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Antibacterial Properties of Local Malaysian *Trigona Sp*. Honey Towards Different of Pathogenic Bacteria in Vitro

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Abstract

Honey is one of the oldest natural medicines known with a very high therapeutic value. Nowadays, in the medical field, several important therapeutic effects of honey have been elucidated. This study was conducted to reveal the antimicrobial activity of the commercially available local Malaysian *Trigona* sp. honey towards different pathogenic bacteria specifically *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, and Salmonella typhimurium.* The Minimum inhibitory concentration and Minimum bactericidal concentration were determined by the disc-diffusion test and agar well diffusion test. Different concentration of the honey was tested in the disc-diffusion and agar well diffusion test. The results of these tests were in terms of Inhibition zone diameter. The results obtained from the current study are the dilution of different concentration of honey from *Trigona* sp. are very significant because the only net concentration of both of honey *Trigon* a sp. possessed antimicrobial properties in term of Minimum inhibitory concentration) and Minimum bactericidal concentration. The result also can say that *Trigona sp*. honey possessed antibacterial properties and can be used as alternative medicine in the veterinary field in the future.

Keywords: Honey, *Trigona* sp., pathogenic bacteria, alternative medicine.

Introduction

Honey is one of the oldest natural medicines known with a very high therapeutic value. Nowadays, in the medical field, several important therapeutic effects of honey have been elucidated. *Staphylococcus aureus and Streptococcus pyogenes* are commonly known to be the primary cause of delayed healing and infection in wounds (Caldwell, 2020). The arisen of multiple drug resistance by nosocomial bacteria, *P. aeruginosa*, and *S. aureus* has been a huge concern internationally and an obstacle in the pharmaceutical industry (Frieden, 2010). *Staphylococcus. aureus* are the most robust and virulent microbes among the Staphylococci family that infect humans. Its tendency to develop antibiotic resistance has been a huge concern to human health. Moreover, externally acquired *Staphylococci* bacteria can cause life-

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threatening complications like pneumonia, endocarditis, meningitis, and osteomyelitis when inoculated into open wounds (David and Daum, 2010). *Escherichia. coli* besides its role in gastrointestinal infections, E. coli can cause infections of the urogenital tract and systemic disease in dogs and cats. Extra-intestinal pathogenic *E. coli* strains from dogs and cats belong to a limited number of serotypes and clonal groups and are frequently found as a part of the normal gut flora of these animals (Aibinu *et al*, 2004). Salmonellosis is an infectious disease of humans and animals which are caused by *Salmonella* mainly *S. enterica* and *S. bongori*. Infection of *Salmonella* is known as host-specific such as *S. Typhiin* humans (causes typhoid fever), *S. Abortusovis* in sheep, *S. Choleraesuisin* pigs, and *S. Dublin* in cattle *S. Pullorum* in avian. While *S. Typhimurium* and *S. Albany* are known to cause only gastroenteritis (Pal *et al*, 2020).

Honey is a rich carbohydrate syrup made by the Bees. Fructose and glucose are the major components of honey. Large numbers of compounds are also present in small quantities. Honey possessed low moisture content and water activity. Honey has been used as medicine since ancient times in many cultures. Honey has been found to heal infected surgical wounds, burns, and decubitus ulcer as published by several researchers (Bunza *et al*, 2019; Divya *et al*., 2018; Abou Zekry *et al*, 2020). The previous study showed that stingless bee honey can act as an anti-inflammatory (Borsato *et al*, 2014), anti-cancer (Al-Ajmi *et al*, 2019), antimicrobial (Kimoto and Amano, 2008; Nolan *et al*, 2019; Fernandes *et al*, 2020) and possessed antioxidant properties (Gośliński *et al*, 2020). In the microbiology field, honey was found to possess good antimycobacterial activity (John-Isa *et al*, 2019). Laboratory studies have revealed that honey is effective against Methicillin-resistant *Staphylococcus aureus* (MRSA), β-hemolytic *Streptococci*, and Vancomycin-resistant Enterococci (VRE) as reported in earlier studies (Allen *et al*, 1991; Lye, 2014). Other than that, geographical distribution and also different floral sources play important role in the antimicrobial activity of honey (Ali ce *et al*, 2011; Taormina *et al*, 2001).

