

ANTI-STAPHYLOCOCCAL ACTIVITY OF MELALEUCA HONEY

Wen-Jie Ng^{1,2} and Mei-Siew Lim¹

¹Faculty of Science, ²Centre for Biodiversity Research, Universiti Tunku Abdul Rahman, Perak, Malaysia

Abstract. Honey is well-known for its antioxidant properties due to the presence of phytochemical compounds, which are also involved in antibacterial activities. In this study, properties (total phenolic compounds, flavonoids, free radical scavenging activity, ferric reducing-antioxidant power, reducing sugar content, and pH) of Malaysian Melaleuca honey were investigated for their anti-staphylococcal activity, against both methicillin-sensitive (MSSA) and -resistant (MRSA) *Staphylococcus aureus* strains using a hole-in-plate diffusion method. The outcome revealed there is a significant positive correlation between the anti-staphylococcal activity and increase in honey concentration [20%-80% (v/v) in distilled water], which also correlated with each of the above mentioned parameter in honey, except for pH value that shows negative correlation. Furthermore, there was no difference in susceptibility to Melaleuca honey between MSSA and MRSA strains. Thus, in addition to being an antioxidant product, Melaleuca honey has a potential as a natural anti-staphylococcal agent.

Keywords: anti-bacterial, anti-staphylococcal, antioxidant honey, Melaleuca

INTRODUCTION

The medicinal significance of honey has been recorded in the world's oldest medical literatures (Maryann, 2000). It is commonly used as a traditional therapeutic agent to cure a wide range of disease conditions, such as wounds since ancient civilization (Hao, 2011). Thus, honey is one of the natural products that has interested researchers regarding its safe medicinal properties and active components.

According to Olaitan *et al* (2007) the antimicrobial properties of honey are due

to its hyperosmolar and acidic properties, as well as its enzymes, especially glucose oxidase. This enzyme is essential for the production of hydrogen peroxide, responsible, in part, in combating bacteria in wounds. However, there are many types of honey, which lack peroxide production or, as in the case of Manuka honey from New Zealand, retain antimicrobial effects even after removal of peroxide compounds (Zainol *et al*, 2013). Hence, these types of honey contain non-peroxide constituents that have antibacterial activity, such as phenolic compounds, flavonoids, methylglyoxal, methyl syringate, antibacterial peptides, and other types of antibiotics (Zainol *et al*, 2013). In addition, honey contains many varieties of antioxidants, both enzymatic (*viz*, catalase) and non-enzymatic (*viz*, phenolics, flavonoids,

Correspondence: Wen-Jie Ng, Faculty of Science, Universiti Tunku Abdul Rahman, 31900, Perak, Malaysia.

Tel: 605 4688888; Fax: 605 4661676

E-mail: ngwj@utar.edu.my

ascorbic acid, amino acids, carotenoids, proteins and Maillard reaction products) (Hao, 2011). There are several factors, which affect the antioxidant capacity of different types of honey, such as seasonal and environmental conditions. However, the floral source of nectar is the predominant factor influencing the antioxidant capacity of honey, with the total phenolic content determining the majority of honey's antioxidant capacity (Beretta *et al*, 2005).

Melaleuca honey or locally known as "Gelang" honey, collected from bee hives in *Melaleuca cajuputi* Powell ("Gelang") trees found naturally in swamp forests between old raised sea beaches (GLOBINMED, 2010), possesses the ability to contract wounds and promote the healing process (Tan *et al*, 2012). Melaleuca honey also shows cytotoxic effect against human colon HT29 cancer cells by suppressing inflammation and inducing apoptosis (Tey *et al*, 2012). In addition, Melaleuca and other kinds of Malaysian honey show antibacterial activity against cariogenic bacteria, vancomycin-resistant enterococci, *Escherichia coli* and *Salmonella* spp (Ng *et al*, 2014a, b).

Staphylococcus aureus is the major cause of nosocomial infection, and owing to frequent exposures to antibiotics has led to the rise and spread of multiantibiotic-resistant strains (Lowy, 2003). In this study, anti-staphylococcal effect and antioxidant capacity of Melaleuca honey were determined.

MATERIALS AND METHODS

Honey sample and bacterial strains

Melaleuca honey samples were collected by authorized bee farmers under the supervision of the Ministry of Agri-

culture and Agro Agro-based Industry, Malaysia. The samples were kept in the dark at room temperature to prevent reduction of glucose oxidase content by photo-oxidation (Nair, 2011). Honey sample was diluted to 80%, 60%, 40%, and 20% (v/v) with distilled water. The pH of each concentration of honey was measured by a pH meter (Sartorius, Grove, IL).

