaste provides an additional benefit to oral hygiene and gingival health. However, triclosan has potential adverse effects on humans and the ecosystem (Dhillon *et al*.,

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2015). Chlorhexidine (CHX) has been known for around three decades as the gold standard of antiplaque treatment, but not without any drawbacks. There are several possible disadvantages to the use of CHX, such as tooth staining, taste disturbance/ alteration, oral mucosa soreness, oral mucosa irritation, mild desquamation, and mucosal ulceration/ erosions, and a general burning sensation and burning tongue (James *et al*., 2017). It is also related to the production of resistant bacteria that impair long-term use (Cieplik *et al*., 2019). Considering these drawbacks of triclosan and CHX, there is a need to develop an alternative antiplaque agent with the use of natural products.

Many researchers have recently been carried out on natural products because of their ability to resist bacteria and mitigate the side effects of the widely used antibiotic (Cunha, 2001; Henriques Normark & Normark, 2002; Liberio *et al*., 2011). Propolis is produced by honeybees for the construction and repair of honeycomb which is collected from exudates of plants (Nicodemo *et al*., 2013). Besides, propolis has pharmacological effects such as antitumor, antimicrobial, immunomodulatory properties, anti-inflammatory, anticytotoxic, and, hepatoprotective (Dobrowolski *et al*.,1991; Banskota *et al*., 2000; Sforcin, 2007). Many studies have reported on the antibacterial activity of propolis extracts from various species against cariogenic bacteria (Mauricio *et al*., 2006; Libério *et al*., 2009).

A decrease in dental biofilm production is seen with the usage of natural products where it has an antimicrobial property to act on dental surfaces. Moreover, these products can prevent the metabolism, growth, and colonization of the bacteria (Selwitz *et al*., 2007). Furthermore, propolis has low toxicity and showed a variety of biological activities that makes it an advantage for its usage in the healthcare field (Libério *et al*., 2009). According to a study by Duailibe *et al*. (2007), propolis extract in Brazil showed antibacterial activity against *Streptococcus mutans* which is a cariogenic bacteria. Similarly, Duarte *et al*. (2006) stated that the occurrence of smooth surface caries was markedly decreased in vivo by using propolis extracts but it does not show a decrease in the number of *Streptococcus sobrinus* in animals.

In this study, propolis from *Trigona thoracica* was used to assess the antibacterial effects against cariogenic microorganisms. In Malaysia, this stingless bee species is called 'Kelulut'. These bees produce Kelulut honey and it has been proven by studies that Kelulut honey has qualitatively antimicrobial excellent potency which is useful medically and therapeutically (Zainol *et al*., 2013). There are many studies regarding propolis from other types of a bee on antibacterial effects against oral pathogens. However, there is limited research on the antimicrobial effects of stingless bee propolis. The goal of this study was therefore to assess the antimicrobial effects of *Trigona thoracica* propolis with ethanol extraction against cariogenic bacteria; *Streptococcus mutans* and *Streptococcus sobrinus.*

The study of the antimicrobial properties of propolis from *Trigona thoracica* could potentially be used as an alternative therapeutic agent against oral microorganisms, particularly *Streptococcus mutans* and *Streptococcus sobrinus.* This study is valuable in the development of alternative therapy in preventing dental caries. It will be benefitted the scientists in findings and formulate the recommended dose for the dental practitioners and help the public to make more efficient use of the beneficial properties of propolis especially in preventing dental caries.

MATERIALS AND METHODS

Preparation of propolis and extract of propolis (EEP)

The raw *Trigona thoracica* propolis was collected from a local stingless beekeeper (Razip International Trade, Kota Bharu, Kelantan). The sample was collected in Kota Bharu, Kelantan, Malaysia, and transported at -20°C in sealed bottles. The sample was stored at -20°C until used for analysis. The preparation of propolis extraction is based on the methods described by Krell (1996) with some modifications. The procedure was performed at Dental School Laboratory, Health Campus, Universiti Sains Malaysia. The sample of propolis was frozen for 24 hr at -20°C and ground into a fine powder. The sample (50 g) was added with 70% ethanol (167 mg) to obtain a 30% (w/w) propolis extract. The mixture was left at room temperature for a week and it was shaken manually for one minute on a moderate basis twice a day. The ethanolic extract was filtered 2 times. The extract was placed in a refrigerator (2–8°C) before the second filtration to remove the wax. The ethanol was removed by using vacuumed rotary evaporator at 35°C. The residual water from the extract was lyophilized using a freezer dryer. The dry extract was kept in an amber glass at -20°C.