The honey that is commonly found in Malaysia is the Tualang honey, Jungle honey, Cerung or Cerang honey, and finally the Kelulut honey (Barakhbah, 2007). Kelulut honey is produced by stingless bees from *Trigona* sp. Kelulut honey is increasingly receiving attention from Malaysians due to its uniquely sour taste and its flavour. The honey is produced believed to have more medical values. Research showed honey of *Trigona. sp*. has antimicrobial properties: high acidic value and low pH value: high saturation of sugar; the antimicrobial activity itself (Shahjahan *et al*, 2007). Even though there are many types of research about *Trigona sp.* worldwide. In Malaysia, there are only a few types of research about the local Malaysian honey of *Trigon* sp. used in the human and veterinary field. Although there are many types of research about the antibacterial activity of Malaysian honey (Tumin *et al*, 2005; Al-kafaween *et al*, 2020), many of them are not used all the types of honey available in Malaysia. Although recently there were researches of comparison of physicochemical properties of Malaysia *Trigona* sp. honey in Peninsular Malaysia, there were no comparison researches about the commercially available Malaysian honey of *Trigona* sp. towards different pathogenic bacteria.

Antimicrobial resistance is becoming a global issue nowadays. So, alternative treatment should be immediately discovered. One of the alternative substances to replace antimicrobials substances is honey. Until now, there were few studies of the antibacterial properties of

different brands of Kelulut honey available in Malaysia on different pathogenic microorganisms. The objectives of this research were to study and compare the antibacterial activity of the different brands of commercially available Kelulut honey in Malaysia.

Materials and Methods

Honey samples

The honey samples (K1-Kelulut Honey Brand 1 and K2-Kelulut Honey Brand 2) were collected from commercial stores located in Kota Bharu, Kelantan, Malaysia between February 2019 and October 2019. It is multi-floral honey from stingless bees and it obtained its name from the bees producing it that are locally known as Kelulut. Kelulut bees are from the genus *Trigona*, the largest genus of stingless bees that are indigenous to Neotropics and Indo-Australian regions (Michener, 2007). Samples were stored in a dark place at room temperature (25-35ºC).

Bacterial Strains

Strains of *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus,* and *Streptococcus pyogenes* were obtained from the faculty of Veterinary Medicine University Malaysia Kelantan, Malaysia.

Disc diffusion method and agar well diffusion method

Filter paper discs of 6 mm diameter were prepared. The discs were impregnated with the different concentrations of each honey (0, 20, 40, 60, 80, and 100%), 0.5 McFarland standard was prepared by the method of Koneman *et al*, (1992) and 5mL was added into a sterile test tube. An inoculum of each isolate was prepared from the subculture of bacterial suspension sterile test tube, 4-5 colonies of each isolate were emulsified in sterile normal saline, and the turbidity adjusted to 1.5×10^8 CFU/mL (corresponding to 0.5 McFarland standers). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the Mueller Hinton agar plates. They were allowed to dry for 3 to 5 minutes. Thereafter, all discs were placed on the plates and pressed gently to ensure complete contact with agar. A distance of at least 15 mm was maintained from the edges of the plates to present overlapping of inhibition zones. Ampicillin disc (5µg) was used as a positive control. Fifteen minutes after the placement of discs, the plates were incubated for 24 h at 37 °C. After incubation, the plates were examined, and three diameters of the inhibition zone were measured in triplicates for each isolate. For the agar well diffusion method, the well had been made by sterilized cork borer to create five well with 2.5mm diameter on the agar (Bauer, 1966).

Minimum inhibitory concentration (MIC).

