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ANTIBACTERIAL ACTIVITY OF MALAYSIAN PRODUCED STINGLESS-BEE HONEY ON WOUND PATHOGENS

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Abstract: The antibacterial activity of Malaysian stingless bee honey was tested on six common wound pathogens using agar well diffusion. All pathogens showed varying degrees of susceptibility to undiluted and diluted honeys produced by *Geniotrigona thoracica of* multifloral source (GTM) and *Melastoma malabathricum* L (Senduduk). Multifloral honey from *Heterotrigona itama* (HTM) failed to inhibit the growth of all pathogens, except for methicillin-resistant *Staphylococcus aureus* (MRSA) which has demonstrated moderate susceptibility to undiluted honey. It was found that the antibacterial activity of GTM and Senduduk honeys were concentration dependent. The minimum inhibitory concentration (MIC) assay showed that a lower value (3.13% v/v) was observed with GTM honey for all pathogens and Senduduk honey for *Streptococcus pyogenes*, MRSA, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively. Interestingly, HTM honey showed MIC between 6.25 to 12.5% (v/v) in microdilution method. The minimum bactericidal concentration (MBC) of GTM honey ranged between 6.25 to 12.5% (v/v), whereas Senduduk and HTM honeys showed MBC of 25% (v/v). The lower MIC and MBC values exhibited by GTM honey indicate ~~s~~ potent antibacterial activity as seen in this honey. This study revealed that the Malaysian stingless bee honeys have promising antibacterial activity against wound pathogens, and this type of honey could be used as an alternative in treating infected wounds.

Keywords: *Geniotrigona, Heterotrigona*, kelulut, medicinal, antibiotic, environmental service, sustainability

Introduction

A wound is the result of physical disruption of the skin together with the destruction of blood vessels. The local conditions favour the bacterial invasion and growth which is inevitable. A severe wound infection, if left untreated, can cause discomfort to the patient due to an unpleasant odour, increased pain and in some cases, under certain conditions, a type of wound (e.g., burn wound) can lead to life-threatening illness and death. Many species of bacteria have been recovered from wounds since most wounds support the colonization of polymicrobial communities (Lasa & Solano, 2018). In most cases, *Staphylococcus aureus* is the most frequently isolated bacteria regardless of the types of wound (Bessa *et al*., 2015). Other common bacteria are *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Proteus* spp., *Streptococcus* spp., Acinetobacter spp., and Enterococci (Rai *et al*., 2017). However, it has been observed that different types of wound harbour different domination of pathogens. For example, MRSA and *P. aeruginosa* are common pathogens associated with burn infections (Dawra *et al*., 2017).

Generally, a wound is treated with antibiotics, either in the form of topical preparation or systemic use to reduce the degree of wound infection, regardless of the etiology of the wound. Antibiotics from penicillin ~~s~~ or beta-lactam group are commonly prescribed. Other groups of antibiotics such as aminoglycosides and macrolides have also been prescribed occasionally. However, there is a worldwide emergence issue that many wound pathogens have been resistant to commercial antibiotics. Resistance of wound pathogens to antibacterial agents has been reported (Mohammed *et al*, 2017). This will make the treatment for wounds more complicated. As a result, the patients need longer hospital stays and it will increase the financial burden ~~of~~ for the treatment (Karimi *et al*., 2015). Therefore, to overcome the resistance problem, alternative natural antibacterial products are being widely explored.

Among the natural products, honey has a good reputation for its outstanding antibacterial property (Meo *et al.*, 2017). Honey is mainly produced from the nectar of flowers or honeydew droplets (Codex Alimentarius, 2001). The bees responsible for honey production are *Apis* species and stingless bees. Honey produced by *Apis* spp. especially *A. mellifera* has been extensively studied and has received high recognition for the medicinal properties (De-Melo *et al.*, 2017). In Malaysia, two species of *Apis* bees are *A. dorsata* and *A. cerana* which are known locally for the production of honey. Tualang honey, honey produced by *A. dorsata*, is commonly used in the Malay community for various medicinal purposes. In addition to antibacterial activity, honey has also been reported to exhibit several

other biological activities such as that ofan antioxidant (Ahmed *et al*., 2018), antitumor (Ahmed & Othman, 2013; Badolato *et al*., 2017), antifungal (Shehu *et al*., 2016), anti-inflammatory (Almasaudi *et al*., 2017), hypoglycemic effect (Bobiş *et al*., 2018), and cardioprotective effect (Khalil *et al*., 2015). Honey has been accepted to be used topically for the treatment of chronic and infected wounds (Jull *et al*., 2015), as well as for the treatment of burns (Aziz & Hassan, 2017). The uses and benefits of honey had been known since ancient times as honey has an inhibitory effect on microorganisms that colonise the wound. Moreover, honey has been regarded as having bacteriostatic and bactericidal effects against a wide range of microbial species, including both Gram-positive and Gram-negative bacteria (Mandal & Mandal, 2011). Apart from that, honey, when used as a dressing on wounds, also promotes epithelization, reduces the unpleasant odour and helps reduce inflammation, edema and exudation (Oryan *et al*., 2016), which in part improves the healing process of the wound. It has been noted that the effectiveness of honey for the treatment of wounds and burns is due to its antibacterial activity (Molan & Rhodes, 2015; Bucekova *et al*., 2017). However, not all types of honey possess similar level of antibacterial activity since the activity is greatly dependent on several factors such as flower sources, location where the honey is collected, and a few intrinsic factors like hydrogen peroxide level, osmotic effect and phytochemicals (Elbanna *et al*., 2014; Matzen *et al*., 2018). To date, only a few types of honey have been approved for therapeutic use, such as Capillano Medihoney, Active Manuka honey and Revamil honey.

