CHAPTER III

EVALUATION OF HONEY AS ANTIBACTERIAL AGENT AGAINST MULTIPLE ANTIBIOTIC RESISTAND PATHOGENS BY DIFFERENT METHODS

3.1. Introduction

Honey are not only a delicious food but are also known for its health and healing properties. The antibacterial effects of honey especially against Gram-positive bacteria are well documented (Molan, 1997; Bogdanov, 1997; Rozaini *et al.*, 2004; Tan *et al.*, 2009). Both bacteriostatic and bactericidal effects of honey against many strains have been reported in many studies especially against pathogenic bacteria (Bogdanov *et al.*, 2008). It is suggested that Manuka honey (6% v/v) can be used against *Burkholderia cepacia* which causes pulmonary infections and chronic granulomatous disease in urinary tract infections and wound infections in hospitalized patients (Cooper *et al.*, 2000a; 2000b).

Honey has significant amount of ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, amino acids and proteins as well as enzymes such as glucose oxidase and catalase, all these components are very important to contribute to honey functional properties (Bogdanov *et al.*, 2008; Perez *et al.*, 2007). Furthermore, honey contains cinnamic acid, antioxidant agent and some flavonoids which have been approved for antibacterial applications (Rahman *et al.*, 2010). Mohammed (2010) reported that

Malaysian honey has antioxidant properties. The antioxidants compounds in honey may play a positive role in food safety beyond food preservation (Taormina *et al*., 2001).

In Malaysia, research on honey has focused on different aspects. It was reported that Tualang honey provide beneficial effects for wound healing and wound burn management (Nasir *et al*., 2010). The effects of different types of honey on tensile strength evaluation of burn wound tissue healing were also evaluated by Rozaini *et al*. (2004). The types of phenolic acidsin Malaysian honey were suggested to be responsible for the antibacterial properties (Aljadi & Yusoff, 2003) while other local Malaysian honey showed antimicrobial activity on some human pathogens (Tumin *et al*., 2005; Hassanain *et al*., 2010; Zainol *et al*., 2013). In addition, propolis of Malaysian honey inhibited the growth of *Staphylococcus aureus* and *Escherichia coli* as reported by Rahman *et al*. (2010). Honey of Malaysia, Libya, New Zealand and Saud Arabia contain lactic acid bacteria with antibacterial activity against selected Multiple Antibiotic Resistant (MAR) Gram-positive bacteria and Gram-negative bacteria (Aween *et al*., 2012a & b).

In fact, two methods that are commonly used to evaluate the antibacterial activity of phytochemicals are disc diffusion method (Bauer *et al*., 1966) and well diffusion (Perez *et al*., 1990). Since honey is a complex of substances, these methods may not reflect the potency of honey as antibacterial agent. Therefore, other methods namely nanophotometer assay, microtiter plates, and microbial plate count methods were used in this study in an attempt to evaluate the effectiveness of the methods for determination of the antibacterial activity of honey on multiple antibiotic resistant pathogenic bacteria.

3.2. Materials and Methods

3.2.1. Honey Samples

Nine samples of honey were obtained from different sources and coded as follows: Tualang honey (H026) and Acacia honey (H030, H031 and H032) from Malaysia, Al-Seder (H025), Kharoob (H028) and Hannon Honey (H020) from Libya and Manuka Honey 5+ (H027) and 10+ (H035) from New Zealand. All samples were kept in glass bottles and stored at room temperature (25 °C) before experiment.

3.2.2. Honey Samples Preparation

Amount of honey used was based on the lowest concentration that showed antibacterial activity. Honey samples were diluted with deionized water and prepared at 0.2 g/mL. All honey samples were heated at 70°C for 10 min using water bath and filtered using 0.45 micron filters (to allow all the compounds in honey to cross through the filter) and then kept at 4°C for further study. Heating removed H_2O_2 and destroyed any contaminating microorganisms that may be present in honey samples.

3.2.3. Cultures of Pathogenic Bacteria

The pathogenic bacteria used in this study were *S. aureus* (ATCC 25923), *S.* Typhimurium (ATCC 13311), *E. coli* (ATCC 25922), *B. subtilis* (ATCC11774) and *P. aeruginosa* (ATCC 27853) obtained from the Microbiology Laboratory Faculty of

Science and Technology, Universiti Sains Islam Malaysia (USIM), Malaysia. The bacteria were grown on nutrient agar (Oxoid, UK), and nutrient broth (Oxoid, UK) at 37ºC for 24 h and then kept at 4ºC before further experiments.

