CHAPTER IV

EFFECT OF PH, HEAT AND ENZYMATIC TREATMENTS ON ANTIBACTERIAL ACTIVITY OF DIFFERENT HONEY SAMPLES

4.1. Introduction

Honey is one of the most common traditional medicine since ancient times and proved by its ability to heal a variety of health problems, ranging from wound infections to gastric bacterial diseases (Dobrowolski *et al*., 1991; Badawy *et al*., 2004; Bankova, 2005). Recently, a number of therapeutic centers around the world start using honey as natural treatment as well as bacterial gastroenteritis to infections (Badawy *et al*., 2004). The antibacterial nature of honey is dependent on various factors working either singularly or synergistically (origin, type of flowers, the region, the nature of bees and the breeding techniques), the most salient of which are H_2O_2, phenolic compounds, specific compounds, pH of honey and osmotic pressure exerted by the honey (Molan *et al*., 2002; Malika *et al*., 2004).

Malaysian wild honey (Tualang honey) showed better activity against different pathogens of wound and enteric bacteria and the activity was comparable to that of Manuka honey from New Zealand. Similarly, Malaysian natural honey collected from different aromatic and medicinal plants have antimicrobial activity against antibiotic resistant bacteria isolated from human; all the honey samples showed strong activity against *E. coli*, *S.*

aureus, B. subtilis and *P. aeruginosa* (Tan *et al*., 2009). Zumla and Lulat (1989) reported that honey is a very good inhibitor to *E. coli*, *Salmonella* and *Shigella.*

Mundo *et al*. (2004) collected honey samples from different sources and evaluated against food spoilage organisms and pathogens namely, *Alcaligenes faecalis, Aspergillus niger, Geotrichum candidum, Penicillium expansum, Lactobacillus acidophilus, P. fluorescens, B. cereus, E. coli* O157:H7, *Listeria monocytogenes, Salmonella enterica Ser. typhimurium, S. aureus, S. aureus* 9144 and *B. stearothermophilus*. The results showed that *B. stearothermophilus* was highly sensitive to honey, *A. faecalis* and *L. acidophilus* were less sensitive, and the fungi *A. niger, P. expansum, G. candidum* and *S. aureus* were unaffected to honey. Tumin *et al*. (2005) investigated the antimicrobial activity of five different types of Malaysian honey and observed that three honey samples showed antibacterial activity against *S. typhi, S. aureus, Shigella sonnie, Streptococcus pyogenes* and *E. coli*.

Salmonella Typhimurium and *E. coli* are of the most common type of pathogenic bacteria around the world. *S.* Typhimurium causes gastroenteritis in human and other mammals. This bacterium usually enters human's body through mouth by contaminating food or water, penetrates the intestinal wall and multiplies in lymphoid tissue. The bacteria could enter bloodstream within 24 to 72 h causing blood poisoning (McCormick *et al*., 1995; Everest *et al*., 1999). *E. coli* is a type of bacteria that can contaminate food such as beef, fruits and vegetables (Todar, 2009). This bacterium is found inside human intestines, where it helps the body breaks down and digests the food. Unfortunately, some types of *E. coli* can get from the intestines into the blood and could cause serious infection such as

E. coli O157:H7. *Staphylococcus aureus* is a commensal bacterium that colonizes, in addition to the nose, the pharynx, axillae, vagina, and skin surfaces (Lowy, 1998). It can survive on disciplined animals, such as dogs, cats, and horses. This bacterium may stay alive for hours to weeks, some cases for months on dry environmental surfaces depending on the strain susceptibility to environmental conditions (Cimolai, 2008). *S. aureus* also can infect infants where it can cause a severe disease- staphylococcal scalded skin syndrome (Curran & Al-Salihi, 1980). *B. subtilis* is not a human pathogen, but it could contaminate several foods and may results in food poisoning in rare cases. It produces proteolytic enzyme subtilisin. The spores are very difficult to be killed by heating during cooking and it may spoil bread dough (Ryan & Ray, 2004). People who suffer from infection due to the contamination by these Gram-positive bacteria were commonly treated by antibiotics by medical practitioners. Therefore, these target pathogenic bacteria may easily become Multiple Antibiotic Resistant (MAR).

