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Short communication

Application of GCMS and FTIR Fingerprinting in Discriminating Two Species of Malaysian Stingless Bees Propolis

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Abstract

Propolis is a sticky substance made up of mixture of plant resin, wax and bee saliva. Propolis has been used in various health related problems as well as cosmetic ingredient. This work was evaluating chemical fingerprint of Malaysian stingless bee propolis produced by two species, *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT). Chemical fingerprint derived from fingerprint region (FR) of ATR-FTIR dataset of two types of extract, methanol (MHI, MGT) and ethanol (EHI, EGT). Principal component analysis and hierarchical cluster analysis was employed to discriminate the two species. The total variances account for FR was 95.63 % (PC1 = 54.01 %, PC2 = 41.61 %). EHI and EGT clustered closely between them and separately from MHI and MGT. HCA conform the grouping by PCA. GC-MS analysis revealed the presence of 20 different phytochemicals in methanol, while ethanol extract shown only 17 different phytochemicals of GT propolis. Meanwhile, 24 different phytochemicals were identified in methanol extract and 13 compounds presence in ethanol extract from propolis samples collected by HI species. Based on the good separation observed in both spectroscopic and chromatographic data, it seems reasonable to use chemical fingerprint coupled with chemometric analysis to differentiate two different species of stingless bee propolis. The findings from this study can be used as identification, classification and quality control methods for the profiling of propolis

Keywords: *Chemical fingerprinting; stingless bees;FTIR; GCMS; PCA; HCA.*

1. Introduction

Malaysia hosts a great number and diverse species of stingless bees which consists of ~33 species [1] that known to be important pollinator in tropical rainforest [2] and also good candidates for providing pollination services in agricultural ecosystem. Stingless bees belong to the Apidae family. Their size ranges from 2mm, and not more than 5mm and they have no stinger [3]. They are reared for harvesting small quantities of highly prized honey, wax and propolis. Product of stingless bees are highly medicinal because they collect nectar and pollen selectively from medicinally important plants and flowers such as coco palm, banana, guava, papaya, jack fruit tree etc., Since stingless bees do not have sting and lacks defence organs, it protects the hive with wax like substance and seals the minute pores of the hive using propolis [4] which it creates on its own by mixing its own body secretion from the salivary glands with the resins collected from the leaves, trees, plants, buds etc.

Malaysian's stingless bee propolis research still at its infancy. With more than 30 species identified, only two species are commercially reared for various products such as honey, propolis and beebread (bee pollen). Those species are *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT). These two species are markedly different from each other. *G. thoracica* have bigger size and be able to travel higher and farther to collect honey and propolis. Meanwhile, *H. itama*, with smaller size, prefer understorey plants. From our observation, *H. itama* collect more honey compare to that of *G. thoracica* and kept in smaller pots (unpublished data). We hypothesized that these two species visit different plants for their honey and propolis. Thus, the chemical composition of their products must be different. We are particularly interested in propolis. Studies from other part of the world discovered that the composition of propolis is highly complex and varies depending on the geographical origin and the collection season [5]. More than 300 chemical compounds have been identified so far from different propolis samples, including flavonoids, aromatic acids, esters, aldehydes, ketones, fatty acids, terpenes, steroids, amino acids, polysaccharides, hydrocarbons, alcohols, hy-droxybenzene, and several other compounds in trace amounts [6, 7]. Propolis also exhibits excellent therapeutic properties such as antimicrobial, antifungal, antiviral and antiparasitic [8, 9, 10], which varies depending on the geographical natural source from where it comes (phytogeography) [11].

Propolis is a waxy substance where extraction can be daunting. In this study, we compared chemical fingerprints of two types of propolis extracts from HI and GT. Chemical fingerprints were derived from GC-MS and FTIR analysis. The analysis alone is insufficient to discriminate the samples. Chemometric method was employed against FTIR data in order to discriminate the propolis sample of different type of extraction from different species.

2. Methodology

2.1. Preparation of Propolis Extract

The raw propolis from two species of stingless bee, *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT) were collected from University's apiary (Besut Campus). Each sample was cleaned, packed in sealed plastic bags and froze in -20 °C. Propolis was then crushes into small pieces and ground to powder. About 30 g of each propolis sample were weighted and macerated in 100 mL of solvent for three days. Two type of solvents were used, methanol and ethanol. After three days, the extract was filtered using Whatman No. 1 filter paper and evaporated using rotavapor under vacuum pressure. The sticky semi-solid crude ethanolic extract of propolis was kept cool in -20 °C prior analysis.

