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CLINICAL INVESTIGATION

Isolation and Identification of Phenolic Acids in Malaysian Honey with Antibacterial Properties

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Abstract: In this study the non-peroxide antibacterial factors in Malaysian honey were isolated and identified. The phenolic components were extracted from two different local floral honeys and their effects on the growth of selected pathogens were examined. A solid-phase extraction procedure was applied for the first time to recover honey phenolics. Identification was carried out via high performance liquid chromatography and gas chromatography analysis. Antibacterial activity was determined via the disc-diffusion and broth dilution assays. The phenolic fractions of gelam and coconut honeys showed potent antibacterial activities. Both honeys contain gallic, caffeic, and benzoic acids. However, gelam honey contains additional phenolic acids, namely ferulic and cinnamic acids. Since phenolic acids are known to exert an antibacterial effect, their presence in honey explains its antibacterial activity.

Key Words: Malaysian honey, phenolics, antibacteria

Introduction

Although the antibacterial action of honey is well established, the mechanism of its action is still a matter of debate. There is a common belief that the antibacterial activity of honey lies partially in its high osmolarity due to its high sugar content (1,2), and in its acidity due mostly to the presence of gluconic acid (1). Hydrogen peroxide, which was described in honey by White et al. (3), is thought to be the main antibacterial factor in honey (1,4); however, the presence of non-peroxide activity was also notable. This activity is usually attributed to the presence of organic components derived from floral sources (1,5).

A number of organic components with antibacterial activity have been identified in the ether extract of honey; these include 3,5-dimethoxy-4-hydroxy benzoic acid (syringic acid), and methyl 3,5-dimethoxy-4-hydroxy benzoate (methyl syringate) (6). By using high performance liquid chromatography (HPLC), some other flavonoids and phenolic acids have also been identified in different honeys, for example, pinocembrin, pinobanksin and chrysin (7-10), caffeic acid and ferulic acid (11), and vanillic acid, cinnamic acid, and benzoic acid (9,10). Despite these findings, Weston et al. (9) found that the phenolic fraction of manuka honey has no significant antibacterial activity and instead the activity was associated with the carbohydrate fraction. Weston et al. (10) then concluded that the phenolic components of manuka honey might contribute to but does not account for the observed non-peroxide antibacterial activity of manuka honey. Moreover, Weston (12) hypothetically concluded that the non-peroxide activity of honey could be interpreted as residual hydrogen peroxide activity, which was probably due to the absence of plant-derived catalase from honey. Hydrogen peroxide may be destroyed by tissue catalase when honey is used as a wound dressing. Therefore, the non-peroxide activity of honey becomes very important. Because of the importance of honey as a whole and of its phenolics as antibacterial factors, especially against antibiotic-resistant bacteria, the present work is an attempt to verify the role of phenolics as antibacterial factors in Malaysian honey.

Materials and Methods

Honey samples: Two of the most common Malaysian Apis mellifera honeys were used in this study; they are named gelam (*Melaluca* spp.) and coconut (*Cocos mucifera*) honeys according to their floral sources.

Bacterial strains: Two strains of standard bacteria, and two strains of pathogenic bacteria isolated from wound swabs were used in this study. They were provided by the Department of Medical Microbiology, Faculty of Medicine, University of Malaya. They included *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) Methicillin-Resistant *Staphylococcus aureus* (MRSA), and Methicillin- Sensitive *Staphylococcus aureus* (MSSA).

All chemicals and reagents used were either of HPLC or analytical grade.