Most of the studies on antibacterial activity of honey and doctors who apply honey as treatment to their patients use raw honey. Some of the conditions which could face honey when applied to infections, serving or mixing with food are mostly pH, heat or/and enzymatic hydrolysis. This chapter investigated the antibacterial activity of Malaysian, Libyan, and New Zealand honey in different conditions of pH (3, 5, 7 and 9), heat (80, 100 and 121 °C), and enzymatic treatments (pepsin, trypsin, chymotrypsin and papain) against selected Gram-positive and Gram-negative bacteria.

4.2. Materials and Methods

4.2.1. Antibacterial Activity of Honey Samples using Microtiter Plates

This experiment was explained in CHAPTER III, Section 3.2.8.

4.2.2. Effect of pH Treatment on the Antibacterial Activity of Honey Against Pathogenic Bacteria

The pH of honey samples was adjusted to different pH values 3, 5, 6, 7 and 9 using 0.1 N HCl and/or 0.1 N NaOH. The adjusted pH honey samples were then tested against the selected pathogenic bacteria in micro-titer plate assay. Next, 100 µl of nutrient broth containing 10^6 pathogenic bacteria/ml was placed in the 96 wells plate and 100 µl pH adjusted honey samples were added into the wells. The plates were incubated in at 37 °C for 24 h. Pathogenic bacteria growth was measured by optical density at OD_{630} nm and visually using Elisa reader (ELx800, BioTek, USA). The percentage of antibacterial activity was calculated using the following formula:

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

4.2.3. Effect of Heating on the Antibacterial Activity of Honey Against Pathogenic Bacteria

Honey samples were heat treated at 80, 100 and 121 °C in autoclave for 10 min and immediately cooled in ice water. The heat treated honey samples were tested against the selected pathogenic bacteria in microtiter plate assay. Initially, 100 µl nutrient broth contains 10^6 pathogenic bacteria/mL were placed in the 96 wells plate and 100 µl of each honey sample was added into the wells. After that, the plates were incubated at 37 °C for 24 h. Pathogenic bacteria growth was measured by optical density at OD_{630}. The percentage of antibacterial activity was calculated using the following formula:

$$\text{Percentage inhibition} = \left(\frac{(\text{Positive control absorbance} - \text{Sample absorbance})}{\text{Positive control absorbance}}\right) \times 100\%$$

4.2.4. Effect of Enzymes on the Antibacterial Activity of Honey Against Pathogenic Bacteria

Honey samples were treated with different enzymes (pepsin, trypsin, chymotrypsin and papain) (Merck, Darmstadt, Germany). 1 µl of each enzyme was added to 3 ml honey samples separately (1 enzyme: 3 honey) and placed in micro-titter plate 96 wells and allowed to react for 1 h. Then each well was inoculated with (10^6 pathogenic bacteria/ml) of the selected pathogenic bacteria and incubated in 37 °C for 24 h. Pathogenic bacteria growth was measured by optical density at 630 nm and visually. The percentage of

activity was calculated using the following formula:

$$\text{Percentage inhibition} = \left(\frac{(\text{Positive control absorbance} - \text{Sample absorbance})}{\text{Positive control absorbance}}\right) \times 100\%$$

4.2.5. Statistical Analysis

Data were analysed with Minitab 16 system to calculate the mean, standard deviation, percentage of inhibition and one way ANOVA test were carried out to show the significance differences in the antibacterial activity.

4.3. Results

4.3.1. Antibacterial Activity of Honey Samples using Microtiter Plates

The results of this experiment were explained in CHAPTER III, Section 3.2.8.