2.2. Fourier Transform Infra-Attenuated Total Reflectance

In this study, FTIR analysis of propolis extracts was carried out using IRPrestige-21 Shimadzu Fourier Transform Infrared Spectrophotometer (Tokyo, Japan) equipped with air-cooled ceramic infrared light source and DLATGS (Deuterated Triglycine Sulfate doped with L-Alanine detector). Two milligrams of propolis extracts of each sample was applied directly on the diamond and tightened with FTIR pressure clamp described by Azemin *et al*. [12] with minor modifications. Propylene glycol spectrum was used as a background. Sample reading was performed in 32 scans at a resolution of 4 cm^{-1}. The analysis for each sample was recorded at the region of 4000-400 cm^{-1}.

2.3. Chemometric Analysis

Chemometrics analysis applied in this study consisted of two tools of unsupervised pattern recognition for multivariate analysis, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA). FTIR spectra were smoothed and normalized with IR Solution version 1.40 software (2002-2007 Shimadzu Corporation, Japan). The spectra were exported as a Spectrum.TXT file, and then transferred to Microsoft Excel 2007. PCA and HCA were analyzed using XLSTAT Pro 2014 (Addinsoft, Paris, France).

2.4. Gas Chromatography and Mass Spectrophotometer (GC-MS)

GC-MS analysis was carried out using Agilent 19091S-433 HP-5MS capillary column (30m x 0.25mm inner diameter, 0.25 µm film thickness) to identify the major compounds. Helium was used as the carrier gas. Each of samples extract propolis (10 mg) were dissolved in 1 mL of methanol and sonicated for 30 minutes. All samples were then centrifuged and supernatant were transferred into new vials. The oven temperature was set for initial temperature at 70 °C (2 min hold) to 280 °C (20 min hold). A split mode of front inlet was used with split ratio 1:1 and the flow rate of helium gas (40 cm/sec) was 1.2 mL/min. Mass Selective Detector (MSD) Transfer Line Heater was 285 °C. Injector temperature was 280 °C and 1 µL of extracts was injected.

3. Results

3.1. Fingerprinting from FTIR analysis

3.1.1. Assignments and Comparison of the ATR-FTIR data

To understand the nature of biomolecules that responsible for the pharmacological activities present in the sample, Fourier infrared spectra of two species stingless bee (*Heterotrigona itama* and *Geniotrigona thoracica*) and extracts type (methanol and ethanol) of propolis sample, were collected by ATR-FTIR using Shimadzu FTIR spectrophotometer in the wavelength range from 4000 – 400 cm^{-1}. The typical FTIR spectra for the methanol and ethanol extracts of two species stingless bee propolis sample were formed to identify the main functional groups are shown in Fig. 1. Through the overall observations, the spectra of all samples appear to be very similar. Within the species and type of extracts, the number of peaks was generally more or less similar as well as for their intensity of the peaks (Fig. 1). However, in spite of the similarity pattern of the peaks, there were some peaks whose presence and/or absence as well as the intensity varied greatly within species and type of extracts, which the most significant peaks can be viewed in all spectrums. It was found that different species and also type of extracts exhibited different fingerprints. The assignments of FTIR spectrum of all samples were shown in Table 1.

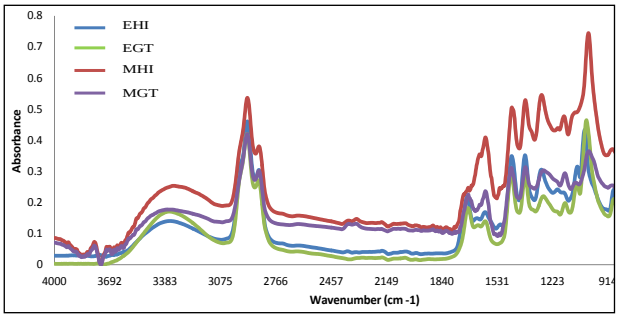

Fig. 1: Overlay of FT-IR spectra of *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT) in methanol and ethanol extracts.

Table 1: The preliminary assignments of FTIR spectrum of propolis extract based on the main signals for phenols and flavonoids.

