CHAPTER V

SUGARS, TOTAL PHENOLIC CONTENT, PROTEINS AND PEPTIDES IN HONEY SAMPLES

5.1. Introduction

Carbohydrates comprise about 95% of the honey dry weight. A daily dose of 20 g honey will cover about 3% of the required daily energy of human body. Honey is a rich source of different sugars consisting fructose, glucose and sucrose in different amounts depending on the origin of honey. Fructose and glucose are quickly transported into the blood during digestion and can be utilized for energy requirements of the human body (Bogdanov *et al*., 2008). There are many types of honey, for example, honeydew is obtained from the secretions of plants or insects live on plants while blossom honey is produced from the nectar of flowers. Doner (1977) detected glucose and fructose are the main sugars in blossom honey while 25 different oligosaccharides are present in blossom honey including isaccharides sucrose, maltose, trehalose and turanose. In comparison, honeydew honey contains higher amounts of oligosaccharides and trisaccharides such as melezitose and raffinose compared to blossom honey (Doner, 1977). The high concentration of sugars in honeys could be one of the responsible factors for the antibacterial activity (Mundo *et al*., 2004).

Honey contains reasonable amounts of phenolic compounds originating from plants and/or flowers. The original source of phenolic compounds from the nectar of plant has

been proposed as important factor for the non-peroxide antibacterial activity of honey. Alvarez-Suarez *et al*. (2010) reported that study on Cuban unifloral honeys contain high phenolic and suggested responsible for the higher antibacterial activity. Honey with high total phenolic content was observed to have antibacterial activity (Russell *et al*., 1990; Molan, 1992; Kwakman & Zaat, 2012), however, no correlation was detected between the phenolic content and the antibacterial activity (Truchado *et al*., 2009). Phenolic fractions from Malaysia honey reported to show antibacterial activity but the identity of the compounds responsible for this activity is unknown (Aljadi & Yusoff, 2003; Kwakman & Zaat, 2012). Additionally, the activity of individual phenolics isolated from honey is too low to substantially contribute to the antibacterial activity (Molan, 1992; Kwakman & Zaat, 2012).

The protein content is about 0.5%, mainly enzymes and amino acids. Research on detection, isolation and characterization of proteins/peptides from honey is very limited. Some of the researchers detected proteins in some honey samples and identified them as honey enzymes are diastase (amylase), decomposing starch or glycogen into smaller sugar units; invertase (sucrase, glucosidase), decomposing sucrose into fructose and glucose, as well as glucose oxidase, producing hydrogen peroxide and gluconic acid from glucose (Bogdanov, 2015). In addition, defensin-1 (Royalisin) peptide was detected in honeybee and in royal jelly but never been detected in honey. Bee defensin-1 has potent antibacterial activity but only against Gram-positive bacteria (Kwakman & Zaat, 2012; Ilyasov *et al*., 2012).

Therefore, this study evaluate the sugar composition analysis, total phenolic content, proteins content and peptides content in different honey samples and their possible contribution to the antibacterial activity.

5.2. Materials and Methods

5.2.1. Sugars Analysis Profile

Sugar profile was analyzed by UNIPEQ, Universiti Kebangsaan Malaysia (UKM), Bangi, Malaysia, following the in-house procedure. Honey samples were diluted with deionized water to make concentration of 20 mg/ml and kept in glass jars and, 20 µl of each sample was injected onto Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm column at 85 °C, with flow rate of 0.6 ml/min. Pure water was used as eluent, and detection carried out on Agilent RI detector by HPLC analytical. All injections were made in duplicate and mean with standard division were calculated.

5.2.2. Determination of Total Phenolic Content

The total phenolic content was determined using Folin-Ciocalteau method modified by Singleton and Rossi (1965). Honey samples (200 µl) were mixed with 2.4 ml of the freshly prepared working solution of Folin-Ciocalteau reagent. Working solution was prepared by diluting the concentrated Folin-Ciocalteau reagent at ration of 1 : 17 with distilled water. After 1 min, 420 µl of sodium bicarbonate (20 %, w/v) was added and the mixture was allowed to stand for 1 h at room temperature (25 ± 3 ^{o}C) in the dark. After

incubation, absorbance of each tube was measured at OD_{765} nm using nanophotometer (IMPLEN, Germany).