4.3.2. Effect of pH on the Antibacterial Activity of Honey Against Pathogenic Bacteria

Adjusting honey samples to pH 3, 5, 7, and 9 affected significantly ($p<0.05$) the antibacterial activity of the different honey samples against the target pathogenic bacteria (Tables 7, 8, 9 and 10). However, all honey samples showed the highest activity at pH 3

(89.8 to 100.0% inhibition). Adjusting pH of honey samples from 5 to 9 showed variable inhibitory activity among the bacteria. *S. aureus* was totally inhibited (100.0%) by honey sample H031 (Acacia honey) at pH 3, while honey sample H030 (Acacia honey) lost the activity (0.0%) at pH 7 against *S. aureus*.

Table 7: Inhibition percentage of target bacteria by honey samples at pH 3 using microtiter plates 24h

Honey sample	**Target bacteria (% inhibition)**				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	90.4	88.7	88.6	87.3	95.5
H025	99.7	92.3	92.7	93.5	79.1
H026	96.3	96.9	96.3	97.8	100.0
H027	88.5	92.4	86.1	89.3	82.1
H028	88.0	92.3	90.0	87.9	85.5
H030	92.3	96.8	92.8	92.6	98.6
H031	100.0	94.8	91.0	93.3	93.1
H032	89.8	95.1	94.5	96.4	99.5
H035	90.0	93.7	91.6	94.8	83.5

Table 8: Inhibition percentage of target bacteria by honey samples at pH 5 using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	35.5	45.6	35.3	48.2	52.0
H025	64.7	67.1	67.9	71.1	77.4
H026	44.0	42.4	52.8	45.8	75.2
H027	64.7	65.7	65.0	64.2	68.6
H028	56.9	64.0	60.2	59.4	91.3
H030	19.3	35.6	31.1	29.9	80.2
H031	44.8	64.3	56.6	59.9	82.2
H032	61.3	70.4	62.8	64.7	73.6
H035	54.7	54.0	50.0	54.4	73.6

Table 9: Inhibition percentage of target bacteria by honey samples at pH 7 using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	35.3	51.8	45.7	53.6	47.7
H025	84.8	65.8	67.6	66.7	63.8
H026	21.4	38.3	23.3	37.3	69.5
H027	80.9	64.8	68.2	61.6	59.7
H028	41.4	57.8	49.5	57.6	35.2
H030	0.0	28.4	20.3	20.9	55.5
H031	75.0	67.0	82.5	62.1	62.1
H032	15.2	54.3	29.7	47.4	61.8
H035	34.7	49.2	51.9	54.4	60.8

Table 10: Inhibition percentage of target bacteria by honey samples at pH 9 using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	61.8	65.8	61.4	67.4	47.8
H025	21.0	57.7	63.5	65.9	55.6
H026	57.2	56.3	45.4	56.6	52.5
H027	54.2	59.5	55.5	52.7	52.3
H028	55.0	62.9	54.2	57.8	23.8
H030	56.5	63.2	50.7	56.9	38.6
H031	83.1	59.5	63.0	53.2	49.8
H032	54.8	60.5	61.8	60.8	50.5
H035	56.0	52.3	59.3	62.3	58.7

4.3.3. Effect of Heating on The Antibacterial Activity of Honey Against Pathogenic Bacteria

The antibacterial activity by all honey samples after heat was shown in Tables 11, 12 and 13. Results indicated that there was no significant difference ($P > 0.05$) of the antibacterial activity between honey heated at 80 °C and 100 °C. The highest antibacterial activity of all honey samples against all tested bacteria was after heating at 121 °C compared to 80 °C and 100 °C. Heating honey at 121 ^{o}C for 10 min resulted in complete inhibition of *S. aureus* (100.0%) for all honey samples, but slight reduction in activity was observed for H028 (Hannon honey), H027 and H035 (Manuka honey) with 99.6, 99.8 and 96.6 %, respectively (Table 13). In general, heating honey samples at 80 and 100^{o}C retained the antimicrobial activity against all target pathogens especially H030

(Acacia honey) (Tables 11 and 12). Honey sample H026 (Tualang honey) showed the highest activity against all target pathogens when the sample was heated at 121 °C.

