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HPTLC PROFILING AND FTIR FINGERPRINTING COUPLED WITH CHEMOMETRIC ANALYSIS OF MALAYSIAN STINGLESS BEE PROPOLIS

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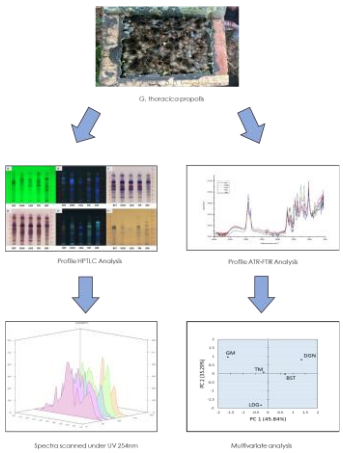
Graphical abstract



Abstract

Propolis is a natural substance produced by the bees to protect their hive. The present work reports the utilization of chemical profiling and fingerprinting combined with multivariate analysis for discrimination of the geographical origin of Malaysian stingless bee propolis. High Performance Thin Layer Chromatography (HPTLC) has been performed to profile the chemical composition of *Geniotrigona thoracica* propolis from different localities in East Coast of Peninsular Malaysia namely Besut, Terengganu (BST), Dungun, Terengganu (DGN), Lundang, Kelantan (LDG), Tanah Merah, Kelantan (TM) and Gua Musang, Kelantan (GM). The obtained HPTLC profiles showed the presence of flavonoids, phenolics and terpenoids in propolis. The chemical fingerprinting was obtained through Fourier Transform Infrared (FTIR) spectroscopy. Chemometric analysis on FTIR dataset using principal component analysis (PCA) and hierarchical cluster analysis (HCA) have classified the propolis into three major groups according to their sampling locations. Chemical fingerprinting analysis on the functional group via FTIR and chemometric revealed that the locations of propolis have direct correlation with the chemical composition, thus affecting the biological activities of propolis. Both chemical marker and chemical fingerprinting analysis are important tools in propolis standardization.

Keywords: Propolis, HPTLC, FTIR, PCA, HCA

Abstrak

Propolis adalah bahan semulajadi yang dihasilkan oleh lebah untuk melindungi sarangnya. Kajian terkini melaporkan penggunaan profil kimia dan cap jari yang digabungkan dengan analisis multivariat untuk mendiskriminasikan geografi asal propolis lebah kelulut di Malaysia. Kromatografi Lapisan Nipis Prestasi Tinggi (HPTLC) telah dilakukan dengan kaedah memprofil komposisi kimia bagi propolis *Geniotrigona thoracica* dari lokasi berbeza di Pantai Timur Semenanjung Malaysia iaitu Besut, Terengganu (BST), Dungun, Terengganu (DGN), Lundang, Kelantan (LDG), Tanah Merah, Kelantan (TM) dan Gua Musang, Kelantan (GM). Profil HPTLC yang diperoleh menunjukkan kehadiran flavonoid, fenolik dan terpenoid di dalam propolis. Cap jari kimia diperoleh melalui Spektroskopi Inframerah Transformasi Fourier (FTIR). Analisis kemometrik pada set data FTIR menggunakan analisis komponen

prinsipal (PCA) dan analisis kluster hieraki (HCA) telah mengelaskan propolis ke dalam tiga kumpulan utama mengikut lokasi pensampelan mereka. Analisis cap jari kimia pada kumpulan berfungsi melalui FTIR dan kemometrik mendedahkan lokasi propolis mempunyai hubungkait secara langsung dengan komposisi kimianya, dengan demikian mempengaruhi aktiviti biologi propolis. Kedua-dua analisis penanda kimia dan cap jari kimia adalah penting dalam pemiawaian propolis.

Kata kunci: Propolis, HPTLC, FTIR, PCA, HCA

1.0 INTRODUCTION

Natural product either from plants or non-plant origin possess wide range of chemical composition. As natural product, chemical composition of propolis highly depends on geographical origin, vegetation types, bee species, harvesting time and extraction method. For number of years, analysis of various samples from different geographical locations revealed the chemical composition of propolis is highly variable. It is established that plant origin of propolis determines its chemical biodiversity. For propolis production, bees use materials resulting from variety of botanical process in different part of the plants. Plants actively secreted exude substances when wounded. These substances can be lipophilic materials on leaves and leaf bud, gums, resin, lattices, etc [1]. These bees also collect propolis from different source of plants by choosing the suitable representative flora available at the ecosystem. Thus, the specificity of the flora at the site collection influences the chemical composition in the propolis including volatile compounds [2].

