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Antibacterial Properties of Kelulut, Tualang and Acacia Honey against Wound-Infecting Bacteria

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ABSTRACT

Bacterial infection is the most common cause of contamination that affects wound healing. This study aims to investigate the bacteriostatic and bactericidal effects of three varieties of Malaysian honey represented by two polyfloral honey varieties - Kelulut and Tualang, as well as one monofloral honey – Acacia, against eight common bacteria that infect wounds. The factors contributing to the antibacterial properties of honey such as acidity, peroxide compounds, and non-peroxide compounds, were determined using the agar well diffusion assay method and compared with medical-grade Manuka honey used in wound care (UMF 18 +). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using honey concentrations of 1.3% to 90% (w/v). The MICs for Kelulut, Tualang, and Acacia ranged from 5% to 12.5% (w/v), 12.5% to 30% (w/v), and 25% to 50% (w/v) respectively. Meanwhile, the MBCs were found to range from 5% to 12.5% (w/v), 12.5% to 90% (w/v), and 25% to 90% (w/v) respectively. Kelulut showed the highest inhibition activity. The antibacterial properties of Malaysian honey were generally comparable to Manuka. However, Kelulut bore the closest resemblance and was highly dependent on an acidic environment as the major antibacterial factor. This effect was further

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supported by the presence of peroxide and non-peroxide compounds.

Keywords: Antibacterial properties, honey, non-peroxide activity

INTRODUCTION

Honey consists of mostly glucose, fructose and trace elements of minerals, vitamins, proteins and amino acids. It can be classified into monofloral and polyfloral honey (Bradbear, 2009) based on botanical resources. Monofloral honey is produced by bees that have been foraging predominantly on one type of plant only, while polyfloral honey originates from several types of plants (Matzen et al., 2018; Ranneh et al., 2018; Samat et al., 2014). Honey possesses a number of pharmacological benefits such antibacterial (Kateel et al., 2018), anti-inflammatory (Liu et al., 2013; Nooh & Nour-eldien, 2016), antioxidant (Liu et al., 2013; Ranneh et al., 2018), and wound healing or tissue repair characteristics (Adewumi & Ogunjinmi, 2011; Rashidi et al., 2016). Recently, the usage of honey as an antibacterial agent in the treatment of ulcers (Kateel et al., 2018), wounds (El-malek et al., 2017), and other surface infections (Mcloone et al., 2016) is gaining traction in the medical industry.

Microbial infections are caused by the presence of microbes such as bacteria, fungi, and viruses; the most common being bacterial infections that often affect wound healing in humans (Yang et al., 2017). Sufficient numbers of bacteria can cause repair mechanisms, such as graft and flap formation, to fail (Sussmann & Bates-Jensen, 2012). Wound infections can be caused by Gram-positive and Gram-negative bacteria. *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., *Escherichia coli*, *Klebsiella* sp., *Proteus* sp. and *Pseudomonas aeruginosa* are among the common causes of bacterial infections in foot ulcers (Kateel et al., 2018), skin ulcers (Yang et al., 2017), post-surgical wounds (Kasithevar et al., 2017) and chronic wounds (El-malek et al., 2017; Nasir et al., 2016; Sienkiewicz et al., 2016). In Malaysia, *Staphylococcus aureus* (Mustafa et al., 2015; Nasir et al., 2010, 2016), *P. aeruginosa* (Nasir et al., 2010, 2016), and *Klebsiella pneumoniae* (Low et al., 2017; Nasir et al., 2010) are commonly associated with wound infections. These bacteria are capable of developing multidrug resistance towards antibiotics, giving rise to superbugs such as methicillin-resistant *S. aureus* (MRSA) (Bereket et al., 2012), multidrug-resistant *P. aeruginosa* (Bereket et al., 2012), and carbapenem resistant *K. pneumoniae* (Low et al., 2017). There has been a call to reduce the usage of antibiotics in order to prevent the emergence of such resistant bacteria. Hence, it is important to explore antibacterial agents that do not involve the usage of antibiotics.

The antibacterial properties of honey are attributed to its osmotic effect (Mandal & Mandal, 2011; Molan, 1992), acidity (Bogdanov, 1997; Molan, 1992), and presence of peroxide and non-peroxide compounds (Kwakman et al., 2010; Zainol et al., 2013). The osmotic effect

of honey is due to its low water content, which is produced by strong interactions between sugar and water molecules, thus reducing the amount of water available for microorganisms (Mandal & Mandal, 2011). During the ripening of nectar, enzymatic action produces gluconic acid, which in turn increases the acidity of the honey (Molan, 1992). Since the optimum pH range for bacteria is from 7.2 – 7.4 (Molan, 1992), the pH of honey, which is between 3.4 and 5.4, inhibits bacterial growth (Bogdanov, 1997). Aside from osmotic and acidic characteristics, peroxide and non-peroxide compounds were identified as dominant bio-active components responsible for the antibacterial properties of most types of honey (Irish et al., 2011; Kwakman & Zaat, 2012; Mandal & Mandal, 2011). Peroxide compounds, usually represented by hydrogen peroxide (H_2O_2), cause an increase in oxidative stress, which is beneficial when it comes to controlling bacterial colonization in wound areas (Brudzynski et al., 2011; Zainol et al., 2013). The presence of non-peroxide compounds, such as phenolic compounds (Kwakman & Zaat, 2012), antimicrobial peptides (AMP) (Kwakman et al., 2011; Kwakman & Zaat, 2012), flavonoids, leptosperin (Roberts et al., 2015), and methylglyoxal (MGO) (Kwakman et al., 2011; Kwakman & Zaat, 2012) are considered unique since the compounds were not presented in all honey for inhibiting bacterial growth. In Malaysia, various types of honey, such as Tualang, Kelulut, Acacia, and Gelam, have been reported to possess antibacterial properties due to the presence of the aforementioned factors (Zainol et al., 2013).

This study aims to explore the antibacterial properties of Malaysian honey against common infectious bacteria. Tualang, Kelulut, and Acacia honey were selected to be evaluated against eight bacterial strains: *S. aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *E. coli*, *P. aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis* and *K. pneumoniae*. These bacteria are widely known to cause wound infections (El-malek et al., 2017; Kasithevar et al., 2017; Kateel et al., 2018; Nasir et al., 2016; Sienkiewicz et al., 2016; Yang et al., 2017) and have the potential to develop drug resistance (Bereket et al., 2012; Low et al., 2017). We believe that this is the first study to consider a large number of bacteria strains associated with wound contamination in local patients (Low et al., 2017; Mustafa et al., 2015; Nasir et al., 2010, 2016) that have not been evaluated with Malaysian Kelulut and Acacia. The antibacterial properties were evaluated based on the bacteriostatic and bactericidal effects through the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The naturally acidic environment, and peroxide and non-peroxide compounds were identified as factors responsible for the antibacterial properties of honey. The antibacterial properties of Malaysian honey were evaluated and compared with the medical-grade Manuka honey used for wound care (UMF 18 +).