Frequency, cm-1	Type of Signal	Type of Link	Main Attribution
3335	Elongation	O-H and N-H	Hydroxyls and Aminos
2917	Elongation asymmetric	C-H	Groups CH2 saturation
2849	Elongation symmetrical	C-H	Hydrocarbons
1699	Asymmetric bending vibration	C=O	Lipids, Flavonoids and Amino acids
1661	Stretching vibration (C-O) and bending (C-OH)	C-O and C-OH	Lipids and tertiary alcohols
1513	Elongation	Aromatics	Flavonoids and aromatic rings
1450	Bending vibration	C-H of CH2 and CH3 , Aromatics	Flavonoids and aromatic rings
1369	Bending vibration	C-H	CH3 group of flavonoids
1269	Bending vibration (O-H) and asymmetrically bending (C-CO)	O-H and C-CO	Hydrocarbons
1088	Stretching vibration (C-C) and bending (C-OH)	C-C and C-OH	Flavonoids and secondary alcohol groups
1043	Elongation (C-C) and bending (C-OH)	=C-O-C, C-C and C-OH	Primary alcohol groups
881	Symmetric stretching	C-C-O	Primary and Secondary alcohols

3.1.2. FTIR spectra of HI and GT Propolis in methanol and ethanol extracts

Ethanol extract

FTIR peaks are attributed to stretching and bending vibrations that characterize the functional groups. As shown in the FTIR spectra of propolis samples from ethanol extract by different species (Fig.

1, there were twelve major peaks existing and it was similar for both species (EHI and EGT). The most stable peaks in the spectrum are: the peaks at 3550-3200 cm^{-1} are assigned to intermolecular hydrogen bonds, the peaks at 2930-2850 cm^{-1} are assigned to the peak at approximately 2917 cm^{-1} is the asymmetric stretching of methylene, and the peak at approximately 2849 cm^{-1} assigned to the symmetric stretching of hydrocarbon [13][14] The peaks at 1750-1735 cm^{-1} are assigned to the C=O stretching vibration of lipids and flavonoids, peaks at 1660-1610 cm^{-1} are assigned to C=O, C=C stretching vibration of flavonoids and also assigned to the N-H asymmetric stretching of amino acids. In addition, there is high correspondence of the signals of the sample analysed with those literature [13] for phenols and flavonoids; this can be considered as a strong indicator of the presence of both types of compounds in the extract, which can be explained with the main characteristics signals for them are stretching vibration and bending at 1450 cm^{-1}, of vibration and bending at 1369 cm^{-1} and of vibration and bending at 1088 cm^{-1}. Peak at 1269 cm^{-1} attributed to asymmetrical O-H and C-CO bending of hydrocarbon. The peaks at 1043 cm^{-1} and 880 cm^{-1} are related to primary and secondary alcohols, and specifically ethanol shows a symmetrical stretching at 881-880 cm^{-1}, which obeys to the fact that the extracts are dissolved into ethanol.

In addition, there were minor peak absorption which is may not be seen clearly around 1513 cm^{-1} that was attributed to stretching vibration of flavonoids and aromatic ring and was only occur in sample of propolis EHI. Since the absorption bands of EHI at 1450 cm^{-1}, 1369 cm^{-1} and 1269 cm^{-1} were more intense than that of EGT, there is a larger amount of phenol and flavonoid compounds (as cited in Cai et al. [14]). Meanwhile, the intense peak near to 1161 cm^{-1} was assigned to the stretching vibration of lipid and bending of tertiary alcohol groups is the most stable peak presence only in sample of EGT. Meanwhile, Shang *et al*. [15] stated that the intensity of the 1160 cm^{-1} band (C-O-C vibration in cellulose and hemicellulose) was higher, which may indicate an increase in the mean degree of polymerization of polycarbohydrates. In addition, according to Nancy et al. (2013), the region peaks at 1659-1651 cm^{-1} attributed of C=C and at 1320-1000 cm^{-1} attributed for C-O from weak absorbance are presence of terpenes compounds and it was shown that the weak absorbance were presences on propolis HI at peak approximately 1637 cm^{-1} and 1161 cm^{-1}.