3.2.4. Antibiotic Resistant Test of Target Bacteria

The target bacteria were tested for their resistant to antibiotics using disc diffusion method as described by Bauer *et al*. (1966). The antibiotics used were vancomycin (5 µm), cephalothin (30 µm), nalidixic acid (30 µm), Gentamycin (10 µm), streptomycin (10 µm), tetracycline (30 µm), bacitracine (10 µm), penicillin G (10 µm), chloramphenicol (30 µm) and polymyxin B (300 µm) (Sigma). The selection of antibiotics in this study was based on the common antibiotics used in medical practice and health therapy.

3.2.5. Antibacterial Activity of Honey Samples using Disc Diffusion Method

Antibacterial activity of selected honey samples were determined by disc diffusion method on Nutrient Agar (NA) agar (Oxoid, UK) (Bauer *et al*., 1966). The pathogenic bacteria cultures were swabbed on NA agar plates. Discs were overlaid with tested honey samples overnight and then dried at 45°C for 24 h using drier oven (BINDER, Germany). Discs were placed on swabbed agars and incubated at 37°C for 24 h. Inhibition zone diameter was carefully measured and the results were expressed in millimeter (mm).

3.2.6. Antibacterial Activity of Honey Samples using Well Diffusion Method

The well diffusion method for antibacterial activity of honey was determined following the method of Perez *et al*. (1990) with slight modifications. Honey samples were prepared with concentration of 0.2 g/mL (w/v) using deionized water. Overnight culture of pathogenic bacteria in nutrient broth (Oxoid, UK) was prepared. Nutrient agar was prepared and once its temperature reached 40°C, 1% of pathogenic bacteria (10^9 CFU/mL) was added and mixed carefully. Amount of 25 mL of the nutrient agar with 1% of pathogenic bacteria was poured to petri dish plates and left under a laminar flow until the plates dried. Wells of 8 mm diameter was made using a sterile cork-borer with 8 mm diameter and the base of the wells were covered with nutrient agar (Oxoid, UK) and left to dry at room temperature. Next, 200 µL of prepared honey samples were poured to the wells individually and kept at 37°C for 24 h. The results were expressed by measuring the zones around the wells after diminution the well diameter. The experiment was done in duplicate and mean with standard deviation were calculated.

3.2.7. Antibacterial Activity of Honey Samples Using Nanophotometer Assay

In Nanophotometer method, concentration of 0.2 g/ 1ml honey samples were prepared using deionized water. Pathogenic bacteria were inoculated to nutrient broth (Oxoid, UK) and kept at 37°C. Prior to analysis, 1 mL of each honey sample was poured to 1 mL of pathogenic bacteria (10^6 CFU/mL) in micro-titer plates and kept at 37°C for 24 h. A 1 mL of each honey sample with 1 mL of nutrient broth without pathogenic bacteria was used as negative control and 1 mL of broth with pathogens with 1 mL of nutrient broth was used as positive control. The reading was determined as bacteria cells using Nanophotometer (IMPLEN, Germany) at wavelength of 600 nm. The results were then calculated using the following formula:

$$\text{Percentage inhibition} = \left(\frac{(+\text{ Control absorbance} - \text{Sample absorbance})}{+\text{Control absorbance}}\right) \times 100$$

3.2.8. Antibacterial Activity of Honey Samples Using Microtiter Plates

Each honey sample was tested against the selected pathogenic bacteria in microtiter plate assay, following the method of Magnusson and Schnurer (2001) with some modifications. 100 µL of nutrient broth containing 10^6 pathogenic bacteria/ml was placed in the 96 wells plate and 150 µL honey samples (0.2 g/mL) were poured into the wells. The plates were incubated at 37°C for 24 h. Optical density of bacterial growth was measured at 630 nm using Elisa plate reader (BIOTEK, USA). Honey with nutrient broth without bacteria was used as negative control and nutrient broth with pathogenic bacteria was used as positive control. The results then were interpreted using the following formula:

$$\text{Percentage inhibition} = \left(\frac{(+\text{ Control absorbance} - \text{Sample absorbance})}{+\text{Control absorbance}}\right) \times 100$$

3.2.9. Antibacterial Activity of Honey Samples Using Total Plate Count Assay

Antibacterial activity of selected honey samples were determined by plate count method on nutrient agar (OXOID) following Aween *et al*. (2012b) with some modifications. 100

µL of overnight pathogenic bacteria culture in nutrient broth were added to 100 µL of honey sample and kept at 37°C for 24 h. After that, 10 µL of the mixture were spread on nutrient agar and incubated at 37°C for 24 h. Pathogenic bacteria in nutrient broth was used as positive control and honey sample with nutrient broth as negative control. The results were taken by counting the number of pathogenic bacteria colonies on nutrient agar plates after 24 h of incubation and expressed as $\log_{10}$ CFU/mL. The enumeration of bacteria on plates followed standard microbiological procedure.

