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Research Article

Antibacterial and Phenolic Content of Propolis Produced by Two Malaysian Stingless Bees, *Heterotrigona itama* and *Geniotrigona thoracica*

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ABSTRACT
Propolis is a sticky material collected by the bees to protect their hive. It has been used for medicinal purposes since ancient time. The methanol extract of Malaysian propolis produced by two commonly found stingless bees, *Heterotrigona itama* (MHI) and *Geniotrigona thoracica* (MGT) inhibited the growth of *Staphylococcus aureus* better than Gram-negative (*Escherichia coli* and *Salmonella typhi*) bacteria. However, the minimum inhibitory concentration (MIC) values showed the most effective propolis was MHI (5 mg/mL) for Gram-positive and 10 mg/mL for Gram-negative compared to MGT has showed less activity against all tested bacteria. Chemical fingerprint was derived from thin layer chromatography analysis. Both extract displayed a characteristic profile and vary from each other. Analysis of Fourier transform Infrared (FT-IR) spectra of both extract tested show similar IR spectral profiles but from the intensities of both extract have significant differences. The results indicate that the differences are caused by group of phenolic and flavonoid. Total phenolic and flavonoid content of MHI of 56.9 ± 0.12 ug/mL and 163.9 ± 0.10 µg/mL, respectively was higher than MGT of 29.1 ± 0.10 ug/mL and 61.5 ± 0.15 µg/mL, respectively. These results showed that the antibacterial activity and chemical analysis of propolis do vary according to its species of stingless bees.

Keyword: Antibacterial, thin layer chromatography, FT-IR, phenolic content, flavonoid

INTRODUCTION

Propolis often referred as bee glue, is a sticky material collected by bees and used to protect their hive from adverse weather conditions or invaders by sealing internal walls, holes and cracks of the beehive in order to prevent hive infections. Chemical composition of propolis is very complex. More than 200 compounds have been identified in many country such as Brazilian[1,2], Europe[3] and Canary Islands[4]. Due to the diversity of its chemical composition, propolis exerts numerous pharmacological activities such as antioxidant, antibacterial, anticancer, antifungal, anti-inflammatory, antiviral, antidiabetic and anticancer activities[5]. It has been observed that geographical locations where the source plants might vary with respect to the local flora at the site collection and seasons[6] affects chemical composition of propolis. Also, the bee species equally contribute to the diversity of chemical content, owing to their flora preferences. Two common stingless bee (locally known as kelulut) species, *Heterotrigona itama* and *Geniotrigona thoracica* are the main polinators in this region. Stingless bee is not popular as it produces only small amount of honey, but it produces propolis in a higher quantity than other bees. Their propolis and honey somehow different from honey bees. It is believe that propolis produced by stingless bees is more potent than that of honey bees. In Malaysia, research on stingless bee propolis are scarce. In this study, we analysed antibacterial activity and chemical analysis of propolis produced by these two species of commonly found stingless bees were compared.

MATERIALS AND METHODS

Sample Collection and Extraction

Propolis that was produced by stingless bees *Heterotrigona itama* and *Geniotrigona thoracica* was collected from AGROPOLIS Apiary, Agriculture Production and Food Innovation Research Institute (AGROPOLIS), Universiti Sultan Zainal Abidin, Tembila Campus, Besut. Each sample was cleaned and froze in -20°C, then ground to powder. Seven grams crude propolis was macerated with 70 mL methanol at least 3 days. The extracts were filtered and dried under pressure using rotary evaporator. Crude propolis and extracts were kept in -20 °C until further analysis. Propolis produced by *H. itama* coded as MHI and propolis produced by *G. thoracica* coded as MGT.

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Bacterial Strains

Gram-positive bacteria such as *Staphylococcus aureus* ATCC 33591, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212 and *Listeria monocytogen* ATCC 7644 and for Gram-negative bacteria such as *Acinetobacter baumannii* ATCC 19606, *Salmonella typhi* ATCC 14028 and *Escherichia coli* ATCC 35218. All microorganisms were provided by Microbiology Laboratory, Universiti Sultan Zainal Abidin, Tembila Campus, Besut.

