MALAYSIAN JOURNAL OF ANALYTICAL SCIENCES

Journal homepage: https://mjas.analis.com.my/

Research Article

^{1}H-NMR metabolomics and molecular networking reveal relationship between metabolite profile and antioxidant activity of Malaysian stingless bee honey

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Received: 28 May 2024; Revised: 27 October 2024; Accepted: 28 October 2024; Published: 12 February 2025

Abstract

This study revealed the relationship between metabolite profiling and the antioxidant activity of honey from Malaysian stingless bee species (*Heterotrigona itama*, *Tetrigona apicalis*, *Geniotrigona thoracica*, *Tetrigona binghami* and *Lophotrigona canifrons*) using the proton nuclear magnetic resonance (^{1}H-NMR) metabolomics approach. To identify the metabolites associated with antioxidant activity, the partial least squares (PLS) model was utilised. Meanwhile, the principal component analysis (PCA) was employed to discriminate stingless bee honey samples based on their species. The results revealed that *H. itama* samples were clearly discriminated from other species. *G. thoracica* and *L. canifrons* had similar chemical characteristics, whereas *T. binghami* and *T. apicalis* shared their similarities. A total of 32 metabolites were identified, and amongst them, amino acids (glutamic acid, glutamine, leucine, alanine, valine, isoleucine and tyrosine), as well as organic acids (methylmalonic acid and citric acid), positively contributed to the antioxidant activity, as indicated by the PLS biplot. According to the PLS biplot, phenolic compounds are also associated with its antioxidant activity. This study found that *T. apicalis* honey exhibited the highest potential as an antioxidant agent with 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibitory activity of 30 mg/mL was 73.36 ± 6.47%. Through liquid chromatography-mass spectrometry (LC-MS) and molecular networking approach, a comprehensive analysis of phenolics and flavonoids of *T. apicalis* was successfully conducted. The identified flavonoids included quercetin, naringenin, kaempferol, genistein, apigenin, hesperetin, isorhamnetin, quercitrin and flavonoid glucosides (such as kaempferol-5-methyl ether 3-galactoside-4'-glucoside and kaempferol-7-O-glucoside), whereas caffeoylquinic acid isomers were the identified phenolic compounds. The methods used in this study are useful in assessing the quality of stingless bee honey that possesses antioxidant activity.

Keywords: metabolite profile, antioxidant activity, stingless bee honey, ^{1}H-NMR, phenolic compounds

Introduction

Stingless bee honey (Kelulut honey) is produced by Meliponine bees of the family Apidae. This species can be found only in tropical and subtropical regions, such as Southeast Asia, Central and South America, and mainland Australia [1]. Compared to honeybee honey from the genus *Apis*, stingless bee honey has entirely different physicochemical properties, such as high moisture content, free acidity and electrical conductivity but low viscosity and glucose [2, 3].

Stingless bee honey has gained increased popularity due to its health-beneficial effects, such as providing natural antioxidant sources. Amongst the known and native species of Malaysian stingless bees are *Heterotrigona itama*, *Geniotrigona thoracica* and *Tetrigona apicalis*. Most research on antioxidant

activity involves the species *H. itama*, which is the most abundant species domesticated by beekeepers. A previous study reported that *H. itama* stingless bee honey showed better antioxidant properties than those of the *Apis* species, although its antioxidant activity was lower than that of Manuka honey [3]. Other studies showed that stingless bee honey has different levels of antioxidant activities depending on several factors, for instance, different species [1, 4], sources of floral [5, 6] geographical origin and climate conditions [1]. These factors influence the levels of metabolite profiles and compositions that are related to antioxidant activities.

Metabolomics can be defined as the study of all metabolites, aimed at investigating the presence of metabolites in a specific organism, cell or tissue [7, 8]. For more than two decades, metabolomics has been successfully applied not only for classification and discrimination purposes using principal component analysis (PCA), partial least squares-discriminant analysis (PLS-DA) [9] and orthogonal partial least squares-discriminant analysis (OPLS-DA) [10–12], but also for regression purposes using the partial least squares (PLS) regression model [13]. By using PLS, metabolomics can provide a relationship between metabolite profiling with antioxidant activities [14, 15] and other biological activities [16–19] in biological samples.