MATERIALS AND METHODS

Honey Samples

Honey samples were obtained from a local apiarist (Bee Park Pahang Sdn. Bhd., Bayu Gagah Marketing Sdn. Bhd., and Federal Agricultural Marketing Authority (FAMA) Corporation Sdn. Bhd., Malaysia) in sterile glass bottles. Prior to obtaining the honey samples, the quality and authenticity of all honey samples were approved by Malaysian Agriculture Research and Development Institute (MARDI), and Food Quality and Safety Research and Development (UNIPEQ). The three types of Malaysian honey considered in this study were Tualang, Kelulut, and Acacia. The commercially available medical-grade Manuka honey (*Comvita*® Wound care UMF 18+, New Zealand) was used as a basis of comparison in order to validate the reliability of this study.

Bacteria

Eight wound-associated bacteria commonly known to infect wounds were used in this study. These bacteria were kindly supplied by the Department of Pathology and Laboratory Medicine, International Islamic University Malaysia Medical Centre (IIUMMC), and Central Laboratory Universiti Malaysia Pahang (UMP), all labelled as standard strains from the American Type Culture Collection (ATCC, US). Three of the eight bacteria were Gram-positive bacteria – *S. aureus* ATCC 6538, *S. pyogenes* ATCC 19615, and *E. faecalis* ATCC 29212, while the other five bacteria were Gram-negative – *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. typhimurium* ATCC 14028, *P. mirabilis* ATCC 12453 and *K. pneumoniae* ATCC BAA 1144. The bacteria were cultured on nutrient or soy agar and incubated at 37ºC for 24 h, during which they were known as primary cultures. Working bacterial cultures were prepared by inoculating a loop of primary culture into the sterile screw-capped test tubes containing 10 mL of nutrient or soy broth. These cultures were incubated in an incubator shaker (Infors AG CH-4103 Bottmingen) for 24 h at 37ºC and rotational speed of 150 rpm.

Preparation of Honey Samples

The honey samples were diluted to a range of concentrations in preparation for future assays. For the evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), Kelulut, Tualang, Acacia, and Manuka samples were first diluted to a range of 30% and 90% (w/v) in water, then further diluted in broth to a final concentration of 1.25% to 30% through a stepwise two-fold dilution. For both evaluations, sugar-based (SB) samples containing the major sugar compounds which are commonly present in honey were used as artificial honey. These sugar compounds are fructose, glucose, maltose, and sucrose (Ahmed & Othman, 2013; Jalil et al., 2017). High concentrations of SB solutions were prepared by mixing 40% (w/v) fructose (Sigma, US), with 30% (w/v) glucose (Sigma, US), 8% (w/v) maltose (Sigma, US), and 2% (w/v) sucrose (Sigma, US).

In addition to MIC and MBC evaluations, efforts were also made to verify the presence of peroxide compounds and the factors contributing to antibacterial properties possessed by each type of honey by studying the effects of acidity, peroxide compounds, and osmotic pressure. In this case, each honey sample was diluted to a final concentration ranging between 30% and 90% (w/v).

Minimum Inhibitory Concentration (MIC)

The MIC of each bacterium was determined by using the method previously described in Tan et al. (2009) and Zainol et al. (2013) with slight modifications. This assay was performed in a sterile 96-well flat-bottomed polystyrene microtitre plates (Nunclon™, Thermo Fisher Scientific). A working bacteria culture was prepared accordingly and adjusted to a final concentration of 1 x 10^8 colony forming units (CFU/mL), which is equal to 0.5 McFarland standard. The adjusted concentration of bacteria at 0.5 McFarland was prepared based on optical density by diluting the working bacteria culture in fresh sterile broth until the absorbance ranged from 0.08 and 0.13 (Franklin et al., 2012). The absorbance of the prepared cultures was measured using UV-viscometer (Shimadzu, Japan) at a reference wavelength of 600 nm. The honey samples, prepared as described in the previous section, were dispensed into the test and control wells. From each concentration of honey sample, 190 µL was aseptically transferred into the prepared 96-well plate containing 10 µL of adjusted cultures. Cultures without honey samples served as the positive control while wells containing only nutrient broth and honey samples served as negative controls.

The samples were incubated in the incubator shaker (Infors AG CH-4103 Bottmingen) at 37ºC, 120 rpm for 24 h. The absorbance of the samples was measured at a time prior to incubation (known as $t = 0$), and at an elapsed time after 24 h of incubation (known as $t = 24$) at a wavelength of 590 nm. The percentage of growth inhibition, also known as MIC was calculated for each sample using Equation (1).

$$MIC = [1 - \frac{Absorbance\ of\ the\ test\ well}{Absorbance\ of\ corresponding\ control\ well}] \times 100\%$$

Eq. 1

The MIC values were expected to fall between 0% and 100%, where 0% indicated no effect on bacterial growth, and 100% indicated detrimental effects on bacterial growth. The MIC value refers to the lowest concentration of a test material which results in up to 95% growth inhibition in the test organism. All MIC values from Kelulut, Tualang, and Acacia were compared to those obtained from Manuka, and SB solutions. In addition, the growth of the bacteria was plotted to study the inhibition profiles of each honey sample. The growth inhibition responses for all tested bacteria were plotted with respect to honey concentrations ranging from 5% to 90% (w/v).

Minimum Bactericidal Concentration (MBC)

The MBC evaluation is a continuation of the MIC assessment. The MIC does not identify whether or not the bacteria is killed. Therefore, an evaluation of MBC was required. To evaluate MBC, the wells which did not show visible growth after the MIC evaluation were taken into consideration. One loopful of suspension from clear wells were subcultured on freshly prepared Trypticase Soy Agar (TSA) using the spread plate method (Zainol et al., 2013). The cultures were spread evenly on the agar surface before being incubated at 37ºC for 24 h. These samples were then examined for colony formation, which were taken to be a sign of bacterial growth. Three biological replicates were performed for each test. The concentration honey was considered as bacteriostatic if growth occurred after being cultured on the TSA, and bactericidal when inhibition of growth persisted (Zainol et al., 2013). The lowest concentration showing no growth of test organisms was considered to be the MBC. All MBC values from Kelulut, Tualang, and Acacia were compared to those obtained from Manuka, and SB solutions.

