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The roles of acidity, peroxide and non-peroxide compounds in antibacterial properties of Malaysian Kelulut, Tualang and Acacia honey

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ABSTRACT

Aims: In this study, three putative factors that commonly contribute to antibacterial properties in honey were determined, namely acidity (pH level), peroxide compounds and non-peroxide compounds.
Methodology and results: Honey samples were prepared based on the known factors of acidity, peroxide compounds, and non-peroxide compounds to identify factors that contribute to the antibacterial properties of the honey based on agar diffusion assay. Liquid chromatography quadropole time-of-flight mass spectrometry was employed to detect and quantify the presence of acidic, peroxide, and non-peroxide compounds in the honey samples. Acidity and non-peroxide compounds were identified as the significant factors contributing to the antibacterial properties of Kelulut, Tualang and Acacia honey. No peroxide compound was detected in this study across all honey samples. In Kelulut, the presence of the additional compounds (reptoside, platycogenic acid and kauranoic acid) may explain its higher antibacterial properties against *Escherichia coli* and *Staphylococcus aureus* as compared to Tualang and Acacia honey based on the inhibition zones on the agar plates.
Conclusion, significance and impact of study: The presence of multiple antibacterial factors in honey is notably important as it gives an advantage of using honey compared to antibiotics in preventing the growth of a wide range of bacterial species with multiple modes of action.

Keywords: Acidity, antibacterial factors, Malaysian honey, non-peroxide, peroxide

INTRODUCTION

Honey is one of the natural resources used for medicinal purposes due to its antibacterial properties (Almasaudi, 2021). Honey has been proven to possess bacteriostatic and bactericidal properties useful for controlling bacterial infection (Ng *et al.*, 2020), including antibiotic-resistant strains (Brudzynski, 2021). Interestingly, limited studies have reported on the resistance of bacteria towards honey to date (Combarros-fuertes *et al.*, 2020). The presence of potent and irresistible antibacterial properties in honey can be understood by identifying the factors that contribute to its antibacterial properties.

The presence of antibacterial properties of honey is mainly to the osmotic effect (Mandal and Mandal, 2011), acidity (Bogdanov, 1997) and the existence of peroxide and non-peroxide compounds (Almasaudi, 2021). The osmotic effect of honey is produced by strong interactions between sugar and water molecules to reduce the amount of water available to microorganisms (Mandal and Mandal, 2011). As for acidity, it is due to enzymatic action to produce gluconic acid during the ripening of nectar, which creates an acidic environment in honey (Molan, 1992). The acidic pH of honey is generally measured between 3.2 and 5.4, which opposes bacterial growth at an optimum pH between 7.2 and 7.4 (Almasaudi, 2021). Apart from the osmotic effect and acidity, the presence of peroxide and non-peroxide compounds in honey were also identified as among the dominant factors responsible for honey's antibacterial properties (Irish *et al.*, 2011; Kwakman and Zaat, 2012). Peroxide compounds, usually represented by hydrogen peroxide (H_2O_2), cause an increase in oxidative stress that is beneficial to control bacterial colonisation in wound areas (Brudzynski *et al.*, 2011; Combarros-fuertes *et al.*, 2020). The presence of non-peroxide compounds is considered unique since

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these compounds were selectively available in honey to inhibit bacterial growth with various capabilities. The non-peroxide compounds are usually represented by phenolic compounds (Kwakman and Zaat, 2012), flavonoids, antimicrobial peptides (AMP) (Kwakman *et al*., 2011), leptosperin (Roberts *et al*., 2015) and methylglyoxal (MGO) (Girma *et al*., 2019; Nader *et al*., 2021).

Numerous types of honey available in Malaysia, including Kelulut, Tualang and Acacia honey, display both bacteriostatic and bactericidal effects (Mohd-Aspar and Edros, 2019; Omar *et al*., 2019). In our recent study, Kelulut honey resulted in lower minimum inhibitory concentration (MIC) to inhibit common pathogenic bacteria species of *P. aeruginosa* indicating higher antibacterial properties compared to manuka honey (Mohd-Aspar *et al*., 2020). There were studies reported on the factors that contribute to the antibacterial properties of Malaysian honey, which are generally due to acidity and the presence of peroxide (Zainol *et al*., 2013; Jalil *et al*., 2017) and non-peroxide compounds (represented by phenolic and flavonoid compounds) (Chua and Ismail, 2015; Tuksitha *et al*., 2018). However, the active contribution of these factors towards the antibacterial properties of the honey was questionable and there are limited data to support the active contribution of the factors on antibacterial properties of Malaysian honey, which limit the understanding (Roslan *et al*., 2015; Shehu *et al*., 2015).