3.2.10. Statistical Analysis

All experiments were done in duplicate and all data were analyzed using Minitab16 system to calculate the mean, standard deviation, MAR index, correlation and percentage of inhibition. The correlation was carried out between the methods (disk diffusion method versus well diffusion method and microtiter plates versus nanophotometer) and the bacteria tested were determined (R= <0 no correlation, R= 0-0.7 poor correlation and R= 0.7-1 strong correlation).

3.3. Results

3.3.1. Antibiotic Resistant of Selected Target Bacteria

The target bacteria showed high resistance to several antibiotics tested. The diameter of inhibition zone varied between 0 and 29 mm (Table 1). All target bacteria were not inhibited by bacitracin, polymyxin B, penicillin G, vancomycin and streptomycin(Table

1). However, *S. aureus* were totally resistant to polymyxin B, while *S.* Typhimurium and *B. subtilis* were resistant tobacitracin, tetracycline, penicillin G, vancomycin, naladixic acid and chloramphenicol. *E. coli* were resistant to bacitracin, gentamycin, penicillin G and vancomycin. Bacitracin, tetracycline, penicillin G,vancomycin and chloramphenicol were not effective against *P. aeruginosa*. MAR index was from 11 to 88%, *S.* Typhimurium and *B. subtilis* showed the highest MAR index percentage (88%) compared to *E. coli* (55%), *P. aeruginosa* (66%) and *S. aureus* (11%).

3.3.2. Antibacterial Activity of Honey Samples Using Disc Diffusion Method

All the nine honey samples showed variable inhibitory activities against tested target bacteria by the disc diffusion method (Table 2). The inhibitory activity was significantly ($p<0.5$) affected by type of bacteria used but not with honey samples. *P. aeruginosa* was greatly inhibited by all honey samples as shown by inhibitory zone greater than 10.5±4.94 mm except H032 which was less than (7.5 ± 0.70 mm). While *E. coli* and *S.* Typhimurium were inhibited but to a lesser extent (Table 2). Tualang honey (H026) from Malaysia showed the highest inhibitory activity against *S. aureus*, *S.* Typhimurium and *B. subtilis* (20.00 ± 1.41, 14.00 ± 4.24 and 17.00 ± 0.00 mm respectively), Acacia honey (H031) from Malaysia showed the highest inhibitory zone of 18.50 ± 3.53 and 17.00 ± 0.00 mm against *E. coli* and *B. subtilis*, while against *P. aeruginosa* Al-Seder honey (H025) from Libya showed the highest activity.

Table 1: The antibacterial activities of selected antibiotics against target bacteria[a]

Antibiotics	Target bacteria				
	S. aureus	*S.* **Typhimurium**	*E. coli*	*B. subtilis*	*P. aeruginosa*
Bacitracin (10 µg)	6.50±0.70	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Gentamycin (120 µg)	11.00±0.00	14.50±0.70	0.00±0.00	13.00±0.00	13.00±0.00
Tetracycline (10 µg)	13.50±2.12	0.00±0.00	8.00±5.65	0.00±0.00	2.50±2.12
Naladixic acid (30 µg)	8.50±0.70	0.00±0.00	18.00±0.00	0.00±0.00	17.00±0.00
Polymyxin B (300 µg)	0.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00	4.00±0.00
Penicillin G (5 µg)	29.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Vancomycin (5µm)	6.00±0.00	0.00±0.70	0.00±0.00	0.00±0.00	0.00±0.00
Streptomycin (10 µm)	6.00±0.00	3.00±0.00	8.50±0.70	3.00±0.00	7.50±0.70
Chloramphenicol (30 µg)	15.00±2.82	0.00±0.00	18.50±4.94	0.00±0.00	0.00±0.00
MAR index %	11	88	55	88	66

