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Chemical Constituents of Malaysian *Geniotrigona thoracica* Propolis

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ABSTRACT

Propolis produced by a stingless bee (*Geniotrigona* spp.), commonly known as "Kelulut" in Malaysia, is known to possess various medicinal values. It is a resinous product that is used to build the beehives. Chemical constituents of propolis differ and depend on many factors such as regions and species. To date, the study of the chemical constituents of *Geniotrigona thoracica* propolis from Malaysia is still lacking. Hence, this study report the chemical constituents from Malaysian *G. thoracica* propolis collected from Kota Bharu, Kelantan, Malaysia. The ethanolic extract of propolis (EEP) was derivatized and analyzed by gas chromatography–mass spectrometry (GCMS). The compounds were later identified by library searching Wiley 275 and NIST 02 mass spectral databases. Out of the 48 individual compounds identified, 30 compounds were identified for the first time from propolis. The main class group compounds were phenolic compounds and terpenoids. 1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl) and fren-9(11)-en-2.alpha.-ol were the main identified phenolic compound and terpenoid, respectively.

Keywords: GCMS, *Geniotrigona thoracica*, Malaysian propolis

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INTRODUCTION

Stingless bees belong to the former genus *Trigona* with many subgenera that have been elevated to generic status such as *Geniotrigona, Heterotrigona, Lepidotrigona, Lisotrigona*, and *Tetragonula* (Michener, 2000). They are commonly known as "Kelulut" in Malaysia. These bees can be commercially reared and stingless bee keeping industry in Malaysia

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has increased drastically in the last 6 years. Currently there are more than 1000 registered farmers nationwide (Harun et al., 2015). In Malaysia, five species of *stingless bees* have been recorded so far (Kelly, Farisya, Kumara, & Marcela, 2014). They are *Geniotrigona thoracica, Heterotrigona itama, Lepidotrigona terminata, Lisotrigona scintillans,* and *Tetragonula laeviceps*. However, stingless bee keeping in Malaysia is limited to *G. thoracica* and *H. itama*.

Stingless bees can provide a lot of propolis per hive compared to honeybees (*Apis* spp.). Propolis is one of the most fascinating bee product, both for hive-building material and defensive substance. It is well known to have various biological activities such as antimicrobial (Shehu et al., 2016; Choudhari, Punekar, Ranade, & Paknikar, 2012), antihyperglycemic (Mahani, Jannah, Harahap, Salman, & Habib, 2013), anticancer (Choudhari, Haghniaz, Rajwade, & Paknikar, 2013), and anti-inflammatory activities (Campos et al., 2015). These activities were attributed to the presence of biologically active compounds such as phenolic compounds (flavonoids, phenolic acids, and their esters), terpenoids, and steroids.

Generally, propolis contains resins that comprised flavonoids, phenolic acids, fatty acids, terpenoids, aromatic acids, pollen, and minerals (Krell, 1996). The chemical composition of propolis depends on a few factors such as bee species, source of plant resin, season, and region. To date, the study of chemical composition in Malaysian stingless bee propolis is still lacking. Previous study of Malaysian propolis (*H. itama*) had analyzed the nonpolar or volatile compounds only (Usman & Mohamed, 2015). Hence, this study was aimed to identify both volatile and nonvolatile compounds of propolis from *G. thoracica* by using gas chromatography–mass spectrometry (GCMS). The propolis extract was derivatised to increase the volatility and thermal stability of the compounds, thus making compounds amenable to GCMS analysis.

MATERIALS AND METHODS

Propolis Sample and Preparation of Ethanolic Extract of Propolis (EEP)

Raw propolis sample from *G. thoracica* was obtained from the local stingless bee keeper (Razip International Trade, Kota Bharu, Kelantan). The sample was collected from Kota Bharu, Kelantan, Malaysia and transported in sealed bottles at −20°C. The extract was prepared based on the methods described by Krell (1996) with some modifications. Propolis sample was cooled in a freezer (−20°C) for a day and was ground into a fine powder. A 50 g of propolis sample was then mixed with 167 mL of 70% ethanol to obtain 30% (w/v) propolis extract. The mixture was shaken moderately by hand twice a day for a week at room temperature. The mixture was then filtered twice through Whatman filter paper No. 1. Prior to the second filtration, the extract was kept in refrigerator (2-8°C) for a day to remove the wax. The ethanol was evaporated by using a rotary evaporator under vacuum at 35°C. The remaining water in the extract was dried by using freeze dryer and the dry extract was stored in the amber glass with a screw cap and put in the freezer (−20°C) until used.