Propolis is reputed to have numerous pharmacological activities such as antiseptic, anti-inflammatory, antioxidant, antibacterial, antimitotic, antifungal, antiulcer, anticancer, and immunomodulatory properties [3]. The biological activities and pharmacological effects of propolis are mainly related to the synergistic effect of the chemical composition [4, 5] which are influenced by species of stingless bee and the plant species where the bees collect the raw materials [2, 6]. Hence a proper method to discriminate the origin of propolis is required since the chemical and biological properties of propolis depend on its geographical locations which could have different approaches for identification, quality assurance and authentication control. Generally, samples that have similar chromatographic or spectroscopic fingerprints have similar composition. There are numerous reports on analytical methods based on chromatography techniques for profiling propolis such as (HPLC and GC), mass spectrometry techniques (ESI-MS, GC-MS and LC-MS) and spectroscopy (NMR and IR) that were used for determination of geographical origin of propolis around the world. In recent years, the combination of chromatography and spectroscopy methods for profiling and chemical fingerprinting with chemometric started to attract the interest of researchers in propolis. For example, a study done by Cheng *et al*., (2013) focused on discrimination geographical origin of propolis in China using GC-MS combined with multivariate analysis [7]. The results obtained confirmed the propolis has been assigned to four larger groups according to their vegetal sampling locations. On the other hand, UV [8, 9] and NMR [10, 11, 12] were also proved to be useful to achieve the same purpose.

High Performance Thin Layer Chromatography (HPTLC) is recognized as a tool for chemical fingerprinting analysis especially for complex mixtures such as food samples [13] or plant extracts [14, 15, 16] due to its simplicity, few requirements and low cost [17]. Moreover, the analysis can be performed simultaneously up to 20 samples under identical condition [18]. Additionally, this technique has been used for fingerprinting analysis of complex matrix of propolis [19, 20, 21, 22]. For instance, Azemin *et al*., (2017) studied the quality of processed and unprocessed stingless bee propolis from Malaysia using HPTLC and had concluded the method of processing influenced the chemical profile of propolis [23]. However, only few studies have been reported on HPTLC profiling of Malaysian stingless bee propolis from different origins.

Fourier Transform Infrared (FTIR) is one of the most widely used spectroscopic technique for profiling and fingerprinting because of its simple, rapid and non-destructive analysis which could provide reliable information on molecular structure and composition [24]. FTIR measures the bonds vibrations between 4000-600 cm^{-1} of functional groups region and produces a spectrum which can be regarded as a metabolite fingerprint [25]. The FTIR spectra is complex, consisting many variables per sample and making visual analysis is very difficult to be performed. Thus, multivariate analysis including principal component analysis (PCA) and hierarchical cluster analysis (HCA) are required to extract useful informations from the whole spectra [26]. The FTIR technique followed by multivariate data analysis have been successfully applied for fingerprinting and discrimination of peaches [27], coconut oil [28, 29,

30], cocoa beans [31] and beer [32, 33]. In addition, it has been used for quality assessment of bee products including honey [34], bee pollen [35] and royal jelly [36]. FTIR analysis has also been employed to identify the geographical origin of propolis samples [37]; however there were few studies aimed to identify and evaluate Malaysian stingless bee propolis concerning its fingerprinting and profiling by FTIR coupling with multivariate analysis.

Thus in the present work, the chemical profiling of *G. thoracica* propolis has been carried out by HPTLC and FTIR coupling with chemometric analysis. We screened the secondary metabolites involved in *G. thoracica* propolis from five different locations in Malaysia by using HPTLC followed by FTIR analysis. The spectroscopy data of FTIR were then submitted to chemometric analysis like PCA and HCA in order to discriminate the propolis samples from different regions in Malaysia.

2.0 METHODOLOGY

2.1 Chemicals

All analytical grade solvents used in the study such as toluene, ethyl acetate, acetic acid and methanol were purchased from Merck located in Darmstadt, Germany. Iron (III) chloride and aluminum chloride were obtained from Sigma-Aldrich Corporation located in St. Louis, Missouri. Vanillin was purchased from R&M Marketing Company located Essex, UK and p-anisaldehyde was obtained from Acros Organics located in New Jersey, USA.

2.2 Propolis Samples

Propolis from stingless bee *G. thoracica* was collected from hive of five different localities in East Coast of Peninsular Malaysia consisting of two states namely Terengganu and Kelantan. The raw propolis collection area and vegetation source available in each location were described in Table 1. The beekeeper scraped off the raw propolis on top of the hive using knife and spatula and collected it in a clean plastic sample bags which were labelled according to their locations. The raw propolis samples were frozen in -80°C before subjected to extraction process.

Table 1 Propolis *G. thoracica* collected from different localities in Kelantan and Terengganu states, Malaysia

Propolis code	Area of collection	Coordinate	Main vegetation source
BST	UniSZA Apiary, Besut, Terengganu	N 05° 75' 96.5' E 102° 63' 84.4''	Acacia, broad-leaved paperbark, cajuput, *Baeckea frutescens*, *Mangifera indica*
DGN	Padang Serai, Dungun, Terengganu	N 04° 71' 21.6'' E 103° 39' 71.9''	Acacia, broad-leaved paperbark, cajuput, *Mangifera indica*
LDG	Department of Agriculture, Lundang, Kelantan	N 06° 06' 12.3'' E 102° 16' 02.3''	Ornamental areca palm, floriculture, *Arabian jasmine*, *Antigonon leptopus*, *Impatiens balsamina*
TM	Pondok Kelewek Village, Tanah Merah, Kelantan	N 05° 49' 08.1'' E 102° 06' 24.6''	Miracle fruit, floriculture, *Antigonon leptopus*, *Clerodendrum thomsoniae*, *Portulaca grandiflora hook*, *Arabian jasmine*, Orchidaceae, Rosaceae, Bougainvillea
GM	Dalam Lah (2) Village, Gua Musang, Kelantan	N 05° 09' 12.5'' E 101° 58' 33.8''	Rubber tree plantation, *Mangifera caesia*, *Bouea oppositifolia*, *Durio zibethinus*, *Parkia speciosa*, Musaceae, *Lansium domesticum*, *Lansium parasiticum*