Malaysia is known to have a diverse species of stingless bees, of about 35 species (Jaapar *et al*, 2016). Some of these species such as *Geniotrigona thoracica* and *Heterotrigona itama* have been successfully domesticated for their honey. Currently, Malaysian stingless bee honey is gaining more attention since this honey has been listed as a Malaysian superfood by the Malaysian Agriculture and Research Development Institute (MARDI), a Malaysian government agency (MARDI, 2016). Hence, the Malaysian stingless bee honey industry has high potential in the near future (Ismail, 2016; Mustafa *et al*., 2018). Not many studies are found on antibacterial activity of honey produced by stingless bee worldwide. This might be due to an assumption that honey produced by stingless bee has so little to offer compared to the honey of *A. mellifera.* In recent years, a few authors have found that honey from stingless bees also possesses antibacterial activity against various pathogens (Irish *et al*., 2008; Kimoto-Nira & Amano, 2008; Ewnetu *et al*., 2013; Zainol *et al*., 2013; Andualem, 2014; Eswaran *et al*., 2015). This indicates that the stingless bee honey has potential as an antibacterial agent and research on this particular field should be explored. Research has shown a wide variation of antibacterial activity in unifloral and multifloral honeys, indicating that botanical origin may influence the antibacterial activity in honey. Honeys from unifloral varieties have been proved to have some distinctive properties that have given prominence to their use for medicinal purposes. They also command a much higher price than multifloral honeys. A clear example is Manuka honey, the unifloral honey from *A. mellifera* which is derived from *Leptospermum scoparium* nectar known to possess exceptional antibacterial activity. However, due to the nature of stingless bees which inhabit the forest and forage multiple flower varieties, the honeys produced are mostly multifloral. It is difficult to get the unifloral honey produced by stingless bees unless the bees are domesticated in the specific plant area for the honey. Only recently, a few studies have found that multifloral honeys from stingless bees exhibited higher antimicrobial activity than unifloral honeys (Ewnetu *et al*., 2013; Andualem, 2014; Massaro *et al*., 2014; Morroni *et al*., 2018). Since then, research has been focusing on the antibacterial property of the honey produced by stingless bees.

Until now, no study has been conducted on honeys produced by *G. thoracica* and *H. itama* from different floral sources against wound pathogens. Therefore, the purpose of this study was to determine the *in vitro* antibacterial activity of these types of honey against common pathogens infecting wounds. The results of this study could provide information on the potential of Malaysian stingless bee honeys as antibacterial agent which may give benefits to both apiary industry and the health care sector.

Materials and Methods

Bacterial Strains

The bacterial isolates representing wound pathogens were obtained from the Microbiology Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The species included MRSA, *P. aeruginosa*, and *K. pneumoniae*. These strains were isolated from the animal specimens. The isolates were identified by the standard bacteriological techniques and confirmed by polymerase chain reaction. On the other hand, *Streptococcus pyogenes* was purchased from the Department of Medical Microbiology and Parasitology, Faculty of Medical Sciences, Universiti Sains Malaysia. The standard isolates used included *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25922). All cultures were maintained in nutrient agar (Oxoid, UK) at 4°C throughout the study.

Preparation of Standard Inoculums

The inoculum suspension for each bacteria was prepared by picking one single colony from the stock of pure colonies maintained in nutrient agar into 10 ml of sterile normal saline. The suspension density was adjusted visually to 0.5 McFarland turbidity standard which gives approximate bacteria suspension of 1.5 x 10^8 CFU/ml. The inoculum suspensions for the test

pathogens were freshly prepared prior to antibacterial assay and used within 30 min after preparation.