Gas Chromatography–Mass Spectrometry (GCMS) Analysis

Sample Preparation for GCMS. The dried extract (0.5 mg) was derivatised with 50 µl *N*-Methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) in a sealed glass tube for 30 min at 60°C. The trimethylsilylated extract was cooled to room temperature before diluting with 50 µl of dodecane. The extract was transferred into crimped cap vial before GCMS analysis. Blank 70% of ethanol (negative control) was dried and treated similarly as the sample.

GCMS Conditions and Parameters. GCMS was carried out using Hewlett Packard 6890 Gas Chromatograph fitted with 5973N Mass Selective Detector. The column used was fused silica capillary, HP-5 column (30 m × 0.25 mm i.d × 0.25 µm film thickness; Agilent Technologies, USA). The carrier gas was helium with flow rate at 1.0 mL/min with the oven temperature was programmed from 50°C (held for 2 min) to 280°C (held for 10 min) at a rate of 10°C/min. The injection and interface temperatures were set at 250°C and 280°C, respectively.

One microliter sample was injected in splitless mode and analyzed in MS full-scan mode (*m/z* 40–650). The electron ionization was set at 70 eV. Acquisition of data was performed using Chemsation software.

Identification of the Chemical Constituents. The National Institute of Standards and Technology (NIST) library of mass spectra was used to match and to identify an unknown chemical in the sample mixture. The mass spectrum produced by a certain chemical compound is basically the same every time. Therefore, the mass spectrum is essentially a fingerprint for the molecule. This fingerprint can be used to identify the compound. The propolis compounds were identified by library searching Wiley 275 and NIST 02 mass spectral databases. The percentage compound was calculated from the summation of the peak areas of the propolis compounds.

RESULTS AND DISCUSSION

Forty-eight individual compounds were identified. From this, 30 compounds are identified for the first time from propolis, that are hydroginkgol, resorcinol, pentadecyl-,1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl), ethyl (3-phenylcyclobutylidene)acetate, 1-(2-Methoxyphenyl)-2,5-dihydro-1H-pyrrole-2,5-dione, nootkatone, fren-9(11)-en-2.alpha.-ol, beta-amyrenol, friedelan-y-al, 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-, cycloeucalenol, 3-(Dimethylphenylsilyl)-2-methylpropionamide, 3,7-Dioxa-2,8-disilanonan-5-one, 2,2,8,8-tetramethyl-, cyclopentane-1-carbonitrile,1-(4-hydroxy-3-methoxyphenyl)-, vinyl palmitate, methyl 9,10-methylene-octadec-9-enoate, oleyl alcohol, 2-(2’,4’,6’-Trichlorophenoxy)-4,5,6-trichlorophenol, 8-methyl(6)

(2,4) thiophenophane, curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo-, methyl ester, 1,8-Dimethyl-3,6-diazahomoadamantan-9-ol,2-Amino-3-methylpyridine -N-oxide, 29,30-Dinorgammaceran-3-one, 22-hydroxy-21,21-dimethyl-, (8.alpha.,9. beta.,1, 12-Oleanen-3-yl acetate, (3.alpha.)-, 8-Amino-2,6-dimethoxylepidine, octahydroisocolumbinic acid methyl ester, 3-hydroxy-4-dimethyloxetanecholest-4-ene, 9,19-Cyclolanost-24-en-3-ol, acetate, (1RS,2SR,4SR,9SR)-1,6,6-trimethyl-9-isopropenyl-10-oxatricyclo[5.3.0.0(2,4)], and cycloisolongifolene, 7-bromo-.