2.3 Propolis Extraction

The propolis was crushed with mortar to form powdered propolis. About 30 g of powdered propolis was weighed and extracted with 70 mL of methanol for at least 3 days using maceration process. The extracts were filtered using Whatman No 1 filter paper. The extracts were evaporated using rotatory evaporator (Heidolph Instruments GmbH 5 & Co. KG Germany) removing all solvents in the samples. Then the crude extracts were transferred to labelled empty weighed vials and were kept in the chiller prior to analysis.

2.4 HPTLC Analysis

HPTLC chromatography was performed on HPTLC glass plate coated with silica gel 60 F_{254} (20 cm x 10 cm, Merck, Germany) followed method by Azemin *et*

al., (2017) with slight modifications [23]. The methanolic propolis extract samples were weighed 30 mg and dissolved in 1 mL of methanol. All samples prepared were sonicated for 30 minutes and centrifuged. The supernatant was spotted on TLC plate using automatic sample spotter (CAMAG, Switzerland) equipped with 25 µL syringe. Samples were applied as 8 mm wide bands (=5 tracks per plate) with a length of 8 mm from the bottom edge, 60 mm from the margin and 20 mm distance between the tracks. The application rate was held constant at 100 nL/s. The plate was developed in a twin trough chamber (CAMAG, Switzerland) up to 85 mm using mobile phase toluene/ ethyl acetate/ acetic acid/ methanol (8: 2: 0.1: 0.1, v/v/v/v) with saturation duration of 30 minutes prior the development. After development, the plate was dried with a dryer for complete removal of mobile phase. The plate was visualized at visible light, 254 nm and 366 nm using CAMAG TLC visualizer (DXA252 Digital Camera). Densitometric scanning was accomplished using TLC scanner (CAMAG, Switzerland) at constant scanning speed 20 mm/s with slit dimensions of 8.00 x 0.20 mm, Macro data resolution 100 μm/step, optical filter (second order), and filter factor (Savitsky-golay 7). The plate was derivatized using vanillin in sulphuric acid, anisaldehyde in sulphuric acid, aluminium chloride and ferric chloride reagents. Data handling and processing were analyzed using winCATS 1.4.10 software.

2.5 FTIR Analysis

The FTIR analysis was carried out using IRPrestige-21 Shimadzu Fourier Infrared Spectrophotometer (Tokyo, Japan) equipped with air-cooled ceramic infrared light source and DLATGS (Deuterated Triglycine Sulfate doped with L-Alanine) detector. The single-reflection attenuated total reflectance (ATR) scan technique was used for analysis. The propolis samples were directly placed on the diamond prism for data collection. The resolution for IR measurement was at 4 cm^{-1} and 16 inferograms were co-added before the Fourier transformation. The background spectra was recorded prior to analysis of sample. The data was recorded at the middle of IR range between 4000 and 400 cm^{-1}. Acquisition of FTIR data was analyzed using Shimadzu IRsolution version 1.40 (Shimadzu Corporation) software for baseline correction, normalization and smoothing. Cleanliness of the diamond was done using soft tissues (Kimtech Science, Kimwipes) with 70% ethanol before application of each sample. Each sample was performed in triplicate.

2.6 Pre-processing Data

The FTIR data sets (1789 x 5 datasets) from Shimadzu IRsolution software from baseline correction, normalized and smoothing were saved in a file.txt and copied manually to Microsoft Excel 2013 as two data sets (rows: samples; and column: wavenumbers) for extracting their numerical values from spectra files. The spectra range 3630-550 cm^{-1} was determined to perform PCA and HCA using XLSTAT Pro 2014 (Addinsoft, Paris, France). For PCA and HCA, the data was aligned in row for wavenumbers and column for samples. The FTIR spectra of each location was plotted using OriginPro 9.0 (OriginLab, Northampton, United States).

2.7 Chemometric Analysis

FTIR spectroscopy coupled with chemometric analysis such as PCA and HCA were used to determine the arrangement of inspected elements into groups based on their similarity. Chemometric could be an effectively used to detect the hidden relationship between elements.

2.7.1 PCA

As chemometric, PCA was used to achieve the reduction of dimensionality of the large data sets to a small set by retaining the variability of the data sets in principal component (PCs) [38]. Commonly, two main PCs were used namely PC1 and PC2 as they contributed to higher variation in a data set. The varimax rotation was performed in the spectra data as the factors were difficult to interpret after analyzation of component. Eigen value more than 1 was considered when performing the varimax rotation. In this work, PCA was performed to evaluate whether the geographical origin of propolis samples could be discriminated with respect to their vibrational functional groups.