Honey Samples

Honey from *G. thoracica* and *H. itama* were used. The Google map was used to provide the Global Positioning System for the honey locations. Two flower sources (multifloral and unifloral) of *G. thoracica* honeys were obtained from Universiti Malaysia Kelantan Agropark, Jeli, Kelantan (5°44'47.0"N 101°52'01.4"E). The unifloral honey was identified from the flowers of *Melastoma malabathricum* L. by pollen analysis. This plant is locally known as Senduduk. For multifloral honey of *G. thoracica*, the major sources of nectar were from the wild flowers from the nearby mountainous forest of Jeli (5°44'45.8"N 101°52'01.7"E). The pollen analysis for both honeys was done by Dr Kumara Thevan Krishan from the Faculty of Agro Based Industry, Universiti Malaysia Kelantan. As a general guidance, the botanical classification of unifloral honey was achieved when the pollen spectrum contained more than 45% of the total number of the predominant pollen and it is vice versa for multifloral honey (Krishnan & Rao, 2017). The *H. itama* honey was obtained from a local *H. itama* beekeeper in Kota Bharu, Kelantan (6°05'26.1"N 102°17'28.4"E), and the flower sources were mainly from various types of fruit trees in the orchards. No pollen analysis was carried out ~~to~~ on *H. itama* honey and it was considered multifloral based on the location of the hives. Honeys were addressed as GTM (multifloral honey from *G. thoracica*), Senduduk (unifloral honey from *G. thoracica*) and HTM (multifloral honey from *H. itama*) throughout the article. Figure 1 shows the regions where the honeys were collected. All three types of honey were harvested between July and August, 2017. The pH of GTM, Senduduk and HTM honeys were 3.22, 3.31 and 3.42, respectively. Table 1 shows the physical characteristics of these honeys. The honeys were kept in tight capped glass bottle in a refrigerator at 4°C throughout the study.

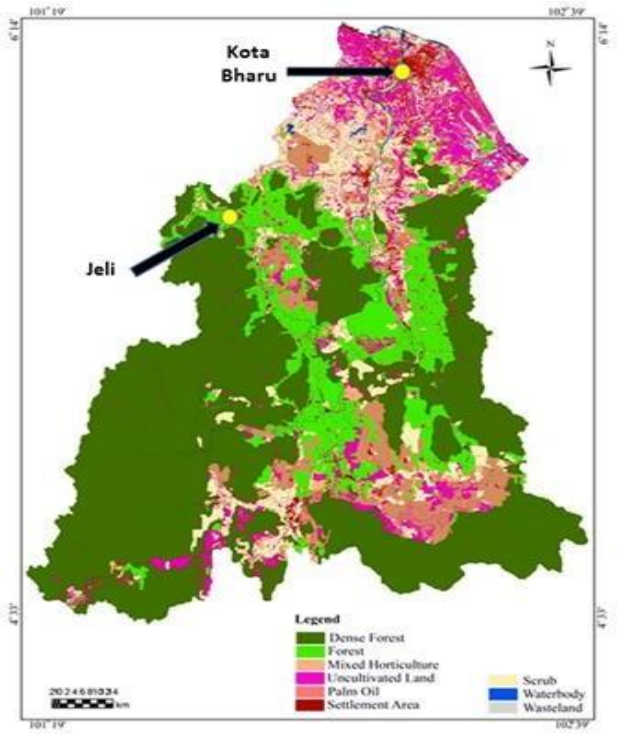

Figure 1: Map of the regions in Kelantan where honey samples were collected. The figure was adapted from Anees *et al*. (2017)

Table 1: Type, origin and colour of stingless bees honey samples used in the study

Honey	Bee species	Colour	Taste	Plant origin	Region
GTM	*G. thoracica*	Light amber	Intense sour	Multifloral	Jeli
Senduduk	*G. thoracica*	Dark purple	Light sour	*M. malabathricum* L	Jeli
HTM	*H. itama*	Light amber	Sweet fruity taste	Multifloral	Kota Bharu

Testing for antibacterial activity was done within 2 weeks after the collection of the honey. Artificial honey was prepared according to Cooper *et al*. (2002) but with slight modifications. Briefly, artificial honey was prepared by dissolving 40.5 g fructose, 33.5 g glucose, 7.5 g maltose and 1.5 g sucrose in sterilised deionised water up to 100 ml. To sterilize the artificial honey, the solution was filtered through 0.22 µm pore size sterile filter. This solution consists of 83% sugar (w/v) which is approximately equivalent to sugar in raw honey. The sugars were selected based on the common sugars which exist in honey. The test with artificial honey was done to distinguish the components that cause antibacterial activity in honey from the osmotic effect.

Susceptibility Assay of Bacteria to Honey

The serial dilutions of each honey using sterilised distilled water were made to give concentrations of 75%, 50%, 25%, 12.50%, 6.25%, 3.13% and 1.56% (v/v). Agar well diffusion assay was employed according to the one described by Nweze *et al.* (2016) but with some modifications. The fresh culture of test organisms (50 µl) was swabbed over the surface of Mueller Hinton agar (Oxoid, UK) plates using sterile cotton swab in eight different angles to ensure that the organism was uniformly distributed on the agar surface. The wells were then punched, at equal distance, on the agar plates with a sterile cork borer (6 mm diameter) at different sites (three wells per plate). Sixty microliter (60 µl) of each honey concentration (i.e., 75% 50%, 25%, 12.50%, 6.25%, 3.13% and 1.56%) including undiluted honey was pipetted into the agar wells. A similar amount (60 µl) of artificial honey was used as a negative control. The disk containing tetracycline (30 µg/disk, Oxoid, UK) was placed on the Mueller Hinton agar plate and served as a positive control. All plates were incubated at 37°C for 24 h. The antibacterial activity was evaluated by measuring the diameter of zone of growth inhibition. Each experiment was repeated three times for each of the six bacteria. The results were recorded as mean ± standard deviation for the test and control groups.

Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations

Following the initial susceptibility test, the Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC) of one honey variety for each pathogen were determined by using the broth microdilution method as described by Grego *et al*. (2016) with slight modifications. The 96 well microplate was used in this study. A hundred microliter (100 µl) of undiluted and concentration of the honeys were placed into the microplate wells. Then, 30 µl of test inoculum suspension was loaded to the content of the wells, together with 100 µl of artificial honey and test inoculum (30 µl) and this served as a negative control. After gently mixing it, the content of the microplate was incubated for 24 h at 37°C. The MIC was determined visually and taken as the lowest concentration of honey that showed clear solution in the wells, indicating that there was no visible growth (no presence of turbidity) for each cultured nutrient broth. The MBC of the honeys against test pathogens was determined by further sub-culturing 30 µl of culture (from the wells that showed no visible growth in the MIC assay) into the wells that contained 100 µl of freshly sterilised nutrient broth (Oxoid, UK). The microplate containing the cultures was then incubated at 37°C for 24 h. The MBC was taken as the lowest concentration or highest dilution of honey that did not show any visible growth on sub-cultured nutrient broth (Zainol *et al*., 2013). Three replicated wells were used ~~at~~ for each concentration of honey per determination. The value for MIC and MBC was expressed in % (v/v) of honey. The results were recorded as mean ± standard deviation of three determinations.

Statistical Analysis

For the results of zone of inhibition, MIC and MBC are expressed as the mean ± standard deviation of three experiments. A one-way Analysis of Variance (ANOVA) followed by Tukey's *post-hoc* test were carried out to determine the effect of honey concentration on antibacterial activity. A p value < 0.05 was considered statistically significant. Data were entered in Microsoft Excel® (2010) statistical package and analysed using SPSS for Windows, Version 21; SPSS Inc., (Chicago, IL, USA).

Results

The antibacterial activity of undiluted and varying concentrations of honeys (3.13-75%) (v/v) against wound pathogens were tested using agar well diffusion method. The results of the susceptibility of pathogens to the honeys are shown in Table 2.

Table 2**:** The zone of inhibition (mm) of GTM, Senduduk and HTM honeys at various concentrations against pathogens. The cultures were incubated at 37°C for 24 hours. Tetracycline was used as a positive control. Results are mean ± standard deviation of three determinations

		Microorganisms					
Honey	**Concentration % (v/v)**	***S. pyogenes***	**MRSA**	***S. aureus***	***P. aeruginosa***	***E. coli***	***K. pneumoniae***
GTM	Undiluted	24.3 ± 0.6^{l}	21.3 ± 1.5^{kl}	15.7 ± 0.6^{efgh}	18.0 ± 1.0^{hij}	12.3 ± 1.5	9.7 ± 1.1
	75	22.3 ± 0.6^{kl}	19.3 ± 0.6^{ijk}	10.7 ± 0.6^{abc}	12.7 ± 0.6^{cde}	-	-
	50	17.3 ± 2.3^{ijk}	14.7 ± 1.2^{defg}	7.3 ± 0.6^{a}	9.3 ± 0.6^{ab}	-	-
	25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
Senduduk	Undiluted	21.3 ± 1.5^{kl}	18.0 ± 1.0^{hij}	14.7 ± 0.6^{defg}	16.7 ± 0.6^{fghi}	10.7 ± 0.6	14.3 ± 0.6
	75	20.7 ± 2.3^{jk}	13.7 ± 0.6^{def}	10.3 ± 0.6^{abc}	12.3 ± 0.6^{bcd}	-	-
	50	16.4 ± 0.6^{fghi}	10.3 ± 0.6^{abc}	7.7 ± 0.6^{a}	9.7 ± 0.6^{abc}	-	-
	25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
HTM	Undiluted	-	13.6 ± 0.6	-	-	-	-
	75	-	-	-	-	-	-
	50	-	-	-	-	-	-
	25	-	-	-	-	-	-
	12.5	-	-	-	-	-	-
Control	Tetracycline (30 µg/disc)	11.0 ± 0.0	24.5 ± 0.7	25.0 ± 0.0	21.0 ± 1.4	17.0 ± 2.8	26.0 ± 1.4

Legend: (-) = no inhibition. There was no inhibition in all honeys at concentrations of 6.25%, 3.13% and 1.56% (v/v) against all pathogens.
Values of zone of inhibition (mean ± SD) with different superscript letters are significant different at $p<0.05$ using ANOVA Tukey's statistical test