[a]Diameter of inhibition zone around the discs (mm)

Table 2: Growth inhibition zone of target bacteria by honey samples by disc diffusion method[a]

Honey sample	Target bacteria				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	16.50±0.70	11.50±4.94	13.00±4.24	15.50±0.70	15.00±0.00
H025	19.50±0.70	11.50±3.53	10.50±2.12	15.50±0.70	17.00±0.00
H026	20.00±1.41	14.00±4.24	13.50±3.53	17.00±0.00	16.00±0.00
H027	17.00±0.00	11.50±2.12	11.00±1.41	15.50±0.70	16.00±0.00
H028	17.00±1.41	10.50±3.53	11.50±3.53	14.00±0.00	15.00±0.00
H030	19.00±0.00	13.50±2.12	15.00±2.82	15.50±0.70	14.50±3.53
H031	16.50±3.53	12.00±1.41	18.50±3.53	17.00±0.00	12.50±4.94
H032	17.00±1.41	11.00±1.41	13.50±2.12	16.50±0.70	7.50±0.70
H035	18.00±1.41	11.50±3.53	14.50±2.12	15.50±0.70	10.50±4.94

[a]Diameter of growth inhibitory zone was measured in mm after 24 h incubation at 37 °C

3.3.3. Antibacterial Activity of Honey Samples Using Well Diffusion Method

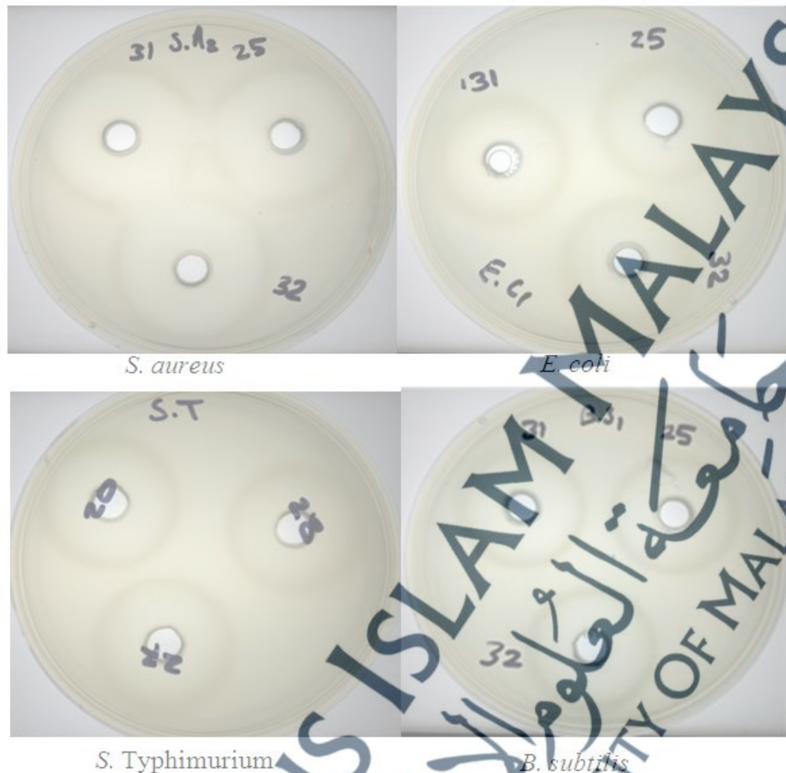
In well diffusion method, all honey samples from different sources showed inhibitory activity against all the target pathogenic bacteria. However, in the current study the degree of inhibition was affected by type of bacteria; the growth inhibitory zone varies between 15.5 and 27.5 mm (Table 3 and Fig. 1). Among the tested bacteria, *S. aureus* was easily inhibited by all tested honey samples, while *E. coli* was the most difficult to be inhibited. Growth of *S. aureus* was easily inhibited by Hannon honey, Libya (H020), Acacia honey, Malaysia (H031), Acacia honey, Malaysia (H032) and Manuka honey, New Zealand (H027) with inhibitory zone of 27.50, 25.00, 25.00 and 25.50 mm, respectively (Table 3 and Figure 1). Growth of *S.* Typhimurium and *B. subtilis* were moderately inhibited by all honey samples with inhibitory zone between 15.50 to 19.50 mm. All honey samples also inhibited *P. aeruginosa* (16.00 to 18.50 mm inhibitory zone). Libyan honey Hannon honey (H020) showed the highest inhibitory activity against all tested pathogenic bacteria (18.50 to 27.50 mm) except against *E. coli* (15.00 mm), while Acacia honey from Malaysia (H032) was effective against *S. aureus* (25.00 mm), *S.* Typhimurium (18.00 mm) and *E. coli* (17.00 mm).