Preparation of Untreated (UT), Peroxide Non-peroxide (PNP), and Non-peroxide (NP) Samples

In this section, an attempt was made to identify the role of acidic pH, and peroxide and non-peroxide compounds that contribute to antibacterial properties of honey. Each honey sample was diluted in deionised water to a final concentration from 30% to 90% (w/v). For this assay, seven honey samples that were not subjected to any catalase treatment were prepared at a volume of 2 mL each and were denoted as untreated honey (UT). In UT samples, the acidic pH, peroxide compounds, and non-peroxide compounds were preserved because no elimination agents were added. This preparation was repeated to evaluate peroxide non-peroxide (PNP), and non-peroxide (NP) activity to verify the role of peroxide compounds in contributing to antibacterial properties. A non-acidic honey sample can be achieved through titration using of 5% (w/v) NaOH until the pH of the honey reached 7.0. A non-acidic, peroxide-free sample requires both titration using 5% (w/v) NaOH, and addition of catalase solution to the honey samples to catalyze the decomposition of hydrogen peroxide to water (Kwakman et al., 2010, 2011). The pH for the prepared solutions were measured. The UT, PNP and NP solutions are described in Table 1.

As for the catalase solution, a concentration of 4000 unit/mL was used (Adams et al., 2008). It was prepared by adding 10 mg of catalase (Sigma, US) to ultra-pure water to make a final volume of 5 mL. It has been suggested that the efficacy of catalase in removing peroxide compounds in water should be tested (Brudzynski et al., 2011; Zainol et al., 2013). To do so, six different sets of experiments were conducted: 1) 50% (w/v) Kelulut, Tualang, Acacia, and Manuka in pure water; 2) 50% (w/v) of honey in pure water with an addition of catalase solution; 3) 50% (w/v) of honey in pure water with an addition of

1% (w/v) hydrogen peroxide (Bendosen, Malaysia); 4) 50% (w/v) of honey in pure water with an addition of 1% (w/v) hydrogen peroxide and catalase solution; 5) 1% of hydrogen peroxide in pure water; and 6) 1% of hydrogen peroxide with an addition of catalase solution. The solutions were tested in the same way as agar well diffusion assay. Each test was carried out in triplicate and the average values were calculated. The catalase solution was considered effective in removing hydrogen peroxide compounds if there was no increase in the inhibition zone of sample after the addition of 1% hydrogen peroxide.

Evaluation of Non-peroxide and Peroxide Activities in UT, PNP, and NP Samples

After the sample preparation, peroxide and non-peroxide activity were determined using the method previously described in Moussa et al. (2012) with slight modifications. These evaluations were performed on one type of Gram-negative bacteria (*S. aureus* ATCC 6538) and one type of Gram-positive bacteria (*E. coli* ATCC 8739). These bacteria were selected for their dominance in wound infection, clear and measurable inhibition zone on agar, and their potential of developing antibiotic-resistance. Nutrient agar was prepared according to the manufacturer's instructions. It was allowed to cool after being autoclaved at 100 kPa, 121ºC for 20 min. After a uniform swirling, the agar was poured into petri dishes and stored at 4ºC for 30 min to solidify. The bacteria cultures were prepared and adjusted to 0.5 McFarland standard, which is equivalent to 1.5 x 10^8 CFU/mL. All agar plates were inoculated using the

Table 1

Detailed description of UT, PNP and NP solutions

Solution	Honey	pH	Activity
UT	Kelulut	2.37 ±0.13	Honey was diluted with deionized water. Solutions with concentrations of 30% to 90% (w/v) of honey samples were prepared.
	Tualang	3.88 ±0.04	
	Acacia	4.25 ±0.09	
	Manuka	3.80 ±0.03	
PNP	Kelulut	7.37 ±0.22	Honey was diluted with deionized water and followed by titration with 5% (w/v) NaOH to neutralize the pH. Solutions with concentrations of 30% to 90% (w/v) of honey samples were prepared.
	Tualang	7.35 ±0.22	
	Acacia	7.27 ±0.11	
	Manuka	7.23 ±0.29	
NP	Kelulut	7.40 ±0.15	Honey was diluted with catalase solution at concentration of 4000 unit/ mL and followed by titration with 5% (w/v) NaOH to neutralize the pH. Solutions with concentrations of 30% to 90% (w/v) of honey samples were prepared.
	Tualang	7.29 ±0.27	
	Acacia	7.30 ±0.22	
	Manuka	7.27 ±0.24	

Note. The symbol ± represents the standard deviation, which was calculated between the three biological replicates

spread plate technique by spreading 100 µL of the adjusted 0.5 McFarland culture on the surface of the agar. After inoculation, wells of 6 mm in diameter were cut into the agar surface and filled with 80 µL of the test solutions. Manuka honey (UMF 18+) was taken to be the positive control, while sterile ultra-pure water and 4000 units/mL catalase solution were used as the negative control wells. Plates were incubated at 37ºC for 24 h. The diameters of the clear inhibition zones were measured in millimeter (mm), inclusive of the diameter of the well. Three biological replicates were performed for each assay.

RESULTS

Results of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Manuka honey has been very well studied in terms of its antibacterial properties against a number of bacteria (Adams et al., 2008; Henriques et al., 2011; Roberts et al., 2015). However, studies on the antibacterial properties of Malaysian honey varieties such as Kelulut and Tualang are limited (Ismail, 2016). Acacia, a monofloral honey available in Malaysia, is produced using the nectar of the *Acacia mangium* tree. It is known for its antioxidant properties and is associated with therapeutic medicinal effects such as inhibition of cancer cell growth (Salleh et al., 2017). Recently, Acacia has been studied for its antibacterial properties against several infectious bacteria and was found to possess both bacteriostatic and bactericidal effects (Zainol et al., 2013). Hence, this study includes Acacia in order to further understand its activities against infectious bacteria.

In this study, the MIC and MBC of Kelulut, Tualang, and Acacia were determined by using Manuka and SB used as the basis of comparison. The minimum bacteriostatic and bactericidal concentrations for all the bacterial strains used in this study are tabulated in Table 2. From the eight listed bacteria, Kelulut was observed to inhibit the growth of *P. aeruginosa*, *E. coli* and *P. mirabilis* growth at low concentrations of 3.75%, 7.5%, and 7.5% (w/v) respectively when compared with Tualang and Acacia. This value was 4.0, 1.3, and 1.7- fold stronger than Manuka. These findings are consistent with results shown by previous studies that examined the antibacterial activity of stingless bee honey from Borneo against bacteria associated with animals (Tuksitha et al., 2018). The MIC of Kelulut was approximately 10% (w/v) for *S. pyogenes*, similar to Manuka. However, other bacteria such as *K. pneumoniae*, *S. typhimurium*, *S. aureus* and *E. faecalis* were 2.0, 1.2, 2.0, and 1.3 times were more susceptible to Manuka respectively when compared to Kelulut. Acacia was the least strong bacteriostatic honey with MIC ranging between 30% and 50% while Tualang had MIC ranging between 20% and 40%. However, the results showed a different pattern for the MBC evaluation. Manuka honey was observed to be a stronger bactericidal agent for all bacteria except *P. aeruginosa* as compared to Kelulut, Tualang and Acacia