The zones of inhibition (ZOI) demonstrated by undiluted honeys produced by *G. thoracica* against all pathogens tested ranged between 9.7 ± 1.1 mm to 24.3 ± 0.6 mm indicating that the pathogens were very susceptible to these honeys. Among Gram-positive bacteria, *S. pyogenes* showed the highest sensitivity to GTM honey and Senduduk honey (ZOI: 24.3 ± 0.6 mm; 21.3 ± 1.5 mm), followed by MRSA (ZOI: 21.3 ± 1.5 mm; 18.0 ± 1.0 mm) and *S. aureus* (ZOI: 15.7 ± 0.6 mm; 14.7 ± 0.6 mm), respectively. For Gram-negative bacteria, *P. aeruginosa* was the most susceptible (ZOI: 18.0 ± 1.0 mm; 16.7 ± 0.6 mm) to this type of honeys, followed by *E. coli* (ZOI: 12.3 ± 1.5 mm; 10.7 ± 0.6 mm) and *K. pneumoniae* (ZOI: 9.7 ± 1.1 mm; 14.3 ± 0.6 mm), respectively. Comparative to the results of GTM honey and Senduduk honey, HTM honey only exhibited antibacterial activity against MRSA (ZOI: 13.6 ± 0.6 mm). The growths of other bacteria were not affected in a noticeable way by HTM honey. When the honeys were diluted, only GTM honey and Senduduk honey showed inhibition against *S. pyogenes, MRSA, S. aureus* and *P. aeruginosa* at concentration of 75% and 50% (v/v). The ANOVA analysis yielded significant variation (p<0.05) among honey concentrations of the same honey type and between GTM and Senduduk against *S. pyogenes*, MRSA, *S. aureus* and *P. aeruginosa.* A *post-hoc* Tukey's test showed that dilution at 75% and 50% (v/v) in GTM and Senduduk honeys gave significant effect to the antibacterial activity (p<0.05) except for *P. aeruginosa* (Table 2). Senduduk honey at 75% and 50% (v/v) ~~also~~ did not give a significant antibacterial activity against *S. aureus*. The test also indicated that MRSA was more susceptible to GTM than Senduduk honey when compared at the same honey concentration (p<0.05).

No inhibition of growth of *E. coli* and *K. pneumoniae* was observed at any concentrations of dilution of GTM honey and Senduduk honey. On the other hand, HTM honey at various concentrations failed to inhibit the growth of all pathogens. No inhibition was observed for artificial honey against all pathogens. The MIC and MBC values of the honeys were determined by using microdilution technique. The MIC and MBC determinations for HTM honey continued even though there was no zone of inhibition produced in agar well diffusion technique in susceptibility testing of this honey. Table 3 shows the MIC and MBC values of the honeys for each of the pathogens. The MIC of GTM honey was found to be at 3.13% (v/v) and consistent to all pathogens, whereas the MICs for Senduduk honey ranged between 3.13 and 6.25% (v/v). While the MICs of HTM honey were a little bit higher than the two honeys produced by *G. thoracica* bee, which were between 6.25 and 12.5% (v/v). The MBCs for GTM ranged between 6.25 and 12.5% (v/v), whereas MBCs for Senduduk and HTM honeys were similar which were at 25% (v/v). All pathogens exhibited growth in artificial honey for MIC determination.

Table 3: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values expressed as % (v/v) of GTM, Senduduk and HTM honeys against pathogens causing wound. Results are the mean of three determinations. Identical results are obtained for each determination, giving the standard deviations (s.d = 0)

Microorganisms	**GTM**		**Senduduk**		**HTM**	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
P. aeruginosa	3.13	6.25	3.13	25	6.25	25
MRSA	3.13	6.25	3.13	25	6.25	25
S. aureus	3.13	12.50	3.13	25	12.50	25
S. pyogenes	3.13	12.50	3.13	25	12.50	25
E.coli	3.13	12.50	6.25	25	12.50	25
K. pneumoniae	3.13	12.50	6.25	25	12.50	25

Discussion

The present study was carried out to investigate the susceptibility of bacteria representing wound pathogens to honey produced by *G. thoracica* and *H. itama* stingless bees. Based on the results in agar well diffusion assay, it was observed that undiluted and diluted honeys exhibited different levels of inhibition against different wound bacterial pathogens as shown in Table 2. In general, all bacterial pathogens showed susceptibility to undiluted honeys of *G. thoracica*, regardless of their floral sources (multifloral or unifloral). It was observed that *S. pyogenes* was highly sensitive to GTM honey and Senduduk honey, and both honeys produced the highest zone of inhibition; 24.3 ± 0.6 mm and 21.3 ± 1.5 mm, respectively. These ZOI values are interpreted as extremely sensitive by Moussa *et al*. (2012). The susceptibility of pathogens is then followed by MRSA and *P. aeruginosa* which have been interpreted as very sensitive, while other pathogens showed moderate sensitivity (Moussa *et al*., 2012) to these honeys. Suntiparapop *et al*. (2012) who tested the antibacterial activity of honey produced by *Tetragonula laeviceps*, the Thai stingless bee, using agar well diffusion found that the honey exhibited moderate antibacterial activity against *S. aureus*, *S. pyogenes*, MRSA, *E. coli*, *K. pneumoniae* and *P. aeruginosa* with the zone of inhibition ranged between 8.0 to 12.0 mm. Comparing to the results of Suntiparapop *et al*. (2012), it was evident that GTM honey and Senduduk honey produced by *G. thoracica* in our study had superior antibacterial activity against similar pathogens tested. However, it should be noted