Table 3: Growth inhibition zone of target bacteria by honey samples using well method[a].

Honey sample	**Target bacteria**				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	27.50±0.70	19.00±1.41	15.00±2.82	19.50±00.70	18.50±2.12
H025	22.50±0.70	16.50±0.70	15.50±0.70	17.50±0.70	16.00±0.00
H026	24.50±0.70	18.00±1.41	16.50±0.70	17.50±0.70	16.00±0.00
H027	25.50±2.12	17.00±2.12	14.00±0.00	19.00±0.00	17.50±0.70
H028	24.50±3.53	15.50±0.70	14.00±0.00	18.00±0.00	16.50±0.70
H030	23.50±0.70	17.50±0.70	15.00±0.00	18.50±0.70	16.00±0.00
H031	25.00±0.70	17.50±0.70	16.50±0.70	17.50±0.70	16.00±0.00
H032	25.00±0.00	18.00±0.00	17.00±0.00	18.00±1.41	16.00±0.00
H035	24.00±0.00	18.50±0.70	15.00±0.70	17.50±0.70	16.00±0.00

[a]Diameter of growth inhibitory zone was measured in mm after 24 h incubation at 37°C, ±: SD.

Figure 1: Growth inhibition zone of honey samples against pathogenic bacteria by well method at 37°C after 24 h of Incubation



3.3.4. Antibacterial Activity of Honey Samples Using Nanophotometer Assay

In nanophotometer assay the survival of target bacteria was determined using nanophotometer and the results were expressed as percentage inhibition from survival (CFU/mL) of the bacteria after treatment with honey (Table 4). The percentage of inhibition of all honey samples ranged from 13.17 to 100%. Al-Seder honey (H025) obtained total inhibition activity value of 100% against *S. aureus, E. coli* and *P. aeruginosa*; Acacia honey (H030) showed inhibitory activity against *S. aureus* and *S.* Typhimurium (100 and 99.09%). *S. aureus* and *E. coli* were easily inhibited by all tested honey samples with percentage of 94.26 to 100 and 83.36 to 100, respectively. All samples of honey showed inhibitory activity against *P. aeruginosa* except H031 which was poor (13.17%).

Table 4: Percentage of inhibition of target bacteria by honey samples using Nanophotometer assay[a]

Honey sample	Target bacteria (% inhibition)				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	94.26	78.47	83.36	82.18	93.92
H025	100.0	83.68	100.0	92.78	100.0
H026	100.0	93.59	99.82	97.81	99.25
H027	99.15	82.28	90.03	84.67	96.75
H028	99.46	87.18	99.17	87.36	97.54
H030	100.00	99.09	97.18	96.06	96.93
H031	97.92	79.57	94.84	95.82	13.17
H032	98.40	88.08	96.92	95.82	93.27
H035	99.15	94.09	92.11	90.64	70.40

[a]Percentage of inhibition= [(+ Control absorbance–Sample absorbance)/+Control absorbance] ×100

3.3.5. Antibacterial Activity of Honey Samples Using Microtiter Plates

Microtiter plates measures growth of bacteria by turbidity at OD630. All tested honey samples exhibited high inhibitory activity against all target pathogenic bacteria. Percentage of inhibition of target bacteria ranged from 60 to 100% by all honey samples within 24 h of incubation (Table 5). The growth of *S. aureus* was totally inhibited (100%) by Tualang honey (H026), while Acacia honey (H032) showed the highest inhibitory activity against *S.* Typhimurium (93.79%). The lowest activity was obtained from Hannon honey (H020) against all tested pathogens (64.04-74.63%).