2.7.2 HCA

Briefly, HCA is one of the methods used in chemometric analysis by clustering the elements into groups based on their similarities within the groups and dissimilarities to each other [39]. HCA was performed on spectra data using single linkage technique to link the cluster and Euclidean distance. Usually, Wards' algorithm is strongly recommended for HCA. In this work, HCA was performed to monitor the related clusters and sub-clusters of propolis samples based on their vibrational functional groups.

3.0 RESULTS AND DISCUSSION

3.1 HPTLC Analysis

The chemical characterization of propolis is difficult to perform as it consists highly variable of chemical composition. TLC fingerprinting technique is widely used for sample pattern recognition as it emphasizes a set of chromatographic signals [19]. Due to this, HPTLC fingerprint was conducted for initial screening of Malaysian stingless bee propolis to verify the

geographical differences among the samples. Visual HPTLC chromatograms of *G. thoracica* propolis extracts from different locations are presented in Figure 1. Each of propolis sample has been marked with corresponding geographical origin. It unveils the occurrence of the secondary metabolites presence in the propolis such as flavonoids, phenolics and terpenoids. Some representative for each type of metabolites that are commonly found in propolis are shown in Figure 2 [40, 41]. The different of spraying reagents and colour of spots according to the presence of phytochemicals are tabulated in Table 2.

Table 2 Detection of secondary metabolites of *G. thoracica* propolis crude extracts with respective spray reagent and the changed colour of the spot

Name of metabolite	Spray reagent	Colour of the spot	
		Visible light	UV 366 nm
Essential oils	Anisaldehyde sulphuric acid reagent	Brown	-
Flavonoids	Aluminium chloride reagent	-	Yellow, blue, brown, yellow-green
	Vanillin sulphuric acid reagent	Pink	-
Phenolics	Ferric chloride reagent	Blue, purple	-
	Anisaldehyde sulphuric acid reagent	Pink, red	-
Terpenoids	Vanillin sulphuric acid reagent	Purple	-
	Anisaldehyde sulphuric acid reagent	Purple	-

HPTLC fingerprint has shown a great diversity in the flavonoids, phenolics and terpenoids profile of these propolis from different locations. *G. thoracica* propolis demonstrated the presence of UV-active compounds including aromatic compounds and compounds with double bonds and extended conjugation under short-waved 254 nm and long-waved 366 nm ultraviolet light as shown in Figure 1A and 1B. In Figure 1A, despite different geographical locations, DGN and LDG propolis had almost identical chromatogram profile when visualized under UV 254 nm. BST propolis showed 9 spots with their corresponding ascending order of R_f values; 0.12, 0.17, 0.23, 0.32, 0.36, 0.47, 0.55, 0.62 and 0.80. DGN propolis revealed the presence of 8 spots with R_f values in the ascending order of 0.06, 0.17, 0.32, 0.41, 0.47, 0.55, 0.62 and 0.70. Next, LDG propolis spotted 9 bands with R_f values in the ascending order 0.06, 0.17, 0.23, 0.32, 0.41, 0.47, 0.55, 0.62 and 0.80 meanwhile TM propolis give 10 spots with ascending R_f values 0.12, 0.17, 0.23, 0.32, 0.41, 0.52, 0.55, 0.62, 0.74 and 0.80. Lastly, GM propolis showed 9 bands with R_f values of 0.17, 0.23, 0.29, 0.32, 0.36, 0.41, 0.52, 0.62 and 0.80. Nevertheless, there are three spots with similar R_f values that were detected in all *G. thoracica* samples from different locations; at R_f 0.17, 0.32 and 0.62.

The HPTLC fingerprint of *G. thoracica* propolis under non-destructive UV 366 nm revealed variety of UV active spots as different locations of propolis displayed different profiles (Figure 1B). BST propolis exposed 7 spots in which were seen at R_f values 0.18, 0.25, 0.31, 0.48, 0.62, 0.80 and 0.83. Next, DGN propolis was found to have 6 prominent peaks at R_f values 0.15, 0.25, 0.31, 0.48, 0.54 and 0.71 meanwhile LDG propolis exerted 7 spots which were seen at R_f 0.07, 0.20, 0.31, 0.42, 0.48, 0.62 and 0.80. TM propolis revealed the occurrence of 9 bands at R_f values 0.15, 0.25, 0.31, 0.42, 0.48, 0.57, 0.62, 0.69 and 0.80. Furthermore, GM propolis also attained 9 bands which R_f values can be seen at 0.18, 0.25, 0.29, 0.31, 0.42, 0.54, 0.62, 0.69 and 0.80. Apparently, there is a peak which is common in all *G. thoracica* propolis at R_f 0.31.