Table 11: Inhibition percentage of target bacteria by honey samples after heating at 80 °C using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	***S.* Typhimurium**	***E. coli***	***B. subtilis***	***P. aeruginosa***
H020	70.2	76.0	77.3	74.9	51.4
H025	65.7	73.2	74.2	75.9	45.2
H026	75.0	78.0	80.8	81.5	49.4
H027	86.3	88.1	88.3	91.3	58.9
H028	75.5	80.2	82.0	84.0	48.5
H030	91.4	90.2	91.7	92.9	60.8
H031	82.4	86.9	85.9	88.5	62.8
H032	84.4	84.5	86.3	86.5	63.2
H035	88.9	86.7	85.3	100.0	51.5

Table 12: Inhibition percentage of target bacteria by honey samples after heating at 100 °C using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	77.2	69.6	72.1	73.2	55.3
H025	82.1	77.2	78.9	77.9	51.7
H026	91.9	81.5	80.8	85.5	56.9
H027	83.9	76.4	76.8	81.7	59.5
H028	84.4	75.7	76.2	81.1	59.4
H030	99.3	88.3	90.7	91.2	85.1
H031	89.0	80.8	81.2	86.0	63.0
H032	80.4	84.0	86.8	86.6	77.4
H035	94.8	78.9	81.0	79.1	63.5

Table 13: Inhibition percentage of target bacteria by honey samples after heating at 121 °C using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	99.6	83.8	75.3	83.4	99.5
H025	100.0	85.9	85.6	87.0	100.0
H026	100.0	99.9	97.6	100.0	100.0
H027	99.8	81.7	77.8	82.0	96.6
H028	100.0	83.7	82.8	84.1	93.8
H030	100.0	94.9	94.0	95.6	100.0
H031	100.0	87.4	84.7	87.5	96.4
H032	100.0	90.6	91.4	92.3	99.9
H035	96.6	80.0	73.5	82.8	93.8

4.3.4. Effect of Enzymatic Treatment on the Antibacterial Activity of Honey against Pathogenic Bacteria

Results of treated honey samples with proteolytic enzymes (pepsin, chymotrypsin, trypsin and papain) maintain the antibacterial activity against target pathogens, but at different percentage inhibition (Tables 14, 15, 16 and 17). There was no significant difference ($P > 0.05$) on the antibacterial activity of honey treated with chymotrypsin and pepsin, while there was significant difference ($P < 0.05$) in the antibacterial activity between honey treated with papain and trypsin. Honey samples H026 (Tualang honey), H030 (Acacia honey) and H032 (Acacia honey) showed higher inhibitory activity against *S. aureus* after treated with pepsin than chymotrypsin, while honey sample H030 (Acacia honey) showed higher inhibitory activity after treated with chymotrypsin against all target bacteria (Table 15). H026 (Tualang honey), H028 (Kharoob honey) and H030 (Acacia honey) lost the activity after treating honey samples with trypsin enzyme (Table 16). *P. aeruginosa* was totally inhibited (100.0%) by H020 (Hannon honey), H026 (Tualang honey), H028 (Kharoob honey), H032 (Acacia honey) and H035 (Manuka honey) after treating honey samples with papain. Similarly, all target pathogenic bacteria were totally inhibited by H020 (Hannon honey) and H028 (Kharoob honey), after papain treatments (Table 17).