Metabolomics has been applied for the authentication and adulteration of stingless bee honey using various analytical tools, such as proton nuclear magnetic resonance (^{1}H-NMR) [20, 21] near-infrared (NIR) s [22] and Fourier-transform infrared (FTIR) spectroscopy [23]. Other studies also showed that metabolomics has succeeded in applying for the classification of stingless bee honey based on species [21, 24], entomological and geographical origins [24, 25] and dehumidification process [25]. While the application of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) metabolomics to find the relationship between metabolite profiling and the antioxidant activity in stingless bee honey has been reported, the use of the ^{1}H-NMR metabolomics approach on the same purpose has not been conducted yet. ^{1}H-NMR metabolomics is the ideal technique for untargeted analysis and has advantages over mass spectrometry (MS) metabolomics, such as being cost-effective, fast, simple in sample preparation and capable of analysing diverse classes of metabolites [7].

Previous research reported the application of LC-MS time-of-flight (TOF) metabolomics to evaluate the metabolites related to antioxidant activity, such as 3-hydroxy-4,4-dimethyloxolan-2-one,2-(hydroxy methyl)-1-methyl-3,4,5-piperidinetriol, 3-carboxy-2,3-dideoxy-pentaric acid, 3-phenyl-2-propenoic acid phenylmethyl ester and 1,3-bis(4-methoxyphenyl)-2-propen-1-one [12]. Nevertheless, to the best of the authors' knowledge, comprehensive identification of other metabolites, such as phenolics and flavonoids, which are known to have a positive correlation to antioxidant activity in Malaysian stingless bee honey has not yet been investigated. A recent approach through the LCMS-MS analysis in combination with molecular networking would be a great benefit to comprehensively identify the phenolics and flavonoids in stingless bee honey. Molecular networking (MN) is a useful tool for the annotation of different metabolite classes and analogues, which can assist in the detection of isomers by comparing the similarity between MS/MS fragmentation patterns and the visualisation of their relationships based on mass spectrometry data [26].

The objectives of this study are to investigate the relationship between metabolite profiling and the antioxidant activities of stingless bee honey from Malaysia and to discriminate stingless bee honey based on their species using ^{1}H-NMR metabolomics. PCA and hierarchical cluster analysis (HCA) were used for the discrimination of five different species of stingless bee honey, including *H. itama*, *G. thoracica*, *T. apicalis*, *Lophotrigona canifrons* and *Tetrigona binghami*. Meanwhile, the PLS model was used to evaluate the relationship between metabolite profiling and the antioxidant activity in stingless bee honey. In addition, the LCMS-MS analysis, in combination with molecular networking, was conducted to comprehensively identify the phenolics and flavonoids of the stingless bee honey that exerted the most potent antioxidant. The results of this study will give an insight into the roles of the identified metabolites in the antioxidant activities of stingless bee honey, which is useful for the evaluation of the quality and antioxidant potential of stingless bee species.

Materials and Methods

Materials and chemicals

Stingless bee honey samples were collected from local farmers in Peninsular Malaysia. The species included *H. itama*, *G. thoracica*, *T. apicalis*, *L. canifrons* and *T. binghami*. At least four samples from different farms were used in this study, except for *L. canifrons* and *T. binghami*, which had three replicates. The details of each different species of stingless bee honey samples are listed in Table S1. All honey samples were stored in the refrigerator (4–6 °C) until further analysis to prevent the degradation of chemical composition and the physical properties of honey samples.

All the chemicals and reagents used in this study were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany). Potassium dihydrogen phosphate (KH_2PO_4), deuterium oxide (D_2O), sodium deuteroxide solution (NaOD) and 3-(trimethylsilyl)propionic acid (TSP) sodium salt from Merck (Darmstadt, Germany) were utilised for the ^{1}H-NMR analysis (Bruker, 400 MHz, Karlsruhe, Germany). Folin–Ciocalteu reagent, analytical grade methanol (Sigma-Aldrich), sodium carbonate solution, gallic acid, quercetin and 2,2-diphenyl-1-picrydrazyl (DPPH) were used in antioxidant assays. Meanwhile, LC-MS grade of water, acetonitrile, methanol and formic acid from Merck (Darmstadt, Germany) were applied for the LC-MS analysis (Thermo Fisher Scientific Inc, Bremen, Germany).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