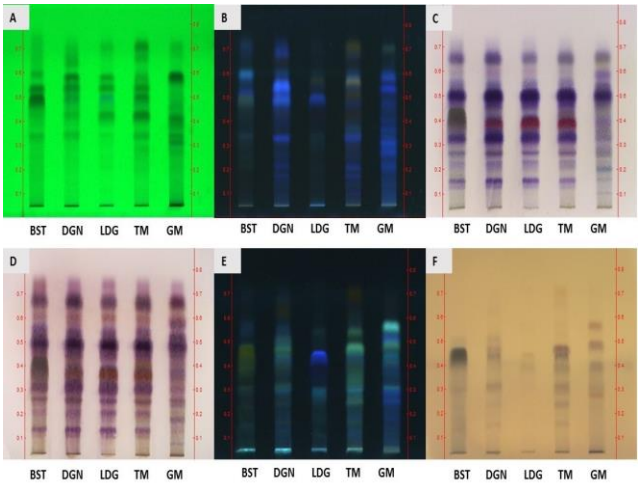


Figure 1 HPTLC fingerprinting profile for various secondary metabolites present in *G. thoracica* propolis from different locations by visualization (A) UV 254 nm (B) UV 366 nm (C) Post derivatization vanillin in sulphuric acid (D) Post derivatization anisaldehyde in sulphuric acid (E) Post derivatization aluminium chloride under UV 366 nm and (F) Post derivatization ferric chloride

The chromatographic fingerprinting of *G. thoracica* propolis under derivatization of vanillin in sulphuric acid reagent showed prominent peaks in all samples as shown in Figure 1C. Purple, pink and brown colour after derivatization confirmed the presence of terpenoids, flavonoids and essential oils respectively. Most of the samples showed similar characteristics of bands except GM propolis. BST propolis revealed 10 bands at R_f values of 0.13, 0.15,

0.23, 0.27, 0.33, 0.42, 0.46, 0.51, 0.55 and 0.66. DGN propolis presented 11 peaks at their R_f values of 0.13, 0.15, 0.23, 0.27, 0.33, 0.39, 0.43, 0.46, 0.51, 0.55 and 0.66. Next, LDG propolis also revealed 11 peaks at R_f values 0.13, 0.15, 0.23, 0.27, 0.33, 0.39, 0.43, 0.46, 0.51, 0.55 and 0.66. In addition, TM propolis demonstrated 11 spots at R_f values 0.13, 0.15, 0.23, 0.27, 0.33, 0.39, 0.43, 0.46, 0.51, 0.55 and 0.66. Lastly GM propolis revealed the occurrence of 11 peaks which were seen at R_f 0.10, 0.15, 0.20, 0.27, 0.33, 0.40, 0.46, 0.51, 0.55, 0.59 and 0.66. Moreover, there are 7 peaks detected in all samples at R_f 0.15, 0.27, 0.33, 0.46, 0.51, 0.55 and 0.66.

The purple, pink or red colour appeared when the HPTLC plate was sprayed with anisaldehyde in sulphuric acid reagent which indicated the presence of terpenes and phenol derivatives respectively. The profile of propolis samples presented similar pattern with slight differences. It can be seen in Figure 1D as BST propolis consisted of 12 prominent peaks at R_f values 0.14, 0.21, 0.27, 0.30, 0.35, 0.42, 0.45, 0.50, 0.52, 0.59, 0.68 and 0.74. DGN propolis detected 12 bands which can be seen at R_f 0.14, 0.21, 0.27, 0.30, 0.35, 0.40, 0.45, 0.50, 0.52, 0.59, 0.68 and 0.74. Next, LDG propolis revealed 12 spots at R_f 0.14, 0.21, 0.27, 0.30, 0.35, 0.40, 0.45, 0.50, 0.52, 0.59, 0.68 and 0.74 while TM propolis exposed 13 bands at R_f 0.14, 0.21, 0.27, 0.30, 0.35, 0.40, 0.41, 0.45, 0.50, 0.52, 0.59, 0.68 and 0.74. GM propolis displayed 10 spots at R_f 0.07, 0.21, 0.30, 0.37, 0.45, 0.50, 0.52, 0.59, 0.68 and 0.74. To conclude, there are 8 bands which can be found in all *G. thoracica* propolis at R_f 0.21, 0.30, 0.45, 0.50, 0.52, 0.59, 0.68 and 0.74.

The HPTLC fingerprint of occurrence metabolites of flavonoids identified in all *G. thoracica* propolis can be seen in Figure 1E when the plate was sprayed with aluminum chloride ($AlCl_3$) reagent then visualized under UV 366 nm. The $AlCl_3$ reagent showed the presence of several fluorescence spots which exposed flavonoids in yellow in the visible and sub-UV 366 nm. Other than that, the change of colour from blue to brown [42, 43] or fluorescent yellow-green [44] confirmed the presence of flavonoids. BST propolis presented 5 spots of flavonoids at R_f 0.16, 0.24, 0.30, 0.45, and 0.55. DGN propolis showed 8 peaks at R_f 0.16, 0.24, 0.30, 0.45, 0.48, 0.49, 0.55 and 0.62. LDG propolis revealed 4 bands which can be seen at R_f 0.18, 0.30, 0.44 and 0.55. TM propolis showed 8 prominent bands at R_f 0.16, 0.24, 0.30, 0.45, 0.48, 0.55, 0.62 and 0.71. Lastly, GM propolis exposed 9 bands of flavonoids at R_f 0.24, 0.26, 0.28, 0.31, 0.41, 0.45, 0.49, 0.52 and 0.57.