Both K1 and K2 honey were tested for the minimum inhibitory concentration test using the broth dilution method according to the method described by Turnidge (2007). This procedure involved the preparation of two-fold serial dilutions of antibiotics in a liquid growth medium dispensed in sterile test tubes. From each folded of serial dilution, the mixture needed to thoroughly mix. A different concentration of the test material (Ampicillin) was obtained by six rows in each containing 50, 100, 150, 200, 250, and 300 mg/mL. Then, 0.5 mL of bacterial suspension was filled into each tube to achieve a final concentration of 1.5 x

10^8 CFU/mL. Two sets of controls were set for each tube which contained; (a) positive control consisting of broth and bacterial suspension and (b) negative control only consisting of broth. Afterward, the tubes were incubated for 24 hours incubation at 37 ºC. Then, the tubes were observed for bacterial growth as evidenced by turbidity. The turbidity of MIC test tubes was measured according to absorbance at a wavelength of 600 using spectrophotometry. The lowest concentration of extracts (antibiotic) which tubes with turbidity indication was recorded as the MIC value. The average values were calculated for the MIC of the test material.

Minimum bactericidal concentration (MBC)

After identification of the MIC, inoculum from each tube was streaked into an agar plate and incubated at 37 ºC for 24 hours. According to Aibinu et al, (2004), the lowest concentration that prevented bacterial growth was recorded as MBC value.

Statistical analysis

Data obtained were analyzed by one-way ANOVA by using SPSS package v.10

Results and Discussions

Disc diffusion test

The results in terms of inhibition zone diameter (IZR). The IZR of both different brands of honey was different. By establishing the column chart, quantitatively can compare the IZR of both of these honey. This also correlates with the minimum inhibitory concentration test because each of the discs also is soaked with different concentrations of honey. The disc diffusion test for 20, 40, and 60% concentration of Kelulut honey 1 and Kelulut honey 2 showed no zone of inhibition against the selected bacteria. At 80% concentration, Kelulut honey 1 and 2 exhibited inhibition zone only against *Streptococcus pyogenes* 2 ± 0.3 and 1.5 ± 0.5 mm respectively (Fig. 1).

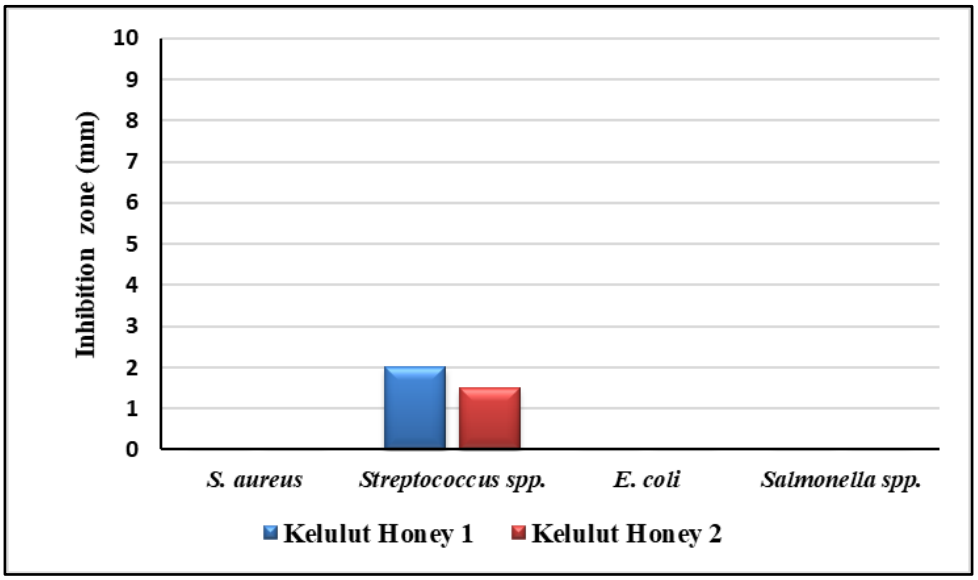

Figure 1. Inhibition Zone Diameter (mm) for 80% Concentration of Kelulut Honey 1 and 2.