Antimicrobial susceptibility testing

Antimicrobial tests were performed at Microbiology Laboratory, Health Campus, Universiti Sains Malaysia.

Preparation of bacterial suspensions

Streptococcus mutans and *streptococcus sobrinus* were grown in blood agar and incubated in anaerobic condition at 37°C for 72 hr. After incubation, the colonies from each bacterium were suspended in 1 mL of peptone water and standardized to 0.5 Mc Farland (1×10^8 CFU/mL) by using a nephelometer.

Preparation of propolis extract and antibiotic solution

A crude extract of dry EEP was dissolved in 70% of ethanol at a concentration of 100 mg/mL which was further diluted with distilled water in a ratio of 1:10 (10 mg/mL). The preparation of antibiotic solution was similar to EEP.

Agar well diffusion assay

Agar well diffusion assay was performed to evaluate the antimicrobial activity of EEP from *Trigona thoracica.* This experiment was performed as described by Ahmad *et al*. (1998) with some modifications. A sterile cotton bud was dipped into *Streptococcus mutans* and *Streptococcus sobrinus* suspension (1×10^8 CFU/mL) and lawned on the surface of the Mueller-Hinton Blood agar (MHBA) media plate. Wells were made by using a sterile glass Pasteur pipette with a diameter of 6 mm and labeled accordingly. Each well was filled up with 100 µL of EEP (10 mg/mL), 7% ethanol, and antibiotic (10 mg/mL). Metronidazole was used as a positive control and 7% ethanol as a negative control. The plates were incubated at 37°C under anaerobic conditions using an anaerobic gas pack for 72 hr. Each experiment was done in triplicates independently and the antimicrobial activity will be expressed as the mean of inhibition diameters (mm).

Determination of minimum inhibitory concentrations

The MIC of EEP was determined by the broth microdilution method with a few modifications (Mohammadzadeh *et al*., 2007; Sarker *et al*., 2007). The prepared solution of the EEP at concentration of 10 mg/mL was two-fold serially diluted into the sterile well plate containing 100 µL of cation-adjusted Mueller-Hinton broth to produce the concentrations of 5000, 2500, 1250, 625, 313, 156, 78, 39, 20, 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, 0.08 and 0.04 µg/mL.

A bacterial suspension of 20 µL (1×10^6 CFU/mL) was added to the test dilutions. Each plate had a set of controls: a column with the standard antibiotic (metronidazole) as a positive control, a column with all solutions except the EEP as a negative control, and a column with all solutions except the bacterial solution (100 µL broth added instead) to check the sterility of the media. The plates containing *Streptococcus mutans* and *Streptococcus sobrinus* were incubated at 37°C for 72 hr under anaerobic conditions. After incubation, 10 µL of resazurin indicator solution (Sigma Aldrich, US) (0.01%) was added to each well and incubated for 2 hr at 37°C under anaerobic conditions. After 2 hr, the lowest concentration at which color change to pink occurred was taken as the minimum inhibitory concentration (MIC).

Statistical analysis

Statistical analysis was done using an IBM SPSS statistics software version 22. The median MIC between EEP and standard antibiotic (metronidazole) against *Streptococcus mutans* and *Streptococcus sobrinus* was compared using a Mann-Whitney test. A p-value of < 0.05 was considered statistically different.

RESULTS

The results of agar well diffusion assay of EEP and metronidazole against selected cariogenic bacteria as shown in Figure 1 and Figure 2. The median of inhibition diameter zone using EEP against *Streptococcus mutans* and *Streptococcus sobrinus* are 14 mm and 18 mm; respectively. Whereas the median inhibition diameter of metronidazole which is the positive control in this study against *Streptococcus mutans* and *Streptococcus sobrinus* is 34 mm. The MIC of EEP against *Streptococcus mutans* and *Streptococcus sobrinus* is 625 µg/mL. The MIC of metronidazole against *Streptococcus sobrinus* is 2 µg/mL whereas against *Streptococcus mutans* is 5 µg/mL. The MIC values between EEP and metronidazole against *Streptococcus mutans* and *Streptococcus sobrinus* are statistically significant as shown in Table 1.