HPTLC fingerprinting for *G. thoracica* propolis of phenolic derivatives was performed using ferric chloride reagent then visualized under visible light (Figure 1F). The colour appeared were blue and purple. BST propolis exposed 4 spots of phenolic at R_f 0.23, 0.31, 0.45 and 0.52. DGN propolis showed 6 spots at R_f 0.16, 0.24, 0.33, 0.45, 0.48 and 0.52. LDG propolis revealed 2 bands at R_f 0.41 and 0.45 while TM propolis displayed 10 peaks at R_f 0.17, 0.25, 0.30, 0.32, 0.40, 0.45, 0.48, 0.58, 0.66 and 0.71. Lastly, GM propolis detected 5 spots at R_f 0.28, 0.32, 0.41, 0.50 and 0.57.

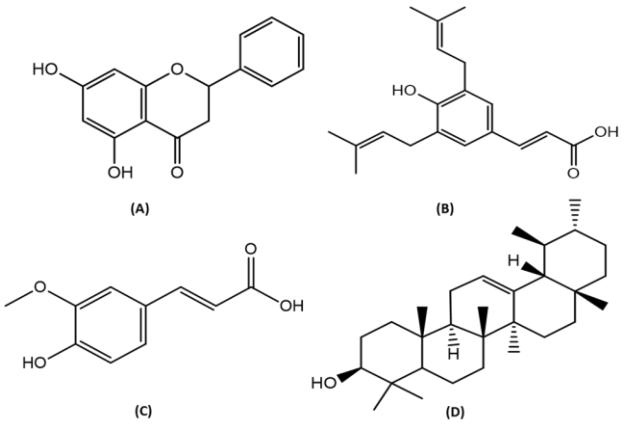


Figure 2 Chemical structures of some representative of flavonoids, phenolics and terpenoids that are commonly found in propolis, (A) Pinocembrin (B) Artepillin C (C) Ferullic acid and (D) β-amyrin

Figure 3 shows HPTLC densitogram of *G. thoracica* propolis from different localities when scanned under UV 254 and 366 nm. The fingerprinting UV spectra of *G. thoracica* propolis displayed variability among the samples under both UV 254 and 366 nm respectively. Despite some differences in the UV spectra, there are slight similarities observed in the propolis samples. There are also a few major spots detected in each propolis sample representing the major constituents.

The results illustrated in Figure 1 indicates the importance of considering the geographical origins of propolis since they demonstrated the variety of secondary metabolites in *G. thoracica* propolis. With regard to the HPTLC fingerprinting *of G. thoracica* propolis, some of the identical characteristics of bands were found in all investigated samples confirming these bands have the potential as chemical markers for Malaysian stingless bee propolis. HPTLC chromatogram visualization suggested that terpenoids are the major components in Malaysian propolis as shown in Figure 1C and Figure 1D compared to flavonoids or phenolics. Our results corroborated with a study by Ibrahim *et al.*, (2016) on the chemical profiling of two species Malaysian stingless bee propolis using Thin Layer Chromatography (TLC) that reported the presence of terpenoids and flavonoids in *G. thoracica* propolis [45]. Terpenoids are secondary metabolites derived from structure consisting of carbon backbones from isoprene units and they are one of the largest families of natural product containing more than 55, 000 components of both primary and secondary metabolism [46]. Other than that, these natural compounds have a broad array of biological activities such as antimicrobial activity [47], anticancer activity [48] and anti-inflammatory activity [49]. Besides that, terpenoids are widely used

in pharmaceutical applications including dietary supplements and drugs. Terpenoids were detected in *G. thoracica* propolis from Malaysia could be an added value of medicinal importance. However, in order to verify the terpenoids as major components of these propolis, further investigations with higher number of samples are needed.

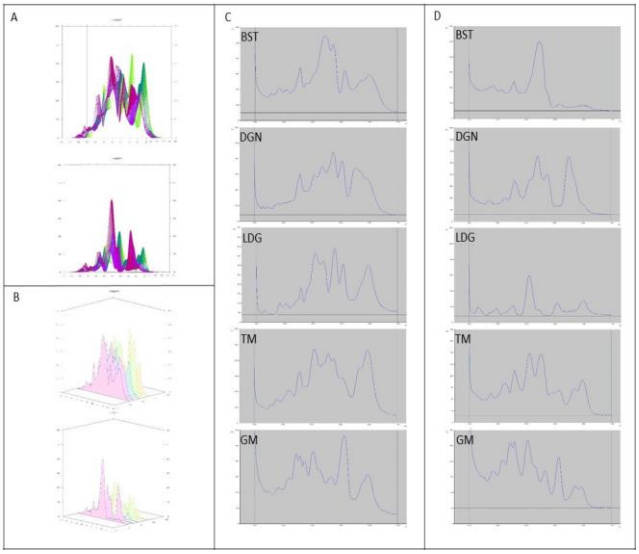


Figure 3 HPTLC densitogram of *G. thoracica* propolis from different localities (A) An overlay of 2D UV 254 nm and 366 nm (B) An overlay of 3D UV 254 nm and 366 nm (C) 2D of UV 254 nm with respective locations and (D) 2D of UV 366 nm with respective locations

3.2 Characterization of FTIR Spectra

The typical FTIR spectra of *G. thoracica* propolis from five localities and overlaid spectra are presented in Figure 4. Despite the general pattern, some differences in the spectra intensity were observed between samples from various locations. The summary of the significant vibrational bands for each of the functional group detected in studied propolis samples are tabulated in Table 3.