DPPH assay was employed to examine the radical scavenging activity of stingless bee honey samples according to a previous study with some modifications [27]. About 50 µL of sample solution (final concentration of 30 mg/mL) was added into each well of the 96-well microplate. After that, 100 µL of DPPH was transferred to each well. The mixture was incubated in the dark at 37 °C for 30 min, and the absorbance was measured at 517 nm using the microplate reader. For each stingless bee honey species, three replicates were performed and quercetin was used as a positive control to assess the validity of the bioassay. DPPH activity was expressed as the mean ± SD of percentage inhibition of DPPH radical scavenging at 30 mg/mL. The results were calculated using the equation shown below:

Percentage of DPPH radical scavenging activity =

$$\frac{Abs\ control - Abs.\ sample}{Abs.control} \times 100 \qquad \text{(Eq. 1)}$$

Total phenolic content (TPC) determination

Folin-Ciocalteu assay was conducted to determine the total phenolic content (TPC) of the stingless bee honey samples according to previous research with slight adjustments [27]. About 20 µL of the honey extract (final concentration of 10 mg/mL) was transferred to a 96-well microplate, followed by the addition of 100 µL of Folin-Ciocalteu reagent. The mixture was incubated for 5 min. Then, 80 µL of 7.5% sodium carbonate was added, and the mixture was incubated for another 30 min to 2 hr in the dark. The sample absorbance was measured at 750 nm using a microplate reader. The analyses were conducted for all honey samples in triplicates. The results were presented as mg of gallic acid equivalents (GAE)/g of sample based on a calibration curve prepared using the gallic acid standard.

^{1}H-NMR sample preparation

The ^{1}H-NMR sample preparation was done following the previously described method with slight modifications [21]. Briefly, 0.1 g of stingless bee honey was mixed with 600 µL of D_2O buffer (pH 6.0) containing 0.1% triphenylphosphine (TSP). The sample solution was vortexed for 5 min and centrifuged at 13,000 rpm for 10 min. Then, the supernatant was transferred into an NMR tube for the ^{1}H-NMR analysis.

^{1}H-NMR data acquisition, pre-processing and multivariate data analysis

The ^{1}H-NMR analysis of stingless bee honey samples was carried out on a 400 MHz Bruker NMR spectrometer based on the following parameters: pulse width (PW) 21.0 µs (90°), relaxation delay (RD) 2.0 s, and number of scans of 256. A ^{1}H-presat experiment was conducted to remove the residual signal of water. TSP was used as an internal reference and for alignment purposes in all ^{1}H-NMR spectra. Spectral pre-processing, including phasing, baseline correction and alignment and binning was performed using the online software NMRProcFlow version 1.3 (https://nmrprocflow.org/). Metabolite identification was performed using the Chenomx Profiler software (version 7.6) and confirmed by comparing the characteristics of metabolite signals in the ^{1}H-NMR spectrum with reference standard signals available in the online library (https://hmdb.ca/) and literature data.

A multivariate data analysis (MVDA) was carried out using SIMCA-P version 14.1 (Umetrics AB, Umeå, Sweden). MetaboAnalyst (https://www.metaboanalyst.ca/) online free software was used for the heat map analysis of stingless bee honey species [28]. Pareto scaling was applied to all NMR data before the MVDA analysis. Various MVDA models, such as principal component analysis (PCA), hierarchical cluster analysis (HCA) and partial least squares (PLS) analysis were used in this study. PLS was employed to correlate between the identified metabolites (X-variables) of the samples and their antioxidant properties, including DPPH inhibitory activity and TPC (Y-variables). Meanwhile, PCA and HCA were applied to discriminate the stingless bee honey samples based on their species.

Liquid chromatography-mass spectrometry (LC-MS) analysis

Before the LC-MS/MS analysis, the honey underwent solid-phase extraction (SPE) to remove carbohydrates and obtain the polyphenol extract. About 20 g of *T. apicalis* honey was mixed with 75 mL of acidified water (deionised water adjusted to pH 2 by adding a hydrochloric acid [HCl] solution) and stirred on a magnetic stirrer for 15 min to ensure complete

dissolution of the honey. Next, an SPE Hypersep C18 cartridge (500 mg) was pre-conditioned with 3 mL of methanol, followed by 3 mL of acidified water and 3 mL of deionised water. Subsequently, the honey solution was loaded and washed with 5 mL of acidified water (to remove all sugars and other polar constituents), followed by 5 mL of deionised water. Afterwards, 20 mL of methanol was added to the SPE cartridge to elute the polyphenol extract, and the extract was concentrated under reduced pressure in a rotary evaporator at 40 °C. The extract was then freeze-dried to remove excess water and stored in a refrigerator at -4 °C before analysis.