The chemical constituents of Malaysian *G. thoracica* propolis as percentage of total ion chromatogram (TIC) are presented in Table 1. The classes of compounds that were identified are phenolic compounds, terpenoids, steroids, fatty acids, and sugar alcohol, which were similar with propolis from other stingless bees (Choudhari et al., 2012).

Table 1
Chemical compounds of Malaysian Geniotrigona thoracica *propolis*

Retention time	Compound names	% of total ion chromatogram
	Phenolic compounds	
7.32	Phenol	0.11%
9.12	Benzoic acid trimethylsilyl ester	0.13%
14.45	Hydroginkgol	0.46%
15.39	Resorcinol, pentadecyl-	0.24%
19.47	1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl)-	1.26%
20.11	Ethyl (3-phenylcyclobutylidene)acetate	0.45%
21.46	1-(2-Methoxyphenyl)-2,5-dihydro-1H-pyrrole-2,5-dione	0.31%
	Terpenoids	
10.62	Delta-cadinene	0.14%
12.46	Nootkatone	0.26%
16.56	Lup-20(29)-ene-3,21-dione, 28-hydroxy-	0.51%
17.98	Fren-9(11)-en-2.alpha.-ol	0.60%
19.42	Beta-amyrenol	0.36%
23.54	Friedelan-y-al	0.17%
	Steroids	
19.58	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	0.48%
19.73	Cycloeucalenol	0.37%
	Sugar alcohol	
10.59	Erythritol, 1,2,3,4-tetrakis-O-(trimethylsily	0.10%
	Fatty acids	
12.06	Myristic acid, trimethylsilyl ester	0.27%
12.79	Palmitic acid, trimethylsilyl ester	0.21%

Table 1 *(continue)*

Retention time	Compound names	% of total ion chromatogram
12.98	Linoleic acid	0.22%
13.55	Octadecanoic acid, trimethylsilyl ester	0.14%
	Others	
8.19	3-(Dimethylphenylsilyl)-2-methylpropionamide	0.53%
8.90	3,7-Dioxa-2,8-disilanonan-5-one, 2,2,8,8-tetramethyl-	0.42%
9.15	Glycerol tms	0.61%
10.95	Diethyl Phthalate	0.27%
11.01	Caryophyllene oxide	0.21%
12.04	Cyclopentane-1-carbonitrile,1-(4-hydroxy-3-methoxyphenyl)-	0.20%
12.35	Hexadecanoic acid, methyl ester	0.29%
12.53	Phthalic acid, butyl isobutyl ester	0.17%
12.60	Vinyl palmitate	0.69%
13.07	Linolenic acid, methyl ester	0.38%
13.16	Ethyl linoleate	0.36%
13.34	Stearic acid, ethyl ester	1.25%
13.50	Methyl 9,10-methylene-octadec-9-enoate	0.21%
13.60	Oleyl Alcohol	0.75%
15.46	Squalene	0.93%
15.61	2-(2',4',6'-Trichlorophenoxy)-4,5,6-trichlorophenol	0.60%
15.66	8-methyl(6)(2,4) thiophenophane	0.68%
16.86	Curan-17-oic acid, 2,16-didehydro-20-hydroxy-19-oxo-, methyl ester	0.46%
17.04	1,8-Dimethyl-3,6-diazahomoadamantan-9-ol	0.28%
17.48	2-Amino-3-methylpyridine-N-oxide	0.36%
18.39	29,30-Dinorgammaceran-3-one, 22-hydroxy-21,21-dimethyl-, (8.alpha.,9.beta.,1	1.05%
18.66	12-Oleanen-3-yl acetate, (3.alpha.)-	1.94%
19.00	8-Amino-2,6-dimethoxylepidine	1.33%
19.67	Octahydroisocolumbinic acid methyl ester	0.58%
20.01	3-hydroxy-4-dimethyloxetanecholest-4-ene	1.18%
21.06	9,19-Cyclolanost-24-en-3-ol, acetate	0.43%
21.18	(1RS,2SR,4SR,9SR)-1,6,6-trimethyl-9-isopropenyl-10-oxatricyclo[5.3.0.0(2,4)]...	0.19%
21.31	Cycloisolongifolene, 7-bromo-	0.29%