Table 2

Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Malaysian honey

	Kelulut		Tualang		Acacia		Manuka		SB	
	MIC†	MBC†	MIC†	MBC†	MIC†	MBC†	MIC†	MBC†	MIC†	MBC†
E. coli ATCC 8739	7.5%	40%	25%	>90%*	40%	>90%*	10%	12.5%	40%	>90%*
P. aeruginosa ATCC 9027	3.75%	12.5%	20%	40%	30%	50%	15%	20%	40%	>90%*
K. pneumoniae ATCC BAA 1144	12.5%	30%	30%	80%	40%	>90%*	6.25%	10%	40%	>90%*
S. typhimurium. ATCC 14028	7.5%	25%	20%	60%	40%	>90%*	6.25%	10%	40%	>90%*
P. mirabilis ATCC 12453	7.5%	25%	25%	90%	40%	>90%*	12.5%	15%	50%	>90%*
S. aureus ATCC 6538	10%	30%	20%	50%	30%	>90%*	5%	5%	50%	>90%*
S. pyogenes ATCC 19615	10%	20%	30%	90%	40%	>90%*	10%	20%	40%	>90%*
E. faecalis ATCC 29212	20%	50%	40%	>90%*	50%	>90%*	15%	25%	50%	>90%*

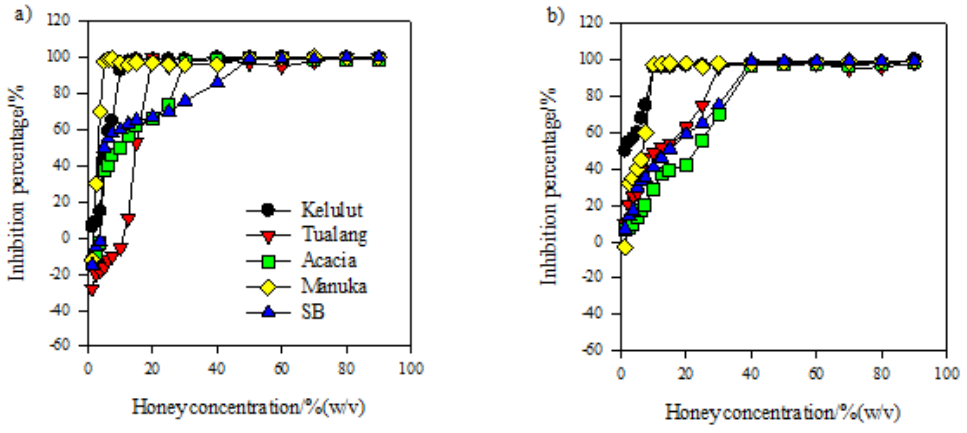
Note. The symbol * represents the highest percentage of honey concentration tested. † denotes a statistically significant difference between the mean of the sampled population and the hypothesised population mean. P-values were calculated based on the Student t-test at confidence interval of 95%. The test was performed on five biological replicates

honey. Kelulut was recorded to be the most potent antibacterial agent to *P. aeruginosa*, indicated by the lowest MIC and MBC values in both evaluations. These results are in good agreement with the previous work (Tuksitha et al., 2018).

Results of Growth Inhibition Profile of Gram-positive and Gram-negative Bacteria

Our second attempt began by studying bacterial growth response when Kelulut, Tualang, and Acacia samples were added to Gram-positive and Gram-negative bacteria. The experiments were performed with Manuka and SB as a basis of comparison. The results obtained were in the form of growth inhibition percentages at concentrations ranging from 1.25% to 90% (w/v). These results are shown in Figure 1 (for Gram-positive bacteria) and Figure 2 (for Gram-negative bacteria). In both figures, negative inhibition percentage may indicate that the concentration of glucose in honey was not enough to inhibit bacterial growth through osmotic pressure, but instead sufficient to support bacterial growth. Similar negative growth inhibition percentages were reported by previous research on bacteria such as *Bacillus cereus*, *Enterobacter clocae*, *P. mirabilis* and *Streptococcus agalactiae* (Tan et al., 2009; Zainol et al., 2013).

Klebsiella pneumoniae. According to Figure 2, Kelulut inhibited most of the Gram-negative bacteria at concentrations as low as 1.25% (w/v) except for *K. pneumoniae*. *Klebsiella pneumoniae* was observed to be more susceptible to Manuka and Kelulut – concentrations of 7.5% (w/v) already began to affect bacteria growth. On the contrary, the growth of this strain was less affected by Tualang and Acacia at concentrations less than 15% (w/v).



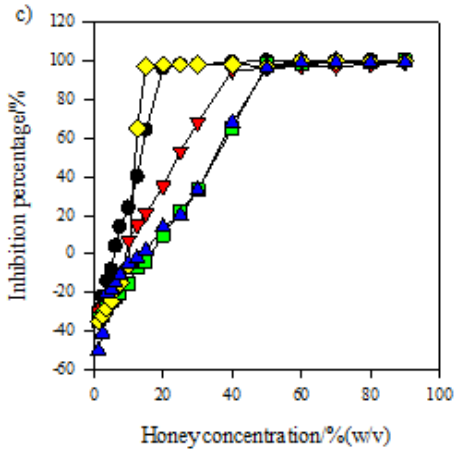


Figure 1. Growth inhibition percentage for Gram-positive bacteria of: a) *Staphylococcus aureus* ATCC 6538, b) *Streptococcus pyogenes* ATCC 19615, and c) *Enterococcus faecalis* ATCC 29212. Kelulut (circle, black), Tualang (triangle downward, red), Acacia (square, green), Manuka (diamond, yellow), and SB (triangle upward, blue)

Escherichia coli and Salmonella typhimurium. Our findings showed that less effort was needed to inhibit the growth of *E. coli* – the lowest concentration of all honey samples and SB solutions produced growth inhibition percentages ranging from 30% (Tualang), to 70% (Kelulut). However, the percentage of inhibition increased gradually when this strain was exposed to Acacia and Tualang. It took a concentration of more than 50% (w/v) for this strain to be 100% inhibited. *S. typhimurium* has similar response trends to *E. coli* when exposed to Tualang and Acacia.

Pseudomonas aeruginosa. As for *P. aeruginosa*, 100% growth inhibition was observed at a 3.75% (w/v) concentration of Kelulut, thus demonstrating its susceptibility to this variety of honey. However, it showed a strong resistance towards Manuka and Tualang. There was no growth inhibition for concentrations less than 12.5% (w/v) of Manuka, but the percentage abruptly jumped to 97% when the concentration was increased to 15% (w/v). A similar pattern was observed in Tualang. A mild resistance was demonstrated towards Acacia at concentrations of less than 5% (w/v).