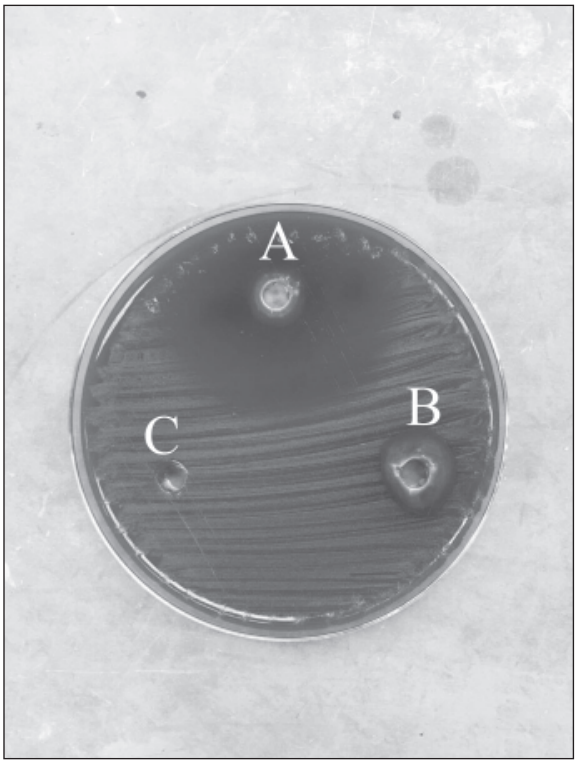

Fig. 1. Zone of inhibition of *Streptococcus mutans* shown by metronidazole (A), ethanol extract of propolis (B), and distilled water (C).

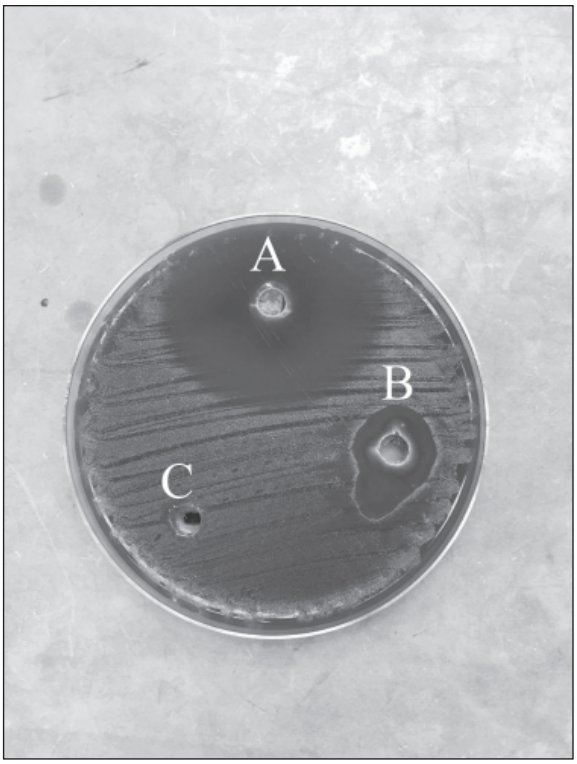

Fig. 2. Zone of inhibition of *Streptococcus sobrinus* shown by metronidazole (A), ethanol extract of propolis (B), and distilled water (C).

Table 1. Comparison of MIC values between EEP and metronidazole against *Streptococcus sobrinus* and *Streptococcus mutans*

	MIC (µg/mL)		Stat[a] Z score	*p*-value
	EEP	Metronidazole		
S. Sobrinus	625	2	-2.236	0.025
S. mutans	625	5	-2.236	0.025

Tests were performed in triplicate.
[a]Mann-Whitney test was applied.

DISCUSSION

The agar well diffusion assay showed EEP has antimicrobial activity against both *Streptococcus mutans* and *Streptococcus sobrinus.* Although the MIC value of metronidazole is higher than EEP against both oral pathogens, the antimicrobial activity of EEP shows promising results because the MIC values against both pathogens were 625 µg/mL. The natural product is considered to have an antimicrobial effect if the MIC value is lower than 1000 µg/mL (Silva *et al*., 2013). Therefore, propolis has a potential alternative to antimicrobial agents against oral pathogens.