T. apicalis honey was analysed using LC-MS as per Romera-Torres et al.'s [29] method. The bioactive compounds were analysed on ultra-high performance liquid chromatography coupled to a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Bremen, Germany). An Acquity UPLC BEH C18 column (1.7 µm, 2.1 mm X 150 mm) (Waters, Ireland, UK) was used as a stationary phase during chromatographic separation. The chromatographic separation was performed using 0.1% of formic acid in water (eluent A) and 0.1% of formic acid in acetonitrile (eluent B) at the flow rate of 0.3 mL/min. The total running time was 60 min, the injection volume was 10 µL and the column temperature was set at 25 °C. The analysis was carried out in an Orbitrap analyser with a heated electrospray ionisation (ESI) interface in positive and negative ion modes. The Xcalibur version 2.2 software (Thermo Fisher Scientific Inc., Waltham, MA, USA) was employed for post-analysis and sample pre-processing.

Molecular networking and metabolite annotation

LC-MS raw data from MS1 and MS/MS mass spectra were converted into the mzXML format using MSConvert software from an open-source Proteowizard package (http://proteowizard.sourceforge.net/). The converted files were then subjected to molecular networking (MN) via the Global Natural Products Social (GNPS) platform (https://gnps.ucsd.edu) [30]. The molecular networking was visualised using Cytoscope software version 3.9.1 (http:/www.cytoscape.org) [31], with a force-directed layout modulated by the cosine score factor. The created molecular network and its parameters can be accessed through this link: https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=ee736556bedf424b928b2bbb733682d7.

Statistical data analysis

Analysis of variance (ANOVA) and Tukey-HSD post-hoc tests were conducted to evaluate the significant differences in DPPH inhibitory activity and TPC amongst the samples using SPSS version 16.0 (SPSS Inc., Chicago, IL). The results are presented as mean value ± SD. The superscript letters (a, b and c) were used to indicate the significant difference (p-value < 0.05) amongst the samples. The Pearson correlation analysis was performed using Rstudio (https://www.r-project.org/) to determine the relationship between TPC and DPPH activity.

Results and Discussion

Antioxidant activity

Honey can be differentiated by its physical and chemical constituents, leading to varying levels of biological activities. These variations may be due to the honey being produced by different bee species [4]. There are also other factors (e.g. floral sources and geographical origin) that can affect the variation in antioxidant activities of stingless bee honey [32, 33]. Numerous studies have reported that flavonoids and phenolic acids contribute to the antioxidant activities of stingless bee honey [34].

In this study, the antioxidant properties of stingless bee honey from different species in Peninsular Malaysia were evaluated using DPPH free radical scavenging assay and total phenolic contents (TPC). The results are presented in **Table 1**. It should be noted that *L. canifrons* samples were not subjected to statistical data analysis due to insufficient data.

Table 1. Antioxidant activity of stingless bee honey from different species

Species	Percentage of DPPH Inhibitory Activity (%) *	TPC (mg GAE/g extract)
Heterotrigona itama	48.04 ± 13.23^{ab}	0.15 ± 0.03^{b}
Geniotrigona thoracica	34.65 ± 6.37^{b}	0.18 ± 0.13^{b}
Tetrigona apicalis	73.36 ± 6.47^{a}	0.61 ± 0.30^{a}
Tetrigona binghami	47.21 ± 18.32^{ab}	0.24 ± 0.12^{b}
Quercetin (1 mg/mL)	82.81 ± 1.41	

*Percentage of DPPH inhibitory activity was calculated at the concentration of 30 mg/mL

The DPPH radical scavenging assay reflected the antioxidant capability of honey to inhibit the formation of free radicals. Free radical scavengers donate hydrogen to DPPH, which consists of unpaired electrons, thereby forming a complete and stable DPPH molecule. This transformation is observed through a change in colour from purple to yellow [35]. *T. apicalis* exhibited the highest radical scavenging activity; however, the antioxidant activity was not significantly different when compared to *T. binghami* and *H. itama*. Meanwhile, *G. thoracica* displayed the lowest radical scavenging activity. Similar observations were made by Abdullah et al. [36] and Maringgal et al. [37], who reported that *T. binghami* had great antioxidant potential compared to other stingless bee honey.