Extraction and recovery of phenolics: Honey samples were prepared, subjected to base hydrolysis and extracted with ethyl acetate (liquid-liquid extraction) as described by Wahdan (11). Phenolics were then recovered using a solid phase extraction (SPE) technique. It was a modification of the method used by Seo and Morr (13) for the recovery of phenolic acids and isoflavonoids from soy bean. In brief, the dry honey extract was redissolved in acidified deionised water (pH 3.5), and the phenolics were adsorbed onto preconditioned isolute C_{18} columns (International Sorbent Tech. Ltd, Hengoed, Mid Glamorgan, UK). The cartridges were preconditioned by sequentially passing 5 ml each of methanol and acidified water (pH 3.5) at a drop-wise flow rate. Phenolic extracts were passed through the preconditioned cartridges at a drop-wise flow rate to provide efficient adsorption of the phenolic compounds. The adsorbed phenolics were then eluted from the cartridges by passing 3-5 ml of 50% (v/v) methanol–water solution at a drop-wise flow rate. The recovered fractions were combined, dried under nitrogen and subjected to further analysis.

Assay of the antibacterial activity: The antibacterial activity of the phenolic extract was assayed by the broth dilution assay (14) and disc diffusion assay (15).

HPLC: Prior to HPLC analysis, the dried phenolic extracts were redissolved in methanol and filtered through a 0.45 mm Millipore filter (Millipore Corp., Bedford, MA). HPLC analysis was performed on an LC-10A series liquid chromatograph (Shimadzu, Japan), equipped with a C_{18} column (150 x 4.6 mm, 5 mm particle size) (Jones Chromatography, UK), thermostated at 35 ºC. A gradient elution (Weston, et al., 2000) was modified to separate the extracted phenolics. Solvent (A) was 1.0% acetic acid in water, and solvent (B) was 1.0% acetic acid in methanol. Elution was performed at a solvent flow rate of 1.0 ml/min. The gradient profile of the system was 3% solvent B at the initial stage, 8% solvent B at 10 min, 31% solvent B at 20 min, 45% solvent B at 30 min, 50% solvent B at 50 min, 97% solvent B at 75 min, and again 3% solvent B at 81 min. The eluted phenolic compounds were monitored at 280 nm, and identified by comparing the chromatographic retention times with those of authentic standards. Quantitative levels were determined from the UV absorption during HPLC, and from the extinction coefficient which was obtained from Beer-Lambert graphs of the external standards (10). Recovery was measured in honey samples by adding pure standards to the extraction solutions prior to sample analysis; percent recovery was estimated by comparing the individual HPLC peaks areas measured for the samples before and after the addition of the standards.

Gas chromatography (GS) and mass spectrometry (MS): Prior to GC, the phenolic extract was methylated with BF_3: MeOH (10). GC-FID analysis was performed on GC-14A series gas chromatography (Shimadzu, Japan) equipped with a CBP1-Shimadzu non-polar column (20 m x 0.2 mm, 0.25 mm film thickness). Separation was carried out using helium as a carrier gas at a rate of 50 ml/min. The column was programmed from 60 ºC (1 min) to 145 ºC at 7 ºC/min, then to 190 ºC at 2 ºC/min and finally to 300 ºC at 10 ºC/min. A combination of GC and MS analysis was carried out similarly using a GC-MS-QP 5050 (Shimadzu, Japan).

Results

A combination of liquid-liquid extraction using ethyl acetate, and solid phase extraction using isolute C_{18} cartridges was used to extract and recover honey phenolics. For both honey types, the recovered fractions from each 10 g of honey yielded a dry residue of 103.8 ± 7.94 mg (1.038%) (mean ± S.D., n = 20). Thus, we assumed that every gram of honey yielded a residue of nearly 10 mg. The antibacterial activity of these phenolic fractions was measured against different bacterial strains.