In this study, the effects of acidity, peroxide, and non-peroxide compounds on the antibacterial properties of Malaysian Kelulut, Tualang and Acacia honey were determined through a successive approach that unravels multifactorial that present in honey. The approach determines the antibacterial properties of honey in the presence of known factors while neutralising the other factors. By doing so, the prominent factor responsible for the antibacterial properties of Kelulut, Tualang and Acacia honey can be identified, compared, and preserved to ensure an optimum effect in implementing the honey as an antibacterial agent.

MATERIALS AND METHODS

Chemicals and reagents

The catalase C9322, 2000-5000 units/mg, was purchased from Sigma, USA and sodium hydroxide (NaOH) solution 0.1 M from Advanced BioMatrix, USA.

Honey samples

In this study, Kelulut, Tualang and Acacia honey were considered to represent honey that is actively harvested in Malaysia. Honey samples were obtained from a local apiarist and aseptically stored in sterile glass bottles. The honey was gathered from various farms in Malaysia, including Kedah, Pahang, and Johor. Information on the collected honey was recorded in the Certificate of Analysis (CoA) obtained during purchasing of the honey, provided by an authorised body, the Malaysian Agriculture Research and Development Institute (MARDI). Additional assay to confirm the honey sample's purity was achieved using the commercially available test kit, RapidRaw™ established by the Malaysia Genome Institute (MGI). The samples of honey obtained were stored at room temperature and kept away from direct sunlight. The commercially available medical-grade honey represented by manuka honey (Comvita® UMF 18+, New Zealand) was used as the basis of comparison to validate the reliability of antibacterial evaluations of the study.

Bacteria

The evaluations were performed on clinically isolated strains, *S. aureus* and *E. coli,* which were obtained from the Department of Pathology and Laboratory Medicine, Sultan Ahmad Shah Medical Centre (SASMEC). The primary culture was prepared by re-culturing the bacteria in nutrient agar and incubating for 24 h at 37 °C. A loop of primary culture was inoculated into sterile screw-capped test tubes containing 10 mL of broth before being incubated at 37 °C in a shaking incubator with the rotational speed of 150 rpm for 24 h to produce a working bacterial culture. The prepared working bacteria cultures were set to 0.5 McFarland standard, equivalent to 1.5×10^8 CFU/mL in every experimental work. It was achieved by diluting the prepared culture with fresh sterile nutrient broth to reach an absorbance between 0.08 and 0.13 (CLSI, 2012). The absorbance of cultures was measured at the reference wavelength of 600 nm using Ultraviolet-Visible Spectrophotometer UV-1800 (Shimadzu, Japan).

Samples preparation

Different types of honey samples were prepared based on the known factors present in honey, referred to as untreated (UT), peroxide non-peroxide (PNP) and non-peroxide (NP) solutions. UT solution is a sample that preserves the acidity, peroxide, and non-peroxide compounds naturally present in honey. Meanwhile, the PNP solution preserves the peroxide and non-peroxide compounds while neutralising the acidity. As for NP solution, only non-peroxide compounds are preserved while the acidity and peroxide compounds are eliminated.

The UT, PNP and NP solutions were prepared for each honey sample at 30% to 90% (w/v) concentration. The UT solution was prepared by weighing 3 g, 4 g, 5 g, 6 g, 7 g, 8 g and 9 g of honey and mixed with deionised water to make a final volume of 10 mL. The acidity, peroxide and non-peroxide compounds were preserved in the UT solution, as no elimination agents were added. The PNP solution was prepared using similar steps as the UT solution preparation and a non-acidic solution was achieved through titration using 5% (w/v) NaOH until the solution reached pH 7.0. Meanwhile, in the NP solution, instead of deionised water, the peroxide-free sample was prepared by weighing 3 g, 4 g, 5 g, 6 g, 7 g, 8 g and 9 g of honey and mixed with 4000 unit/mL catalase solution to catalyse the decomposition of hydrogen peroxide to water (Adams *et al*., 2008; Kwakman *et al*., 2011). This was

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followed by titrations using 5% (w/v) NaOH to neutralise the acidity. The pH of the prepared solutions is measured and described in Table 1.

Evaluation of antibacterial properties of UT, PNP and NP solutions

The antibacterial properties of UT, PNP and NP solutions were determined through the measurement of inhibition zones. The prepared working bacteria culture was inoculated using the spread plate technique by spreading 100 µL of the culture onto the surface of the nutrient agar. After inoculation, wells of 6 mm in diameter were cut on the agar and filled with 80 µL of the test solutions. The UT, PNP and NP solutions for manuka honey (UMF 18+) were included for comparison. Plates were incubated for 24 h at 37 °C. The diameters of the clear inhibition zones were measured in millimetres (mm), including the diameter of the 6 mm well created. Each assay was measured with three biological replicates. A high concentration of sugar base (SB) solution was included to represent the sugar osmotic effect in honey. The SB was 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).