For Disc diffusion test neat (100%) of Kelulut honey 1 exhibited better antibacterial outcome than Kelulut honey 2 and the clear zone produced by neat honey against *Staphylococcus aureus and E.coli*, were 3 ± 0.5 and 4 ± 0.2 mm respectively comparing 2 ± 0.5 and 3 ± 0.5 mm respectively (Fig. 2).

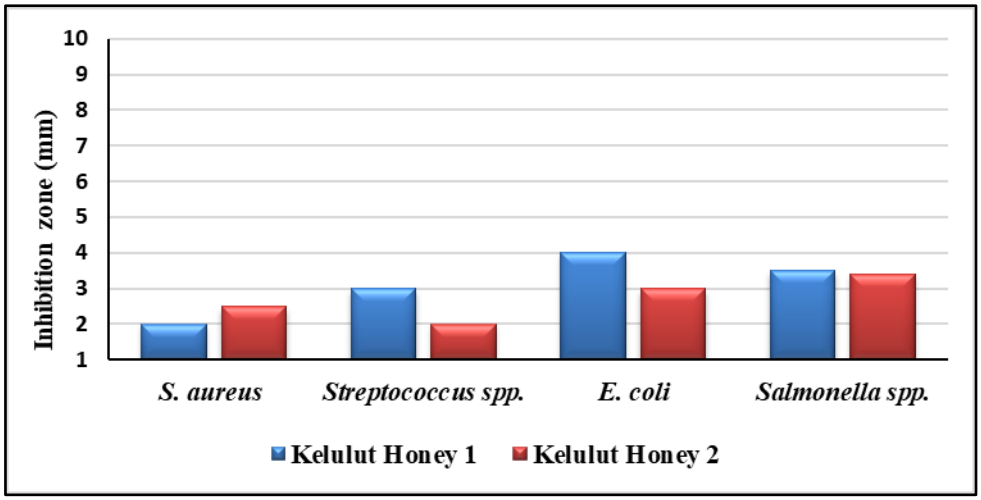

Figure 2. Inhibition zone diameter (mm) for 100% concentration of Kelulut honey 1 and 2.

Agar well diffusion test

Agar well diffusion test for 20, 40, and 60% concentration of Kelulut honey 1and Kelulut honey 2 showed no zone of inhibition against the selected bacteria. At 80% concentration, Kelulut honey 1and 2 showed zone of inhibition against *Staphylococcus aureus* and *Streptococcus pyogenes*, Kelulut honey 1 exhibited 3 ± 0.5 and 2.3 ± 0.2 mm respectively whereas Kelulut honey 2 exhibited inhibition zone against *Staphylococcus aureus* and *Streptococcus pyogenes* 2.4 ± 0.6 and 2.0 ± 0.5 mm respectively (Fig. 3). For agar, well diffusion test neat of Kelulut honey 1 exhibited better antibacterial outcome than diluted honey, and the clear zone produced by neat honey against, *Streptococcus pyogenes*, *E.coli* and *Salmonella typhimurium* were 3.4 ± 0.6, 2.4± 0.6, 9.3 ± 0.2, and 8.1 ± 0.4 mm respectively. Agar well diffusion test neat of Kelulut honey (2) exhibited better antibacterial outcome than diluted honey and the clear zone produced by neat honey *Staphylococcus aureus*, *Streptococcus pyogenes*, *E.coli* and *Salmonella typhimurium* were 3.7 ± 0.3, 1.6 ± 0.4, 8.2 ± 0.3, and 7.2 ± 0.3 mm respectively (Fig. 4).