To perform the broth dilution assay, five dilutions were prepared from each of the phenolic extract residues in sterile broth. They were prepared so that the addition of bacterial inoculum would give the following concentrations: 0.65, 1.3, 1.95, 2.6 and 3.2 mg/ml; these dilutions are equivalent to honey dilution series from 5% (v/v) to 25% (v/v), (the calculations were made on the basis that each gram of honey yielded an average dry extract of nearly 10 mg, and that the average weight of 1 ml of honey is 1.3 g); broth dilution assay was then performed for these dilutions. Table 1 shows the growth of *E. coli, S. aureus,* MRSA and MSSA in these preparations. The growth of all these bacterial strains was partially inhibited by the phenolic extract of gelam honey at 1.3 mg/ml, and completely inhibited at ≥1.95 mg/ml. On the other hand, the growth of all strains was not inhibited by the phenolic extract of coconut honey at a concentration of ≥1.3 mg/ml; however, the growth of *S. aureus* and MSSA was partially inhibited at a concentration of 1.95 mg/ml, but all the strains were completely inhibited at ≥ 2.6 mg/ml.

For the disc diffusion assay; discs of Whatman paper containing 0.65, 1.3, 1.95, 2.6 and 3.2 mg of phenolic extracts, which are equivalent to honey dilution series from 5% (v/v) to 25% (v/v), were assayed against the bacterial strains mentioned above. Table 2 and 3 showed that the phenolic extracts of both honeys exhibited various degree of activity against the different strains tested as indicated by the inhibition zones around the paper discs. Filter paper discs saturated with methanol (negative controls) showed no sensitivity. A linear relationship ($r > 0.9$) was obtained by plotting log

Table 1. The antibacterial activity of the ethyl acetate extracts of coconut and gelam honey assayed against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), MRSA, and MSSA using broth dilution assay. The growth of bacterial cultures(inoculum 5 x 10^5) of different strains in broth containing various concentrations of the extract, incubated at 35 ºC for 24 h. Lack of visible turbidity was considered an absence of growth. These results are the summary of five independent experiments.

Conc. (mg/ml)	Control (0.0)		0.65		1.3		1.95		2.6		3.25	
	C	G	C	G	C	G	C	G	C	G	C	G
E. coli	++	++	++	++	++	+	+	x	x	x	x	x
S. aureus	++	++	++	++	++	+	x	x	x	x	x	x
MRSA	++	++	++	++	++	+	+	x	x	x	x	x
MSSA	++	++	++	++	++	+	x	x	x	x	x	x

Key: C: Coconut; G: Gelam; ++: growth; +: partially growth; x: no growth.

Table 2. The antibacterial activity of the ethyl acetate extracts of coconut honey assayed against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), MRSA and MSSA. The activity was assayed by the disc diffusion method as described in the text, and expressed as the diameter (mm) of the inhibition zone obtained. These results are the mean of five replicates ± standard deviation (a: n = 10; b: n = 15; c: n = 20; d: n = 10; e: n = 5).

Disc Content (mg)	Control				0.65	1.3	1.95	2.6	3.25
	Am	G	PG10	M					
E. coli	21.3 ± 0.82[a]	25.2 ± 0.92[c]	nt	nil	9.6 ± 0.54	10.8 ± 0.84	13.4 ± 0.55	14 ± 0.71	16.2 ± 0.45
S. aureus	nt	27 ± 0.46[d]	nt	nil	11.6 ± 0.89	13.2 ± 0.83	15.2 ± 0.71	19.5 ± 0.44	22.4 ± 1.14
MRSA	nil	nt	nil	nil	9.8 ± 0.45	12.4 ± 0.65	15 ± 0.67	18.2 ± 0.5	22 ± 0.75
MSSA	21.8 ± 0.77[b]	25 ± 0.71[e]	nt	nil	10.1 ± 0.42	13 ± 0.79	17.2 ± 0.91	19.1 ± 0.74	21.5 ± 0.93

Key: A_m: Ampicillin µg; G: Gentamicin µg; PG: Penicillin G 10 units; M: filter paper discs saturated with methanol and dried before being applied; nil: absence of inhibition zone; nt: not tested.

Table 3. The antibacterial activity of the ethyl acetate extracts of gelam honey assayed against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), MRSA and MSSA. The activity was assayed by the disc diffusion method as described in the text, and expressed as the diameter (mm) of the inhibition zone obtained. These results are the mean of five replicates ± standard deviation (controls are same as in Table 2).