Proteus mirabilis. Unlike other Gram-negative bacteria, *P. mirabilis* showed a strong resistance to Acacia honey. The addition of this honey only began to take effect at a concentration of 15% (w/v). The strongest growth inhibition for this strain occurred at the lowest concentration of Kelulut, which inhibited growth by 76%.

Staphylococcus aureus. This strain has different responses to different types of honey. The growth of *S. aureus* was more susceptible to Kelulut, starting at the lowest concentration of 1.25% (w/v). However, it was less susceptible to Tualang, requiring concentrations above 12.5% (w/v) for growth inhibition to begin. There was an abrupt increase in growth inhibition from 10% to 100% when the concentration of Tualang was increased from 12.5% to 20% (w/v). Interestingly, rather than reaching a plateau at absolute inhibition, the percentage dropped by ±5% at 95% and then slowly increased to 100% inhibition. This suggests that this strain may have developed resistance towards Tualang in the 25 - 70% (w/v) concentration range.

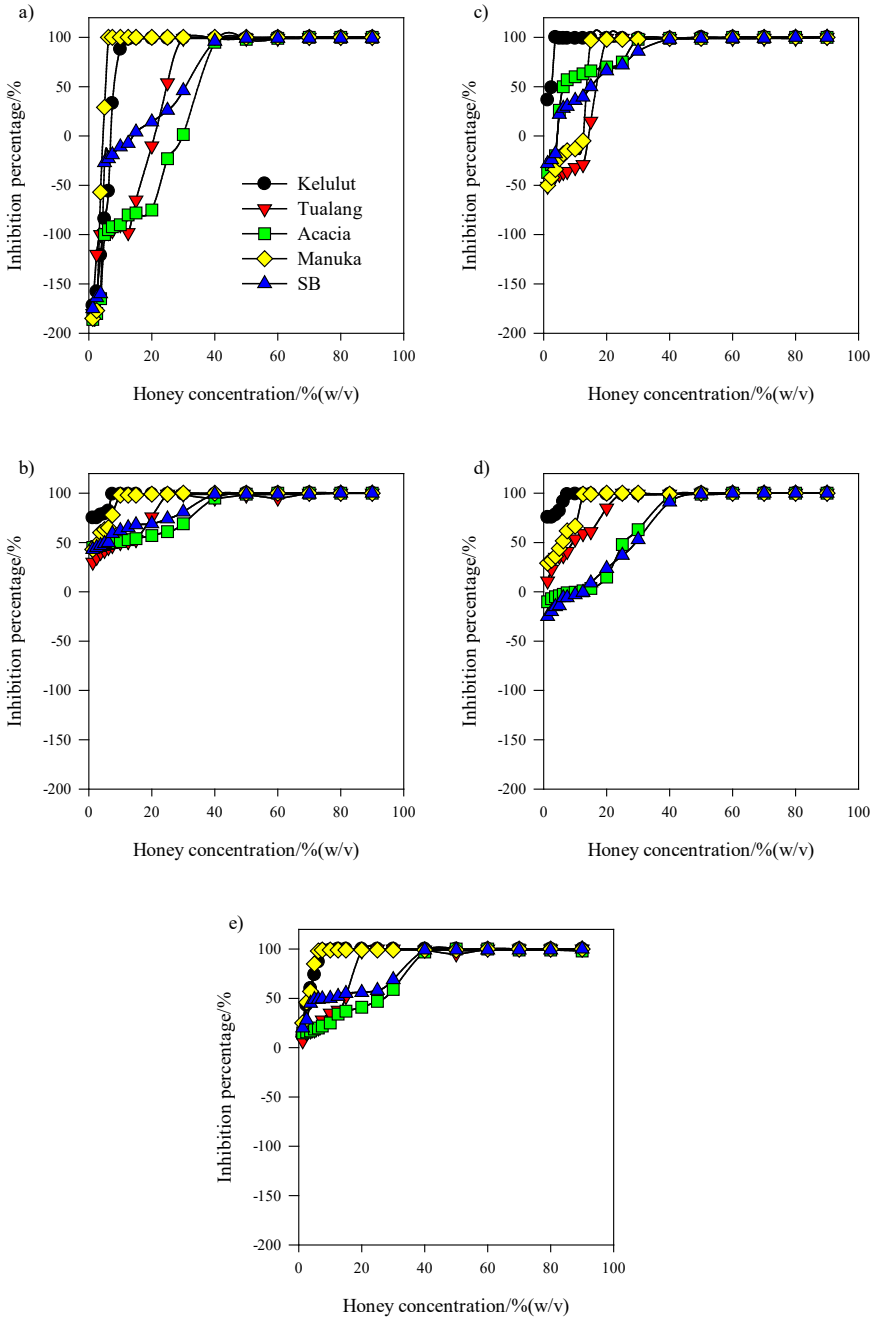


Figure 2. Growth inhibition percentage for Gram-negative bacteria of: a) *Klebsiella pneumoniae* ATCC BAA 1144, b) *Escherichia coli* ATCC 8739 c) *Pseudomonas aeruginosa* ATCC 9027, d) *Proteus mirabilis* ATCC 12453, and e) *Salmonella typhimurium* ATCC 14028. Kelulut (circle, black), Tualang (triangle downward, red), Acacia (square, green), Manuka (diamond, yellow) and SB (triangle upward, blue)

***Streptococcus pyogenes*.** The growth inhibition of *S. pyogenes* was promising when cultured with Kelulut and Manuka. There was a gradual increase in growth inhibition as the concentration increased. Besides that, this strain has shown similar trends of growth inhibition when exposed to Tualang and Acacia, with slightly lower percentage for Acacia.

***Enterococcus faecalis*.** This strain appeared to be the strongest of all the Gram-positive bacteria tested in this study when cultured with the different varieties of honey. Growth inhibition of *E. faecalis* occurred at 6.25%, 12.5%, 10%, and 20% (w/v) for Kelulut, Manuka, Tualang, and Acacia respectively, all requiring concentrations of more than 6.25%.

When exposed to SB solutions, two out of five Gram-negative bacteria showed strong resistance towards SB solutions. These bacteria were *P. mirabilis* and *K. pneumoniae*. Their growth inhibitions only started at 15% (w/v). Meanwhile, growth inhibition was only shown at concentrations of 5% (w/v) in *P. aeruginosa*, indicating a possible mild resistance. Both *E. coli* and *S. typhimurium* showed similar growth inhibition trends with a slightly lower growth inhibition percentage for *S. typhimurium*. As for Gram-positive bacteria, SB solutions have a are able to begin inhibiting the growth of *S. pyogenes* starting with the lowest SB concentration. The patterns of growth inhibition by SB solutions are similar to that of Acacia honey for both Gram-positive and Gram-negative bacteria, suggesting that the antibacterial properties of Acacia are more likely caused by the presence of high sugar content. The factors contributing to antibacterial properties of Kelulut and Tualang were then investigated.