that Chanchao (2009) found that the undiluted *T. laeviceps* honey gave very high ZOI against *S. aureus* (38.0 ± 0.4 mm) and *E. coli* (29.4 ± 0.1 mm). The variation in the antibacterial capacity in honey, even if produced by similar bee species, could be due to different geographical locations where the honey is collected, seasonal conditions, flower source, processing, storage conditions and the sensitivity of the bacteria to antibacterial compounds in honey (Schneider *et al*., 2013; Pimentel *et al*., 2013). This is evident in our study that GTM honey possess stronger antibacterial activity than Senduduk honey against MRSA at any concentrations (Table 2). The results of this study also demonstrated that the antibacterial activities of GTM honey and Senduduk honey are directly proportional to their concentration, as shown by a marked decrease of ZOI as honeys are diluted as depicted in Table 2. In comparison with the undiluted honeys, the diluted honeys had less antibacterial activity. However, only a few pathogens such as MRSA, *S. pyogenes*, *P. aeruginosa* and *S. aureus* showed sensitivity when honeys were diluted up to 50% (v/v). The dilution of both honeys did not inhibit the growth of *E. coli* and *K. pneumoniae*. This result is in agreement with the research carried out by Chanchao (2009) who studied the antimicrobial activity of undiluted and diluted (i.e., 25%, 50% and 75% v/v) honey of *T. laeviceps* against *S. aureus* and *E. coli and* found that the antibacterial activity of this honey became less when honey was further diluted. Furthermore, many studies showed that some honeys, regardless of their entomological origins, lost their antibacterial activity through dilution (Pimentel *et al*., 2013; Ewnetu *et al*., 2013; Zainol *et al*., 2013; Eswaran *et al*., 2015; Nweze *et al*., 2016).

The possible explanation for these results is the reduced concentration of antibacterial compounds left in honey upon dilution. Even though the exact compounds or factors that are responsible to the antibacterial activity were unknown in this study, it has been well established that the antibacterial activity of honey is contributed by some markers such as hydrogen peroxide (Brudzynski *et al*., 2011; Cooke *et al*., 2015; Sowa *et al*., 2017), osmotic effect due to high sugar content in honey (Molan & Rhodes, 2015), low pH and phytochemicals (Kateel *et al*., 2017). Bang *et al*., (2003) have reported that the concentration of hydrogen peroxide was optimal at honey concentrations between 15 to 67% (v/v) over 30 min of incubation. If the antibacterial activity was due to hydrogen peroxide, the increment in zone of inhibition in higher dilution of honey would be seen for MRSA, *P. aeruginosa*, *S. aureus* and *S. pyogenes*. However, in our study, there was no increment of ZOI against those pathogens at honey concentrations between 25% and 50% (v/v), indicating that antibacterial activity of GTM honey and Senduduk honey observed in this study might not be due to hydrogen peroxide. This observation was qualitative as we did not measure the actual level of hydrogen peroxide formation in honey solutions or adding catalase to honeys prior to antibacterial assay to diminish the hydrogen peroxide in order to rule out its contribution to the antibacterial activity observed. We assumed that the formation of hydrogen peroxide in these honeys had occurred. This is because the glucose oxidase in honey is only active in producing hydrogen peroxide when honey is diluted (Bucekova *et al*., 2014; Strelec *et al*., 2018). However, the concentration of hydrogen peroxide formed in these honeys might not reach the levels that give a significant antibacterial effect. There are a few possible factors affecting the level of hydrogen peroxide in honey and one possible explanations is that the level of glucose oxidase varies between honeys depending on honey phytogeographical origin (Bucekova *et al*., 2014) which results in different levels of hydrogen peroxide formation in honeys (Matzen *et al*., 2018). On the other hand, the activity of glucose oxidase could also be suppressed by the low pH of honey (Sumaiya & Trivedi, 2015). Those possibilities complicate the determination of hydrogen peroxide as a sole agent for antibacterial activity observed in the present study. The osmotic pressure was not supposed to be a contributor to the antibacterial activity observed as GTM honey and Senduduk honey were more watery (less viscous), indicating that the concentration of sugar was low. Furthermore, the osmotic pressure would definitely get weaker upon dilution. The concentration of artificial honey with 83% w/v of sugars is enough to inhibit the growth of many pathogens (Schneider *et al*., 2013), but it did not inhibit the growth of pathogens in this study. The undiluted artificial honey gave no antibacterial activity, therefore, the observed results in the present study may not be attributed to an osmotic effect.