A standard curve using gallic acid solutions (31 .2, 62.5, 125, 250, 500 µg/ml) was constructed to calculate the total phenolic content of the sample. The total phenolic content was expressed as µg gallic acid equivalents (GAE) per g of honey.

5.2.3. Protein Content of Freeze Dried Honey Samples using Bradford Method

The method of Bradford (1976) was followed with some modifications to determine the protein content of honey. Preparation of protein reagent was carried out by dissolving 100 mg of Coomassie Brilliant Blue G-250 (BIO-RAD, USA) in 50 mL 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 L. Final concentrations in the reagent were 0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid. Bovine serum albumin (BSA) (BIO-RAD, USA) was used as standard. The preparation of BSA standard was done by dissolving 0.144 g of BSA in deionized water with final volume of 100 ml (Stock solution), and then serial dilutions were prepared 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 and 0 mg/ml (Table 18) and measured at OD_{595} nm using nanophotometer (IMPLEN, Germany) in 1 ml cuvettes to construct the standard curve. Blank was prepared by mixing 0.1 mL of deionized water and 1 mL of Bradford reagent. Calculation of protein content in honey was calculated using the standard curve BSA.

Table 18: Preparation of bovine serum albumin (BSA) standard

Blank	Concentration of BSA (mg/ml)	Amount of BSA stuck solution (ml)	Amount of deionized water (ml)
1	0	0	1.00
2	0.2	0.143	0.857
3	0.4	0.286	0.714
4	0.6	0.429	0.571
5	0.8	0.571	0.429
6	1.0	0.714	0.286
7	1.2	0.857	0.143

5.2.4. Peptides Content of Freeze Dried Honey Samples using OPA (O-phthalaldehyde) Method

The OPA reagent was prepared essentially as described by Church *et al*. (1983) with some modifications. The OPA solution was made by combining the following reagents and diluting to a final volume of 50 mL with deionized water as follows: A 25 ml of 100 mM sodium tetraborate; 2.5 ml of 20% (wt/wt) SDS; 40 mg of OPA (dissolved in 1 ml of methanol); and 100 µl of β-mercaptoethanol. To assay honey peptides, undiluted honey (30 µl) was added directly to freshly prepared OPA reagent, whereby 1.0 ml of OPA reagent in a 1.5 ml quartz cuvette. The solution was mixed briefly by inversion and incubated for 2 min at ambient temperature (25±3^{o}C), and the absorbance at 340 nm was measured using nanophotometer (IMPLEN, Germany). The reagent blank was prepared from 0.030 mL of the appropriate buffer and 1 mL of protein reagent.

Glutathione was used as standard. The glutathione stock solution of 1 mM concentration was prepared by dissolving 0.307 mg of the glutathione in water and then

make to final volume of 100 mL. Then serial dilutions of glutathione solution were prepared as shown in Table 19.

Table 19: Preparation of Glutathione standard

Blank	Glutathione (mg/ml)	Glutathione stock solution (ml)	Deionized water (ml)
1	0	0	1.0
2	0.2	0.2	0.8
3	0.4	0.4	0.6
4	0.6	0.6	0.4
5	0.8	0.8	0.2
6	1.0	1.0	0.0

5.2.5. Protein Profile using Sodium Dodecyle Sulphate (SDS-PAGE) for Freeze Dried Honey Samples

The SDS-PAGE profile was carried out for both freeze dried honey samples and Sephadex collected fractions to confirm the proteins/peptides content and their range of molecular weight. SDS-PAGE was done by the discontinuous buffer system as described by Laemmili (1970) with some modifications. Electrophoresis was carried out at a constant voltage of 120 V for 120 min onto 12, 16.5 and 20 % polyacrylamide gels under denaturing conditions. The gels were calibrated with standard molecular weight proteins (high and low ranges: 250, 200, 150, 100, 75, 50, 37, 25, 20, 15 and 10 kDa). Protein bands were visualized using Coomassie Blue dye staining. Calculations were determined using regression analysis as described in the manufacturer's procedure manual. The procedure for preparation of the gels and buffer solution is as in Appendix 6.