Results of Role of Peroxide and Non-peroxide Compounds in Antibacterial Properties of Honey

The previous section has shown that the antibacterial properties possessed by Acacia could be due to its high sugar content. Here, an attempt was made to study how peroxide and non-peroxide compounds in Kelulut, Tualang, and Acacia affected the growth of *E. coli* and *S. aureus* as compared to the effects of Manuka. The inhibition zones of UT, PNP, and NP are indicated in Figure 3 for *S. aureus* (Figure 3a to 3c) and *E. coli* (Figure 3d to 3f).

UT solution preserved all factors when no elimination agents were added. As for the prepared PNP solution, it preserved peroxide and non-peroxide compounds while neutralizing the natural acidic characteristic of honey. Lastly, the prepared NP solution only preserved non-peroxide compounds and eliminated acidic and peroxide compounds.

In general, the UT solutions of Kelulut, Tualang, and Manuka showed similar patterns of inhibition in which the growth inhibition started at a concentration of 30% (w/v), and inhibition activity increased as the concentration increased. The growth inhibition of Acacia UT solution, however,

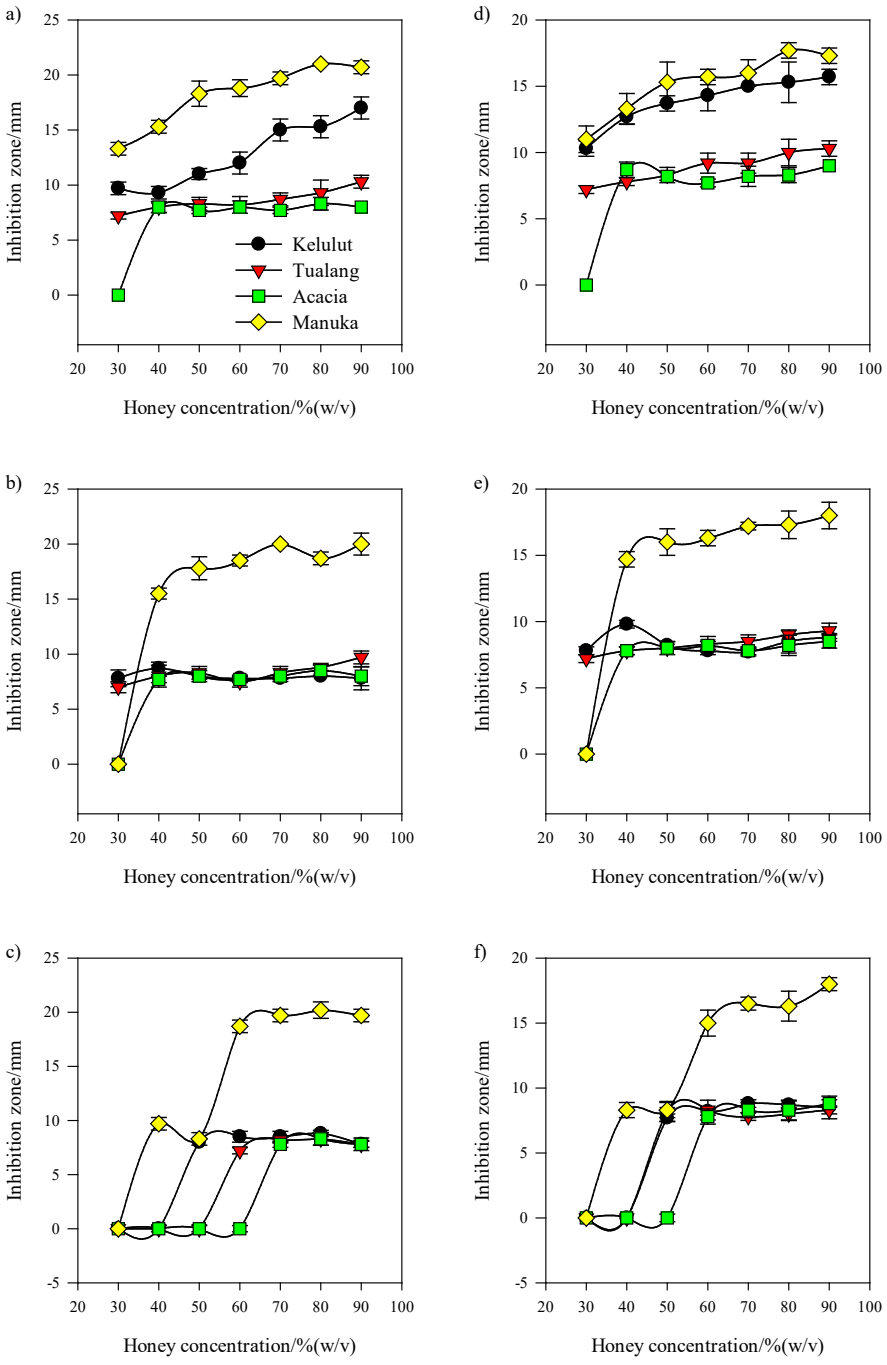


Figure 3. Inhibition activity of UT, PNP, and NP solution against *Staphylococcus aureus* ATCC6538 [(a) to (c)], and *Escherichia coli* ATCC8739 [(d) to (f)]. Kelulut (circle, black), Tualang (triangle, red), Acacia (square, green) and Manuka (diamond, yellow)

only began to appear at a concentration of 40% (w/v), and an increase in inhibition continued at higher concentrations. Among the Malaysian honey samples, both *S. aureus* and *E. coli* were most susceptible to the Kelulut UT solution, in which the inhibition activity was noticed to be as high as 1.7-fold compared with Tualang and 2.1-fold compared with Acacia.

The PNP solution demonstrated inhibition activity similar to the UT solution for all tested honey samples with no significant difference ($p>0.05$), except for Kelulut. The UT solution of Kelulut was dominated the growth inhibition assay when compared with other Malaysian honey varieties. However, the Kelulut PNP solution showed a pattern similar to that of to Tualang and Acacia instead of being comparable to Manuka PNP solution. Even so, large inhibition zones of 8.7 ±0.58 mm and 9.8 ±0.58 mm were recorded against *S. aureus* and *E. coli* by Kelulut PNP solution at a low concentration of 40% (w/v). These actions were noticed to be as high as 1.1-fold higher than the inhibition activity against *E. coli* when compared with *S. aureus*.

NP solutions for Kelulut, Tualang, and Acacia showed inhibition activities at different concentrations. The NP solution for Kelulut was the first to demonstrate inhibition activity against *E. coli* at a concentration of 50% (w/v). It was then followed by Tualang at 60% (w/v) and Acacia at 70% (w/v). For *S. aureus*, both NP solutions for Kelulut and Tualang began to inhibit growth at 50% (w/v), followed by Acacia at 60% (w/v). However, despite the inhibition of bacterial growth, these Malaysian honey samples showed no increment in the diameter of inhibition zone when higher concentrations of solutions were used for both *S. aureus* and *E. coli*. In contrast, the NP solution for Manuka began to show inhibition activity at a concentration of 40% (w/v) which continued to rise at higher concentrations against both *S. aureus* and *E. coli*.