Antibacterial Activity

Antibacterial activity of the MHI and MGT propolis extracts was determined by the disc diffusion method with a modification[7]. All the bacterial test strains maintained on nutrient agar were freshly subcultured for 24-48 hours at 37ºC. Saline suspension of each test strain was prepared and turbidity matched to 0.5 McFarland standards to yield a bacterial suspension of 1.5 x 108 cfu/mL. Freshly prepared Mueller Hinton Agars (MHA) plates were seeded with the test inoculums to obtain a lawn culture. Sterile Whatmann No. 1 filter paper discs (5mm diameter) were loaded with 50 µL of propolis extract dilutions Discs were dried for 5 hours at 37ºC in a sterile incubator and then placed on seeded agar plates. Five percent (5 %) DMSO served as negative control, Ampicillin (10 µg) and Tetracycline (30 µg) were used as standard antibiotics. Plates were incubated at 37 ^{0}C for 24 hours and inhibitory zone diameters were measured around the disc. MIC endpoints were also read as the lowest concentration of propolis that resulted in no visible growth on the surface of the culture medium.

Thin layer Chromatography

Accurately weighed 50 mg each of extract and dissolved in 1 mL of HPLC grade methanol and sonicated for 5 min to ensure complete dissolution. TLC analyses of MHI and MGT were performed on 20 cm x 10 cm silica gel aluminium plate $60F_{254}$ (Merck, Darmstadt. Germany). Two microliters of each extract were deposited and developed of hexane: ethyl acetate: formic acid, 7:10:0.1 (v/v/v), as mobile phase, in a glass twin-trough chamber previously saturated with mobile phase vapour for 20 min. After development, the plates were dried with a hair dryer and then visualized under UV 254, 366 and after derivatisation in vanillin-sulphuric acid and anisaldehyde spray reagent. R_f and color of the spots were recorded.

Fourier Transform-Infra Red Analysis

Two milligrams of propolis was prepared for each sample and was put in Fourier Transform-Infra Red Analysis (FT-IR) sample holder. Propylene glycol spectrum was used as a background. Sample reading was performed in 32 scans at a resolution of 4 cm^{-1}. For each sample, analysis was performed in eight replications within the region 400-4000 cm^{-1}. FT-IR spectra are smoothed and normalized with Spectrum Version 3.02 (Perkin-Elmer Inc.).

Determination of Total Phenolic Compounds

Phenolic compounds from propolis samples were estimated by a modified spectrophotometric Folin-Ciocalteu method[8]. Briefly, 200 µL of propolis extract were mixed with 1 mL Folin and Ciocalteu's phenol reagent. After 3 min, 1 mL of 10 % Na_2CO_3 solution was added to the mixture and adjusted to 10 mL with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm by a spectrophotometer (UV-vis mini 1240 Shimadzu Co.). Gallic acid was used to calculate the standard curve (12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 µg/mL, r^2 = 0.997). Estimation of the phenolic compounds was carried out in triplicate. The results were reported as the mean ± standard deviations and expressed as micrograms of gallic acid equivalents (GAEs) per mL propolis.

Determination of Total Flavonoids Compounds

Total flavonoid content (TFC) of MHI and MGT propolis was determined according to the colorimetric assay methods[9]. Propolis extract (200 µL) was mixed with 4 mL of distilled water. At baseline, 0.3 mL of $NaNO_2$ (5 %, w/v) was added. After 5 min, 0.3 mL $AICI_3$ (10 % w/v) was added, followed by the addition of 2 mL of NaOH (1 M) 6 min later. Immediately after that, the volume was increased to 10 mL by the addition of 2.4 mL distilled water. The mixture was vigorously shaken to ensure adequate mixing, and the absorbance was read at 510 nm. A calibration curve was prepared using a standard solution of catechin (12.5, 25.0, 50.0, 100.0, 200.0 and 400.0 µg/mL, r^2 = 0.997). The results were also expressed as micrograms of catechin acid equivalents (CEQ) per mililitre propolis.

Statistical Analysis

Triplicate determinations, mean and standard deviation were calculated. Calibration curve of standard was obtained for concentration versus absorbance.