5.3. Results

5.3.1. Sugars Profile of Honey Samples

All honey samples contained variable amounts of fructose (37.8 to 46.3 g/100g), glucose (23.9 to 33.0 g/100g), sucrose (2.0 to 5.8 g/100g) except maltose (0.2 to 1.25 g/100g). However, maltose was not detected in four honey samples namely, H020 (Hannon honey), H030 (Acacia honey), H031 (Acacia honey) and H035 (Manuka honey) as shown in Table 20. The amount of fructose, glucose, sucrose and maltose in the different honey samples were significantly different (P>0.05) to each other. Honey sample H027 (Manuka honey) showed the highest amount of fructose (46.30 g/100g), followed by H035 (Manuka honey) (44.9 g/100g) compared to other honey samples. The highest amount of glucose was detected in H030 (Acacia honey) (33.0 g/100g) followed by H035 (Manuka honey) (30.5 g/100g). The amount of sucrose in H025 was 05.8 g/100g, while the amount of maltose in H028 was 01.50 g/100g). Honey samples H030 (Acacia) and H031 (Acacia) contain high amounts of fructose (>42.4 g/100g) and glucose (>29.5 g/100g).

Table 20: Sugar content of honey samples (g/100g)*

Honey sample	Sugar			
	Fructose	**Glucose**	**Sucrose**	**Maltose**
H020	43.60±0.14	24.10±0.98	04.75±0.35	00.00±0.00
H025	44.15±0.35	28.40±1.13	05.80±0.28	00.20±0.00
H026	40.55±2.47	26.10±2.40	02.00±0.00	01.25±0.35
H027	46.30±0.00	28.40±0.00	03.25±0.35	01.00±0.00
H028	42.90±0.70	23.95±1.06	03.00±0.00	01.50±0.00
H030	42.45±0.35	33.00±0.28	03.40±0.00	00.00±0.00
H031	43.35±0.21	29.55±0.35	02.25±0.35	00.00±0.00
H032	37.80±0.70	27.40±0.70	04.60±0.14	00.00±0.00
H035	44.95±0.63	30.55±1.76	02.40±0.00	01.00±0.00

* Results are stated as mean ± standard division (SD)

5.3.2. Total Phenolic Content

The phenolic content was calculated using gallic acid as the standard (R^2= 0.9988, y = 0.005x) as shown in Table 21 and Figure 32 (Appendix 3). The phenolic content of the samples was from 43.80 µg/ml for H032 (Acacia honey) to 147.20 µg/ml for H030 (Acacia honey). H035 (Manuka honey) and H020 (Hannon honey) showed high amounts of phenolic content of 115.00 and 106.60 µg/ml, respectively. However, sample H025 (Alseder honey) and H026 (Tualang honey) showed similar amounts of phenolic which was 50.80 and 50.00 µg/ml, respectively (Table 22).

Table 21: Absorbance of Gallic acid standard at 765 nm

Tube no.	Gallic acid (mg/ml)	Absorbance at 765 nm
1	00.00	0.000
2	31.25	0.163
3	62.50	0.283
4	125.0	0.626
5	250.0	1.272

Table 22: Phenolic content as calculated using standard formula (Y = 0.005x)

Sample code	Absorbance at 765 nm	Phenolic (µg/ml)
H020	0.533	106.6
H025	0.254	50.80
H026	0.25	50.00
H027	0.448	89.60
H028	0.408	81.60
H030	0.736	147.20
H031	0.471	94.20
H032	0.219	43.80
H035	0.575	115.0

5.3.3. Protein Content of Freeze Dried Honey Samples using Bradford Method

The protein content of honey samples was calculated using the standard formula

(Y=0.5259x + 0.0029) as shown in Table 23 and Figure 33 (Appendix 3). All freeze dried honey samples contained proteins with different concentrations depending on the source of honey sample. The proteins content of honey samples was in the ranged from 0.315 to 1.426 mg/mL (Table 24). The highest concentration was from H026 (Tualang honey) (1.426 mg/mL) followed by H020 (Hannon honey) and H032 (Acacia honey), 1.289 and 1.203 mg/mL, respectively. It was observed that the lowest protein content was from H030 (Acacia honey) (0.315 mg/mL) followed by H031 (Acacia honey) and H035 (Manuka honey), 0.567 and 0.570 mg/mL.