The sensitivity of bacteria towards honey was determined based on the inhibition zones measured that were categorised as not sensitive, sensitive, very sensitive and extremely sensitive. The term not sensitive was denoted by the diameter of the inhibition zone lower than 8 mm, sensitive for a diameter from 8 to 14 mm, very sensitive for a diameter from 15 to 19 mm and extremely sensitive for a diameter of 20 mm and above. This category was used to describe the sensitivity of bacteria towards the honey according to the diameter inhibition zone measured, as suggested in the previous study (Moussa *et al*., 2012).

Identification of compounds by liquid chromatography quadrupole time-of-flight mass spectrometry

The active contribution of acidity, peroxide and non-peroxide compounds in honey samples used were confirmed through compounds identification using Vion IMS Liquid Chromatography Quadrupole Time-of-flight Mass Spectrometry (LC-QToF-MS) (Waters®, Milford, USA). This test was performed at the Central Laboratory Universiti Malaysia Pahang (UMP). Each honey sample used, i.e., Kelulut, Tualang and Acacia was sent for compound identification. Chromatographic separation was achieved on an ACQUITY UPLC HSS T3 column (2.1 × 100 mm, 1.8 µm pore size). The injection volume was 5 µL. The mobile phase consisted of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in acetonitrile (mobile phase B) with a flow rate of 0.6 mL/min. In mobile phase A, the mobile phase gradient (0%-99% A) steps were applied as follows: 0-16 min, 99% A; 16-18 min, 65% A; and 18-20 min, 0% A. As for mobile phase B, the mobile phase gradient (1%-100% B) steps were applied as follows: 0-16 min, 1% B; 16-18 min, 35% B; and 18-20 min, 100% B. In both phases, the total analysis time was 20 min. The sampling rate was set at 10 points/sec, the capillary voltage at 2 kV, the cone gas flow at 50 L/h and the desolvation gas flow at 800 L/h. The samples were run for positive and negative ionisation modes in characterising the numerous compounds in honey based on the ionisation charge of the compound. The available compounds were identified based on their mass spectra, retention time and retention time and compared with the standard library information that includes 500 established compounds.

Table 1: The pH of UT, PNP and NP solutions.

Solution	Honey	pH
UT	Kelulut	2.37 ± 0.13
	Tualang	3.88 ± 0.04
	Acacia	4.25 ± 0.09
	Manuka	3.80 ± 0.03
PNP	Kelulut	7.37 ± 0.22
	Tualang	7.35 ± 0.22
	Acacia	7.27 ± 0.11
	Manuka	7.23 ± 0.29
NP	Kelulut	7.40 ± 0.15
	Tualang	7.29 ± 0.27
	Acacia	7.30 ± 0.22
	Manuka	7.27 ± 0.24

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

RESULTS

Effect of acidity, peroxide, and non-peroxide compounds in honey

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of Kelulut, Tualang and Acacia honey samples used in this study were determined previously (Mohd-Aspar and Edros, 2019) to indicate the presence of bacteriostatic and bactericidal effects in the honey. Here, the factors in the Kelulut, Tualang and Acacia honey samples were identified, considering the acidity, peroxide, and non-peroxide compounds as the factors. The results are shown in Figure 1, with manuka and SB solutions used as the basis for comparison.

In general, the UT solutions of Kelulut, Tualang and manuka began to show inhibition on both *S. aureus* and *E. coli* at the lowest concentration of 30% (w/v) and the inhibition zone increased as the concentration increased until it reached 90% (w/v). As for the Acacia UT solution, the zone of inhibition began to appear at the concentration of 40% (w/v) and the inhibition zone remained constant with ± 5% of the inhibition zone measured at higher concentrations. Among the types of Malaysian honey, both *S. aureus* and *E. coli* were more susceptible to Kelulut UT solution, where the zones of inhibition were 1.7-fold larger than Tualang and 2.1-fold larger than Acacia on average.

