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Antibacterial and Antibiofilm activities of Malaysian *Trigona* honey against *Pseudomonas aeruginosa* ATCC 10145 and *Streptococcus pyogenes* ATCC 19615

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Abstract

This study aimed to investigate the antibacterial and antibiofilm activities of *Trigona* honey against *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. The antimicrobial and anti-biofilm activities were examined by agar well diffusion assays, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), time-kill curve, biofilm formation in 96-well plates and scanning electron microscope (SEM). Larger zones of inhibition were recorded from the agar well diffusion method. *Trigona* honey samples showed clear zones of inhibitions against *P. aeruginosa* and *S. pyogenes*, 25.2±0.6mm and 26.7±1.0mm respectively. *Trigona* honey possessed the lowest MIC value against *P. aeruginosa* and *S. pyogenes* was 20% (w/v) and MBC was 25% (w/v). In addition, MIC_{50} was between 10%-12.5% (w/v) and MIC_{90} was between 20%-25% (w/v) concentration of honey for both bacteria. In time-kill curve, *Trigona* honey inhibited *P. aeruginosa* and *S. pyogenes* in a 3 $\log_{10}$ at 18 hours, and total viable counts (TVCs) were killed after 24 hours at honey concentration of 25%. In biofilm degradation assay, *Trigona* honey degraded 70% of *P. aeruginosa* and 68% of *S. pyogenes* biofilm. Also in biofilm inhibition assay, *Trigona* honey inhibited 91% of *P. aeruginosa* and 89% of *Streptococcus pyogenes* biofilms. SEM images of *P. aeruginosa* and *S. pyogenes* showed that *Trigona* honey changed shape, size of cells, destroyed cell wall integrity and lysed the cells in both bacteria. Scanning electron microscope images for biofilm of *P. aeruginosa* and *S. pyogenes* showed that *Trigona* honey decreased cell density, and cells appeared curved of *P. aeruginosa* and rough, holes and crevices of *S. pyogenes*. In sum, *Trigona* honey disrupted and damaged biofilm formation. This study demonstrated that *Trigona* honey has high antibacterial and antibiofilm activities against both bacteria *in vitro* and showed the efficacy of honey against biofilm in different degrees of potential effect. The study supports previous finding that *Trigona* honey can be used as an alternative medicine for various bacterial infections.

Keywords: *P. aeruginosa*, *S. pyogenes*, Antibacterial, Antibiofilm, *Trigona* honey, Scanning electron microscope (SEM).

1. Introduction

Antibiotics have been extensively used to prevent bacteria in modern medical treatments. The efficiency of antibiotics is highly significant; however, it leads to the emergence of antibiotic-resistant for some bacteria (Badet and Quero, 2011; Nassar *et al.,* 2011). Resistant bacteria are not easily eliminated because of evolvement in adaption model and surviving pattern against antibiotics (Lee *et al.*, 2011). Survival of bacteria in a patient could lead to persistent infection by forming bacterial biofilm. Biofilm is a type of self-produced extracellular matrix which is embedded by the bacteria to provide a protective environment for them to grow (Al-Saadi *et al.,* 2016; Jaffar *et al.,* 2016). The biofilm supplies nutrient to the bacteria and protects them from eradication by the drugs (Costerton *et al.*, 1999; Doern *et al.*, 2009).

Antimicrobial agents are important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished (Levy and Marshall, 2004; Mandal *et al.,* 2009). An alternative antimicrobial agent is urgently needed, and these circumstances have led to a re-evaluation of the therapeutic use of ancient remedies such as plants and honeys (Fatima *et al.,* 2018.; 2019). The use of traditional medicine to treat infection has been practiced since the existence of mankind and is one of the oldest traditional medicine which is important for the treatment of human ailments (Boorn *et al.,* 2010). Many researchers have reported the antibacterial activity of honey with broad-spectrum activity after being tested against pathogenic bacteria, oral bacteria as well as food spoilage bacteria (Boorn *et al.,* 2010; Lusby *et al*., 2005; Mundo *et al* ., 2004; Roberts *et al.,* 2012). In most ancient cultures, honey has been used for both nutritional and medical purposes. Currently, an alternative medicine using bee based products as a treatment for human diseases, known as apitherapy, is being practiced (Alandejani *et al.,* 2009; Ng *et al.*, 2017; Shahjahan and Halim, 2007).