Table 5: Percentage of inhibition of target bacteria by honey samples using microtiter plates

Honey sample	Target bacteria (% inhibition)				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	70.14	74.63	70.93	74.44	64.04
H025	98.48	86.52	85.43	89.17	93.73
H026	100.0	92.38	90.69	95.22	89.08
H027	99.33	89.22	96.89	96.25	97.49
H028	80.37	85.06	83.28	85.17	74.5
H030	89.00	92.32	88.47	91.02	80.05
H031	70.61	77.15	76.12	74.14	73.25
H032	98.48	93.79	93.54	95.58	87.56
H035	97.53	89.45	82.52	86.40	75.76

[a]Percentage of inhibition= [(+ Control absorbance–Sample absorbance)/+Control absorbance] ×100

3.3.6. Antibacterial Activity of Honey Samples Using Total Plate Count Assay

Survival of target bacteria as evaluated by plate count method was significantly affected by honey samples and type of bacteria (Table 6). Tualang Honey (H026) inhibited all tested pathogenic bacteria, followed by H030 and Acacia honey (H032) which inhibited all tested bacteria except *P. aeruginosa*. Growth of *E. coli* was totally inhibited by all honey samples except Acacia honey (H031) and Manuka honey (H035). *S. aureus* was not sensitive to Al-Seder honey (H025) and Manuka honey (H027), but inhibited by all the others. Hannon honey (H020) and Al-Seder honey (H025) were not active against *S.* Typhimurium and *B. subtilis*. In general, all honey samples found to have a good antibacterial activity against tested pathogenic bacteria compared to the positive control.

Table 6: Growth inhibition of target bacteria by honey samples using total plate count method[a]

Honey sample	Target bacteria (CFU/mL)				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	<10 (est.)	10^5	<10 (est.)	10^5	10^5
H025	10^5	10^5	<10 (est.)	10^5	<10 (est.)
H026	<10 (est.)	<10 (est.)	<10 (est.)	<10 (est.)	<10 (est.)
H027	10^5	10^5	<10 (est.)	10^5	10^5
H028	<10 (est.)	10^5	<10 (est.)	<10 (est.)	<10 (est.)
H030	<10 (est.)	<10 (est.)	<10 (est.)	<10 (est.)	10^3
H031	10^3	10^3	10^5	10^3	10^5
H032	<10 (est.)	<10 (est.)	<10 (est.)	<10 (est.)	10^5
H035	<10 (est.)	10^3	10^5	10^5	10^5
+ Control	10^{16}	10^{15}	10^{15}	10^{15}	10^{16}

[a]10: number of colonies.

3.4. Discussion

The occurrence of MAR bacterial strains is a public health concerns due to the bacteria are not easily killed by common antibiotics that normally used for health therapy. Some strains of *Staphylococcus* species were resistant to several antibiotics (Salvatore *et al*., 2010); *S. aureus* was resistant to ampicillin, kanamycin and oxytetracycline. The *S. aureus* used in this study demonstrated low MAR index of 11% compared to *E. coli* and

P. aeruginosa with 55 and 66%, respectively. *S.* Typhimurium and *B. subtilis* showed highest MAR index (88%) compared to other tested bacteria. Similarly, multiple antibiotic resistant bacteria was shown by several tested pathogens including *S.* Typhimurium ATCC13311 that was resistant to 10 antibiotics especially to bacitracin, cephalothin, penicillin G, vancomycin and strepromycin; *E. coli* ATCC25922 was resistant to bacitracin, penicillin G and vancomycin (Aween *et al.*, 2012b).

The presence of multiple resistance pathogenic bacteria has led to the investigation of natural effective alternatives to common antibiotics used in medical practice and health therapy. The results of present study showed that honey available in Malaysia can be used as antibacterial agent to prevent and control infections which are caused by pathogenic bacteria. Five different methods were used to evaluate the antibacterial activity of different honey samples and the results showed no correlation (R=<0) between disc diffusion method and well diffusion method. However, the antibacterial activity of honey samples against *S.* Typhimurium, *E. coli* and *P. aeruginosa* showed poor correlation between these two methods with all values were R = 0.310, 0.505 and 0.316, respectively. Well diffusion method was more suitable method for detecting the susceptibility of bacteria to antibacterial substances compared to disc diffusion. In contrast, the disc diffusion method is mainly used as a qualitative test for detecting the susceptibility of bacteria to antimicrobial substances (Mandal & Mandal, 2011). Disc diffusion and well method is based on the ability of molecules to diffuse into the agar; while, nanophotometer assay and microtiter plates allowed direct contact of the bacterial surfaces to the compounds. It was also observed that there was a positive correlation (R= 0.308 to 0.767) between nanophotometer assay and microtiter plates method.