Disc Content (mg)	Control				0.65	1.3	1.95	2.6	3.25
	Am	G	PG10	M					
E. coli	21.3±0.82a	25.2 ± 0.92c	nt	nil	9.9 ± 0.41	13.1 ± 0.55	16.2 ± 0.91	18.9 ± 1.02	20 ± 0.79
S. aureus	nt	27 ± 0.46d	nt	nil	16.2 ± 0.57	18.4 ± 0.65	20.4 ± 0.42	25.1 ± 0.74	26.5 ± 0.35
MRSA	nil	nt	nil	nil	13.3 ± 0.75	17.8 ± 0.27	20 ± 0.71	25 ± 0.65	26.2 ± 0.76
MSSA	21.8 ± 0.77b	25 ± 0.71e	nt	nil	12 ± 0.93	16.9 ± 0.74	20.8 ± 0.84	21.9 ± 0.89	24.3 ± 0.57

Key: A_m: Ampicillin µg; G: Gentamicin µg; PG: Penicillin G 10 units; M: filter paper discs saturated with methanol and dried before being applied; nil: absence of inhibition zone; nt: not tested.

concentrations of the phenolic extract of both honey types versus inhibition zone diameters (Fig. 1). Both assays showed that gelam honey extract has a more pronounced activity than coconut, and *E. coli* is the most resistant strain to the activity of both honeys.

HPLC Analysis

Some commercially available phenolic compounds with antibacterial activity that had been reported to be present in honey were used for the tentative identification of the unknown peaks. These included benzoic acid, caffeic acid, cinnamic acid, ferulic acid, gallic acid, and kaempferol. A mixture was prepared containing these six standards and it was analysed via HPLC under the conditions described in the Materials and Methods. The proposed extraction procedure allowed the extraction of these phenolics with recovery values ranging from 86.7% (±6.4) to 98.2% (±0.86) as illustrated in Table 4. Honey samples of gelam and coconut types were then analysed via HPLC under the same chromatographic conditions (Fig. 2 and 3). Five phenolic acids were identified in gelam honey extract: benzoic acid, gallic acid, cinnamic acid, caffeic acid and ferulic acid. On the other hand, three phenolic acids were identified in coconut honey extract, namely benzoic acid, gallic acid and caffeic acid; however, cinnamic acid and ferulic acid were not detected. In addition, kaempferol was not detected in any of these honey samples (Table 5). Chromatographic comparisons revealed that both honey samples exhibit similar phenolic profiles, but their relative amounts are different.

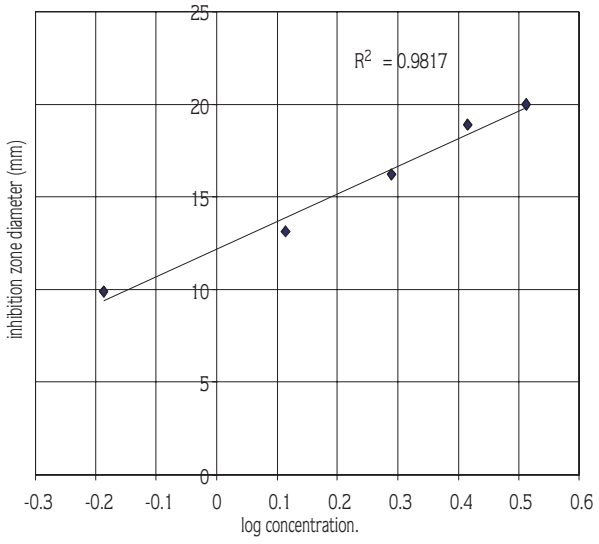

Figure 1. Typical relationship found between the concentration of honey extract and the diameter of the inhibition zone against different bacterial strains R=0.991. Each point represents the mean of five replicates.

Table 4. Recoveries of the standards added to honey after solid-phase extraction procedure.