The broad bands at 3358, 3354, 3361, 3363 and 3369 cm^{-1} were assigned to O-H stretching vibrations of alcohol groups [50] that present in all propolis samples. Two respective bands detected in all propolis samples (BST: 2926 and 2854 cm^{-1}, DGN: 2922 and 2854 cm^{-1}, LDG: 2931 and 2875 cm^{-1}, TM: 2914 and 2846 cm^{-1} and GM: 2916 and 2848 cm^{-1}) corresponded to CH_2 and CH_3 stretching vibrations. The significant band at 1715 cm^{-1} could be attributed to C=O stretching of saturated aliphatic ketone vibrations which detected only in propolis from BST. The peaks observed at 1693, 1689, 1699, 1683 and 1689 cm^{-1} resulting from C=O stretching of conjugated acid vibrations. The peaks at 1627, 1639, 1610, 1624 and 1614 cm^{-1} corresponded to C=C alkenes stretching vibrations.

Bands at 1597, 1593, 1591, 1587 and 1589 cm^{-1} could be attributed to C=C-C aromatic ring stretching vibrations, at 1463, 1454, 1444, 1465 and 1446 cm^{-1} related to CH_3 asymmetrical bending vibrations, at range between 1375 to 1369 cm^{-1} corresponding to the CH_3 symmetrical bending vibrations. The O-H in plane bending for primary or secondary alcohol was observed at 1288, 1274, 1280, 1265 cm^{-1} regions. The peak around 1194 cm^{-1} is corresponded to C-O stretching vibrations of the phenolics that present in BST sample. In addition, the bands at 1159, 1157, 1168, 1155 and 1165 cm^{-1} could be assigned to C-O stretching vibrations of tertiary alcohols. The C-O stretching of secondary alcohol was identified at 1113 and 1103 cm^{-1} in BST and LDG samples respectively. On the other hand, bands at 1024, 1029, 1016, 1028 and 1022 cm^{-1} due to C-O stretching vibration of primary alcohol, C=C alkene bending vibration was observed at 887 cm^{-1} in BST and LDG samples respectively. Lastly peak at 875 cm^{-1} is assigned to C-C stretching vibration of LDG propolis.

Table 3 Summary of identified distinct bands in FTIR spectra in all *G. thoracica* propolis extracts

Functional group vibration mode	Bands (cm^{-1})
O-H stretching	3500-3200
CH_2 and CH_3 stretching	2931-2914, 2875-2846
C=O stretching of saturated aliphatic ketone	1715
C=O stretching of conjugated acid	1699-1683
C=C stretching alkenes	1639-1610
C=C-C aromatic ring stretching	1597-1587
CH_3 asymmetrical bending	1465-1444
CH_3 symmetrical bending	1375-1369
O-H in plane bending for primary or secondary alcohol	1288-1265
C-O stretching of phenol groups	1194
C-O stretching of tertiary alcohol	1168-1155
C-O stretching of secondary alcohol	1113-1103
C-O stretching of primary alcohol	1029-1016
C=C bending alkene	887
C-C stretching	875

The characterization of functional groups in FTIR suggests that propolis from *G. thoracica* species contain mainly alcohols, acids, ketone, phenolics and aromatic functional groups indicated the presence of aromatic acids, terpenes, flavonoids and phenolic acids which could be corresponding to the various biological activities. Previously reported *G. thoracica* propolis possess antioxidant activity [51, 52], cytotoxic activity [53] and antimicrobial activity [54]. Note that, the data indicated fingerprint of FTIR spectra for *G. thoracica* propolis are identical with slight differences despite different in locations.

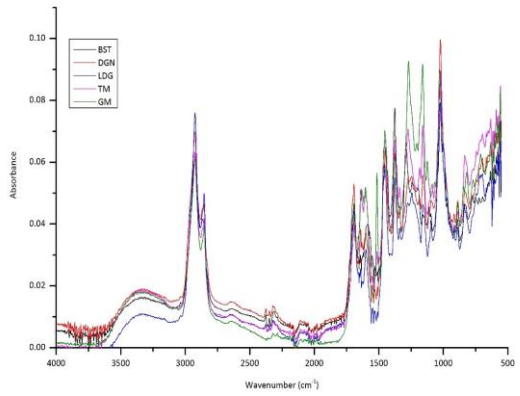


Figure 4 An overlay of FTIR spectra of *G. thoracica* propolis extracts from different locations at mid infrared region (4000- 550 cm^{-1})

3.3 Chemometric Analysis of FTIR

FTIR spectroscopy in combination with chemometric are found to be an efficient approach to discriminate propolis from different locations. As for Malaysian propolis there is a little knowledge regarding the correlation between chemical composition and sites of propolis collection. We observed PCA of FTIR spectra showed the scattering of propolis samples along the principal component 1 and 2 based on functional groups with variance at 81.13%. The first and second PCs displayed variability of 45.84% and 35.29% respectively (Figure 5A). The obtained results from factor score classified BST, DGN and LDG in PC1 while TM and GM in PC2 (see Figure 5B).