Stingless bees are found in tropical and subtropical regions including central and south America, Africa, Asia,

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and northern Australia (Guerrini *et al.*, 2009; Shahjahan and Halim, 2007). *Trigona* bee which is known as 'Kelulut' is a commercial stingless bee species abundantly found in Malaysia. This bee produces Kelulut honey, a multi floral honey which is stored in cluster of small resin dome of their nests. Kelulut honey has been reported to have excellent antibacterial properties and is useful medically and therapeutically (Fabíola *et al.*, 2014; Fabíola *et al.*, 2016; Shahjahan and Halim, 2007). Honey from stingless bees has a distinct taste and aroma, is more fluid in texture, and has low crystallization. Stingless bee honey has variable and broad-spectrum activities against many different kinds of wound and enteric bacteria (Anthimidou and Mossialos, 2013; Ramalivhana *et al.*, 2014).The removal of exudate after wounds dressing with honey was increasing healing process in inflamed wounds (Ahmed *et al.*, 2003). Honey is hygroscopic, which enables dehydrating bacteria by decreasing the moisture of the environment. Its high sugar content and low pH level might prevent the growth of microbes (Eswaran *et al.*, 2015; Nishio *et al.*, 2016). An injured intestinal mucosa may be healed by using honey because it stimulates the growth of new tissues and works as an anti-inflammatory agent (Kek *et al.*, 2014). This study was carried out to investigate the antibacterial and antibiofilm activities of *Trigona* honey against *P. aeruginosa* and *S. pyogenes*.

2. Materials and Methods

2.1. Culture bacteria

Two strains of *Pseudomonas aeruginosa* (ATCC 10145) and *Streptococcus pyogenes* (ATCC 19615) were used for this study. Working bacterial culture was prepared by picking up 2-4 morphologically identical colonies from stock culture and then was suspended in 10 mL of sterile Mueller Hinton Broth (MHB) in sterilized universal bottles. The inoculum was incubated at 37°C for 24 hours before proceed to the subsequent assay (Shehu *et al*., 2016; Zainol *et al*., 2013).

2.2. Honey samples

Fresh *Trigona* honey samples were obtained from a bee farm located in Kelantan a state in East Coast of Peninsula Malaysia. Honey samples were stored in the dark at room temperature.

2.3. Agar well diffusion assay

Sterile distilled water was used to dilute *Trigona* honey to achieve 75%, 50%, 25%, and 10% (w/v) concentration of honey. A few single colony of *P. aeruginosa* and *S. pyogenes* were aseptically picked from the fresh culture plate using sterile cotton swab and then were suspended into 10 mL of saline solution. The inoculum density was adjusted to 0.5 McFarland (Hudzicki, 2009; Zainol *et al*., 2013). A sterile cotton swab was then dipped into the bacterial suspension and was rotated onto the tube with firm pressure to remove excess fluid. The swab was streaked over the entire surface of plate for three times and each time the plate was rotated approximately 90º to ensure even distribution of bacterial suspension onto the Mueller-Hinton agar surface. A sterile 9 mm cork borer (Fisher Scientific, UK) was used to create six wells of agar plate. The wells of agar plate were labelled and were added with 170 µL of the five different honey concentration; 100%, 75%, 50%, 25%, and 10% (w/v). The well with distilled water was used as a negative control. The agar plates were then incubated at 37ºC for 24 hours. Digital venire calliper (mutiarasaintifik, Malaysia) was used to measure the zones of inhibition. The assay was carried out in triplicate for each of the test organism.