Total phenolic content (TPC) is a direct method used to measure the phenolic content, which is presumably related to its antioxidant activity. According to the findings in **Table 1**, *T. apicalis* exhibited the highest TPC, followed by *H. itama*, *T. binghami* and *G. thoracica*. *H. itama*, across various batches of samples, consistently showed TPC results ranging from 0.14 to 1.5 mg GAE/g extract when compared to other species. These TPC findings closely align with the results reported by Fadzilah et al. [38] and Wong et al. [39]. Furthermore, the results indicated that higher TPC corresponded to increased antioxidant activity in stingless bee honey.

Variations in the DPPH activity were observed within the same species of stingless bee honey. This outcome was likely due to the fact that the honey samples were collected from different geographical origins with varying floral sources (Supplementary Table S1). These factors significantly influence the chemical composition of the honey, affecting its antioxidant properties and overall bioactivity. Additionally, the Pearson correlation analysis was performed (Supplementary Figure S1), revealing a positive correlation between TPC and DPPH (0.44) and indicating that higher total phenolic content was associated with greater DPPH activity. This moderate correlation was reasonable, given that phenolic compounds were present as minor constituents in honey, with other compounds, such as amino acids, also contributing to its overall antioxidant activity. This finding is further supported by our ^{1}H-NMR metabolomics study, which highlighted amino acids as additional contributors to DPPH activity.

^{1}H-NMR spectral data of different species and the identification of metabolites

Different species of stingless bee honey samples were subjected to the ^{1}H-NMR analysis for metabolite identification. The samples included *L. canifrons*, *T. binghami*, *T. apicalis*, *G. thoracica* and *H. itama*. The ^{1}H-NMR spectra of the analysed samples are shown in **Figure 1**. A total of 32 known metabolites were identified by referring to compound references from the database (https://hmdb.ca/) available via Chenomx Profiler software and previous literature [21, 40]. The detailed information on ^{1}H-NMR identification metabolites is shown in Table S2.

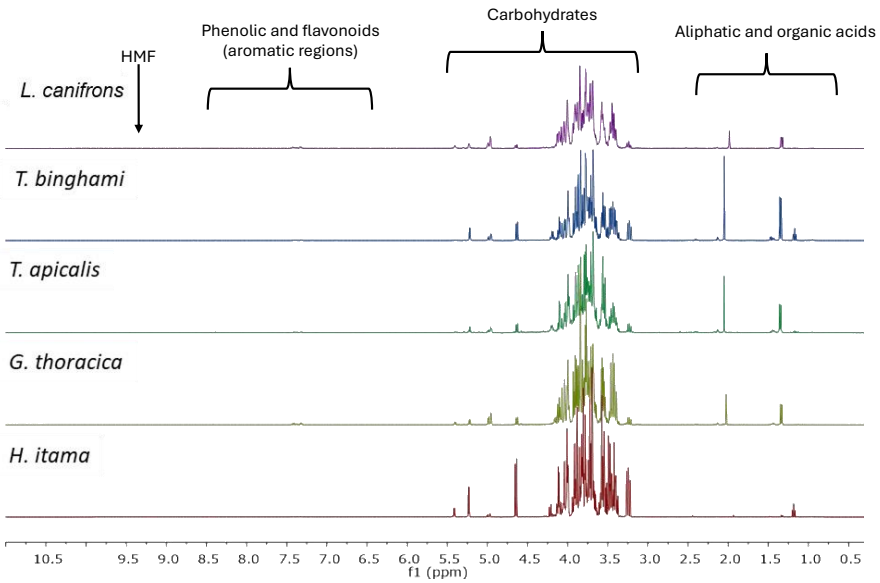


Figure 1. ^{1}H-NMR spectra representation of stingless bee honey of *H. itama*, *G. thoracica*, *T. apicalis*, *T. binghami* and *L. canifrons* species