Earlier reports showed that Egyptian clover honey tested for its antibacterial effect against antibiotic resistant strains of *E. coli* and *S.* Typhimurium using disc diffusion method was more pronounced on *E. coli* than *S.* Typhimurium (Badawy *et al.*, 2004); the Zone Diameter of Inhibition (ZDI) of different honey samples against *E. coli* was 12 to 24 mm and *S.* Typhimurium was 0 to 20 mm. Manuka honey has been demonstrated to be effective against several human pathogens *S. aureus*, *S.* Typhimurium, *E. coli* and *E. aeruginosa* using agar diffusion method (Lusby *et al.*, 2005; Visavadia *et al.*, 2006). The potency of six varieties of honey from different regions in Algeria were determined against *P. aeruginosa* using disc diffusion method and results showed that Sahara honey have inhibitory activity against tested pathogens and the authors suggested that Sahara honey could be used to manage the wounds and burns infected by *P. aeruginosa* (Boukraa & Niar, 2007). Recently, Boorn *et al.* (2010) tested eleven samples of stingless bee honey and the antibacterial activity was assessed using agar diffusion method which showed inhibitory activity against Gram-negative and Gram-positive bacteria including *S. aureus*, *S. epidermidis*, *S.* Typhimurium, *E. coli* and *P. aeruginosa*. Nilgiris honeys showed Zone Diameter of Inhibition (ZDIs) of 20-21 mm for *S. aureus*, 15-16 mm for *P. aeruginosa* and 13-14 mm for *E. coli* (Rajeswari *et al.*, 2010). RS and Manuka honeys killed *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. aureus* after 24 h of incubation (Kwakman *et al.*, 2011) using disc diffusion method. In this study using disc diffusion method, it was observed that Tualang and Acacia honeys showed higher inhibitory activity compared to Nilgiris honeys, but comparable with the activity from Egyptian clover honey. Tualang and Acacia honeys from Malaysia are able to inhibit the growth of MAR target bacteria using disc diffusion method. Considering the antibacterial activity of honey it was observed that all different honey samples possessed antibacterial activity against target Gram-negative and Gram-positive pathogenic bacteria as evaluated by disc diffusion and well methods. There were significant differences ($p>0.05$) between target bacteria using disc diffusion method but there was no significant differences ($p<0.05$) between honey type used (Table 2).

The well method indicated that all honey samples were significantly ($p<0.05$) inhibited target bacteria, but inhibitory activity varied between bacteria (Table 3 and Figure 1) with diameter of inhibition between 15.00 and 27.50 mm. The highest inhibition was shown by H020 from Libya against *S. aureus* (27.50 ± 0.70 mm), *S.* Typhimurium (19.00 ± 1.41 mm), *B. subtilis* (19.50 ± 00.70 mm) and *P. aeruginosa* (18.50 ± 2.12 mm) and from H032 from Malaysia against *E. coli* (17.00 ± 0.00 mm). Zainol *et al*. (2013) tested the antibacterial activity of several Malaysian honeys including Acacia and Tualang and one New Zealand honey (Manuka 18+) using well method against *S. aureus*, *E. coli*, *P. aeruginosa* and *B. cereus*; the results varied from 7.59 to 27.35 mm diameter, the highest inhibitory zone was obtained from Manuka honey against *S. aureus* (19.81 mm) and *E. coli* (14.04 mm), comparable inhibitory activity from Tualang honey against *P. aeruginosa* (16.22 mm) and *B. cereus* (27.35 mm), while Acacia honey showed the lowest activity compared to other samples. In the present study the ability of Al-Seder honey and Acacia honey to kill target bacteria using well method was higher than what observed by Tualang, Acacia and Manuka 18+ as reported by Zainol *et al*. (2013) against all MAR target bacteria. The well method tends to give a higher inhibitory activity for all honey compared to disc diffusion method and might be due to the direct attach of the tested compound in liquid form to the agar.

In contrast, when the antibacterial activity of the honey samples were evaluated using Nanophotometer assay no significant difference ($p>0.05$) was observed for both honey and target pathogens. This method used Nanophotometer (IMPLEN) that reported the number of target bacteria cells (both dead and alive) in the sample. All target pathogen reduced in numbers after 24 h incubation compared to control (without honey) (Table 4) ranging from 70 to 100% reduction after 24 h of incubation as shown by Al-Seder honey, 100% (H025), Tualang honey, 100% (H026) and (Acacia honey, 100% (H030) against *S. aureus*.