2.4. Minimum inhibitory concentration (MIC) assay

To determine the MIC of *Trigona* honey, a broth micro dilution method was used. A few single colonies of *P. aeruginosa* and *S. pyogenes* isolates were aseptically picked from the fresh culture plate by using a sterile loop and then were suspended into 10 mL of MHB and incubated for 24 hours at 37°C. The inoculum density was diluted with MHB to achieve to 0.5 McFarland at 600 nm by using a spectrophotometer (Genesys 20, Thermo Scientific) (Hudzicki, 2009; Zainol *et al.*, 2013). The honey samples were freshly prepared for MIC. A bacterial suspension was inoculated together with nine different concentrations of honey (50%, 25%, 20%, 12.5%, 10%, 6.3%, 5%, 3.1% and 1.6%). After that, 200 µl of each concentration of honey with inoculum was added to wells. Column number 10 was filled with 200 µl of honey as a corresponding negative control, column number 11 was filled with 200 µL of inoculum as a bacterial growth control, and finally, column number 12 was filled with 200 µl of broth as a sterility control. The plates were incubated at 37°C for 24 hours. After 24 hours, visual inspection was done. Bacteria growths were assessed by turbidity in the wells and were compared to the positive and negative controls. Absorbance was measured by using the microtiter plate reader (Tecan Infinite 200 PRO, Austria) at 570 nm. The MIC_{50} and MIC_{90} was determined by using the following formula (Bouacha *et al.*, 2018; Lye, 2015; Zainol *et al.*, 2013):

$$\% \text{ Inhibition} = 1 - \frac{\text{OD of the test well} - \text{OD of corresponding negative control}}{\text{OD of bacterial growth control} - \text{OD of sterility control}} \times 100$$

2.5. Minimum Bactericidal Concentration (MBC) Assay

The MBC was determined after obtaining the MIC results. After 96-well plate was incubated overnight, the well without turbidity was selected for MBC assay. A sterile wire loop was gently and aseptically dipped into the selected wells (once at a time to ensure sterility before getting dipped into another well to avoid possible contamination) and then sub-cultured on fresh nutrient agar plate. The plates was labelled and incubated at 37ºC for 24 hours. Plate free of any bacterial growth was recorded as the MBC value (Zainol *et al.*, 2013).

2.6. Determination of the time-kill curve

Bactericidal or bacteriostatic of *P. aeruginosa* and *S. pyogenes* was determined by the time-kill curve. Firstly, 0.5 g of honey samples were mixed with 1.5 mL of inoculum and then were adjusted to 10^4 CFU/mL to achieve 25% concentration of *Trigona* honey. Inoculum without honey was used as a positive control. Broth without bacteria was used as a negative control. All inoculums were incubated at 37°C. Inoculums were collected at different time points, serially diluted in broth, plated on nutrient agar and cultured for 24 hours at 37°C to determine the total CFUs in each tube (Bonapace *et al.*, 2000; Boorn *et al.*, 2010; Bouacha *et al.*, 2018). Mean of Log_{10} CFU/mL against time were plotted for each

inoculum. The Log Reduction (LR) was calculated for each inoculum by subtracting the Log_{10} CFU at zero time and the Log_{10} CFU at 24 hours of incubation. The experiments were performed in triplicate at three different time (Bouacha *et al.*, 2018; Mandal and Mandal, 2011).

2.7. Scanning Electron Microscopy (SEM)

The effects of *Trigona* honey on the morphology of *P. aeruginosa* and *S. pyogenes* was examined by SEM (JEOL 6360LA, Japan). Firstly one tube containing 0.4 g of honey was mixed with 1.6 ml of inoculum and then was adjusted to 0.5 McFarland to reach 20% (w/v) concentration of *Trigona* honey. The second tube was filled with 1 mL of inoculum and was used as a control. Subsequently, the tubes were incubated at 37˚C for 24 hours. All samples were then centrifuged at 3500 rpm for 5 minutes. Pellets were collected and were fixed overnight with 2.5% (v/v) glutaraldehyde in 0.01 M phosphate buffer solution (PBS). The samples were washed three times with 0.01 PBS for 10 minutes followed by deionized water for 10 minutes. All samples were dehydrated with ascending concentrations of ethanol for 10 minutes as following; 25% (v/v), 50% (v/v), 75% (v/v), 95% (v/v) and 100% (v/v). After dehydration, the samples were transferred to the carbon tape on copper stage and were graduated to obtain an even and thin layer of sample. The samples were coated with platinum for 1 minute before were placed onto the copper stage holder and were viewed by the SEM (Ng *et al*., 2017). In addition to planktonic stage, the effects of *Trigona* honey of *P. aeruginosa* and *S. pyogenes* biofilms were also examined by SEM.