It has been proven that a huge spectrum of biological curative effects for example antifungal, antimicrobial, and antiviral properties has been found in most propolis variants (Toreti *et al*., 2013; Banskota *et al*., 2001; Burdock, 1998). This leads to an idea where propolis can be used in any anti-cariogenic product since it inhibits the major bacteria that is responsible for dental caries which include *Streptococcus mutans* and *Streptococcus sobrinus*. There are many studies done in support of the statement where propolis can be used as an anti-cariogenic agent. According to De Luca *et al*. (2014), propolis varnish showed satisfactory effects as an antimicrobial agent against the oral pathogen and provides minimal cytotoxicity effect on the osteoblasts (<50%). A study from Machado *et al*. (2016) concluded that daily mouthwashes with propolis are effective in decreasing *Streptococcus mutans* level in the oral cavity. The results are identical to a study done by Anauate-Netto *et al*. (2013) where they carry out a randomized, double-blind, placebo-controlled clinical trial on the antibacterial activity of a mouth rinse containing propolis at 2% on mutans streptococci and lactobacilli with the comparison to chlorhexidine 0.12% and placebo. The study found that mouthrinse containing propolis at 2% was statistically significant in suppressing the salivary levels of mutans streptococci and lactobacilli compared to CHX mouth rinse in the period between 14 - 28 days, and the residual effects persist until 45 days. On top of that, patient satisfaction and acceptability were highest and excellent for the propolis mouth rinse compared to CHX and placebo mouth rinses. On the other hand, EEP was found to possess antimicrobial effects against biofilms of Streptecocci, *P. gingivalis*, *A. israelii*, and *C. albicans* as good as CHX (Akca *et al*., 2016). Interestingly, the authors found that EEP had better results against Lactobacilli and *P. intermedia.*

The complex mechanism of propolis antibacterial action, though not fully understood, may be different according to its contents (Gebara *et al*., 2002). Based on the study done, flavonoids are the substance that gives the antimicrobial actions of propolis (Temiz *et al*., 2011). The mechanism of antimicrobial actions associated with the high contents of flavonoids may be similar to crude propolis. Flavonoids inhibit the synthesis of nucleic acid, energy metabolism, and function of the cytoplasmic membrane (Cushnie & Lamb, 2005). However, in another study, the anti-cariogenic effect of propolis is also by inhibition of glucosyltransferase activity and synthesis of extracellular polysaccharide (Bozcuk Erdem & Ölmez, 2004). The enzyme glucosyltransferases act on sucrose to produce insoluble glucans (Libério *et al*., 2009). These glucans are essential in biofilm formation from cariogenic microorganisms by early steps of colonization and accumulation (Libério *et al*., 2009). Thus, the control of caries can be approached

effectively by the inhibition of glucosyltransferase activity (Libério *et al*., 2009).

From a safety aspect, propolis is safe in low doses (Castaldo & Capasso, 2002). Although hypersensitivity reactions to *Apis* propolis have been reported in some studies (Callejo *et al*., 2001; Walgrave *et al*., 2005), it is known that no toxic effects are produced by propolis (Özen *et al*., 2004; Bhadauria *et al*., 2008). Based on an animal study done by Almeida *et al*. (2008), the toxicity study only showed an increment of the urea level but did not reach the limit of toxicity which excludes renal toxicity. Besides, the use of propolis revealed no significant imbalance in the oral microorganism (Arslan *et al*., 2012). The prolonged use of metronidazole as a standard antibiotic reported many side effects to the central nervous system for example encephalopathy (Heaney *et al*., 2003; Patel *et al*., 2008; Sarna *et al*., 2009; Kuriyama *et al*., 2011) and seizure (Patel *et al*., 2008; Sarna *et al*., 2009) since metronidazole is a well-recognized neurotoxin. Moreover, it was found out that common adverse reactions which are gastrointestinal upset and dry mouth are seen in a patient taking metronidazole (Patel *et al*., 2008). Although the current research concluded that the propolis is safe and less toxic compare to other synthetic medicine when using standardize preparation, the toxic effects of propolis in humans should be further investigated.

There is the potential of using propolis in the dentistry field because of its antimicrobial activity towards oral pathogens, and other biological properties, including anti-inflammatory, and anti-oxidant (Banskota *et al*., 2000; Ahn *et al*., 2004; Oršolić & Bašić, 2005; Sforcin, 2007). Moreover, *Trigona thoracica* bees are widely available throughout Malaysia. The bees can be commercially reared and produce a lot of propolis per hive. Since there are a lot of advantages in using propolis compared to metronidazole, further research needs to be done so that propolis can be incorporated into dental products such as toothpaste, gels, varnish, mouth rinse, and foam.

CONCLUSION

This study has proved that propolis from stingless bee *Trigona thoracica* can act as an antibacterial agent against cariogenic bacteria such as *Streptococcus mutans* and *Streptococcus sobrinus*. However, there is a need for further investigations on the possibility of toxicity as well as side effects that might occur. Further research is needed to standardize it chemically and to establish the most effective and safest concentration level, hence, propolis can be utilized on a larger scale for the prevention of dental caries.

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