RESULTS AND DISCUSSION

Antibacterial Activity

It is well observed that stingless bees produced propolis to seal their hive and to prevent the decomposition of creatures which have been killed by stingless bees after invasion of the hive. Studies have shown that propolis produced by *A. mellifera* possess antimicrobial potential[10] as well as propolis produced by *Melipona scutellaris*[11]. However, antimicrobial activities of other types of propolis especially from Malaysia have been sparsely studied. In this study, antibacterial activity of propolis produced by two commonly found stingless bees, *Heterotrigona itama* and *Geniotrigona thoracica* were analysed against seven strains of organism using disc diffusion method by three different concentrations. As presented in Table 1, both MHI and MGT propolis showed inhibition zone against all tested strains. In comparison to the inhibition of both propolis, MHI shown better inhibition (6-14 mm) compared to that of MGT extract (inhibition zone 6-7 mm). Among the bacteria strain studied, both propolis inhibited the growth of *Staphylococcus aureus* better than Gram-negative (*Escherichia coli and Salmonella typhi*) and observed that the antibacterial activity is mainly due to polar phenolic compounds[12]. The minimum inhibitory concentration (MIC) values ranged from 5 mg/mL to 40 mg/mL (Table 2) and were showed that Gram-negative bacteria were less suspectible to lower MIC than Gram-positive strains. The MIC values of the most effective propolis (MHI) were 5 mg/mL for *Staphylococcus aureus*, *Listeria monocytogen* and *Enterococcus faecalis* and 10

Table 1: Antibacterial activities of MHI and MGT propolis, ampicillin, tetracycline and 5% DMSO against various indicator bacteria by disc diffusion method

Bacteria	MHI (mg/disc)			MGT (mg/disc)			Amp. (µg/ disc)	Tetra. (µg/ disc)	DMSO 5%
	20	15	10	20	15	10	10	30	
Gram-positive bacteria									
Staphylococcus aureus	14	12	10	7	-	-	20	22	-
Bacillus subtilis	11	9	8	6	-	-	20	22	-
Enterococcus faecalis	10	10	9	6	-	-	20	28	-
Listeria monocytogen	11	11	10	6	-	-	21	24	-
Gram-negative bacteria									
Acinetobacter baumannii	10	8	7	6	-	-	-	21	-
Salmonella typhi	10	8	6	-	-	-	12	20	-
Escherichia coli	11	7	6	-	-	-	-	17	-

*No inhibition zone detected. Values expressed are averages of three replicates

Table 2: Minimal Inhibitory Concentration (MIC) of MHI and MGT propolis extracts

Bacteria	MHI (mg/mL)	MGT (mg/mL)
Staphylococcus aureus	5	20
Bacillus subtilis	10	20
Enterococcus faecalis	5	20
Listeria monocytogen	5	20
Acinetobacter baumannii	10	20
Salmonella typhi	10	>40
Escherichia coli	10	>40

Values expressed are averages of three replicates

mg/mL for *Bacillus subtilis*, *Acinetobacter baumannii*, *Salmonella typhi* and *Escherichia coli*. Propolis of MGT has showed less activity against all tested bacteria. The MIC values of MGT were 20 mg/mL for all gram-positive bacteria and for all gram-negative bacteria were inhibited at concentration higher than 40 mg/mL. Many studies have shown that fatty acid esters, phenolic compounds and cinnamic acid were the main propolis constituents and some of them were shown to possess antibacterial activity[13]. Propolis collected from MGT hives showed a weaker antibacterial activity than MHI. The greater activity of the MHI propolis sample on bacteria than MGT samples may be attributed to its different chemical composition and concentrations of compounds. The experiments revealed that there could be minor differences in antibacterial activity of propolis extracts depending on the different species of stingless bees.

Thin Layer Chromatography

Thin-layer chromatography (TLC) is a chromatography technique used to separate non-volatile mixtures[14]. TLC methods were done for the separation of compound in the MHI and MGT extracts. The mobile phase used was Toluene/ ethyl acetate/ formic acid with ratio 8: 2: 0.1 (v/v). Based on the Figure 1, it was shown that the compound was visible under short wave and long wave of UV light. The TLC plates normally contain fluorescent indicator which makes them glow under 254 nm visualization of UV light. The plate also visualized by the

Table 3: Total flavonoid and phenolic content of the MHI and MGT propolis extracts

Chemical content	MHI	MGT
Total phenolic content, ug.mL^{-1}	56.9 ± 0.12[1]	29.1 ± 0.10
Total flavonoid, ug.mL^{-1}	163.9 ± 0.10	61.5 ± 0.15