2.8. Biofilm degradation assay

The different series concentration of honey samples were prepared as follows;100%, 90%, 80, 70, 60%, 50%, 40%, 30%, 20% and 10% (w/v).To form *P. aeruginosa* and *S. pyogenes* biofilms, 200 µL inoculums were pipetted into 96-well plate. The well with inoculum only was used as a positive control, the well with broth only was used as a sterility control and the well with honey only was used as a corresponding negative control. The plates were initially incubated at 37°C for 72 hours. Subsequently, the 200 µL of planktonic cells were carefully removed before being replaced with 200 µL of honey at different concentration as mentioned above for overnight incubation. The wells were washed by using sterile distilled water for three times, and then were stained with 200 µL of 0.1% (w/v) crystal violet for 10 minutes before washing with PBS for three times (Jaffar *et al*., 2016). The plates were kept at room temperature for air dried and were reconstituted with 200 µL of 95% ethanol. Finally, the degradation of biofilms was measured by using micro plate reader (Tecan Infinite 200 PRO, Austria) at the wavelength of 570 nm. The experiments were performed in triplicate (Cooper *et al*., 2014; Cooper *et al*., 2011).

2.9. Biofilm inhibition assay

Inoculums with nine different concentrations of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10% were prepared and then 200µL of each inoculums were pipetted into a 96-well plate. The well with inoculum only (100% concentration) was used as a positive control, the well with broth only was used as a sterility control and the wells with honey only was used as a corresponding negative control. The plate was incubated at 37°C for 72 hours. After incubation, the inoculums were removed and the remaining biofilm was washed three times with sterile distilled water. The biofilm was then stained with 200 µL of 0.1% (w/v) crystal violet for 10 min before washing with PBS for three times. The plate was then kept at room temperature. The dried stained biofilm was reconstituted with 200 µL of 95% ethanol and was incubated for 5 min. The inhibition of biofilm was measured by micro plate reader at the wavelength of 570 nm. The experiments were performed in triplicate (Cooper *et al.*, 2014; Jaffar *et al*., 2016; AL-Kafaween *et al*., 2019 a, b).

2.10. Degradation and inhibition biofilm analysis

The degradation and the inhibition of biofilm were calculated as following (Cooper *et al.*, 2014; Cooper *et al*., 2011; Low, 2013; Lu *et al*., 2014; Ng *et al*., 2014).

$$\text{Biofilm (\%)} = \frac{\text{OD (positive control)} - \text{OD (treatment)}}{\text{OD (positive control)}} \times 100\%$$

3. Results

3.1. Agar well diffusion assay

Agar well-diffusion assay shows the zone of inhibition for *P. aeruginosa* and *S. pyogenes* after being treated with *Trigona* honey. *Trigona* honey exhibits a great inhibition zone on *P. aeruginosa* and *S. pyogenes* cultures. The MIC of *Trigona* honey against *P. aeruginosa* and *S. pyogenes* was 20% as shown in Figure 1. MIC_{50} was between 10%-12.5% concentration of honey and MIC_{90} was between 20%-25% for *P. aeruginosa* and *S. pyogenes* as shown in Figure 2.

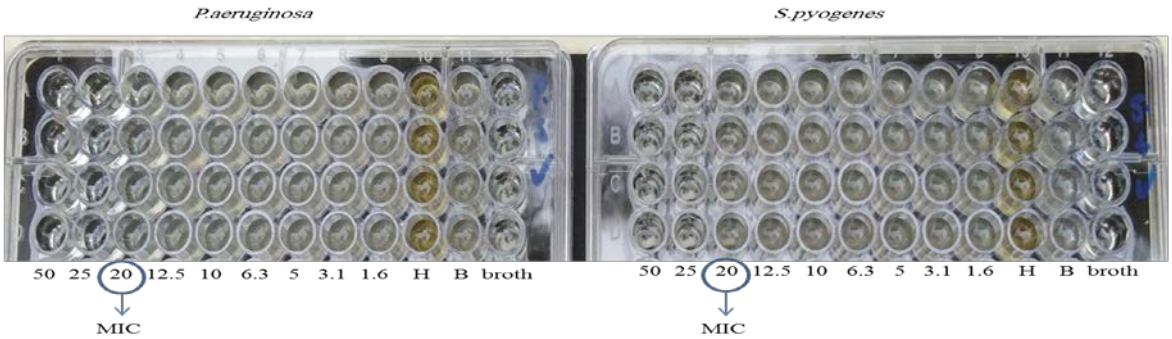

Figure 1. MIC of *Trigona* honey against *P. aeruginosa and S. pyogenes.* H: *Trigona* honey. B: Bacteria (inoculum)