Staphylococcus aureus methicillin-sensitive (MSSA) strains (ATCC 25923 and ATCC 6538) and methicillin-resistant (MRSA) strains (ATCC 33592 and ATCC 33591) were cultured and maintained on nutrient agar (Merck, Darmstadt, Germany).

Antibacterial assay

Antibacterial activity of the honey samples was evaluated using a modified hole-plate diffusion method (Saravankumar *et al*, 2009). In brief, each *S. aureus* strain was inoculated into Muller-Hinton broth (BD Bioscience, Sparks, MD) and its turbidity was adjusted to 0.5 McFarland standard unit and then added to molten Muller-Hinton agar and allowed to solidify. Honey samples were placed into the wells (5 mm in diameter) made in the agar. After incubation overnight at 37°C, the diameters of clear zones were measured using a Vernier caliper.

Analysis of antioxidant capacity

Determination of total content of phenolic compounds. Concentration of total phenolic compounds in honey was determined using a modified Folin-Ciocalteu method (Khalil *et al*, 2011). In short, 0.5 ml aliquot of each concentration of honey sample (adjusted to 0.2 g/ml) was mixed with 0.5 ml of Folin and Ciocalteu's phenol reagent, and after 3 minutes, 0.5 ml aliquot of 10% (w/v) sodium carbonate solution was added and the volume adjusted to 5 ml

with distilled water. The reaction mixture was incubated in the dark for 90 minutes and then absorbance at 725 nm was measured in spectrophotometer (Thermo Scientific, Hudson, NH). A standard curve of gallic acid solution (20, 40, 60, 80 and 100 µg/ml) was generated, and total phenolic content is expressed as mg of gallic acid equivalent (GAE)/kg of honey.

Determination of total flavonoids content. Total flavonoids content of the honey sample was determined by a modified spectrophotometric assay of Khalil *et al* (2011). In brief, 1 ml aliquot of each concentration of honey sample (adjusted to 0.2 g/ml) was mixed with 4 ml of distilled water and 0.3 ml of 5% (w/v) sodium carbonate solution, and after 5 minutes, 0.3 ml aliquot of 10% (w/v) aluminum chloride solution was added, and then after 6 minutes 2 ml of 1 M sodium hydroxide solution were added. The total volume was made up to 10 ml with distilled water, mixed and the absorbance at 510 nm was measured as described above. A standard curve of catechin (20, 40, 60, 80 and 100 µg/ml) was generated and total flavonoids content is expressed as mg of catechin equivalents (CEQ)/kg of honey.

Free radical scavenging activity assay. Antioxidant capacity of the honey sample was measured based on its scavenging activity of free radicals generated from 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Aljadi and Kamaruddin, 2004). A 0.75 ml aliquot of each concentration of honey sample (adjusted to 0.1 g/ml) was mixed with 1.5 ml of methanolic DPPH solution (90 µg/ml) and the mixture was allowed to stand for 30 minutes in the dark at room temperature. Then the absorbance was measured at 517 nm as described above and free radical scavenging activity is expressed as percent radical scavenging activity (% RSA) using the

formula: % RSA = $[(1 - A_s)/A_c] \times 100$, where A_s = absorbance of sample and A_c = absorbance of negative control (water).

Ferric reducing-antioxidant power (FRAP) assay. FRAP assay was modified from that of Benzie and Strain (1999). FRAP reagent [10 ml aliquot of 300 mM acetate buffer pH 3.6 was mixed with 1 ml of 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) solution and 1 ml of 20 mM $FeCl_3 \cdot 6H_2O$] was incubated at 37°C for 30 minutes, and then 1.5 ml aliquot of FRAP reagent was added to 200 µl of each concentration of honey sample (adjusted to 0.1 g/ml). The reaction mixture was incubated at 37°C for 4 minutes and absorbance at 593 nm measured as described above. A standard curve of ferrous sulfate solution (100, 200, 400, 600 and 1000 µM) was generated and FRAP is expressed as µM ferrous equivalent [µM Fe (II)]/kg of honey.