Table 14: Inhibition percentage of target bacteria by honey samples after treating with chymotrypsin enzyme using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S. Typhimurium*	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	76.1	64.6	55.5	64.1	51.7
H025	74.9	67.7	58.2	72.3	81.3
H026	82.0	74.1	62.3	80.0	80.6
H027	81.4	72.4	63.1	72.7	79.3
H028	74.3	71.4	61.1	72.7	81.0
H030	87.3	80.6	70.5	79.9	86.0
H031	75.8	76.3	64.0	76.7	46.8
H032	79.0	73.5	62.1	72.6	85.8
H035	70.1	72.5	54.0	72.6	85.8

Table 15: Inhibition percentage of target bacteria by honey samples after treating with pepsin enzyme using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S. Typhimurium*	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	80.5	71.3	60.8	75.3	59.8
H025	76.4	72.3	65.4	81.5	94.8
H026	100.0	75.0	67.0	85.2	100.0
H027	77.0	75.2	63.2	69.7	94.5
H028	75.4	76.3	65.6	73.8	95.2
H030	89.3	79.7	75.4	79.9	95.3
H031	76.7	75.2	62.3	73.8	94.5
H032	74.4	76.3	62.0	76.4	94.2
H035	79.8	75.5	63.4	83.3	99.2

Table 16: Inhibition percentage of target bacteria by honey samples after treating with trypsin enzyme using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	70.4	62.3	40.9	60.4	40.3
H025	67.5	54.4	32.0	54.4	28.7
H026	70.4	60.5	35.8	61.2	0.0
H027	68.9	58.9	37.0	61.6	28.7
H028	65.2	55.2	33.4	61.9	0.0
H030	73.7	65.7	43.2	67.3	0.0
H031	64.9	56.6	33.7	58.2	66.6
H032	70.0	64.2	38.8	65.0	18.5
H035	68.3	58.8	41.5	60.6	30.6

Table 17: Inhibition percentage of target bacteria by honey samples after treating with papain enzyme using microtiter plates 24h

Honey sample	Target bacteria (% inhibition)				
	S. aureus	*S.* Typhimurium	*E. coli*	*B. subtilis*	*P. aeruginosa*
H020	100.0	100.0	100.0	100.0	100.0
H025	81.2	80.1	57.3	76.7	82.2
H026	99.1	89.6	72.4	92.7	100.0
H027	79.1	73.8	51.8	74.3	67.2
H028	100.0	100.0	100.0	100.0	100.0
H030	91.2	84.6	61.5	88.7	81.0
H031	80.4	85.2	55.1	85.5	98.3
H032	87.3	84.1	79.6	81.6	100.0
H035	84.4	75.6	55.2	72.1	100.0

4.4. Discussion

The presence of multiple resistance pathogenic bacteria led to the investigation of natural effective alternatives to known antibiotics. Honey is known to have antimicrobial activity. High osmolality, acids, flavonoids, hydrogen peroxide, phenolic compounds, proteins, enzymes, peptides, and amino acids are well known for their antimicrobial activity. Most studies reported on the antimicrobial activity of raw of honey. Treating honey such as heating, pH and enzymes may change the nature of antimicrobial activity.

Antimicrobial activity of honey sample is generally evaluated using disc diffusion and well diffusion methods. These methods usually used amounts of 50 and 100 µL. However, using microtiter plates method allows evaluation of the antimicrobial activity of honey in small amount lower than the normal MIC of raw honey (<20% w/v) as described in CHAPTER III, Section 3.2.8. Additionally, the results can be expressed quantitatively and qualitatively. In this study, the percentage of inhibition of diluted honey samples using microtiter plates ranged from 60.0 to 100.0% (Table 5, CHAPTER III). Microtiter plates method was used by Muhialdin *et al*. (2011a & b) to evaluate the antimicrobial activity of cell free supernatant from *Lactobacillus* species against food fungi, and the results were presented in percentage of growth. Aween *et al*. (2012b) reported the presence of LAB in honey collected from different sources, and the LAB supernatant showed high antibacterial activity against *S. aureus*, *S.* Typhimurium, *B. subtilis*, and *E. coli* using microtiter plate method.