DISCUSSION

Manuka honey is a monoflora honey produced from the nectar of *Leptospermum scoparium*, also known as the Manuka tree. This honey is widely known for its antibacterial properties due to the presence of an active non-peroxide compound called methylglyoxal (MGO) (Adams et al., 2009). MGO is a major bactericidal factor in Manuka (Kwakman et al., 2011). Besides MGO, osmotic pressure, acidic pH, leptosin, and hydrogen peroxide are also factors responsible for the antibacterial properties in Manuka (Carter et al., 2016; Roberts et al., 2015). Manuka has been found to have both bacteriostatic and bactericidal effects on numerous Gram-positive and Gram-negative pathogenic bacteria (Kwakman et al., 2011). Other than being antibacterial, Manuka has been reported to possess various pharmacological effects such as healing properties (Carter et al., 2016), antiviral properties (Watanabe et al., 2014) and antiulcer properties (Almasaudi et al., 2016). To date, Manuka is a widely accepted honey variety that has been accepted for usage during the medical care of wounds

due to its potent antibacterial properties. The term Unique Manuka Factor (UMF) is a global standard used to classify bioactive compounds that exist in Manuka honey. Manuka honey with a UMF value of more than 10 has been strongly recommended for wound care instead of honey of low or unknown potency (Tan et al., 2009). As a result, Manuka has been frequently used as a basis of comparison when it comes to determining the potency of antibacterial properties in honey.

In Malaysia, the distribution of Kelulut honey is lower compared to that of the common honeybee. Limited knowledge about this type of honey has resulted in less popularity in industrial production, and lack of research regarding its medicinal properties (Jalil et al., 2017). Kelulut honey has been found to possess beneficial effects such as antibacterial, antioxidant and anti-inflammatory properties (Jalil et al., 2017), and was proven to have bacteriostatic and bactericidal effects on various Gram-positive and Gram-negative bacteria (Tuksitha et al., 2018; Zainol et al., 2013). This study attempted to evaluate the antibacterial properties of Kelulut against bacteria commonly associated with wound infection. Several earlier studies have investigated the antibacterial properties of Kelulut against other pathogenic bacteria. When the results of this study were compared with the results of previous studies, the MIC of Kelulut in this study was found to be within the same range, studies – 3% to 20% (Tuksitha et al., 2018; Zainol et al., 2013). The MBC was also within the same range as previous studies – 1% to 32% (Tuksitha et al., 2018; Zainol et al., 2013). The MIC values obtained in this study were between 5% and 20%, while the MBC values were found to be between 12.5% and 50%.

Among the infectious bacteria tested, *P. aeruginosa* and *Acinetobacter baumannii* were found to be most susceptible to Kelulut. The MIC and MBC of *P. aeruginosa* were the lowest when compared with other honey samples including Manuka. Previous studies have reported that Kelulut is capable of stopping the growth of *P. aeruginosa* at a range between 5% and 10%, and kills the bacteria after 10% (Tuksitha et al., 2018). Meanwhile, other studies have reported that Kelulut can simultaneously stop and kill the growth of *P. aeruginosa* at a concentration of 20% (Zainol et al., 2013). In this study, the MIC and MBC values were recorded at 3.75% and 12.5% respectively. *Pseudomonas aeruginosa* is a ubiquitous opportunist pathogen that is distributed throughout the environment, particularly in moist habitats. It is the cause of many illnesses such as endocarditis, folliculitis, keratitis, meningitis, pneumonia, urinary tract infections, and wound infections. Wound infections due to *P. aeruginosa* have given significant rise to persistent infections in burn patients and patients with chronic venous leg ulcers because it is a multidrug-resistant organism. The bacteriostatic and bactericidal abilities of Kelulut should be investigated further as a possible antibacterial agent against drug-resistant, wound-infecting bacteria such as *P. aeruginosa*. Kelulut was the only local

honey in this study that possessed both bacteriostatic and bactericidal effects against all the strains of bacteria tested. Similar effects were absent for Tualang and Acacia. This was consistent with findings reported by previous antibacterial studies (Tuksitha et al., 2018; Zainol et al., 2013).

Tualang is a popular polyflora Malaysian honey produced by *Apis dorsata* (Boukraâ, 2014). It has been reported to possess various pharmacological benefits such as antibacterial, antioxidant, and anti-inflammatory properties (Ahmed & Othman, 2013). Tualang was found to possess both bacteriostatic and bactericidal effects against numerous pathogenic Gram-positive and Gram-negative bacteria (Tan et al., 2009; Zainol et al., 2013). In this study, the antibacterial properties of Tualang were evaluated against bacteria commonly known to infect wounds. The MIC of Tualang was found to be within the previously reported range when tested against general pathogenic bacteria – 6.25% to 25% (Tan et al., 2009; Zainol et al., 2013). In previous studies, Tualang produced higher or unidentified MBC values between 12.5% and 50% when tested against the pathogenic bacteria (Tan et al., 2009; Zainol et al., 2013). After analyzing both MIC and MBC values, the ranges obtained for Tualang in this study were found to be slightly higher when compared with the reported range for general pathogenic bacteria. These differences may be due to the difference in origin of nectar, batch of honey collected, and technical variation while performing the experiment such as the amount of bacterial suspension used, the type of agar or broth, and the diluent used. Among the tested bacteria, *P. aeruginosa* recorded the lowest MIC and MBC, thus being the most susceptible to Tualang. The comparison between Gram-positive and Gram-negative bacteria showed that they were equally susceptible to Tualang. This finding was concurrent with some of the previous reports (Tan et al., 2009).

Acacia honey demonstrated a similar inhibition pattern to sugar based solutions. Acacia honey is a monofloral honey produced by *Apis mellifera* or *Apis cerana* using nectar from the plant *Acacia mangium* (Bradbear, 2009; Samat et al., 2014). Acacia is one of the most widely commercially available types of honey in Malaysia reported to contain various pharmacological benefits such as antibacterial properties. Previous antibacterial studies against pathogenic bacteria reported that Acacia honey possessed both bacteriostatic and bactericidal effects with a MIC value between 15% and 25%, and MBC value between 25% and 50% (Zainol et al., 2013). In this study, we found that Acacia possessed the least potent antibacterial properties against the bacteria tested. This was based on the high MIC values, and the high or unidentified MBC values observed. This study found that the MIC and MBC for Acacia ranged from 30% to 50%, and 50% to 90% respectively. Similar to Tualang, it was apparent that the ranges of MIC and MBC were higher in this study when compared with previous antibacterial studies.