Methanol extract

In contrast, there were ten similar peaks were observed that stable in the spectra of the different species of methanol extracts, MHI and MGT (Fig. 1). As similar to ethanol extract, the peaks at 3550-3200 cm^{-1} are assigned to intermolecular hydrogen bonds and peaks at 2930-2850 cm^{-1} are assigned to the peak at approximately 2917 cm-1 is the asymmetric stretching of methylene (CH2). According to Kothai & Jayanthi [17], the peaks approximately at 3379 cm^{-1}, 3412 cm^{-1}, 3423 cm^{-1} corresponds to hydrogen bonded O-H, N-H, C-H stretching vibrations of alcohols, phenols, amides and alkanes. Meanwhile, the peak at approximately 2849 cm^{-1} assigned to the symmetric stretching of hydrocarbon (CH) of aromatic rings, methylene, methyl and O-H groups of acids. Meanwhile, the peaks at 1660-1610 cm^{-1} are assigned to C=O, C=C stretching vibration of flavonoids and also assigned to the N-H asymmetric stretching of amino acids. The presences of phenols and flavonoids compounds at peaks 1450 cm^{-1}, 1369 cm^{-1} and 1088 cm^{-1} were attributed to vibration and bending. In addition, peak at 1269 cm^{-1} attributed to asymmetrical O-H and C-CO bending of hydrocarbon and the peak near to 1161 cm-1 was assigned to the stretching vibration of lipid and bending of tertiary alcohol groups. Similar to ethanol extract, the peaks at 880 cm^{-1} are related to primary and secondary alcohols, and specifically methanol shows a symmetrical stretching at 881-880 cm^{-1}. The region of 1750-1735 cm^{-1} which are assigned to the C=O stretching vibration of lipids and flavonoids were absence on propolis MHI but only presence in propolis MGT. However, the intense peak approximately 1088 cm^{-1} was observed contain a higher amount concentration of phenols and flavonoids on propolis HI which assigned to the vibration and bending for two compounds according to Yan-Wen et al. [13]. The occurring of peak around 881-880 cm^{-1} (related to primary and secondary alcohols) was observed as more intense in propolis MHI and less intense in propolis MGT.

Thus, the significant difference between the ethanol and methanol extracts was the absence of the regions at 1750-1735 cm^{-1} for species HI only in methanol extract which are assigned to the C=O stretching vibration of lipids and flavonoids. Besides, the occurring of peak approximately at 1043 cm^{-1} (C-O stretch in primary alcohol) was observed as strong absorption in ethanol extract (more intense) and weak absorption in methanol extract. The absorption at regions of 3000 cm^{-1} to 2800 cm^{-1} (groups of saturation hydrocarbon C-H) of HI species from both extract are more intense than that of GT, so there is a larger amount of long-chain alkyl compounds in HI species at this region. From the qualitative point of view, the main differences between the samples were observed in the region between 1800 and 1000 cm^{-1}, which is known as the "fingerprint" region.

3.1.3. Chemometrics Analysis of FTIR Data

PCA-FTIR

PCA was performed in this study to see the global clusterification between two species of propolis. FTIR spectra for samples were shown in Fig. 1 where the main differences between the samples were observed in the region between 1800 and 1000 cm^{-1}, which is known as the "fingerprint region", respectively. Fig. 2 shows the PCA scores plot and loading plot for two species stingless bee (Heterotrigona itama and Geniotrigona thoracica) and extracts type (methanol and ethanol) of propolis sample, which were analysed based on FR.

PCA was performed to this study to establish the similarities or the differences between the HI and GT propolis in ethanol and methanol extract. Natural grouping of four samples were visualized in two-dimensional scores plot, as shown in Fig. 2. The total variances account for FR was 95.63% (PC1 = 54.01 %, PC2 = 41.61 %). EHI and EGT clustered closely between them and separately from MHI and MGT. It means that for the ethanol extracts, HI and GT propolis have similar compositions that attribute into their clusterification. However, in methanol extract, HI and GT propolis give a significant where MHI and MGT have clear separation between each other. Presumably, their chemical composition had attributed into their clusterifcation.

In addition, the interpretation of PCA scores plot along with loading may give a better understanding and clarification on the clustering of the samples. Due to the huge datasets of factor loading, the strong loading (≥ 0.75) of FR (1800-600 cm^{-1}) variables were summarized in Table 2 in order to identify the most influence variable that clustered four sample propolis extracts. Table 2 (FR) shows that there were five variables (wavenumber ranges) that give strong loading into VF1 and VF2. VF1 and VF2 were given the most attention as they have the highest percentage of variability and the most important information. Strong loading of FR give out influence variable range only in PC1 that was found in peaks around 1799-1737cm^{-1} and 1660-1610 which are assigned to the C=O stretching vibration of lipids and flavonoids. Besides, the strong loading also give influence variable range in the presences of phenols and flavonoids compounds at peaks 1450 cm^{-1}, 1369 cm^{-1} and 1088 cm^{-1} which attribute to the clusterification in Fig. 3. Furthermore, the peaks at range 833-540 cm^{-1} is related to primary alcohols, and specifically ethanol shows a symmetrical stretching at 880 cm^{-1}, which obeys to the fact that the extracts are dissolved into ethanol are presence as the influence variables.