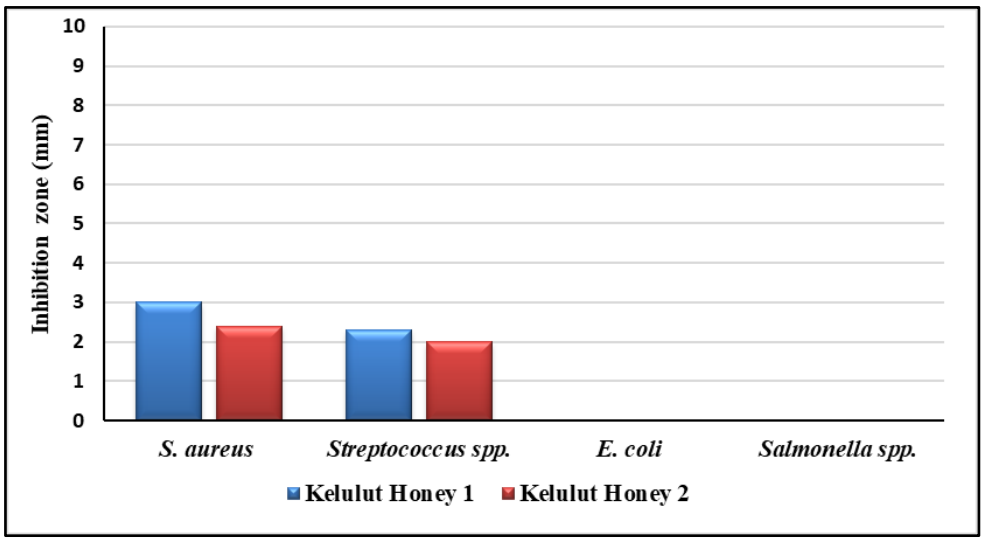

Figure 3. Inhibition zone diameter (mm) for 80% concentration of Kelulut honey 1 and 2

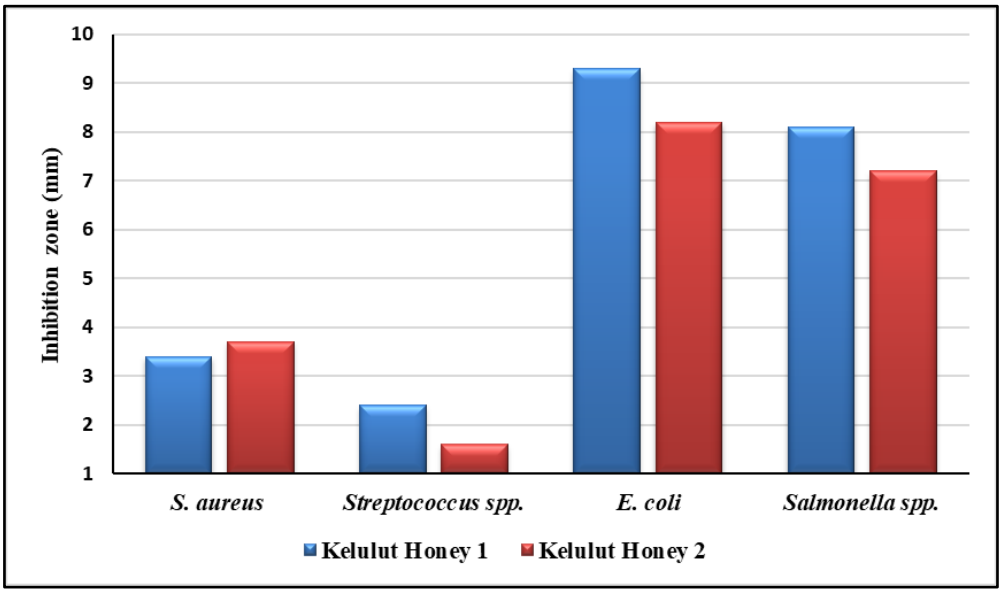

Figure 4. Inhibition zone diameter (mm) for 100% concentration of Kelulut honey 1 and 2