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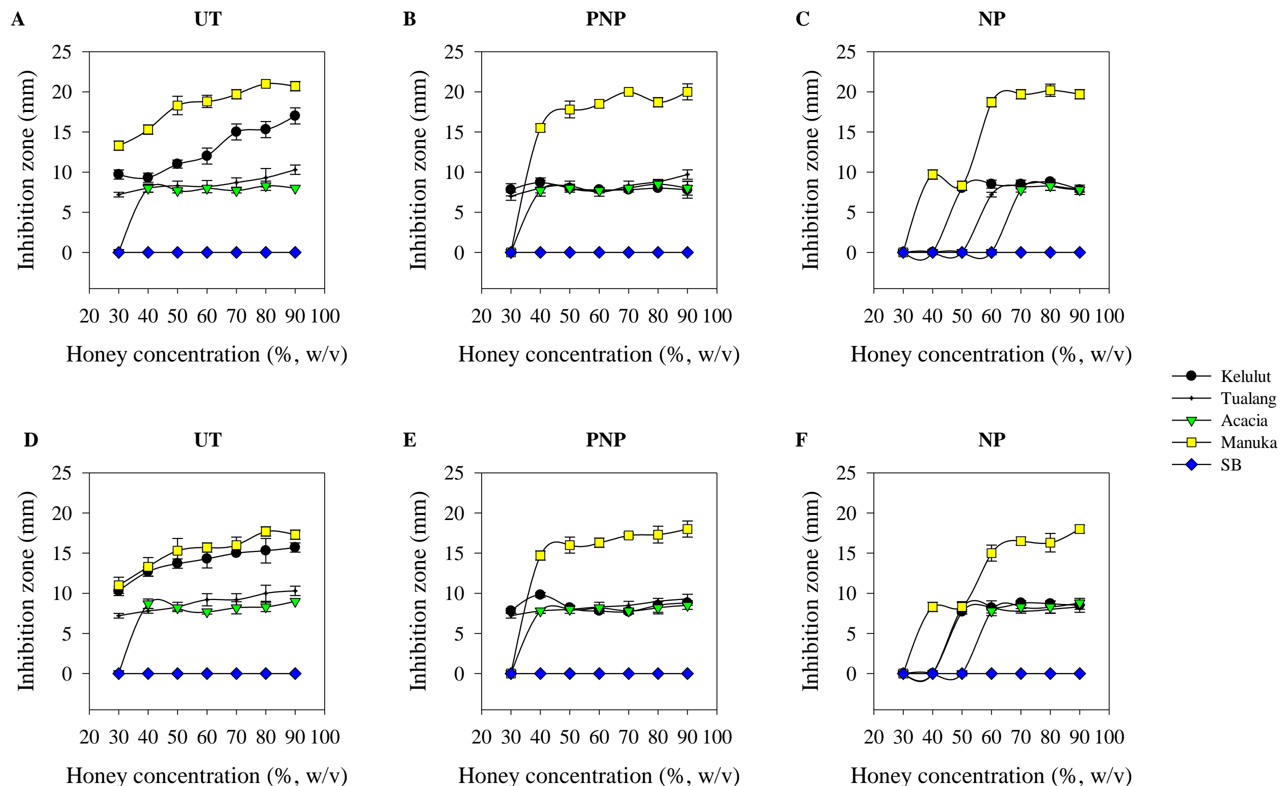

Figure 1: Inhibition zones of UT, PNP and NP solutions on *S. aureus* (A to C) and *E. coli* (D to F). Error bars indicate the standard deviation calculated from three biological replicates.

The PNP solution of Tualang and Acacia produced inhibition zones similar to that obtained from the UT solution. This indicates that acidity does not majorly affect the inhibition zones of Tualang and Acacia. In the Kelulut PNP solution, the inhibition zones were reduced to a similar level obtained from Tualang and Acacia PNP solutions. The elimination of acidity has reduced the inhibition zone of Kelulut, indicating that acidity majorly contributed to the potent antibacterial properties of Kelulut. However, at the concentration of 40% (w/v), significantly larger inhibition zones were recorded by the Kelulut PNP solution compared to Tualang and Acacia (0.015, $p<0.05$). This may occur due to the activation of peroxide compounds which are active in Kelulut at the concentration of 40% (w/v) (Molan, 1992; Kwakman and Zaat, 2012). As for manuka, the zone of the inhibition began to appear at 40% (w/v) and the inhibition zone continued to increase until it reached the largest inhibition zone at 90% (w/v).

The NP solutions of Kelulut, Tualang and Acacia began to show inhibition zones at various concentrations between 50% and 70% (w/v). Once the inhibition zones were observed, the diameters of the inhibition zone remained unchanged within ± 5% until it reached the concentration of 90% (w/v), indicating an equal antibacterial potency in the honey samples. In the manuka NP solution, the inhibition zone began to form at a concentration of 40% (w/v). The zone of inhibition showed no change at a concentration ranging from 40% to 50% (w/v) on both *S. aureus* and *E. coli*. However, it increased at a higher concentration between 60% and 90% (w/v) for both bacterial strains. In contrast, no inhibition zones were detected in the SB solution at any concentration between 30% and 90% (w/v). This indicates that osmotic pressure alone does not contribute to the inhibition of bacterial growth at a concentration between 30% and 90% (w/v).

Identification of compounds by liquid chromatography quadrupole time-of-flight mass spectrometry

The identified compounds were generally classified as acidic, peroxide, or non-peroxide compounds. The compounds with the R-O-O-R chemical structure were denoted as peroxide, while the compounds without the R-O-O-R chemical structure were denoted as non-peroxide. The identified compounds based on the intensity and retention time (min) are shown in Figure 2 for positive and Figure 3 for negative ionisation modes, respectively. The summary for the identified compounds is shown in Table 2.