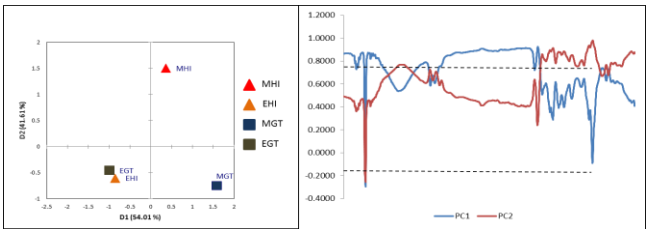

Fig. 2: PCA score plot and its loading plot of overall sample propolis from HI and GT propolis in methanol and ethanol extract.

Table 2: Summary of strong loading (≥ 0.75) of FR (1800-600 cm−1) variables correspond to the assignment of ATR -FTIR spectra on the PCs for HI and GT propolis in methanol and ethanol extract.

Variables (cm^{-1})	Varimax Factor	Assignments of FTIR
1799-1737	VF1	C=O stretching vibration of lipids and flavonoids
1660-1610	VF1	C=O, C=C stretching vibration of flavonoids
1455-1450	VF2	N-H asymmetric stretching of amino acids stretching vibration and bending CH3, CH2, flavonoids, aromatic rings
1369-966	VF2	vibration and bending CH3, flavonoids
833-540	VF2	symmetrical stretching flavonoids, primary alcohol

HCA-FTIR

Fig. 3 shows cluster analysis for fingerprint region of FTIR data set. HCA dendrograms of HI and GT propolis in methanol and ethanol extract of FTIR data were grouped into three groups, as similar result in that of PCA. They were; Cluster 1 (EGT and EHI), Cluster II (MHI) and Cluster III (MGT). The dotted line in the dendrograms represents the automatic truncation which cutting off hierarchical tree for significant cluster. Cluster 2 and 3 shows the highest similarity based on all peaks of compound presence in samples. This might indicate that the compound presence in samples in both cluster are about 50% similar. Cluster 1 shows the highest dissimilarity (10000-12000%) compared to the other cluster, which indicates the highly distinct compound presence in the samples.

3.2. GC-MS analysis

The GC-MS analysis in this study was carried out to analyse and identify major peaks representing volatile compounds in all four samples of propolis. The assigned peaks were detected by MS detector and the total ion chromatogram of each peak were compared and matched with the library search report based on National Institute of Standards and Technology (NIST) library database (Library ID: NIST02.L). The peaks that matched with NIST database in the quality 80% and above were considered as the particular compounds. All the analysed extracts showed good separation in chemical composition. Comparison of GC-MS chromatogram and phytochemical compounds for ethanol and methanol extract for each species, HI and GT are shown in Table 1 and Table 2, respectively.

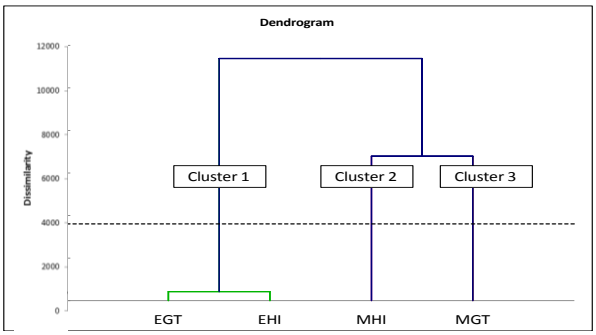

Fig. 3: HCA dendrograms of HI and GT propolis in methanol and ethanol extract of FTIR data.

3.2.1. GC-MS of *Heterotrigona itama*

Identification of peaks was based on Probability Based Matching (PBM) library search system using a NIST MS Spectral database as library references in the quality 80 % and above. Twenty four different phytochemicals were identified in methanol extract, while 13 different phytochemicals presence in ethanol extract from propolis samples collected by HI species (Table 3). The difference in total compound number, type and amount of the various phytochemical compounds identified in methanol and ethanol of Malaysian stingless bee propolis showed their difference in terms of solubility. Amongst these 24 phytochemicals in methanol extract, 9,19-Cyclolanost-24-en-3-ol (14.11 %) and 13,27-Cycloursan-3-one (11.48 %) were found to constitute major amount, whereas others were found to be present in trace. Similar in ethanol extract also found structure of compound 9,19-Cyclolanost-24-en-3-ol (11.63 %) as major amount. Based on the present of area, it can be said that in the HI species for both extract of Malaysian stingless bee propolis dominated by similar compounds 9,19-Cyclolanost-24-en-3-ol or cycloartenol.