Honey antibacterial properties can be attributed to acidic pH, and presence of peroxide and non-peroxide compounds. In this study, we considered these properties to determine the factors contributing to antibacterial properties in honey. Among the Malaysian honey samples, Kelulut was found to have the most potent antibacterial properties against all tested Gram-positive and Gram-negative bacteria associated with wound infection. By evaluating the contributing factors, the potent antibacterial properties of Kelulut were attributed mostly to its naturally strong acidic environment (pH 2.37 ±0.13). A previous study showed that the acidic pH found in Kelulut ranged from 3.29 to 3.71 (Chan et al., 2017), about 1.6-fold higher than in this study. When we compared the pH of Kelulut to Tualang and Acacia samples, the Kelulut had the lowest pH, about 1.8 to 1.6-fold lower than the other samples. The strong acidic environment of Kelulut may provide a partial explanation for its potent antibacterial properties. The strong acidity of Kelulut was found to equally affect both Gram-positive and Gram-negative bacteria which were represented by *S. aureus* and *E. coli* in this study.

In addition to pH, other factors such as peroxide and non-peroxide compounds could be major contributors towards the antibacterial properties of honey. Peroxide compounds are usually represented by hydrogen peroxide (H_2O_2) (Irish et al., 2011; Kwakman & Zaat, 2012; Mandal & Mandal, 2011). In order to evaluate the presence of peroxide compounds, the honey was diluted to a concentration of 30% to 50% (w/v) (Kwakman & Zaat, 2012; Molan, 1992). By doing so, the enzyme glucose oxidase was activated, thus oxidizing glucose into gluconic acid and H_2O_2 (White et al., 1963). Out of all the Malaysian honey samples tested, Kelulut demonstrated a higher inhibition activity at a concentration between 30% and 50% (w/v). The active peroxide activity in Kelulut noticeably affected the Gram-negative *E. coli*, more than the Gram-positive *S. aureus*. The inhibition activity demonstrated was 1.1-fold higher against *E. coli* compared to *S. aureus*. The results shown in this study support the active contribution of H_2O_2 towards the antibacterial properties of honey at concentrations of 30% to 50% and simultaneously demonstrated that peroxide compounds do contribute to the antibacterial properties of Malaysian honey against wound-infecting bacteria. However, a larger number of infectious bacteria should be investigated further to better understand the effect of peroxide activities on wound-associated bacteria.

As for non-peroxide factors, honey reportedly possesses compounds such as MGO, bee-defensin-1, leptosperin, phenolic acids, flavonoids, and jelleins (Ahmed & Othman, 2013; Jalil et al., 2017; Roberts et al., 2015; Salleh et al., 2017). Compounds such as MGO were extensively studied and were revealed to cause various antibacterial mechanisms including cell wall disruption and lysis (Henriques et al., 2011; Nishio et al., 2016), disruption in gene expression patterns (Blair et al., 2009) and DNA degradation (Brudzynski et al., 2011). It

is important to identify the availability of non-peroxide compounds and their actions as they may contribute to the production of potent antibacterial properties in honey (Kwakman et al., 2010, 2011). In this study, we removed acidic and peroxide compounds to determine the effect of non-peroxide activity in the honey samples. This technique of neutralizing the known factors was suggested by previous studies that determined the action of the compound that contributed to antibacterial properties of honey (Kwakman et al., 2010, 2011). By doing so, the presence of non-peroxide compounds and their role in producing antibacterial properties in Malaysian honey against wound-infecting bacteria can be observed. This study proved the presence of active non-peroxide compounds in Kelulut, Tualang, and Acacia. Our conclusion is that the non-peroxide activity may be due to the presence of flavonoid and phenolic compounds previously identified in these honey varieties (Ahmed & Othman, 2013; Jalil et al., 2017; Salleh et al., 2017). We found that non-peroxide activity equally affected Gram-positive and Gram-negative bacteria. However, it may be unfair to simply conclude the effect of non-peroxide activity in Malaysian honey in this study since only a single representative species for each Gram-positive and Gram-negative bacteria were considered. Nevertheless, we recommend further investigation on non-peroxide compounds in Malaysian honey against more species of wound-infecting bacteria in order to thoroughly understand the effects of these compounds. With all due consideration, this study has successfully demonstrated the presence of active non-peroxide compounds that contribute to antibacterial properties in Malaysian honey against two common infectious bacteria, *S. aureus* and *E. coli*.

As with any study, there are a few limitations that have to be considered during the course of this study, thus making further investigation crucial for building a complete understanding of the properties of Malaysian honey varieties. In the present study, drug resistant bacteria strains were not considered during the evaluation of honey's antibacterial properties. Further studies should evaluate the antibacterial properties of honey against these drug-resistant strains. The efficacy of honey as an antibacterial agent can be compared to the drugs commonly used to treat drug-resistant strains of bacteria such as Methicillin Resistant *Staphylococcus aureus* (MRSA). The outcome will confirm whether Malaysian honey is a suitable alternative for antibiotics, and whether the bacteria can develop resistance against honey. Should Malaysian honey proves to be an efficient and effective solution, it can slow the emergence of drug-resistant bacterial strains due to overuse of antibiotics. It can then be used to treat drug-resistant bacterial infections such as those caused by MRSA (Bereket et al., 2012), multidrug resistant *P. aeruginosa* (Bereket et al., 2012), carbapenem resistant *K. pneumonia* (Low et al., 2017), and B-lactam resistant *E. coli* (Jacoby & Sutton, 1985).

The antibacterial properties of honey are attributed to the presence of active compounds in the honey, partially due to the pollen and nectar collected by the corresponding bees. The present study considered a single sample of Malaysian Kelulut, Tualang and Acacia for antibacterial evaluation. Honey samples collected from different regions of Malaysia (e.g. Borneo and Peninsular Malaysia) may have variations in available compounds due to the variation in pollen and nectar sources for each region. Further studies should consider evaluating the antibacterial properties of honey samples from different regions of Malaysia to identify the available compounds and their effect on the antibacterial properties of honey.

CONCLUSION

Malaysian honey, especially Kelulut, proved to be dependent on acidic environment as a major antibacterial factor. This is further supported by the presence of peroxide and non-peroxide compounds. The antibacterial properties of Malaysian honey varieties were generally comparable to Manuka. The closest resemblance was demonstrated by Kelulut. In some cases, Kelulut showed equivalent or better antibacterial activity than Manuka, especially against *P. aeruginosa*. The antibacterial potency of Malaysian honey against microorganisms associated with wounds suggests the potential of honey as an alternative therapeutic agent, particularly for wound infection. Hence, this study proposes the usage of Kelulut at a concentration of 50% (w/v) or more to simultaneously stop and kill bacteria that are commonly found to infect wounds.

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