Based on the obtained results, none of the peroxide compounds was identified in Kelulut, Tualang and Acacia

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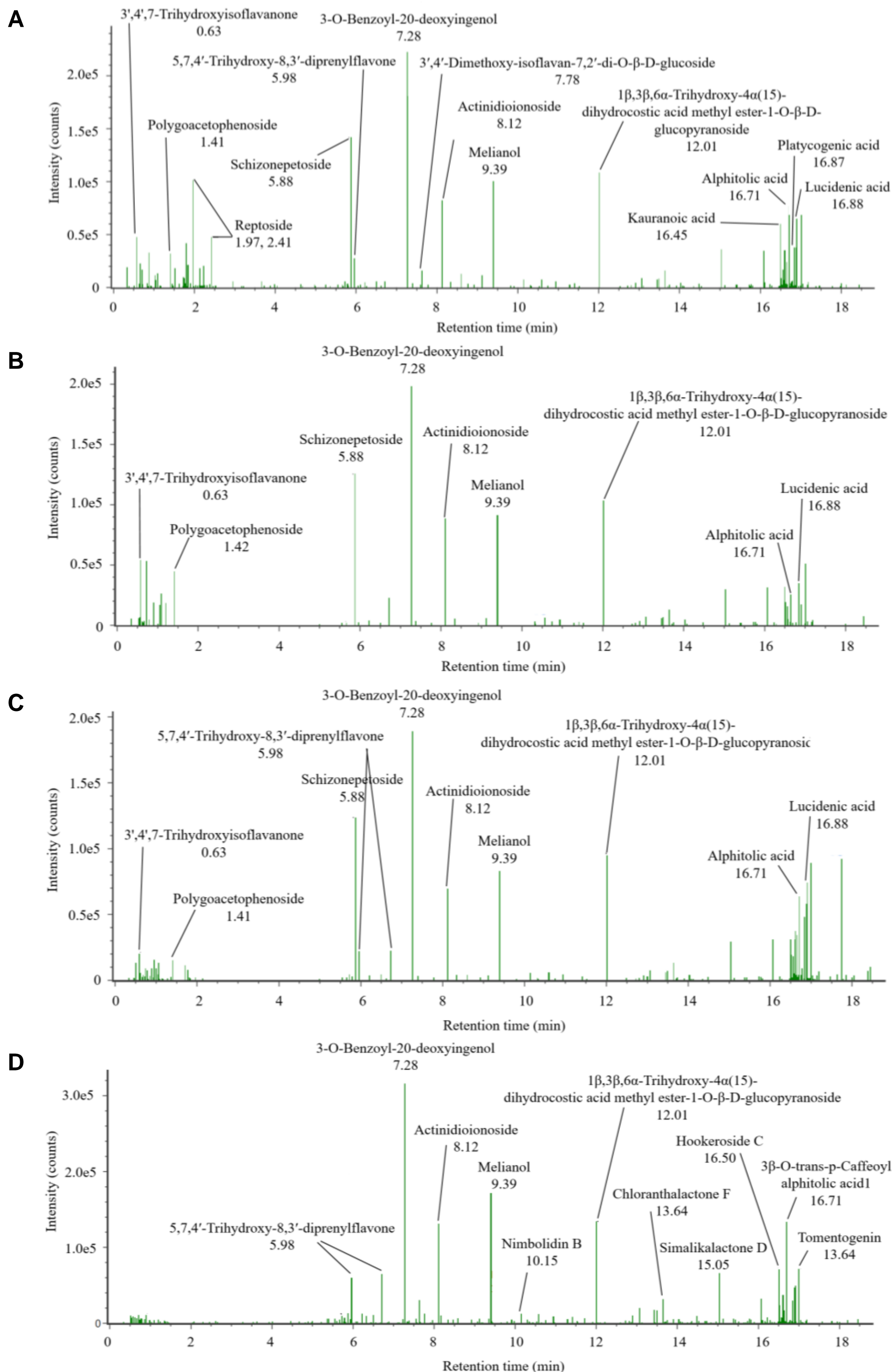

Figure 2: LC-QToF-MS chromatogram of honey samples for the positive ionisation mode. (A) Kelulut, (B) Tualang (C) Acacia and (D) Manuka.