Cycloartenol is a component of making K-liquid chlorophyll which is a health beverage preparation that believed to help detoxify and reduce toxins in the body. Drink supplements that contain efficacious cycloartenol also increase nutrient intake in the blood to increase oxygen in the blood which help to regenerate of red blood cells as well as being inhibitors of bacterial growth [18]. In methanol extract, peak to 23 and 27 have the same structure as 9,19-Cyclolanost-24-en-3-ol. At the peak to 23, the retention time of 19.83 minutes and have 14.11 percent area, while the peak to 27 has a retention time of 21.17 minutes with 1.57 percent area. For ethanol extract, the retention time of 19.83 minutes and have 11.63 percent area. In addition to containing cyclolanost, HI species for both extract of propolis also contains other compounds that are similar to the 9,19-Cyclolanostan-3-ol.24-methylene which has a retention time of 20.58 minutes, 2.18 percent area for methanol extract and 1.57 percent area for ethanol extract. Compound 9,19-Cyclolanostan-3-ol.24-methylene as an anti-HIV compound used to prevent the HIV virus [19].

There are also compounds in propolis that presence for both extract which have similarity with compound of paraffin wax group (1-Octadecane) at peak to 7 and compound of fatty acid group (9,12-Octadecenoic acid) at peak to 8. 1-Octadecane appears through the peak to 7 with a retention time of 11.50 minutes, an area of 0.13% in methanol extract and 0.21% in ethanol extract. Meanwhile, compound 9-Octadecenoic acid appears through peak to 8 with a retention time of 11.58 minutes, an area 0.29% in methanol extract and 0.24% in ethanol extract. 9-Octadecenoic acid is a compound that has a biological activity as hepatoprotective, antihistaminic, hypocholesterolemic, anti-eczemic, antioxidant and anti-cancer properties. At peak 20 and 24, compound that only presence in methanol extract of propolis samples collected by HI species has the similarity with the structure of compound lanosterol or Lanosta-8.24-dien-3-ol. At the peak to 20, the retention time of 19.29 minutes and 2.92 percent area. Peak to 24 had a retention time of 19.91 minutes with an area of 4.29 percent area. Lanosta-8.24-dien-3-ol is equivalent to the 4.4.14 alpha-trimethyl-5-alpha-cholesta-8.24-dien-3beta-ol or also called cryptosterol of family lanosterol is a precursor compound for cholesterol metabolism and cucurbitacins [20]. Besides, these compounds together with cycloartenol were formed from the conversion of acetic acid through a mevalonic acid and squalene (a terpenoid) in a series of steroid biosynthesis. The structure resembles a triterpenoid cycloartenol [21].

Among the 13 identified phytochemicals in the ethanol extract, there is a triterpene compound that have a similar structure with 4, 4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9, 10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one which is the predominant compound. This compound appears through the peak to 19, with a retention time of 19.07 minutes and the percent area of 4.29%. As mentioned above, the difference in total com-

pound number, type and amount of the various phytochemical compounds identified in methanol and ethanol of Malaysian stingless bee propolis may probably depend on the compounds solubility. These findings raised the possibility that this source of chemical composition of propolis varies greatly due to the species of bees and nature of the solvents used for the extraction.

Table 3: GC-MS of HI propolis in methanol and ethanol extract.

Peak	Retention Time (min)	Name of Component	Area (%) MeOH	Area (%) EtOH
1	4.47	2,6-Dimethyl-2-trans-6-octadiene	-	0.20
2	4.72	Cyclohexene, 1-methyl-4-(1-methylethyl)-	-	0.07
3	6.25	1,2-Benzendiol	1.10	3.11
4	8.30	Naphthalene	0.05	-
5	8.60	Naphthalene	0.12	-
6	10.50	Cyclotetradecane	0.14	0.18
7	11.50	1-Octadecene	0.13	0.21
8	11.58	9,12-Octadecenoic acid	0.29	0.24
9	11.89	Ethyl Oleate	0.09	0.08
10	12.48	11-Eicosenoic acid	0.10	-
11	12.58	Eicosanoic acid	0.09	-
12	13.31	Phenol, 3-pentadecyl-	0.20	0.22
13	13.39	Docosanoic acid	0.14	-
14	14.51	13-Docosenamide	0.35	-
15	14.61	1,3-Benzenediol, 5-pentadecyl-	0.59	-
16	14.73	2,6,10,14,18,22-Tetracosahexaene	0.57	-
17	14.73	18-Nonadecen-1-ol	0.71	-
18	15.79	5-Heptylresorcinol	3.35	-
19	19.07	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2H-picen-3-one	-	4.29
20	19.29	Lanosterol	2.92	-
21	19.39	β-Amyrin	4.37	3.54
22	19.62	13,27-Cycloursan-3-one	11.48	-
23	19.83	9,19-Cyclolanost-24-en-3-ol	14.11	11.63
24	19.91	Lanosterol	4.29	-
25	19.98	α-Amyrin	5.87	5.91
26	20.58	9,19-Cyclolanostan-3-ol.24-methylene	2.18	1.57
27	21.17	9,19-Cyclolanost-24-en-3-ol	1.57	-