[1]Each value is the average of three analyses ± standard deviation

derivative by naked eye (Figure 1). There were two reagent used during this step which were anisaldehyde and also vanillin-sulphuric acid. There are six major spots occur in MHI, at R_f of 0.12, 0.30, 0.55, 0.72, 0.91 and 0.96 visualized under UV 254 nm. Whilst, only five major spots occur in MGT at of R_f 0.35, 0.45, 0.50, 0.60 and 0.70, visualized under the same wavelength. Derivatisation with 5 % vanillin-sulphuric acid and anisaldehyde, revealed complex mixture of compounds which exhibit different colored reactions. There were some differences as well as similarities between to samples. From the Figure 1(c), MHI revealed three dark purple violet with R_f value at 0.1, 0.4 and 0.7 which were also observed in MGT. Taganna et al. (2011)[15] stated that the classes of compounds in the extracts include terpenoids (purple or bluish purple). It is interesting to note that the dominant dark red purplish band (R_f 0.55) of terpenoid or polyphenols[16] was only present in MHI. Phenolic compounds such as flavonoid (yellow, pinkish or orange), stibenes (bright red to dark pink color) were appeared after derivatisation with vanillin-sulphuric acid[17] at R_f 0.48 for both extract and second spot (R_f 0.31) was only detected in MHI and the third one (R_f 0.91) only presence in MGT. From the Figure 1(d), both samples turn purple upon derivatisation with anisaldehyde-sulphuric acid reagent. Overall, from TLC analysis, the chemical composition and chemical fingerprint of propolis produced by H. itama are more complex compare to propolis produce by *G. thoracica.*

FT-IR Analysis of MHI and MGT

Propolis is a complex resinous mixture which contains approximately 50 % resin that composed of flavonoids and related to phenolic acids regarded as the polyphenolic compounds, 30 % wax, 10 % essential oils, 5 % pollen and 5 % various organic compounds[18]. The chemical composition of propolis reportedly depends on the