Reducing sugar content assay

Reducing sugar content assay was performed according to a modified method of Lee (2013). A 3 ml aliquot of each concentration of honey sample was mixed with 3 ml of DNS reagent [1 g each of 3,5-dinitrosalicylic acid (DNS) and sodium hydroxide were dissolved in 100 ml of distilled water and added with 0.125 g of sodium sulfite] and the reaction mixtures were incubated at 95°C for 10 minutes, and then cooled to room temperature. One ml aliquot of 40% Rochelle salt (QReC) solution was added to each solution and absorbance at 540 nm was measured as described. A standard curve of glucose solution (0.2-1.0 mg/ml) was generated and reducing sugar content is expressed as mg of glucose/ml of honey.

Statistical analysis

Each assay was carried out in triplicate and the data were reported as

Table 1
Effect of Melaleuca honey on *S. aureus* growth.

Honey concentration (%, v/v)	Zone of inhibition (mm)			
	ATCC 25923	ATCC 6538	ATCC 33591	ATCC 33592
20	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
40	2.0 ± 3.5	2.0 ± 3.5	2.3 ± 4.0	2.0 ± 3.5
60	7.7 ± 2.1	7.3 ± 1.1	7.7 ± 2.2	8.1 ± 2.5
80	10.7 ± 1.5	10.0 ± 1.0	13.0 ± 1.0	13.7 ± 0.6
100	8.9 ± 1.8	9.0 ± 1.0	12.0 ± 1.0	12.3 ± 1.1

Data are expressed as mean ± SD.

Table 2
Total phenolic compounds and flavonoids content, DPPH inhibition, FRAP value, pH, and reducing sugar concentration of Melaleuca honey.

Honey (v/v)	Total phenolic compounds (mg GAE/ kg honey)	Total flavonoids (mg CEQ/kg honey)	DPPH inhibition (% DPPH)	FRAP value (μ M Fe (II)/ 100 g honey)	pH	Reducing sugar concentration (mg/ml)
20	158.3 ± 1.5	238.9 ± 9.6	15.8 ± 0.1	196.0 ± 0.7	3.9 ± 0.0	0.47 ± 0.00
40	279.5 ± 0.4	255.6 ± 19.2	33.7 ± 0.1	355.1 ± 1.7	3.8 ± 0.0	0.66 ± 0.01
60	407.9 ± 0.7	427.8 ± 9.6	46.5 ± 0.0	516.7 ± 1.8	3.7 ± 0.0	0.79 ± 0.01
80	463.3 ± 0.8	444.6 ± 9.4	47.7 ± 0.1	564.0 ± 1.3	3.7 ± 0.0	0.91 ± 0.02
100	513.8 ± 0.4	488.9 ± 9.6	48.0 ± 0.2	643.3 ± 0.0	3.7 ± 0.0	0.99 ± 0.01

Data are expressed as mean ± SD. CEQ, catechin equivalent; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalent.

means ± standard deviation (SD). The Pearson's correlation test was employed with IBM Statistical Packages for Social Science (SPSS) version 20.0 software (IBM, Armonk, NY) to determine the correlation coefficients among means of antioxidant assays, pH and reducing sugar content of Melaleuca honey with zone of inhibition against *S. aureus*. Statistically significant difference between means is considered at $p < 0.05$.

RESULTS

The four tested *S. aureus* strains

showed similar susceptibility pattern towards different concentrations of the honey sample (Table 1). No zone of inhibition was observed at 20% (v/v) honey concentration, however, 80% (v/v) concentration showed the largest zone of inhibition, which is also significantly larger than 60% (v/v) concentration. Although the diameters of inhibition zones exhibited by 100% (v/v) concentration of honey were smaller than 80% (v/v) but they are not significantly different.

The levels of total phenolic compounds, flavonoids and reducing sugar

Table 3
Statistical correlations between *S. aureus* growth (zone of inhibition) and total phenolic compounds and flavonoids content, DPPH inhibition, FRAP value, pH, and reducing sugar concentration of Melaleuca honey.

	Pearson correlation	Total phenolic compounds	Total flavonoids	DPPH inhibition	FRAP	pH	Reducing sugar concentration
Zone of inhibition	0.964	0.964	0.961	0.917	0.952	-0.986	0.959

DPPH, 2,2-diphenyl-1-picrylhydrazyl;FRAP, ferric reducing antioxidant power.

increased with increase in concentrations of honey samples (Table 2). In addition, higher DPPH free radical scavenging activities and FRAP values were also observed in higher concentrations of honey. Besides that, lower pH values (3.7) were observed at higher concentrations (60% and 80% v/v) and these values are not significantly different from that of the undiluted honey sample.