Standard	Mean(%) ± SD
Gallic acid	95.4 ± 3.1
Caffeic acid	98.2 ± 0.86
Ferulic acid	90.5 ± 5.2
Benzoic acid	86.7 ± 6.4
Cinnamic acid	94.4 ± 3.5
Kaempferol	95.8 ± 2.8

Mean values ± standard deviations (n = 5).

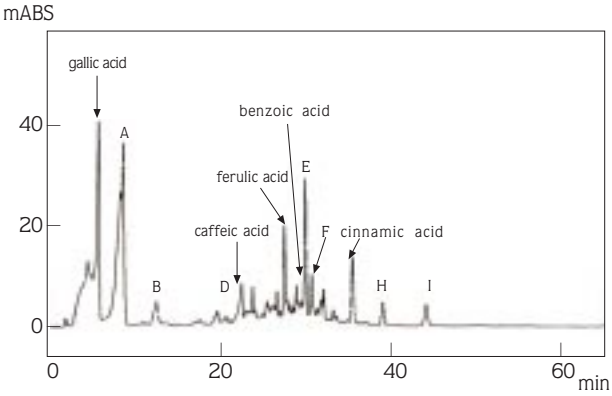

Figure 2. HPLC chromatogram of gelam honey phenolics. Detection at 280 nm; other HPLC conditions are described in the Materials and Methods.

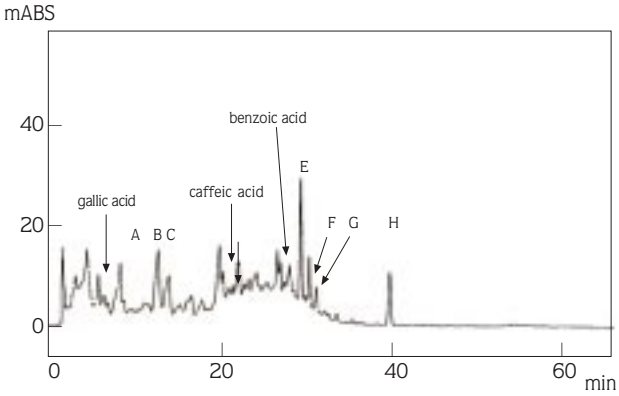

Figure 3. HPLC chromatogram of coconut honey phenolics. Detection at 280 nm; other HPLC conditions are described in the Materials and Methods.

Table 5. Phenolic compounds identified in coconut and gelam honeys using HPLC. Detection at 280 nm; other HPLC conditions are described in the Materials and Methods (mean ± standard deviations n = 5).

Compound	R.T.(min)	Quantity (µg/100g honey	
		Coconut	Gelam
Gallic acid	6.04	82.2 ± 4.92	330.4 ± 12.7
Caffeic acid	22.13	67.9 ± 6.63	69.023 ± 6.84
Ferulic acid	27.71	-	53.3 ± 4.72
Benzoic acid	28.4	184.3 ± 11.8	79.7 ± 4.31
Cinnamic acid	35.81	-	19.0 ± 2.59

Table 6. Phenolic acids identified in coconut and gelam honeys using GC & GC-MS; under conditions described in the Materials and Methods (mean ± standard deviations n = 5).

Compound	R.T.(min)	Honey	
		Coconut	Gelam
Caffeic acid	24.4	+	+
Ferulic acid	30.1	-	+
Benzoic acid	8.13	+	+
Cinnamic acid	14.22	-	+

+: detected; - : not detected.

Generally gelam honey contains higher levels than coconut as calculated from the peak areas; however, some differences were also observed. For example gallic acid, caffeic acid and benzoic acids are present in both honeys in different quantities, but cinnamic acid and ferulic acid were detected only in gelam honey. On the other hand, while unknown compounds A, B, D, E, and H were present in both honeys, unknown compounds C and G were present only in coconut honey, and unknown compound I was present only in gelam honey (Figures 2 and 3).