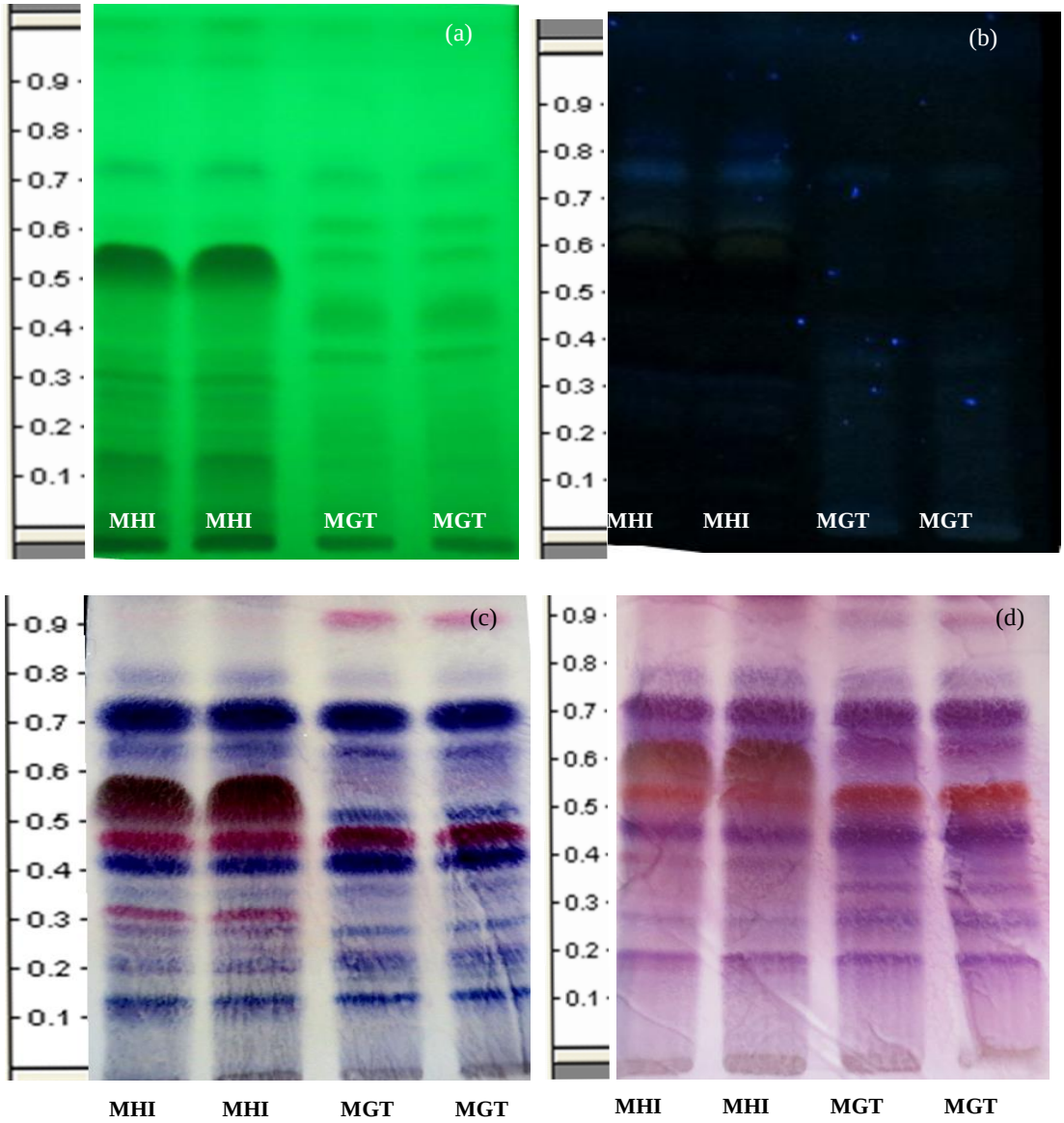

Figure 1: TLC photodocumentation of methanolic extract HI and GT propolis by visualization (a) UV 254 nm (b) UV 366 nm (c) Post derivatisation vanillin-sulphuric acid (d) Post derivatisation anisaldehyde

specificity of the local flora at the site collection and also the stingless bee species. FT-IR was chosen to record the chemical composition of each propolis samples for its simplicity and ability to provide fingerprints information of the measured samples. The FT-IR spectra of the representative MHI and MGT samples are shown in Figure 2. Since MHI and MGT are both complex systems, the broad absorption bands in their FT-IR spectra represent the substantial overlap of each absorption bands of various components with different contents. The preliminary assignments of the spectrum of MHI and MGT are showed the existence of phenolic compounds or its ester (O-H at 3307-3368 cm^{-1} and C-O at 2879 cm^{-1}). The presence of flavonoids was also indicated (C=O at 1651-1659 cm^{-1} and 2972, 2933, 2879 cm^{-1}, C-H, C-H aromatics and O-H at 3500-3200 cm^{-1}, C-O at 1320-1000 cm^{-1} and C=C aromatics at 1500-1600 cm^{-1}). The presence of amino acids and amino acids aromatic were shown by the absorbance of symmetric N-O and C-N at 1334-1290 cm-1, N-H at 3400-3250 cm-1. While the absorbance of C-O and O-H at 1200-1000 cm-1 indicated the presence of fatty acids, stilbenes, steroids and carboxylic acids[19]. The high similarity in the FT-IR spectra of MHI and MGT shows the very similar chemical compositions in the two natural mixtures. However, a slight difference was observed in the spectra of MHI and MGT. In the range from 1320 cm^{-1} to 1000 cm^{-1} of the two spectra, there are two sharp peaks (C-O) and (O-H) groups of flavonoids and alcohol[20]. Since the absorption bands of MHI are more intense than that of MGT, there is a larger amount of flavonoids compounds in MHI.

Total phenolic and flavonoid content

Total phenolic content of MHI and MGT were summarized in Table 4. The obtained results confirmed that the MHI and MGT propolis contains considerable amounts of phenolic compounds. Gallic acid was used as the positive control. As shown in Table 4, amount of phenolic content were found to be low in MGT (29.1 ± 0.10 µg GAE/mL)