Table 23: Absorbance of BSA using Bradford assay

No. of standard	Concentration of BSA (mg/mL)	Absorbance at 595 nm
1	0.00	0.000
2	0.20	0.097
3	0.40	0.217
4	0.60	0.308
5	0.80	0.467
6	1.00	0.531
7	1.20	0.609

Table 24: Protein concentration of honey as calculated from standard curve using the formula Y=0.5259x+0.0029

Sample code	Absorbance at 595 nm	Concentration of protein (mg/ml)
H020	0.681	1.289
H025	0.452	0.854
H026	0.753	1.426
H027	0.521	0.985
H028	0.409	0.772
H030	0.169	0.315
H031	0.301	0.567
H032	0.636	1.203
H035	0.303	0.570

5.3.4. Peptides Content of Freeze Dried Honey Samples using OPA (O-phthalaldehyde) Method

The peptide content of honey samples were calculated using the standard formula: y= 0.2924 x -0.01 as shown in Table 25 and Figure 34 (Appendix 3). The amount of peptides in honey varied between all samples. The peptides were not detected in H020 (Hannon honey), H027 (Manuka honey) and H031 (Acacia honey), but peptides were detected in H026 (Tualang honey, 1.542 mg/mL), H032 (Acacia honey, 1.140 mg/mL) and H035 (Manuka honey, 0.076 mg/mL) (Table 26).

Table 25: Absorbance of Glutathione standard determined at $OD_{340\ nm}$

Blank	Concentration of Glutathione (mg/ml)	Absorbance at 340 nm
1	0	0
2	0.2	0.036
3	0.4	0.102
4	0.6	0.177
5	0.8	0.215
6	1	0.287

Table 26: Peptide concentration of honey samples

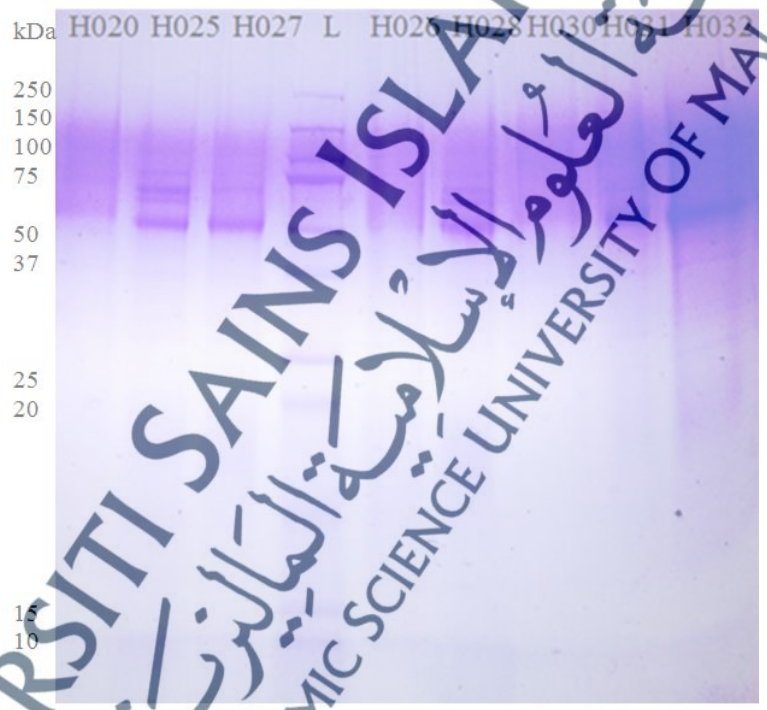
Honey sample code	Absorbance (340 nm)	peptide (mM/ml)	peptide (mg/ml)
H020	0.000	0.000	0.000
H025	0.185	0.666	0.204
H026	1.464	5.041	1.542
H027	0.000	0.000	0.000
H028	0.131	0.482	0.147
H030	0.677	2.349	0.721
H031	0.000	0.000	0.000
H032	1.076	3.714	1 .140
H035	0.063	0.249	0.076

5.3.5. Protein Profile using SDS-PAGE for Freeze Dried Honey Samples

The protein content of all freeze dried honey samples was confirmed by the SDS-PAGE

analysis (Figure 2). The protein of freeze dried honey samples separation ranged from 250 to less than 10 kDa. The molecular weight (MW) of proteins ranged from 250 to 5 kDa and, clear bands were observed between 37 to 150 kDa of protein bands, especially by samples H025 (Alseder honey), H027 (Manuka honey), H028 (Kharoob honey), H031 (Acacia honey) and H032 (Acacia honey).