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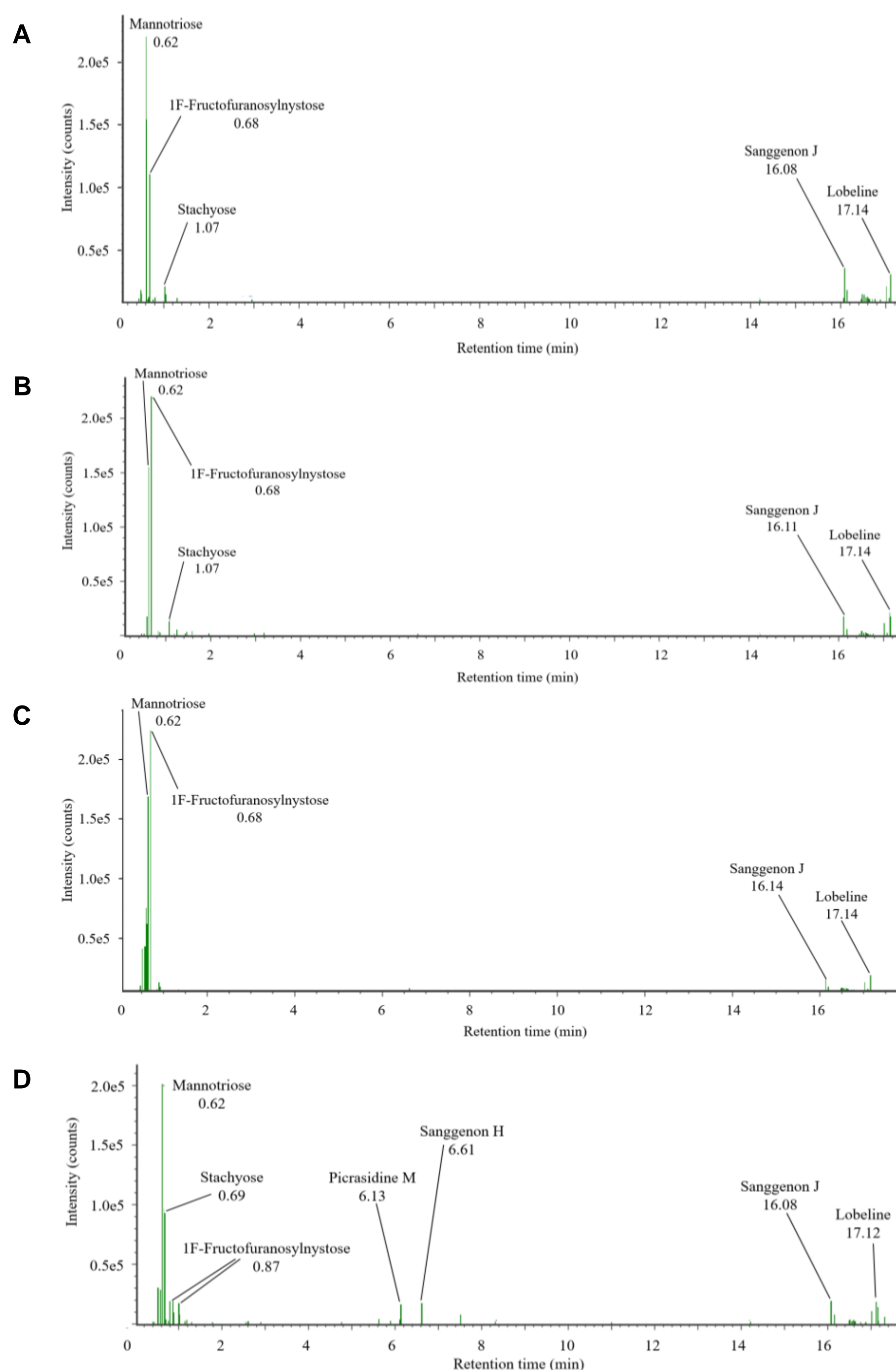

Figure 3: LC-QToF-MS chromatogram of honey samples for the negative ionisation mode. (A) Kelulut, (B) Tualang (C) Acacia and (D) Manuka.

honey. As for the non-peroxide compounds, Kelulut, Tualang and Acacia honey were subjected to the presence of the flavonoids, i.e., 3',4',7-trihydroxyisoflavanone, 5,7,4′-trihydroxy-8,3′-diprenylflavone and 3′,4′-dimethoxy-isoflavan-7,2′-di-O-β-D-glucoside at a retention time of 0.63, 5.98 and 7.78 min, respectively. Kelulut, Tualang and Acacia were found to contain compounds other than flavonoids, including mannotriose, 1F-fructofuranosylnystose, stachyose, polygoacetophenoside, schizonepetoside, actinidioionoside, melianol, 1β,3β,6α-trihydroxy-4α(15)-dihydrocostic acid methyl ester-1-O-β-D-glucopyranoside

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Table 2: List of identified compounds in Kelulut, Tualang and Acacia honey.