The current study indicated that Kelulut honey alone was less potent in inhibiting the growth of all the pathogenic bacteria *Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, and Salmonella typhimurium* when dilution of the honey was done regardless of the concentration. These also include both brands of the commercially available Kelulut honey. The disc diffusion test and agar well diffusion test showed no zone of inhibition was formed by the honey of *Trigona* alone after dilution was made. This result is in agreement with a study done by Shahjahan *et al.* (2007) who showed that neat honey exhibited better antibacterial outcome than diluted honey and the clear zone produced by neat honey against *Salmonella typhi*, *Shigella sonnei*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus group* A were 30, 28, 26, 23 and 24mm respectively. And the MIC value for the bacteria tested was between 8.5 to 70 mg/mL. The efficacy of undiluted honey was superior against bacteria tested with a greater inhibitory effect than diluted honey. Another study also reported that 100% concentration of honey formed the largest zone of inhibition than 50% honey concentration and among all the bacteria investigated, all honey concentrations showed inhibitory effect and only *S. aureus* showed no zone of inhibition (Al-Naama, 2009). The divergence of results might be due to several reasons such as the different botanical origins and processing methods. Honey's antibacterial efficacy is enhanced by the presence of hydrogen peroxide, an essential antibacterial enzyme that also serves as an oxidizing and sanitizing agent. (Latkal *et al*., 2020). Hydrogen peroxide, phenolic compounds, and other flavonoids are responsible for honey's biological activity, which includes antioxidant, antimicrobial, anti-inflammatory, antiproliferative, and anticancer (Alzahrani *et al*, 2012; Godocikova *et al*, 2020). Latkal *et al*, (2020) reported that the concentration of honey produces total elimination of bacterial growth for *E.coli* 30%, *S. aureus* 50%, and *Streptococcus pyogens* 50%. Furthermore, several studies also showed that 50% concentration of honey of T*rigona* exhibited greatest antibacterial action than other concentrations (Ewnetu *et al*, 2013; Eswaran *et al*, 2015). However, the results obtained from the present study did not correspond with other previous studies. The divergence of results might be due to several reasons such as different botanical

origin and processing methods. Besides that, several limitations of the agar well-diffusion test were discovered including the insensitivity in detecting the low level of antimicrobial activity, variation in the experimental conditions, and permeability of nonpolar components. Thus, agar well-diffusion test may not be the most appropriate method to evaluate the antibacterial activity of honey (Kimoto-Nira and Amano, 2008).

In terms of a specific type of bacteria, the results show that the IZD for *E.coli* and *Salmonella spp.* was greater than *S.aureus* and *Streptococcus spp*. The results were applied to both the disc diffusion test and agar well diffusion test. However, the account the chemistry and biotransformation of honey must be taken after ingested by the patient. This will change the efficacy of honey in terms of antimicrobial activity. The variation in the antimicrobial potential of honey used in the present study as compared to the others might be due to differences in the growth rate of pathogens, inoculum size, and the test method itself, as well as the source of the microorganisms (Andualem, 2014). Honey is produced from many sources, and its antimicrobial activity varies greatly with origin and processing. Also, it might be the fact that the type of honey produced by honeybees is dependent on the natural vegetative flowers blooming in different seasons and different places, and thus the flowers from which bees gathered nectar to produce the honey may contribute to the difference in the antimicrobial activities of honey (Machado *et al*, 2019).

Conclusions

This study has proven that dilution of honey of *Trigona* will be decreased or eliminate its antimicrobial activity. *Escherichia.coli* and *Salmonella spp*. were more susceptible to honey of *Trigona* than *S.aureus* and *Streptococcus spp*. in vitro. Finally honey *Trigona* had a potential value to be one of the sources for medical alternative treatment to treat skin infection and gastroenteritis in animals or human but without dilution (as it is).

Conflict of interests

The authors declare no conflict of interest.

Acknowledgments

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