The antibacterial activity of honey is affected by different conditions given to honey such as pH adjustment, heating, and enzymatic treatments. Adjusting the pH of honey samples

still maintain the antibacterial activity at different rates. There was significant difference ($P < 0.05$) in the antibacterial activity between adjusted honey samples to pH 3, and 5, 7 and 9 (Tables 7, 8 and 9). Adjusting the pH of honey samples at 3 showed increase in the antibacterial activity of all tested honey samples. In addition, honey samples H031 (Acacia honey) and H026 (Tualang honey) showed total inhibition against *S. aureus* and *P. aeruginosa*, respectively. In contrast to the report by Kwakman *et al*. (2010), the antibacterial activity of medical-grade honey reduced after adjusting honey pH at 3 against antibiotic resistant *S. aureus*, *E. coli*, *B. subtilis*, and *P. aeruginosa*. Adjusted honey samples at pH 5, 7, and 9 showed a decrement in the antibacterial activity against all target pathogenic bacteria except for honey sample H030 (Acacia honey) at pH 7 which totally lost the antibacterial activity against *S. aureus*. Similar observation on medical-grade honey were reported that decrease in the antibacterial activity was shown after adjusting the pH of honey from 4 to 7 against antibiotic resistant *S. aureus*, *E. coli*, *B. subtilis*, and *P. aeruginosa* (Kwakman *et al*., 2010).

All heated honey samples in this study showed increase in the antibacterial activity compared to honey without heat treatment (Table 11, 12 and 13). The highest percentage of bacterial growth inhibition was detected after heating honey samples at 121 °C for 10 min compared to heating at 80 and 100 °C for 10 min. However, Bogdanov (1997) reported that heating unifloral honeys and mixed honey at 70 °C for 15 min reduced the antibacterial activity against *S. aureus* and suggested that the non-peroxide antibacterial activity honey was slightly affected while the peroxide activity was damaged resulting in loss of the antibacterial activity of honey. Hydrogen peroxide is normally present in honey and its amount increase and active in diluted honey. Hydrogen peroxide has the

ability to inhibit microbes and it can be deactivated and damaged by heating. This study suggested that the antibacterial activity of all nine honey samples (Hannon, Alseder, Tualang, Kharoob, Acacia and Manuka) is from non-peroxide antibacterial activity since the activity was not reduced or lost after heating honey samples but had increased. Currently, limited studies on the effect of heating on the antimicrobial activity of honey have been reported.

The antibacterial activity of honey samples was affected by the enzymatic treatments of chymotrypsin, pepsin, trypsin and papain, and the target pathogenic bacteria (Table 14, 15, 16 & 17). There was no significant difference ($P<0.05$) on the antibacterial activity of honey treated with chymotrypsin and pepsin, while there was significant difference ($P > 0.05$) of honey treated with papain and trypsin. Treating honey samples with papain showed the highest antibacterial activity compared to treating with the other enzymes (chymotrypsin, pepsin and trypsin). The results after the enzymatic treatments suggest the presence of proteins that are hydrolyzed by the specific enzymes resulting in peptides that contribute to the antibacterial activity. Additionally, this study suggests the possible role of protein/peptide in enhancing the antibacterial activity of honey. Presently, no data published on the effect of enzymes on the antimicrobial activity of honey.

This study is useful in understanding the variable mechanisms of antibacterial activity of honey when used internally and externally as natural medicine, as well as when used in food industry. Honey should be viewed as not only as sweetening or flavoring agents but a combination of compounds that are responsible for it to function as natural remedy and preservative.

4.5. Conclusion

All honey samples exhibited antimicrobial activity at low concentration (12%) as evaluated using microtiter plates, especially Tualang honey and Acacia honey. Different honey samples affected differently against pathogenic bacteria. The antibacterial activity of honey samples was affected by pH (activity increased at pH3 and decreased at 5, 7 and 9), heating honey samples increased the activity of at all temperatures used), while enzymatic treatments provide variable antibacterial activity which prove the presence of proteins and peptides in honey. Thus, this study concluded that the antibacterial activity of honey was affected by several treatments given to the honey.