GC and GC-MS

After the methylation of honey extracts, they were subjected to GC analysis. Four phenolic acids were identified in the extract of gelam honey: caffeic acid, ferulic acid, cinnamic acid and benzoic acid; whereas two phenolic acids were identified in coconut honey extract: benzoic acid and caffeic acid (Table 6). These phenolic acids were identified by direct comparison with authentic standards using GC-FID and by their mass spectra using GC-MS; however, the identity of the caffeic acid was confirmed only by direct comparison with its authentic standard through GC-FID. On the other hand, neither GC-FID nor GC-MS was able to detect the presence of gallic acid in honey samples or in the authentic standard solution.

Discussion

Two Malaysian honeys were used for the first time in this study; their activities were assumed to be different from each other due to their different floral sources. The antibacterial activity of gelam honey is higher than that of

coconut honey(unpublished work). The non-peroxide antibacterial activity of honey is usually attributed to the presence of organic components originating from floral sources (1,5). A number of organic ingredients with antibacterial activity have been extracted from honey using different solvents. Most of these componetnts are phenolics (flavonoids or phenolic acids) in nature and are of plant origin (6,7,9-11). In the present study, honey phenolics were extracted using ethyl acetate (liquid-liquid extraction) and SPE. Zaghloul, et al. (16) used four organic solvents for the same purpose: n-hexane, diethyl ether, chloroform and ethyl acetate. Among all these solvents only ethyl acetate extract showed marked antibacterial activity against all the microorganisms studied; however, diethyl ether extract showed a slight antibacterial activity against *E. coli*, but it showed no activity against the other organisms tested, including *S. aureus*. These results indicated that the choice of solvent is very important for extracting honey phenolics, since different solvents give different results. In the present study, the antibacterial activity of ethyl acetate extract was assayed against *E. coli, S. aureus,* MRSA, and MSSA. As tested by the broth dilution assay, gelam extract showed complete inhibition against all strains tested at ≥1.95 mg/ml, which corresponds to 15% (v/v) honey; however, it showed partial inhibition at 1.3 mg/ml, which corresponds to 10% (v/v). On the other hand, coconut extract showed partial inhibition against *S. aureus* and MSSA at 1.95 mg/ml, and complete inhibition against all the strains at ≥2.6 mg/ml, which corresponds to 20% (v/v) honey. These results indicated that the antibacterial activity of gelam extract is higher than that of coconut extract. Therefore, the variation in the antibacterial activity of these honeys could be attributed to their phenolics, which is in agreement with the conclusion of Molan (17). Similar differences were shown by the disc diffusion assay; the antibacterial effect of gelam extract was more pronounced than that of coconut, as indicated by the diameter of the inhibition zones. This shows the involvement of the phenolic contents in the variation between the antibacterial action of different honeys. A linear relationship ($r > 0.9$) was found by plotting log concentrations of ethyl acetate extract versus the diameter of the inhibition zone. In contrast to our results, Weston, et al. (9) used the disc diffusion assay to test the antibacterial activity of diethyl ether extract from 'active' and 'inactive' manuka honeys along with honeydew honey; they found that the degree of inhibition was the same for all honeys tested. In addition, they also found that the phenolic fractions from 'active' manuka honey fractionated on Amberlite XAD-2 resin or on polyacrylamide (Biogel P-2) had no significant activity, whereas the activity was associated with those fractions containing carbohydrate. The differences between our results and theirs are due mostly to differences in the extraction procedure.

Ferreres, et al. (8) fractionated honey solution through an XAD-2 column, and they used diethyl ether to extract honey phenolics from the phenolic fraction. The same procedure was followed by Weston et al. (9); based on this, phenolic acids, which are known as antimicrobial agents (15), seem to be eluted with the sugar fractions during the fractionation of honey on the XAD-2 column, as Ferreres et al. (8) mentioned that sugars and polar compounds were washed with water. In addition, by using diethyl ether, they aimed to eliminate the non-flavonoid phenolic compounds which contaminated the flavonoids peaks; thus the main diethyl ether extract contents were flavonoids. These observations could explain the results shown by Weston et al. (9), who found the antibacterial activity of honey was eluted with the carbohydrate fractions, whereas the phenolic fractions had no significant activity. They also suggested that an antibacterial substance is carried by the monosaccharides which form the bulk of the honey's mass.