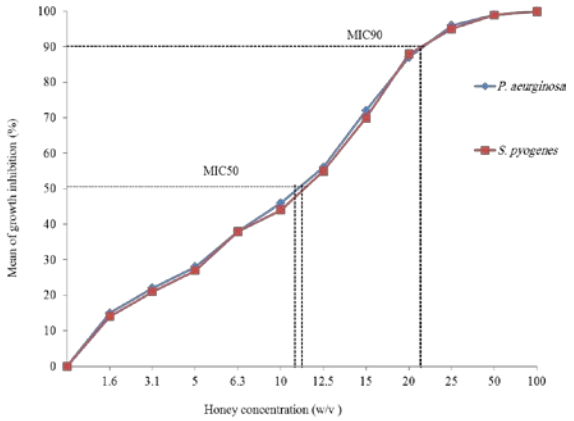

Figure 2. The percentage of bacterial growth inhibition by *Trigona* honey.

3.2. Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined as the lowest concentration of honey that enables abolishing bacterial growth. The MBC of *Trigona* honey against *P. aeruginosa* and *S. pyogenes* was 25% (Appendix 1-4)

3.3. Time-Kill Curve for P. aeruginosa and S. pyogenes

The time-kill curve clearly shows the increased number of *P. aeruginosa* and *S. pyogenes* cultures as shown in Figure 3 and 4. However, after getting treated with *Trigona* honey, *P. aeruginosa* and *S. pyogenes* demonstrate 1 $\log_{10}$ reduction in total viable counts (TVCs) at 6 hours. At 12 hours *P. aeruginosa* and *S. pyogenes*were decreased 2 $\log_{10}$ reduction in TVCs and at 18 hours they decreased 3 $\log_{10}$ reduction. *P. aeruginosa* and *S. pyogenes* were killed after 24 hours and were decreased 4 $\log_{10}$ reduction.

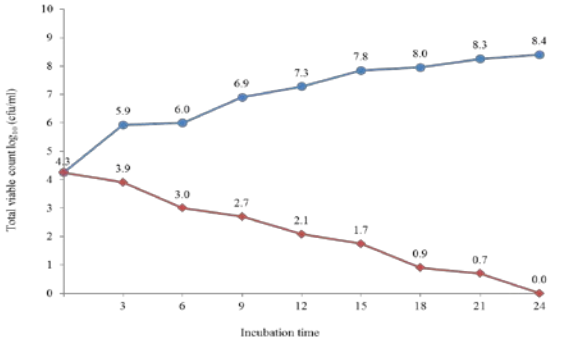

Figure 3. Time-kill curve of *P. aeruginosa*. TVCs of *P. aeruginosa* in the absence (blue) and presence of *Trigona* honey (red).

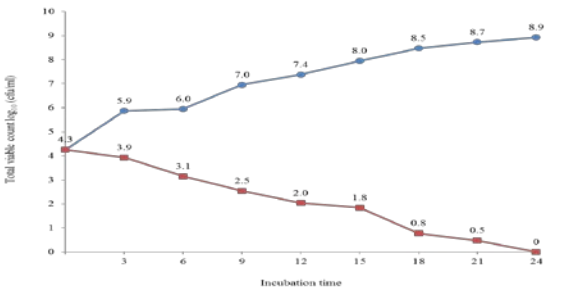

Figure 4. Time-kill curve of *S. pyogenes*. TVCs of *S. pyogenes*in the absence (blue) and presence of *Trigona* honey (red).