The discrimination observed in PCA results suggest that geographical origins influence the presence of functional groups in *G. thoracica* propolis. Examination of factor loading in PC1 suggests that this discrimination based on the functional groups located at regions 2951-2991 cm^{-1} and 1735-2156 cm^{-1} (Figure 5C). Propolis molecules absorbed infrared radiation at 2951-2991 cm^{-1} indicating the CH_2 and CH_3 stretching however there are no significant peaks detected at 1735-2156 cm^{-1}. On the other hand, factor loading for PC2 suggests that this separation based on functional groups located in regions 3041-2997 cm^{-1} and 1700-600 cm^{-1}. Absorptions within 3041-2997 cm^{-1} region corresponded to O-H stretching vibration. The large region of 1700-600 cm^{-1} can characterize different stretching vibrations where C-O stretching of primary alcohol and CH_3 asymmetrical bending occurred in this region. Peaks in the 910-650 cm^{-1} region provide information for determination of the ring substitution pattern on benzene [48].

Figure 5D shows the propolis extracts which were clustered into three main groups based on HCA plot. The dot lines in dendrogram showed the cut off for automatic truncation to determine the clustering of samples. The first cluster consisted of samples originating from BST and DGN while the second cluster was from LDG sample. The third cluster comprised of samples from TM and GM. Samples with high similarity were clustered in the same group.

The grouping of *G. thoracica* propolis in HCA (Figure 5D) are based on the functional groups and the intensities of their FTIR signals as well as plants source available in each location. BST and DGN propolis were classified into the first cluster because of high similarity of functional groups and intensities shown in FTIR signals. Besides that, the plants source at BST and DGN have similarities with minor differences. BST and DGN are located at coastal forest where most of the plants are of coastal forest species. The small differences of plants source in BST and DGN produced four additional functional groups in BST which are C=O stretching of saturated aliphatic ketone, C-O stretching of phenol groups, C-O stretching of secondary alcohol and C=C bending vibrations. However, the additional functional groups with minor differences of plants source did not affect the grouping of BST and DGN as they are in the same cluster.

LDG propolis was classified into second cluster because most of the plant species in LDG are ornamental and orchard plants compared to other locations. TM and GM propolis were clustered into the third cluster because these two locations had the highest intensities of functional groups at regions C=C stretching alkene, O-H in plane bending for primary or secondary alcohols and C-O stretching of tertiary alcohol vibrations as compared to other locations. Furthermore, TM and GM consisted major plant species from dipterocarp forest. There are also some ornamental and orchard plants species in the vicinity of TM but it did not affect the grouping of these two locations. One can conclude that the high similarity obtained from PCA and HCA results confirmed that propolis samples were grouped according to their respective vegetation sampling locations and/or geographical areas.

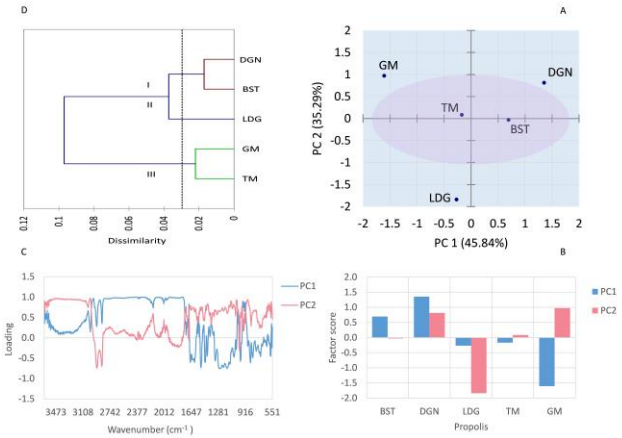


Figure 5 Principal component analysis and hierarchical clustering of *G. thoracica* propolis extract from different locations. (A) Score plot of PC1 vs. PC2 (B) Factor score of PC1 vs. PC2 (C) Factor loading for PC1 and PC2 (D) Dendrogram for HCA plot

4.0 CONCLUSION

The research work has shed light on the importance of HPTLC profiling and FTIR fingerprinting coupled with chemometric analysis for discrimination of propolis based on geographical locations. In this study, the chemical profiling of *G. thoracica* propolis using HPTLC and FTIR analysis combined with chemometric (PCA and HCA) were successfully performed. The obtained HPTLC results showed variety of secondary metabolites including flavonoids, phenolics and terpenoids in *G. thoracica* propolis with terpenoids as the major components. The significant spots observed in all propolis samples could lead for isolation and identification of chemical markers for Malaysian stingless bee propolis. The similarity observed in the fingerprint of propolis corroborated the reliability of FTIR technique for assessing the quality of propolis. All samples have been successfully assigned to three larger groups based on their respective vegetation sampling locations and/or geographical areas indicated the chemometric analysis may be a promising approach for propolis quality control.

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