Note: The total ion chromatogram were matched with the library search report based on National Institute of Standards and Technology (NIST) library database (Library ID: NIST02.L) in the quality 80% and above.

3.2.2. GC-MS of *Geniotrigona thoracica*

According to the Table 4, the methanol extract propolis revealed the presence of 20 different phytochemicals while ethanol extract revealed only 17 different phytochemicals of propolis samples collected by GT species which were characterized and identified by comparison of their mass fragmentation patterns with the similar in NIST database library in the quality 80% and above. At the peak to 15, there is a component compounds in propolis samples collected by GT species that has highest abundance is the similarity with the structure of compound 9,19-Cyclolanost-24-en-3-ol or Cycloartenol for both extract as the major amount. The existence of these compounds in propolis samples appears with a retention time of 19.86 minutes, the percent area in methanol extract 19.78 %, while in ethanol extract 21.07 %.

Among the 22 phytochemicals propolis samples collected by GT species in methanol and ethanol extract, one is terpene group that has similar structure with compound Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-. This compound appears through the peak to 1 with a retention time 8.00 minutes, the percent area of 0.04% in methanol extract and percent area of 0.05 % in ethanol extract. Compound Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)- or α-Bergamotene that has a biological activity as anti-oxidant and anti-microbial. At the peak to 18, 21 and 22, there is a component compounds in propolis samples that have a same structure with fatty acid group (1-Benzazirene-1-carboxylic acid). At the peak to 18, the retention time of 20.37 minutes and 0.74 percent area in methanol extract, meanwhile 0.96 percent area in ethanol extract. Peak to 21 had a retention time of 21.88 minutes with an area of 0.93 percent area in methanol extract and 0.80 percent area in ethanol extract. While the peak to 22 has retention time of 23.44 minutes with 0.56 percent area that only presence in ethanol extracts.

Furthermore, compound that only presence in ethanol extract of propolis samples collected by GT species has the similarity with the structure of compound lanosterol or Lanosta-8.24-dien-3-ol. This compound appears through the peak to 13 with a retention time 18.84 minutes and the percent area of 1.95 %. Compounds lanosterol also exist in propolis samples collected by HI species but only presence in methanol extract (Table 3). Difference in propolis samples collected by GT species, were in ethanol extracts. Among the 20 phytochemicals identified in the methanol solvent collected by GT species, there are also compounds in propolis which has similarities with fatty acid group (9,12-Octadecadienoic acid) which has a retention time of 11.56 minutes, an area of 0.11 %. The existence of these compounds in propolis sample collected by GT species only presence in methanol extract, however, compound 9,12-Octadecadienoic acid presence for both extract in propolis samples collected by HI species. The findings from this study raised the possibility that this source of chemical composition of propolis varies greatly due to the species of bees and nature of the solvents used for the extraction.

Table 4: GC-MS of GT propolis in methanol and ethanol extract.

Peak	Retention Time (min)	Name of Components	Area (%) MeOH	Area (%) EtOH
1	8.00	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	0.04	0.05
2	8.30	Naphthalene	0.09	0.08
3	8.40	Eudesma-4(14),11-diene	0.06	0.05
4	8.60	Naphthalene	0.07	0.06
5	10.72	Pentadecanoic acid	0.07	-
6	11.56	9,12-Octadecadienoic acid	0.11	-
7	12.32	1,15-Hexadecadiene	0.12	0.10
8	12.82	Bicyclo[10.8.0]eicosane, cis-	0.05	-
9	13.30	Phenol	1.23	0.79
10	14.51	13-Docosenamide	0.65	0.47
11	15.73	1,3-Benzenediol	1.79	3.67
12	18.43	Ursa-9(11),12-dien-3-ol	0.57	-
13	18.84	Lanosterol	-	1.95
14	19.39	β-Amyrin	6.23	7.42
15	19.86	9,19-Cyclolanost-24-en-3-ol	19.78	21.07
16	19.93	Ergosta-8,24(28)-dien-3-ol, 4,14-dimethyl-, (3.beta.,4.alpha.,5.alpha.)-	6.49	-
17	19.99	α-Amyrin	7.56	7.01
18	20.37	1-Benzazirene-1-carboxylic acid	0.74	0.96
19	20.58	9,19-Cyclolanostan-3-ol	3.12	3.20
20	21.16	Taraxasterol	1.04	1.56
21	21.88	1-Benzazirene-1-carboxylic acid	0.93	0.80
22	23.44	1-Benzazirene-1-carboxylic acid	-	0.56