3.4. SEM for P. aeruginosa and S. pyogenes

SEM micrographs of *P. aeruginosa* demonstrate the rod shaped with regular structure and normal size as shown in Figure 5 (A). After being treated with honey, there were changes in shape and size of the cells and the cell wall was destroyed. Deformation and lysis of cells were also observed in Figure 5 (B). *P. aeruginosa* biofilm shows hundreds of bacterial cells are connected by a substantial amount of extracellular matrix producing stringy morphology and covering most of the area as shown in Figure 5 (C). The images of biofilm obtained by SEM provided convincing evidence of loss and disruption on viability and integrity of biofilm after being triggered by *Trigona* honey. The biofilm was noticeably shorter, the cell density was decreased, composed of layers of rod shaped cells, appeared in curved and distorted as shown in Figure 5 (D). SEM micrographs of *S. pyogenes* demonstrate the regular cocci with chain structure were observed in Figure 5 (E). After adding the *Trigona* honey, the cells were enlarged than normal size and closed each other as shown in Figure 5 (F). The morphological changes, such as abnormal shape and cell division, incomplete separation of cocci, ruptured and swelling cell were observed in Figure 5 (F). *S. pyogenes* biofilm shows numerous cells and diverse thickness connected to each other by extracellular matrix as shown in Figure 5 (G). Microscopic *S. pyogenes* biofilm with honey shows uneven shape of bacteria, and also the cell surfaces appear in rough, with holes and crevices. The structure of cells was damaged as shown in Figure 5 (H).

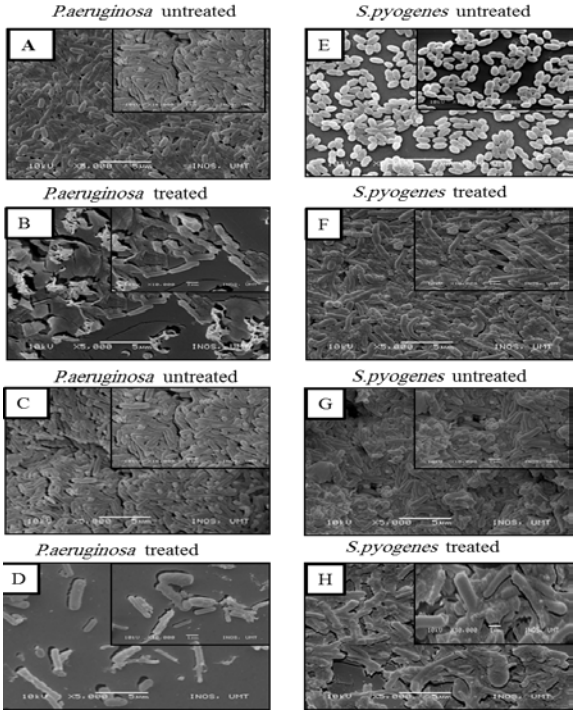

Figure 5. SEM of *P. aeruginosa* at planktonic stage (A) and after adding with *Trigona* honey (B).*P. aeruginosa* biofilm (C) and after honey treatment (D). SEM of *S. pyogenes* at planktonic stage (E) and after adding with *Trigona* honey (F). *S. pyogenes* biofilm (G) and after honey treatment (H).Viewed at 5000x and 10000x magnification. Scale bar 5µm.

3.5. Biofilm degradation assay

Trigona honey samples were able to reduce the biofilms mass of *P. aeruginosa* and *S. pyogenes.* Different concentrations of *Trigona* honey samples cause's different effects on biofilm degradation. *Trigona* honey was able to reduce 70%, 61%, 54%, 45%, 40%, 38%, 29%, 24%, 12% and 8 % biofilm mass of *P. aeruginosa* after being treated with 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10% (w/v) of honey concentration respectively. Similarly, *Trigona* honey was able to reduce 68%, 60%, 52%, 40%, 36%, 32%, 26%, 25%, 13% and 9 % biofilm mass of *S. pyogenes* at honey concentration of 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10 % (w/v) respectively.

3.6. Biofilm inhibition assay

Trigona honey samples at different concentrations are able to inhibit biofilms formation of *P. aeruginosa* and *S. pyogenes* at different levels. All of honey concentrations show the inhibition effect on biofilm formation. *Trigona* honey inhibits 91%, 84%, 78%, 72%, 49%, 27%, 26%, 13% and 9% of *P. aeruginosa* biofilm at honey concentration of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10% (w/v) respectively. Similarly, *Trigona* honey inhibits 89%, 81%, 73%, 68%, 45%, 23%, 19%, 11% and 7% of *S. pyogenes* biofilm at honey concentration of 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20% and 10 % (w/v) respectively.