As a result, at this point, it could be partially suggested that the antibacterial activity exerted by GTM honey and Senduduk honey would be most likely due to the phytochemicals or other factors in honey which produced antibacterial effect. Honey has been known to contain high phenolic compounds. Some phenolic compounds such as flavonoids have been studied to possess antibacterial activity (Xie *et al*., 2015). Mazol *et al*. (2016) have demonstrated that the phenolic fractions of polyfloral, rape and buckwheat honeys have antibacterial activity against *K. pneumoniae* and *E. coli*. Escuredo *et al*. (2012) had tested the phenolic compounds from *Rubus* honey against several bacteria and found that *Bacillus cereus* and *Proteus mirabilis* were the most susceptible bacteria to the phenolic fraction. Yap *et al*. (2015) had also tested the phenolic fractions of some Malaysian honeys against *E. coli*, *S. aureus*, *Salmonella enteridis*, *Bacillus cereus* and *B. subtilis*, and found that the phenolic compounds have antibacterial effect on these pathogens. Other factors such as the bacterial isolates in honey that produced antibacterial compounds (Olofsson *et al*., 2016) and organic compounds such as glycoproteins (Brudzynski *et al*., 2015) have been reported to have this effect. Further investigations should be carried out in future to ascertain whether the

antibacterial effect was due to hydrogen peroxide, phytochemicals or additional substituents in these honeys. Nevertheless, it is worth noting that the differences in antibacterial activity between GTM and Senduduk honeys against MRSA could be due to the differences in nectar sources, due to the fact that GTM was a multifloral honey, where the sources of nectar were from flowers in the forest, while Senduduk honey was derived from the flower of *M. malabathricum* L. but geographical and entomological factors may not give any significant influences since both honeys were collected from the same region (Jeli) and from the same bee tribe (Meliponini).

The MICs and MBCs of the three honey varieties were determined using microdilution method. The broth microdilution method was used due to the efficiency of this method to indicate quantitative results compared to the agar diffusion method (Jantakee & Tragoolpua, 2015). In general, all honeys tested in the present study showed MICs in the range of 3.13 to 12.5% (v/v). The GTM honey inhibited all of the pathogens at a lower MIC (3.13% v/v) than Senduduk honey (MIC 6.25% v/v). HTM honey showed higher MICs (12.5% v/v) than GTM and Senduduk for all pathogens tested. The MIC values obtained in this study were in line with other findings on stingless bee honeys. Study by Boorn *et al*. (2010) reported that *Trigona carbonaria* honey (Australian stingless bee honey) exhibited MICs at 4 to 16% (w/v) against *S. aureus* and MRSA by broth microdilution method. The Ethiopian honey, Tenegn (*Trigona* sp.) honey showed MIC against *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) at 6.25% (v/v) (Andualem, 2014). Earlier on, Ewnetu *et al*. (2013) had tested the Ethiopian stingless bee honey and found the MICs against the sensitive strains of *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) were similar to Andualem (2014), while the MICs against resistant strains of *E. coli*, *K. pneumoniae* and *S. aureus* were 6.25, 6.25 and 12.50% respectively. Recently, Nweze *et al*. (2016) reported that the MICs of *Hypotrigona* and *Melipona* spp. honeys against isolates of multidrug resistant *S. aureus*, *E. coli* and *P. aeruginosa* ranged between 12.50 to 25% (v/v) and 6.30–25% (v/v), respectively. In comparison to the study by Zainol *et al*. (2013) who found the MIC values of Kelulut honey produced by Malaysian *Trigona* spp. bee against *S. aureus*, *E. coli* and *P. aeruginosa* were at 20% (v/v), the MIC values for all honeys in this study were considered low. This indicates the strength of these honeys which require low concentration to inhibit the growth of the pathogens. In this study, the MBC values of GTM honey were 12.50% (v/v) except for MRSA and *P. aeruginosa* which were 6.25% (v/v). The MBCs for Senduduk honey and HTM honey were 25% (v/v) against all pathogens. The MBC values of the honeys in this study were in line with the findings of others who found the MBCs of stingless bee honeys were to be in the range of 3.13% to 50% (v/v) (Zainol *et al*., 2013; Andualem, 2014; Nweze *et al*., 2016). It is interesting to note that the MBC value of GTM honey was lower than that in Tualang honey which possessed the MBC of 25% (w/v) (Tan *et al*., 2009). This could be an indication that honey from stingless bee is better in its bactericidal effect than other honeys produced by *Apis* spp.