In order to confirm the inhibitory potency of honey, the growth of target pathogens were evaluated after 24 h incubation in microtiter plates. All honey samples showed antibacterial activity against the target pathogenic bacteria evaluated in which growth was inhibited between 64 to 100% after 24 h of incubation (Table 5). Honey sample Tualang honey (H026) completely inhibited the growth of *S. aureus*, while Acacia honey (H032) showed the highest activity among the evaluated honey samples. Among the pathogens evaluated, *P. aeruginosa* which was more difficult to be inhibited by all the honey samples. It is interesting to note that this test (microtiter plates) give a different results from above (Agar diffusion, well diffusion and Nnanophotometer methods). Honey samples showed significant differences ($p<0.5$) in antibacterial activity but there was no significant differences ($p>0.5$) between target pathogens in microtiter plates and similar to the results obtained by Nanophotometer method.

Other researchers who evaluated antimicrobial activity from sources other than honey, such as extracts from plants or microbial metabolites have included microdilution assay and microtiter plates in addition to the disc and well methods. The disc diffusion method and well method are the two most commonly used to determine antimicrobial properties of honey (Badawy *et al*., 2004; French *et al*., 2005; Lusby *et al*., 2005; Visavadia *et al*., 2006; Boukraa & Niar, 2007; Boorn *et al*., 2010; Rajeswari *et al*., 2010; Kwakman *et al*., 2011; Mandal & Mandal, 2011). Based on the results obtained in this study it is suggested that either Nanophotometer or microtiter plate assay be included for the evaluation of potency of honey as antibacterial agent.

All methods used in this study (agar diffusion, well diffusion, Nanophotometer and microtiter plate methods) did not evaluate the bactericidal effect of honey; thus plating of survivors of target pathogens after treatment with honey was included by using plate count method (Table 6). Acacia H032 and Tualang honey H026 showed total inhibition (survivors <10 est.) against all target pathogenic bacteria. *E. coli* was totally inhibited (survivors <10 est.) by all tested honey samples except H031 and H035 which allow recovery of pathogen and reached 10^5 CFU/ml after 24 h incubation. The potency Tualang honey (H026) and Acacia honey (H032) which showed their ability to kill the Multiple Antibiotic Resistant (MAR) bacteria including *S. aureus*, *B. subtilis*, *S.* Typhimurium, *E. coli* and *P. aeruginosa* suggests their potential to be used as an alternative therapeutic agent in certain medical conditions, particularly wound infection as well as in preservations to control food spoilage.

Many reported that honey was used as it is without heating and at concentrations (w/v) of 15-80% as used by Mavric *et al*. (2008); 50% by Boorn *et al*. (2010), 17.40, 19.20, 20.80, 23.80% by Voidarou *et al*. (2011) and 10, 30, 50, 70, 100% by Moussa *et al*. (2012). The antimicrobial activity was dose-dependent, the higher concentrations the greater the activity affecting both Gram-positive and Gram-negative bacteria. Compounds like glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, organic acids, amino acids and proteins were found present in honey in substantial amounts and can be responsible for the activity of honey (Bogdanov *et al.*, 2008; Perez *et al.*, 2007). However, in this study honey samples were heated at 70°C for 10 min and then filtered using 0.45 micron membrane filters. Heating at 70°C was reported to decrease glucose oxidase but not totally destroyed the enzyme (Kretavicius *et al.*, 2010), whereas hydrogen peroxide activity can be destroyed by heat, light or storage (Bogdanov, 1997). Additionally, all the honey samples were also diluted with deionized water at 200 mg/ 1 mL, a much lower concentration than that used by others and the antibacterial activity was observed. This study suggested that many compounds including heat stable compounds are responsible for the antibacterial activity and cannot be attributed to one or two main compounds present in honey.

3.5. Conclusion

The antibacterial activity of honey cannot be confirmed by using one single method. While disc diffusion and/or well diffusion method are useful for initial screening, other methods such as microtiter plates, Nanophotometer assay and/or plate count method should be included. To ascertain the potency of honey, the survival of target pathogens after treatment with honey should be carried out as well. This study also confirmed that even at low concentration of honey, it still has the potency to inhibit growth of MAR pathogens and this activity could be contributed by water soluble compounds present in honey. Malaysian honeys namely, Tualang and Acacia tend to give the better antibacterial activity compared to other honey samples evaluated. This work further supports that honey could be used as antibacterial agent.