As shown in Table 3, antioxidant properties of Melaleuca honey, including total phenolic compounds, flavonoids, free radical scavenging activity and FRAP value, together with reducing sugar concentration, show strong positive correlations with zone of staphylococcal inhibition, however, pH value of the honey is negatively correlated with the diameter of inhibition zone.

DISCUSSION

Based on the results, 80% (v/v) concentration of Melaleuca honey exhibited the biggest zone of staphylococcal inhibition, almost equal to that of undiluted honey. This may be due to the high viscosity of undiluted honey limiting diffusion of bactericidal substances (Valgas *et al*, 2007).

This study confirmed the significant importance of phytochemical components in honey as putative antibacterial factors against *S. aureus*. The unique presence of ferulic acid and gallic acid in Melaleuca honey makes it an outstanding antibacterial agent compared to other types of honey (Lee, 2011). That is attributed to the effective free radical scavenging property of gallic acid by chelate divalent redox metals ions involved in the Fenton reaction, which leads to lipid peroxidation as well as other oxidant processes (Lee, 2011). Ferulic acid, a monohydroxylated phenolic

compound, is also involved in scavenging reactive nitrogen species (RNS) (Lee, 2011). In addition, the presence of caffeic acid and benzoic acid in honey contributes to the antibacterial activity, in particular against MSSA and even MRSA (Aljadi and Kamaruddin, 2002).

Catechins can damage bacterial membranes by penetrating directly into the lipid bilayer and perturbing the barrier function, and by causing membrane fusion resulting in syncytium formation (Ikigai *et al*, 1993). The additional hydroxyl groups in flavonoids, especially at 2-position of isoflavones, exert a greater inhibitory effect on bacteria after honey is diluted with water (Sato *et al*, 1996). Furthermore, the additional hydroxyl group at 5-position of flavones and flavavones contribute to their inhibitory effects against MRSA (Alcaraz *et al*, 2000). Certain flavonoids inhibit β -hydroxyacyl-acyl dehydratase and DNA gyrase (topoisomerase II) (Paiva *et al*, 2010). Statistical analysis reveals linear positive relationships suggesting that the anti-staphylococcal effect of Melaleuca honey is due to a great extent to its antioxidant capacity.

Acidic pH value was found in every concentrations of Melaleuca honey which could be due to the presence of high level of organic acid specifically known as gluconic acid which is generated by glucose oxidase enzymatic reaction (Office of Complementary Medicines, 1998). Acidity is believed as one of the anti-bacterial properties of Melaleuca honey as greater anti-staphylococcal effects were observed in lower pH values.

Generally, glucose and fructose are the only reducing sugars found in honey (Moniruzzaman *et al*, 2013). High reducing sugar content leaves few available 'free water molecules' results high

osmolarity, leading to diffusion of water molecules from bacterial cells and cell death (Molan, 2009). Although hydrogen peroxide is the main antibacterial agent in diluted honey sample, but in pure honey more important roles are played by other non-peroxide components of honey and by dehydration.

In summary, other than hydrogen peroxide, this study demonstrated the anti-staphylococcal effect of Melaleuca honey was due to other factors, including phytochemicals and reducing sugars.

ACKNOWLEDGEMENTS

The authors thank all individuals involved in this project and the Universiti Tunku Abdul Rahman (UTAR) for support and facilities.

REFERENCES

- Alcaraz LE, Blanco SE, Puig ON, Tomas F, Ferretti FH. Antibacterial activity of flavonoids against methicillin-resistant *Staphylococcus aureus* strains. *J Theor Biol* 2000; 205: 231-40.
- Aljadi AM, Kamaruddin MY. Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties. *Turkish J Med Sci* 2002; 33: 229-36.
- Aljadi AM, Kamaruddin MY. Evaluation of phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chem* 2004; 85: 513-8.
- Benzie IFF, Strain JJ. Ferric reducing/ antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol* 1999; 299: 15-27.
- Beretta G, Granata P, Ferrero M, Orioli M, Facino RM. Standardization of antioxidant properties of honey by a combination of