Groups	Compound	Acacia	Tualang	Kelulut	Manuka
Peroxide	Hydrogen peroxide	NP	NP	NP	NP
Nonperoxide	3′,4′,7-trihydroxyisoflavanone	P	P	P	P
	Polygoacetophenoside	P	P	P	P
	Reptoside	NP	NP	P	NP
	Schizonepetoside	P	P	P	P
	5,7,4′-trihydroxy-8,3′-diprenylflavone	NP	NP	P	P
	3-O-Benzoyl-20-deoxyingenol	P	P	P	P
	3′,4′-dimethoxy-isoflavan-7,2′-di-O-β-D-glucoside	NP	NP	P	P
	Actinidioionoside	P	P	P	P
	Melianol	P	P	P	P
	1β,3β,6α-Trihydroxy-4α(15)-dihydrocostic acid methyl ester 1-O-β-D glucopyranoside	P	P	P	NP
	Mannotriose	P	P	P	P
	1F-fructofuranosylnystose	P	P	P	P
	Stachyose	NP	P	P	P
	Lobeline	P	P	P	P
Acid	Kauranoic acid	NP	NP	P	P
	Alphitolic acid	P	P	P	P
	Platycogenic acid	NP	NP	P	NP
	Lucidenic acid	P	P	P	P

*Presented (P); not presented (NP).

and lobeline. These compounds were identified at retention times of 0.62, 0.68, 1.07, 1.41, 5.88, 8.12, 9.39, 12.01 and 17.14 min, respectively.

As for the acid, two acid compounds, i.e., lucidenic acid and alphitolic acid, were identified in the Kelulut, Tualang and Acacia samples at retention times of 16.71 and 16.88 min, respectively. In Kelulut, several other acid compounds, such as platycogenic acid and kauranoic acid, were identified at 16.45 and 16.87 min, respectively. Also, reptoside was recognised exclusively in Kelulut honey at retention times of 1.97 and 2.41 min.

DISCUSSION

Acidity is among the factors that influence the antibacterial properties of Kelulut, Tualang and Acacia honey (Tumin *et al.*, 2005; Jalil *et al.*, 2017). At low pH, proton concentration is high to cause protonation of biological molecules affecting both structure and function (Lund *et al.*, 2020). The lower the substance's pH, the higher the degree of protonation exists. In this study, a strongly acidic environment was recorded in Kelulut, with a pH of 2.37, compared to Tualang and Acacia, with a pH of 3.88 and 4.25, respectively (Table 1). The lower pH of Kelulut can cause a higher percentage of protonation to inhibit the growth of bacteria. In addition, a pH of 4 or less may contribute to the increment of antimicrobial properties of flavonoids (Sánchez-Maldonado *et al.*, 2011). This was expected to be the additional factor that enhanced the antibacterial properties of Kelulut compared to Tualang and Acacia. The strong acidic environment owned by Kelulut honey can also be explained by the presence of the additional acid compounds, i.e., platycogenic acid and kauranoic acid. These acids have been associated with various biological properties, including antibacterial properties (Lee *et al.*, 2017; *Cör et al.*, 2018), which react by interfering with bacterial metabolism (Çiçek *et al.*, 2020; Wilkens *et al.*, 2002) and breakdown of the cell wall mechanism (Basnet *et al.*, 2017).

The peroxide compounds are denoted mainly by H_2O_2 in honey (Brudzynski *et al.*, 2011; Kwakman and Zaat, 2012). Previous studies have reported the presence of H_2O_2 through qualitative measurement in Kelulut (Nishio *et al.*, 2016) and Tualang (Tumin *et al.*, 2005) honey to prevent the growth of bacteria, i.e., *E. coli* and *S. aureus*. In this study, the sudden increase in inhibition zones at concentrations of 40% (w/v) in the Kelulut PNP solution might be explained by the contribution of peroxide compounds since peroxide compounds are active in diluted honey at a concentration between 30% and 50% (White *et al.*, 1963; Molan, 1992; Kwakman and Zaat, 2012). The effect of peroxide in the Kelulut PNP solution was observed to have a higher impact on *E. coli* due to the 12.6% larger inhibition zone measured at 40% concentration compared to *S. aureus*. This may be due to the morphological aspect of *S. aureus*, i.e., a Gram-positive bacterium that consist of a thick peptidoglycan cell wall to resist the penetration of peroxide compounds compared to *E. coli*, a Gram-negative bacteria that have a thinner cell wall (Horn *et al.*, 2018). As for Tualang and Acacia, the effect of peroxide compounds was absent, indicating the least or lack of peroxide activity in these types of honey.

However, based on the compound identification using LC-QToF-MS, none of the peroxide compounds was detected in Kelulut, Tualang and Acacia honey. This might be due to a low amount of the H_2O_2, which is beyond the limit of detection by LC-QToF MS. A meagre amount of H_2O_2 was identified in honey, i.e., 900-fold

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lower than the 3% of H_2O_2 solution effectively used for wound cleansing (Urban *et al.*, 2017). In addition, the undetectable H_2O_2 level might also be due to the instability of the H_2O_2 compound that decomposes by excessive heat (temperature of 75 °C and above) and low water activity (less than 18% water content) (Kwakman and Zaat, 2012). Preservation of H_2O_2 in honey requires proper supervision upon storing and diluting the honey. In this study, due to an unidentified compound, the presence of H_2O_2 in Kelulut, Tualang and Acacia honey could not be verified quantitatively. The outcome was in line with the previous studies, which were only successful in confirming the presence of H_2O_2 qualitatively (Sherlock *et al.*, 2010; Roslan *et al.*, 2015). Further identification using the advanced mass spectrometer, such as a triple quadrupole mass spectrometer (QQQMS) or through quantitative measurement as described in the previous studies (Kwakman *et al.*, 2010; Brudzynski *et al.*, 2011) are recommended to affirm the presence of H_2O_2 in Kelulut, Tualang and Acacia honey.