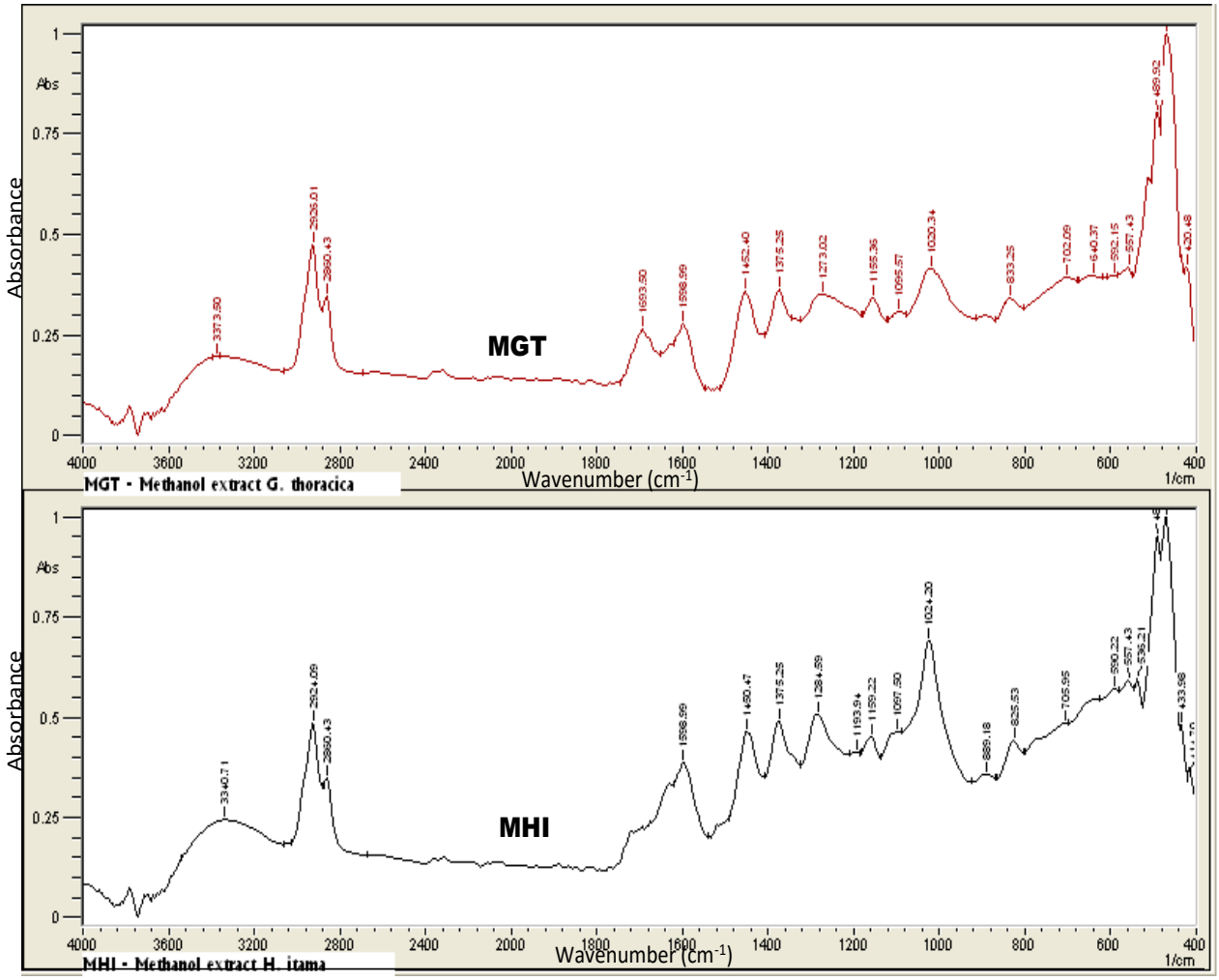

Figure 2: FT-IR spectra of the representative MHI and MGT propolis extracts

when compared to the total phenolic content, which was higher in MHI (56.9 ± 0.12 µg GAE/mL) propolis samples. Similarly, MHI propolis (163.9 ± 0.10) possesses large quantities of flavonoids compared to MGT propolis (61.5 ± 0.15) as seen in Table 3. The total flavonoid contents of MHI and MGT were expressed in catechin equivalents. It is clearly understood that phenolic compounds and flavonoids are chief components of propolis and the account for antioxidant properties. Propolis contains a wide variety of phenolic compounds, mainly flavonoids. Variation in the flavonoid content of propolis is mainly attributable to the difference in the preferred regional plants collected by stingless bees. Contents of flavonoid and other phenolic substances have been suggested to play a preventive role in the development of cancer and heart disease[21].

CONCLUSION

From this study, it shows that bee species play role in determining the chemical content and biological activity. This is the first report on the antibacterial activity of Malaysian propolis, particularly produced by two commonly found stingless bees, *H. itama* and *G. thoracica*. The data presented in this study will shed some light on the potential of Malaysian propolis.

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