Comparative to GTM honey and Senduduk honey, HTM honey did not inhibit the growth of pathogens except for MRSA which only showed moderate sensitivity to undiluted honey. Five out of six pathogens did not show any susceptibility to HTM honey either in undiluted or diluted form when tested using agar well diffusion technique. However, when HTM honey was proceeded for MIC and MBC determinations using broth microdilution, it was evident that all pathogens showed the sensitivity to honey when the MICs and MBCs could be detected visually. Therefore, based on the differences in results of these two techniques for HTM honey, the results showed by agar well diffusion should not be immediately misinterpreted as that pathogens were resistant to this honey. This is an important limitation in this study that must be addressed. Many honey researchers have taken note that there are some limitations to the technique based on diffusion of honey through agar, either using paper disc or well (Kwakman & Zaat, 2012; Zainol *et al*., 2013). Boorn *et al*. (2010) have shown that the technique based on diffusion through agar has limitations in its detection and does not necessarily generate results that are representative of total antimicrobial activity. The ZOI can be affected by several factors such as the solubility, size and the rate of diffusion of the antibacterial compounds through agar matrix. For instance, Kwakman & Zaat (2012) mentioned that the antibacterial components in honey need to diffuse through the agar matrix and the rate of diffusion for high molecular weight antibacterial components through the agar matrix will be delayed. In addition to that, Zainol *et al*. (2013) commented that the diffusion technique could lead to the exclusion of large molecules which are not properly absorbed through agar and may contribute to inaccurate results. Furthermore, the hydrophobic compounds which have antibacterial property should have limitation in migrating through agar which is hydrophilic in nature. Therefore, based on these factors, it is expected that different types of honey with possibly different characteristics of antibacterial compounds might contribute to the variations in ZOI. The agar diffusion technique is widely employed and considered a practical approach to study the antibacterial activity of honey due to easy handling and its relatively cheap cost, however, the results could be mistakenly interpreted. This is of importance when one is doing separation work in order to determine the antibacterial compounds using bio-guided antibacterial activity assay. In this study, we could partially conclude that agar well diffusion might not be the appropriate technique in assaying antimicrobial activity in these

honeys. This is evident since we could determine MIC and MBC values of all honeys for all pathogens tested when using broth microdilution. This technique allows ~~a~~ direct contact between honey compounds and pathogens in the medium culture which eliminates the factor of the migration rate of honey compounds through agar medium (Zainol *et al.*, 2013). A clear example for this case is that our study showed the minimal concentration of GTM and Senduduk honeys that inhibited the growth of *S. pyogenes*, MRSA, *S. aureus* and *P. aeruginosa* in agar well diffusion was

The management of pathogens infecting wound is of great importance. *P. aeruginosa* and *S. aureus* are always in association ~~of~~ with polymicrobial infection (Bessa *et al*., 2015) and delay the wound healing. Meanwhile, *S. pyogenes*, of clinical significance in wounds can initiate infection, destroy skin grafts and persist as a biofilm (Brouwer *et al.*, 2016). The presence of a considerable percentage of MRSA may complicate the treatment, especially in burn injuries. Our results clearly show that the GTM honey has significant antibacterial effect than Senduduk honey against MRSA which add value to the potential use of this multifloral honey in combating MRSA infection. The fact that the MICs and MBCs of this honey against these pathogens are low (3.13% and 6.25–12.50 % v/v) indicate the potency of GTM honey.

Conclusion

In this study, it was clear that honeys from different entomological and floral sources from two regions of Kelantan have variable antibacterial activity against pathogens tested. Honey produced by *G. thoracica* was seen to possess better antibacterial activity regardless of their floral sources than honey produced by *H. itama*. The effectiveness of honey produced by *G. thoracica* from the Jeli region as an antibacterial agent may be related to their floral sources. It is known that mountainous tropical ecosystems are highly loaded with floral diversity. This includes *M. malabathricum* L, a tropical small shrub plant that is commonly found throughout Malaysia which grows wild ~~ly~~ and are abundant in the moist areas and mountain forests. Due to the origins of the floral sources that may influence the antibacterial property of the GTM and Senduduk honeys and the nature of stingless bees which prefer to inhabit the forest, it is therefore vital to sustain the ecosystem by preventing human activities such as logging, in order to preserve the quality of these honeys. Future investigations should include comparisons of hive products from many stingless bee species, phytogeographic regions, flowering seasons and their physicochemical properties such as higher acidity and hydrogen peroxide level to reaffirm the specific factors which dictate the antibacterial activity of honey of the stingless bee species. Nevertheless, this study scientifically shows the potential use of Malaysian stingless bee honeys as an alternative therapeutic agent for treating wound infections.

50% (v/v) which was higher than in broth microdilution (3.13% v/v). This is in agreement with the suggestions of previous authors (Pimentel *et al*., 2013; Akujobi & Njoku, 2010) that the broth microdilution is more sensitive and detection of antibacterial activity could be made at low honey concentrations. Even though the results of these two techniques were incomparable due to differences in mechanism of action, the results obtained from broth microdilution were considered more accurate (Othman *et al*., 2011).

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