Figure 2: Protein bands of freeze dried honey using SDS-Page 12% gel with 10 to 250 kDa Marker



5.4. Discussion

Honey is a rich source of sugar; the fructose, glucose and sucrose content in honey samples were significantly different ($P<0.05$) and, it was observed that fructose was

highest in all honey, followed by glucose and sucrose (Table 20). Relatively small amount of maltose was detected in five of nine honey samples namely, H020, H026, H027, H028 and H035. Similarly, Buba *et al*. (2013) reported the honey samples from North-eastern Nigeria contain fructose that was significantly ($P<0.001$) higher than the glucose and sucrose contents and the amounts ranged from 37.68 to 40.31 g/100 g, glucose from 27.25 to 39.56 g/100 g and sucrose from 0.53 to 3.29 g/100 g.

However, the amount of sugar in the honeydew samples evaluated was higher than those reported by White and Doner (1980). They reported that honeydew samples contain fructose and glucose in amounts of 2.91 to 38.12 g/100g and 19.23 to 31.86 g/100g, respectively; sucrose and maltose were at lower concentration, 0.44 to 1.14 g/100g and 5.11 to 12.48 g/100g, respectively. The sugar content of a number of floral honey samples contains fructose, glucose, sucrose and maltose contents were ranged from 27.25 to 44.26 g/100g, 22.03 to 40.26 g/100g, 0.25 to 7.5 g/100g and 2.74 to 15.98 g/100g, respectively (White & Doner, 1980). The differences in sugar content were related to the different sources of honey evaluated.

The phenolic content of honey depends on its source, season and/or flora and plant types (Molan, 1992; Al-Mamary *et al*., 2002; Yao *et al*., 2003; Estevinho *et al*., 2008; Kwakman & Zaat, 2012). In this study, the highest total phenolic content (147.20 µg/ml) was present in H030 (Acacia honey) compared to other honey samples (Table 22). Interestingly, H035 (Manuka honey) and H020 (Hannon honey) contained high concentration of phenolic, 115.00 and 106.60 µg/ml, respectively. In contrast the lowest low total phenolic content (43.80 µg/ml) was detected in other acacia honey samples. The difference in the amounts of phenolic compounds could be related to the source of honey,

the method used and/or storage time. Alvarez (2010) reported that the amount of phenolic compounds in buckwheat, blueberry and wildflower honeys were variable and the contents were 386.00, 163.00 and 138.00 µg/ml, respectively. It was observed that the total phenolic content of honey in the current study were lower than what was reported by Alvarez (2010). However, Khalil *et al*. (2012) found that the total phenolic content of Tualang honey samples ranged from 188.62 to 465.96 mg /kg, lower than values reported in this study. Similarly, Buba *et al*. (2013) reported that honey samples from North-eastern Nigeria contain lower phenolics compounds and ranged from 59.86 to 72.41 mg/100g. Environment and flower sources in which the bees were reared were reported responsible for the differences in phenolic contents.

Generally, honey contains low proteins (0.1 to 0.4%) (James *et al*., 2009; Buba *et al*., 2013). The protein content of the honey samples was ranged from (0.315 to 1.426 mg/ml) (Table 24), the highest protein content was shown by honey sample H026 (Tualang honey) (1.426 mg/ml). Honey sample H032 (Acacia honey) showed high content of proteins, 1.203 mg/ml. Similarly, Azeredo *et al*. (2003) reported that the proteins in honey samples of different floral origins which commercialized in different states in Brazil and found that high values of protein was detected in honey samples of *Borreria verticillata* (2236.00 µg/g). Lower than those reported by Khalil *et al*. (2001), five different honey samples from northern region of Bangladesh contained proteins with range of 0.655 to 0.744 g/100 g. However, Liberato *et al*. (2013) reported that the protein content in Brazilian honey varied among the honey samples, the highest protein content was from *Anacardium occidentale* honey sample (1121.00 µg/g), followed by *Myracrodruon urundeuva* honey sample (845.80 µg/g).