The major compounds identified were phenolic compounds that make up 2.96% of TIC. The main identified phenolic compounds were 1H-Pyrrole-2-carboxylic acid, 1-(2-hydroxy-2-phenylethyl) (1.26%), hydroginkgol (0.46%), and ethyl (3-phenylcyclobutylidene) acetate (0.45%). Interestingly, all these compounds are identified for the first time in propolis. In contrast, phenolic compounds were the minor compounds that were found in other species (*H. itama*) of stingless bee reared in Kelantan (Usman & Mohamed, 2015). Phenolic compound has relatively low volatility and is not suitable for direct GC analysis and this factor could be the contribution to the discrepancies. In the present study, the propolis extract was derivatised to increase their volatility and thermal stability. Compounds that are adequately volatile and stable in high temperature in GC conditions can be analyzed appropriately by GCMS.

After phenolic compounds, terpenoids were clearly the dominant compounds in the propolis (2.04%). Terpenoids are one of the important classes in the propolis compounds due to their valuable biological activities. The main terpenoids identified in this study were fren-9(11)-en-2.alpha.-ol (0.60%), lup-20(29)-ene-3,21-dione, 28-hydroxy- (0.51%), and beta-amyrenol (0.36%). Fren-9(11)-en-2.alpha.-ol and beta-amyrenol are identified for the first time in propolis. Other terpenoids that has been identified from this propolis was nootkatone. Recent study of this compound showed that it has an anti-inflammatory effect (Choi, Lee, & Jung, 2014).

Steroids are the other main compounds identified from propolis that make up 0.85% of TIC. The steroids that were discovered are 9,19-cyclolanostan-3-ol, 24-methylene-, (3.beta.)- and cycloeucalenol. Both of these steroids are identified for the first time in propolis. Study of cycloeucanol revealed that it has a hypoglycemic effect (Ragasa, Lim, Shen, & Raga, 2015). In contrast, steroids were not detected from other Malaysian *G. thoracica* propolis (Ibrahim et al., 2016). Although the bee species are similar, the main plant source, shrubs and fruit orchards surrounding the two apiaries may differ and contribute to the discrepancies.

Similar with propolis from other stingless bee species, erythritol was also discovered in this study that made up 0.10% of TIC. Erythritol is a sugar alcohol that tastes like sugar but has no calories. It has been approved for use as a food additive in the United States and throughout the world. The other compounds that were identified were fatty acids. The amount of fatty acid identified was 0.84% of TIC. The compounds identified were myristic acid, trimethylsilyl ester, palmitic acid, trimethylsilyl ester, linoleic acid, and octadecanoic acid, trimethylsilyl ester. Similarly, these fatty acid compounds are also identified in propolis from other stingless bee.

Diethyl phthalate was identified in this propolis sample most probably due to the plastic container that was used to transport this sample. It is a synthetic substance that is commonly used to make plastics.

The various polarities of the chemical compounds of propolis thus affect their method of extraction. As ethanol has a polar end, the hydroxyl group (OH) and a nonpolar end, the ethyl group (C_2H_5), it can dissolve both polar and nonpolar compounds. Therefore, it is important that solvents for the identification of propolis chemical constituents should be able to extract both polar and nonpolar compounds. In this study, 70% of ethanol has been used to extract the propolis.

CONCLUSION

In the present study, 48 chemical compounds were identified from Malaysian *G. thoracica* propolis. From this, 30 compounds were identified for the first time from propolis by using GCMS analysis. Most of the identified compounds are known to have biological properties. Furthermore, stingless bee keeping industry in Malaysia has been increasing in trend. Hence, it is needed to further identify the biological properties of this Malaysian propolis that may be beneficial for the human nutrition and health.

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CONFLICT OF INTEREST

None.

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