4. Discussion

Antibacterial activities of honey have been broadly discussed among researchers worldwide. It is postulated to be close on several factors such as osmolarity, pH and other major constituents such as phenolic acids and flavonoids (Fatima *et al*., 2018; 2019; Zainol *et al*., 2013). Limitations of some antibacterial assay such as agar well-diffusion test were discovered including the insensitivity in detecting low level of antimicrobial activity, variation in the experimental conditions and permeability of non-polar components. Agar well-diffusion test may not be the most appropriate method to evaluate the antibacterial activity of honey (Balouiri *et al*., 2016).

Micro-broth dilution was performed to determine the MIC for antibacterial activities of honey toward all the tested bacteria. MIC is the lowest concentration of honey solution required to inhibit 99% of bacterial growth. MBC is defined as the lowest concentration of honey required to kill at least 99% of the tested bacterial strains (Zainol *et al*., 2013). MIC and MBC values are 20% and 25% (w/v), respectively, for both bacteria. Previous studies by (Abbas, 2014; Bouacha *et al*., 2018; Shenoy *et al.*, 2012; Zainol *et al.*, 2013) demonstrated that MIC for *Kelulut* honey, *Algerian* honey, *Manuka* honey *and Egyptian clover* honey against *P. aeruginosa* was at 20% concentration and MBC was at 25% concentration. Other studies showed that MIC for *Manuka* honey against *S. pyogenes* was at 20% concentration and MBC was at 25% concentration (Maddocks *et al*., 2012; Mandal and Mandal, 2011). Also a study by (Roberts *et al.*, 2012) reported that MIC for *Manuka* honey against *P. aeruginosa* was at 12% concentration and MBC was at 16% concentration.

The time kill curve is used to determine the bactericidal or bacteriostatic activity of antimicrobials. It is analyzed by plotting $\log_{10}$ CFU/mL versus time. Total cell count is defined as the total number of both dead and living cells in the sample, whereas total viable count (TVC) is defined as the number of living cells (Singleton, 2004). To maintain and minimize the impact of time-kill variables, several factors should be considered when performing time-kill studies. These variations affect the results and their interpretation. Firstly, the initial or starting inoculum of 10^4 to 10^7 CFU/mL should be applied. Secondly, the samples should be incubated at 37°C. Thirdly, the assay should be continued up to 24 hours (Klepser *et al.*, 1998). In this study, all these conditions were applied in the time-kill assays. The $\log_{10}$ CFU/mL for *P. aeruginosa* and *S. pyogenes* treated with *Trigona* honey was noticed at 12 hours which is almost half of *P. aeruginosa* and *S. pyogenes* were killed ($\log_{10}$ CFU/mL=2.1) and ($\log_{10}$ CFU /mL=2.0) respectively. Similarly, at 24 hours almost 100% of *P. aeruginosa* and *S. pyogenes* were killed ($\log_{10}$ CFU/mL=0) and ($\log_{10}$ CFU/mL=0) respectively.

SEM is an essential tool to observe the structural and physical changes that occur to cells after adding certain agents. In this study, SEM was used to determine membrane integrity, morphological changes of cells and evidence of cell division before and after exposure to honey. The mechanism of antimicrobial effects of honey is not yet fully understood. The effects of honey on bacteria could be complicated because of the complexity of honey compound. Nevertheless, observation of the bacterial structures and morphological variation presents valuable knowledge of complete understanding of antimicrobial and antibiofilm actions of *Trigona* honey on both bacteria.