- spectrophotometric/fluorimetric assays and chemometrics. *Analyt Chim Acta* 2005; 533: 185-91.
- Global Information Hub on Integrated Medicine (GLOBinMED). *Melaleuca cajupati* Powell (Myrtaceae). GLOBinMED, 2010. [Cited 2014 May 15]. Available from: http://www.globinmed.com/index.php?option=com_content&view=article&id=79128:melaleuca-cajuputi-powell-myrtaceae&Itemid=139
- Hao W. The study of antioxidant activity of phenolic components of Manuka honey. Hamilton: University of Waikato, 2011. 22 pp. MSc Thesis.
- Ikigai H, Nakae T, Hara Y, Shimamura T. Bactericidal catechins damage the lipid bilayer. *Biochim Biophys Acta* 1993; 1147: 132-6.
- Khalil MI, Alam N, Moniruzzaman M, Sulaiman SA, Gan SH. Phenolic acid composition and antioxidant properties of Malaysian honeys. *J Food Sci* 2011; 76: C921-8.
- Lee JH. Low concentrations of honey reduce biofilm formation, quorum sensing and virulence in *Escherichia coli* 0157:H7. *Biofouling* 2011; 27: 1095-104.
- Lee YH. The study of synergistic action of starch and honey against *Staphylococcus aureus* in correlation with diastase number. Kuala Lumpur: University of Tunku Abdul Rahman, 2013. 37-40. BSc Thesis.
- Lowy FD. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest* 2003; 111: 1265-73.
- Maryann N. Honey as medicine has a long history--New Zealand honey is focus of intensive research. High Beam Research; 2000. [Cited 2014 May 20]. Available from: <http://www.highbeam.com/doc/1G1-66520151.html>
- Molan PC. Honey: antimicrobial actions and role in disease management. In: Ahmad I, and Aqil F, eds. New strategies combating bacterial infection. Weinheim: Wiley VCH, 2009: 229-53.
- Moniruzzaman M, Sulaiman SA, Khalil MI, Gan SH. Evaluation of physicochemical and antioxidant properties of sourwood and other Malaysian honeys: a comparison with Manuka honey. *Chem Central J* 2013; 7: 1-12.
- Nair S. Shelf life of honey. Buzzle, 2011 [Cited 2014 June 27]. Available from: <http://www.buzzle.com/articles/shelf-life-of-honey.html>
- Ng WJ, Ken KW, Kumar RV, Gunasagaran H, Chandramogan V, Lee YY. *In-vitro* screening of Malaysian honey from different floral sources for antibacterial activity on human pathogenic bacteria. *Afr J Tradit Complement Altern Med* 2014a; 11: 315-8.
- Ng WJ, Chin TJ, Khoo, BHY. Antioxidant properties, enzyme activities and inhibitory effects of Melaleuca honey against cariogenic bacteria growth and biofilm formation. *Adv Environ Biol* 2014b; 8(18) Special: 1-7.
- Office of Complementary Medicines. Honey scientific report. TGA, 1998. [Cited 2013 Nov 8]. Available from: <http://www.tga.gov.au/pdf/archive/report-honey-9812.pdf>
- Olaitan, PB, Adeleke, OE, Ola, IO. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *Afr Health Sci* 2007; 7: 159-65.
- Paiva P, Gomes F, Napoleao T, Sa R, Correia M, Coelho L. Antimicrobial activity of secondary metabolites and lectins from plants. *Curr Rese Technol Educ Top Appl Microbiol Microb Biotechnol* 2010; 1: 396-406.
- Saravanakumar A, Venkateshwaran K, Vanitha J, Ganesh M, Vasudevan M, Sivakumar T. Evaluation of antibacterial activity, phenol and flavonoid contents of *Thespesia Populnea* flower extracts. *Pakistan J Pharmaceut Sci* 2009; 22: 282-6.
- Sato M, Fujiwara S, Tsuchiya H, et al. Flavones with antibacterial activity against cariogenic bacteria. *J Ethnopharmacol* 1996; 54: 171-6.
- Tan MK, Adli DSH, Tumiran MA, Abdulla MA, Yusoff KM. The efficacy of Gelam honey

- dressing towards excisional wound healing. *Evid Based Complement Alternat Med* 2012; 2012: 1-6.
- Tey CPW, Hussein SZ, Abdullah S, Karim NA, Makpol S, Yusof YAM. Gelam and Nenas honeys inhibit proliferation of HT 29 colon cancer cells by inducing DNA damage and apoptosis while suppressing inflammation. *Asian Pac J Cancer Prev* 2012; 13: 1605-10.
- Valgas C, Souza SMD, Smânia EFA, Junior, A.S. Screening methods to determine antibacterial activity of natural products. *Brazilian J Microbiol* 2007; 38: 369-80.
- Zainol MI, Yusoff KM, Yusof MYM. Antibacterial activity of selected Malaysian honey. *BMC Compl Alternat Med* 2013; 13: 2-10.