Note: The total ion chromatogram were matched with the library search report based on National Institute of Standards and Technology (NIST) library database (Library ID: NIST02.L) in the quality 80% and above.

4. Discussion

Due to the complex multivariate of propolis variables in term of wavenumber assigning to particular vibrational functional groups, the application of FTIR spectroscopy technique was coupled with

chemometric analysis. This application may provide pattern recognition of the samples based on the whole profile and/or the selection region, thus turn out to observe the discriminant between the samples. In this study, PCA and HCA were used in determining the differentiation between two species of stingless bee that create propolis for both extracts using FTIR multivariate data. The results of FTIR-chemometric analysis revealed that the grouping of propolis samples using the datasets obtained from spectroscopic technique of FTIR was successfully done in order to differentiate of propolis samples. From PCA point of view, VF1 consisting the most important information which can describe the most variance in PCA, while VF2 is predominant to use to describe the differences between the samples or to discriminate the samples. As for different species of HI and GT in methanol and ethanol extract, they were successfully discriminate into three groups where EHI and EGT clustered together but separately from MHI and MGT which are clustered alone. This result indicates there should be specific chemical characteristics that clustered them apart. PCA and HCA were cluster these four samples propolis into similar of these three groups. There were two regions that responsible in giving the most important in describing the most variance (VF1) of propolis clustering. They were 1799-1737^{cm-1} and 1660-1610^{cm-1} which attribute to flavonoids compound. Therefore, it is recommended to apply this method to perform the quality control and spectroscopy analysis of complex natural products.

The high variability in sample composition according to bee species opens the possibility to develop selective products from Malaysian stingless bee propolis and to diff erentiate propolis types according to the constituent profile. As compared to ethanol extract of HI propolis from Umar and Mahaneem [22], twenty five chemical compounds were detected which are different from this study. On the other hand, it has been reported that there are twenty chemical compounds found in ethanol extract of Brazilian red propolis while twenty four and eighteen chemical compounds in two ethanol extract from samples of Turkish propolis as compared to this study. Furthermore, beta- amyrin and alpha-amyrin found in this study for both species of Malaysian stingless bee propolis were also detected in ethanol extract of Brazilian and Turkish propolis. Besides, Uzel et al. [23] analysed propolis samples from four different Anatolian regions using GC-MS and concluded that the majority of compounds were flavonoids. The reason is that the plants from which bees collect the propolis, vary greatly from region to region resulting in a large variety of possible flora producing propolis compositions [24, 25].

5. Conclusion

Spectroscopy and chromatography techniques of FTIR and GC-MS analysis were applied in establishing the chemical profile of two different species of stingless bees, *Heterotrigona itama* (HI) and *Geniotrigona thoracica* (GT) for quality control evaluation. GCMS analyses without using any marker compounds were found more than 32 volatile compounds from chemical composition of HI and GT propolis which were present and/or absent in both methanol and ethanol extract by gcms. The similar profile pattern as well as the stable marker compounds in propolis samples may be used as quality control approach for establishment chemical profiles of malaysian stingless bee propolis. FTIR analysis coupled with chemometric revealed propolis samples comes out with three different groups, where MHI was found different from other samples of propolis due to its specific unique chemical constituents, respectively. Overall, according to the results obtained from chemical profiling, malaysian stingless bee propolis can be classified into two main groups, namely phenolic-rich and triterpene-rich samples. This finding demonstrated that propolis varied depend on the bee species according to their specific parameters and separation of compounds. Based on the good separation observed in both spectroscopic and chromatographic data, it seems reasonable to use chemical fingerprint coupled with chemometric analysis to differentiate two different species of stingless bee propolis. The findings from this study can be used as identification, classification and quality control methods for the profiling of propolis.

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