Honey ethyl acetate extract was shown to be much more effective in terms of antimicrobial activity than diethyl ether extract (16). Accordingly, an extraction procedure combining the advantages of ethyl acetate and the solid phase extraction technique was developed in this study for the extraction and recovery of honey phenolics (see Materials and Methods). Ethyl acetate was used successfully for the extraction of hazelnut phenolics (18). On the other hand, solid phase extraction was used extensively for the recovery of phenolics from different fruits and food materials (13,19). A similar technique was used by Suarez-Luque et al. (20) to extract organic acids such as malic, citric and succinic acids from honey.

Solid phase extraction is a simple technique using inexpensive disposable extraction columns, and provides many advantages such as reduction of solvent consumption and high recoveries of the analytes. When this modified procedure was applied to honey samples the recovery was within the range 86.7% (benzoic acid) to 98.2% (caffeic acid), indicating the suitability of this procedure for the recovery of honey phenolics.

HPLC, which is the method of choice for food phenolic analysis, was used in this study for the identification of honey phenolics. The eluted compounds were detected at 280 nm because most of the phenolic compounds show reasonably high absorbance at this value (13). A variety of phenolic acids were tentatively identified in gelam and coconut honey. Their identities were confirmed by direct comparison with authentic standards on the basis of their retention times, which were remarkably stable and reproducible, although some other compounds were not identified. The phenolic acids that were detected in gelam honey include gallic, caffeic, ferulic, benzoic and cinnamic acids; whereas only gallic, caffeic and benzoic acids were detected in coconut honey. Kaempferol was not detected in either honey, presumably due to its low concentration. With the exception of gallic acid, which is rarely reported in honey, the other phenolic acids have been identified in honey (9-11). The levels of benzoic acid and cinnamic acid reported in this study are higher than those reported by Weston et al. (9), probably due to the extraction procedure. Although there is a similarity between the HPLC chromatograms of both honeys tested, quantitative and qualitative differences were also shown. In general, the levels of most identified and unidentified compounds are higher in gelam than coconut honey as determined from their peak areas; however, some compounds are present in both honeys while others are present in only one. The higher proportion of phenolic compounds detected in gelam honey could explain its more potent antibacterial activity. GC-FID analysis confirmed the results obtained from HPLC analysis: caffeic, ferulic, benzoic and cinnamic acids were detected in gelam honey extracts, whereas caffeic and benzoic acids were detected in coconut honey. Combined GC-MS analysis also provides the mass spectra for ferulic, benzoic and cinnamic acids; however, the identity of caffeic acid was confirmed by direct comparison with an authentic standard. On the other hand, neither GC-FID nor GC-MS was able to detect the presence of gallic acid in honey samples or even that present in the authentic standard solution, presumably due to methylation defects or to other technical reasons.

The antibacterial activities of the phenolic acids detected in this study have been reported previously (15,21). Therefore, honey phenolics seem to contribute individually or collectively to the antibacterial activity of honey.

In conclusion, the present study established the antibacterial activity of Malaysian gelam and coconut *Apis mellifera* honeys against standard and pathogenic bacterial strains, including MRSA. The activity of honey extract against MRSA is of great importance since these strains cause major problems in hospitals. Honey phenolics are partially responsible for the variation in the antibacterial activity of the honeys tested. A better procedure for the recovery of antibacterial phenolics was also developed. Using that procedure some of the phenolic acids responsible for part of the antibacterial activity have been identified; however, further work needs to be done to identify the rest of the components present. Honey can be recommended as an alternative treatment for infected wounds or ulcers, especially those that are caused by antibiotic- resistant bacteria.

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