Non-peroxide compounds, such as flavonoids and phenolic acids, were identified in Malaysian Kelulut (Chan *et al.*, 2017; Tuksitha *et al.*, 2018), Tualang (Ranneh *et al.*, 2018) and Acacia honey (Salleh *et al.*, 2017). These compounds are among the factors that contribute to the presence of antibacterial properties in honey (Irish *et al.*, 2011; Shehu *et al.*, 2015). In this study, the contribution of non-peroxide compounds in Kelulut, Tualang and Acacia was confirmed through the manifestation of inhibition zones against *S. aureus* and *E. coli*. The inhibition zones by the non-peroxide compounds were detected at the concentration of 50% for Kelulut and Tualang, and 60% (w/v) for Acacia, respectively. Once observed, the inhibition zone remained unchanged until it reached 90% (w/v) concentration. The unchanged diameter of inhibition zones might be due to the limited amount of non-peroxide compounds to cause a change in the diameter of inhibition zone with increasing honey concentration (Johnston *et al.*, 2018; Ranneh *et al.*, 2018) and also might be due to the structure of agar, which limits the penetration of non-peroxide compounds across it.

Flavonoids are a group of non-peroxide compounds consisting of two benzene rings linked with a heterocyclic pyran ring and have been associated with bioactive properties, including antibacterial (Shehu *et al.*, 2015; Górniak *et al.*, 2019), which disrupt microbial membranes and inactivate microbial adhesins, enzymes and cell envelop transport proteins (Kumar and Pandey, 2013). Based on the presence of flavonoid compounds, as listed in Table 2, this study supports the contribution of non-peroxide compounds in the antibacterial properties of Kelulut, Tualang, and Acacia honey, as previously reported in several studies (Salleh *et al.*, 2017; Ranneh *et al.*, 2018). Other than flavonoids, lobeline and mannotriose have also been indicated with the antibacterial properties, which react by inhibiting the synthesis of the bacterial cell wall (Oyedemi *et al.*, 2020) and microbial enzyme (Furuya and Ikeda, 2009).

In Kelulut, the presence of additional non-peroxide compounds, i.e., reptoside, platycogenic acid and kauronic acid, were discovered. Among these compounds, i.e., reptoside, is a compound derived from the group of iridoids that has been associated with antibacterial properties, which react as the deoxyribonucleic acid (DNA) damaging agent (Ni *et al.*, 2015; Kim, 2018; Toiu *et al.*, 2018). The presence of additional compounds in Kelulut may be attributed to the smaller size of stingless bees (ranging from 2 to 14 mm), which allows them to collect pollen and nectar from a broader range of flower sizes, including both small and large flowers. This is in contrast to Tualang and Acacia, which are primarily pollinated by honeybees (*Apis dorsata*) that are larger in size (17-20 mm) and may have difficulty collecting nectar and pollen from larger flowers (Bradbear, 2009; Mohd Norowi *et al.*, 2010).

CONCLUSION

Acidity and non-peroxide compounds were observed to actively contribute to the antibacterial properties of Kelulut, Tualang and Acacia honey. This is due to the formation of inhibition zones measured by the prepared honey solutions incorporating acidity, peroxide, and non-peroxide compounds as the contributing factors. The presence of flavonoids, i.e., 3′,4′,7-trihydroxyisoflavanone, 5,7,4′-trihydroxy-8,3′-diprenylflavone and 3′,4′-dimethoxy-isoflavan-7,2′-di-O-β-D-glucoside and acids, i.e., lucidenic acid and alphitolic acid, verifying the contribution of non-peroxide compounds and acidity in Kelulut, Tualang and Acacia honey to inhibit the growth of bacteria. As for the peroxide compound, further, identification is recommended to affirm the presence of H_2O_2 in Kelulut, Tualang and Acacia honey. In Kelulut, the presence of the additional acid compounds, i.e., platycogenic acid and kauranoic acid, explains the stronger acidic environment in Kelulut compared to Tualang and Acacia, which contributes to the prevention of bacterial growth.

The presence of multiple antibacterial factors in honey is notably essential. It gives an advantage compared to antibiotics, as honey can prevent the growth of a wide range of bacterial species. On the contrary, most antibiotics depend on a single mode of action for their antibacterial activity, which is easily resisted by bacteria, i.e., beta-lactam in penicillin (Peacock and Paterson, 2015).

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