Cell destruction and lysis were observed in *P. aeruginosa* and *S. pyogenes*, which affected the structure of the cell wall. In this study, MIC 20% was used for SEM because it is the lowest concentration that enables inhibiting planktonic and biofilm. A similar study has reported that *Manuka* honey at concentration of 20% affected the structure and viability of *P. aeruginosa* and distorted the cells (Henriques *et al.*, 2011). Another study using *Manuka* honey showed that concentration of 10% honey affected the structure of *Staphylococcus aureus* (Henriques *et al*., 2010). Studies by (Nishio *et al.*, 2016; Zakaria, 2015) showed that Stingless bees honey and Sider omani honey have disrupted the cell wall and inhibited cell division of *P. aeruginosa*, *S. pyogenes* and *Staphylococcus aureus.*

The four major biological activities of honey are acidity, non-hydrogen peroxide activity, high osmotic effect, and the presence of phytochemical components which is beneficial in controlling bacterial colonization and additionally disruption of biofilms (Al-Waili *et al.*, 2011; Zainol *et al.*, 2013). High osmotic effect of honey due to the high contents of sugar in honey also plays a role in reducing biofilm mass (Fatima *et al.*, 2018). Besides high osmotic effect of *Trigona* honey, acidity of honey is assumed to be a role in reducing biofilm mass as well. Acidity of *Trigona* honey, which is within the range of pH 3.2 to 4.5, creates an unfavourable environment for bacterial growth whereas their optimum pH for growth is about pH 7.2 to 7.4 (Fatima *et al.*, 2018).

5. Conclusion

The study revealed antibacterial and antibiofilm activities of *Trigona* honey against *P. aeruginosa* and *S. pyogenes*. Agar well diffusion, MIC, MBC, time-kill curve, biofilm degradation and biofilm inhibition proved that *Trigona* honey has a high antibacterial and antibiofilm activities against both bacteria. Moreover, SEM images showed that *P. aeruginosa* and *S. pyogenes* of planktonic and biofilm were lysed and disrupted after being treated with concentration 20% (w/v). Our finding suggests the consumption of 100% concentration of *Trigona* honey will degrade 70% of *P. aeruginosa* and 68% *S. pyogenes* biofilms. Also, consumption of 100% concentration of *Trigona* honey will inhibit 91% of *P. aeruginosa* and 89% *S. pyogenes* biofilms. Therefore, the Malaysian Trigona honey shows promising potential as an antibacterial agent.

Acknowledgments

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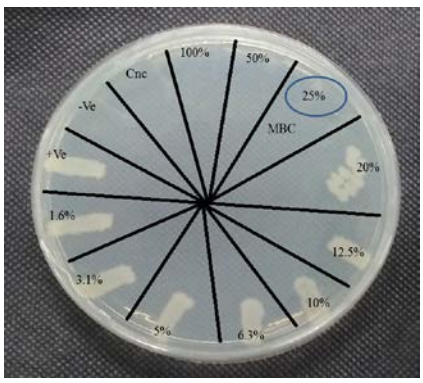
Appendix

Appendix 1: MBC determination for *Trigona* honey against *P.aeruginosa*



Appendix 2: MBC determination for *Trigona* honey against *S.pyogenes*

Appendix 3: Log reduction (LR) for *P.aeruginosa* after 24 hours exposure to 25% of *Trigona* honey.

Time (hours)	Log_{10}CFU/ ml(A)	Log_{10}CFU/ ml+ honey(B)	LR=log_{10}(A) -log_{10}(B)	*P*- value
0	4.3	4.3	0	Initial
3	5.9	3.9	2	0.041
6	6.0	3.1	2.9	0.018
9	7.0	2.5	4.5	0.011
12	7.4	2.0	5.4	0.019
15	8.0	1.8	6.2	0.014
18	8.5	0.8	7.7	0.008
21	8.7	0.5	8.2	0.003
24	8.9	0	8.9	0.002

Appendix 4: Log reduction (LR) for *S.pyogenes* after 24 hours exposure to 25% of *Trigona* honey.

Time (hours)	Log_{10}CFU/ ml (A)	Log_{10}CFU/ ml+ honey(B)	LR=log_{10}(A) - log_{10}(B)	*P*- value
0	4.3	4.3	0	Initial
3	5.9	3.9	2	0.041
6	6.0	3.0	3	0.023
9	6.9	2.7	4.2	0.035
12	7.3	2.1	5.2	0.024
15	7.8	1.7	6.1	0.006
18	8.0	0.9	7.1	0.001
21	8.3	0.7	7.6	0.003
24	8.4	0	8.4	0.004