Detection and purification of proteins and/or peptides from other sources such as honeybee, bee pollen, propolis are reported by several researchers (Matsuyam & Natori, 1988; Casteels *et al*., 1989; Jr *et al*., 1995; James *et al*., 1996; Xu *et al*., 2009; Ilyasov *et al*., 2012). However, this study observed that honey contains peptides (Table 26). Honey sample H026 (Tualang honey) showed the highest content of peptide (1.542 mg/ml) compared to other tested honeys. Acacia honey sample H032 showed high content of peptide (1.140 mg/ml). H025 (Alseder honey), H028 (Kharroob honey) and H035 (Manuka honey) showed amounts of peptides but at lower levels, 0.204, 0.147 and 0.076 mg/ml, respectively. Peptide was not detected present in H020 (Hannon honey), H027 (Manuka honey) and H031 (Acacia honey).

The freeze dried honey samples was found to contain protein bands with molecular weight ranging from 10-150 KDa, and all samples showed clear bands at 50 KDa. However, there seems to be differences in the molecular weight of the protein bands. The freeze dried samples H025 (Alseder honey), H027 (Manuka honey), H031 (Acacia honey) and H032 (Acacia honey) showed clear bands between 37 and 75 KDa, between 15 and 25 KDa and also at 10 KDa. The results indicate that all honey contain protein of 50 KDa but may also contain lower amounts of other proteins of smaller molecular weight which may contribute to the different properties of the honey.

The antimicrobial activity of honey has been contributed by a number of compounds present in the honey such as high content of sugars, phenolic compounds, flavonoids, hydrogen peroxide and/or methylglyoxal (Section 2.5., CHAPTER II). The antibacterial activity of honey samples may not be from the high concentration of sugar in honey and

could be related to compounds other than sugar since low concentration of honey was used to evaluate the antibacterial activity of honey (CHAPTER III and IV).

The total phenolic content in the raw honey samples without dilution ranged from 43.80 to 147.20 µg/ml and the honey samples were diluted with a dilution factor of 1:5 before the evaluation of antibacterial activity (CHAPTERS III and IV). The phenolic content of evaluated honey samples would be in the ranged from 8.76 to 29.44 µg/mL. Al-Maliki (2011) reported that total phenolic content of honey samples was positively correlated to the antimicrobial activity against target bacteria and, the minimum inhibitory concentration (MIC) of phenolic compounds was 12.5 mg/mL against both Gram-positive and Gram-negative pathogenic bacteria. In addition, *in vitro* study carried out by Nitiema *et al*. (2012) and reported that MIC of some phenolic compounds (coumarin and quercetin) ranged between 0.625 and 5.0 mg/mL. In this study all honey samples at a dilution of 1:10 showed high total phenolic content and good antimicrobial activity. Additionally, MIC of honey sample was found to be lower in the ranged from 8.76 to 29.44 µg/ml then previously reported by others. It is possible that combination of phenolic compounds and other minor compounds contributed to the low MIC of the honey samples. This is as shown by Manuka honey which contained low protein and peptide content but the antimicrobial activity mostly related to methylglyoxal (Section 2.5.2., Chapter II).

Proteins and peptides from sources other than honey are known for their ability to inhibit microorganisms depending on their type and origin (Matsuyam & Natori, 1988; Casteels *et al*., 1989; Jr *et al*., 1995; James *et al*., 1996; Xu *et al*., 2009; Ilyasov *et al*., 2012;

Rybal'chenko *et al*., 2013; Vriens *et al*., 2014; Muhialdin *et al*., 2015). However, there are reports on the isolation and purification of proteins and peptides from honeybee (Casteels *et al*., 1989; Qu *et al*., 2008; Xu *et al*., 2009; Aronstein *et al*., 2010; Ilyasov *et al*., 2012) compared to honey. This study confirmed the presence of proteins and peptides in honey samples from Malaysia, Libya and New Zealand and therefore, it is suggested that the proteins and peptides although present in small amount could be equally responsible for the antibacterial activity of honey.

5.5. Conclusion

The honey samples contained sugars, phenolic compounds, proteins and peptides in variable amounts depending on types and sources of honey. It can be concluded that the antibacterial activity of honey samples may not be related to the sugars and phenolic compounds alone, but could also be contributed by the proteins and peptides even at low concentrations.