According to **Figure 1**, carbohydrates were found in all stingless bee honey species, including glucose, fructose, sucrose, maltose, arabinose and rhamnose, at the region of δH 3.20 – 5.42 ppm, which is consistent with previous findings [21]. In this study, *H. itama* exhibited the most prominent signals in the carbohydrate region. Additionally, 2-hydroxymethyl-5-furfural (HMF) was detected in all samples in both the aldehyde region (δ 9.44 ppm) and the aromatic region (δ 7.52 and 6.66 ppm). Other significant metabolites, such as amino acids (phenylalanine, tyrosine, sarcosine, glutamine, glutamic acid, alanine, leucine, valine, 4-hydroxy-L-proline and isoleucine), were found in the spectra at the regions of δH 0.91 – 3.44 ppm and δH 6.87 – 7.47 ppm. Specifically, 4-hydroxymandelic acid signals at δH 6.30 and 7.47 ppm were detected in *H. itama* honey, while trigonelline signals at δH 8.07 – 8.82 ppm and δH 9.10 ppm were observed in *L. canifrons*. Nevertheless, ^{1}H-NMR detected several unknown signals that could not be identified due to a lack of information, as tabulated in Table S2. Therefore, further analysis using other spectroscopy techniques (e.g., LC-MS/MS analysis) is essential to identify the bioactive compounds of Malaysian stingless bee honey. Additionally, the discrimination of Malaysian stingless bee honey samples was performed to gain an overview of the differences amongst the different species.

Discrimination of stingless bee honey species

Unsupervised principal component analysis (PCA) was conducted to discriminate the stingless bee honey based on its species. Figure 2 shows the score plots of PCA, HCA and heat map analyses based on the different species of stingless bee honey. In summary, PCA showed R2X and Q2 cumulative values of 0.778 and 0.661, respectively, indicating a reasonably good model. Throughout the observation on PCA, PC1 (54.9% of variation) and PC2 (22.9% of variation) demonstrated a significant difference between the experiment groups. The segregation amongst the groups revealed that *G. thoracica* and *T. binghami* had similar chemical fingerprints as well as for *T. apicalis* and *L. canifrons*. The *H. itama* cluster was well separated from the others, indicating a significant difference in terms of their chemical profile. This result was supported by the HCA and heatmap analyses in **Figure 2**, which showed three main classes of stingless bee honey, indicating the chemotaxonomic relationship amongst them. Based on the finding above, it can be stated that the combination of PCA, HCA and heatmap was reliable and could be applied in further identifying differential metabolites of stingless bee honey from different species. According to the authors' knowledge, this is the first study that reports the discrimination of five different species of Malaysian stingless bee honey. Previously, ^{1}H-NMR metabolomics has been used to discriminate three species (*H. itama*, *T. apicalis* and *G. thoracica*) of Malaysian stingless bee honey [21].

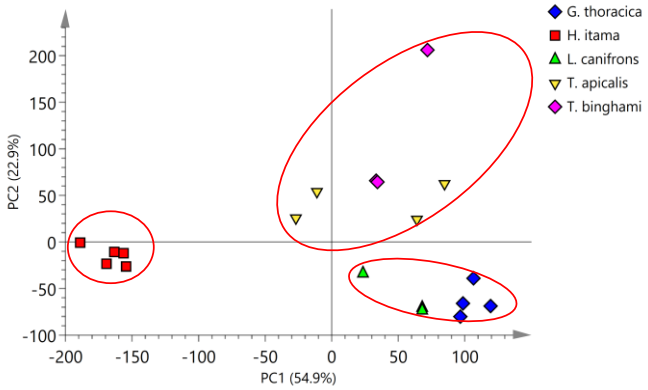
Based on the loading plot results, important metabolites, including alanine, leucine, valine, glutamic acid, methylmalonic acid, isoleucine and lactic acid, were identified as the metabolites that had a great influence on the separation of stingless bee honey species (Figure S2). The alanine level was found to be higher in *T. binghami*, while *H. itama* recorded the lowest level. *T. binghami* also recorded the highest concentration of amino acids, such as leucine, valine, isoleucine, 4-hydroxy-L-proline, glutamine and glutamic acid. Nevertheless, it had the lowest concentration of sucrose in the samples as compared to *H. itama*, which demonstrated the most concentration of saccharides (fructose and sucrose). Another remarkable compound that was only found in the *L. canifrons* species was trigonelline based on its NMR spectrum. Trigonelline could be the representative unique marker of *L. canifrons*. Apart from that, the strong acidity taste of *T. apicalis* might come from the contribution of citric acid, which reported the highest concentration within the species, opposite to *G. thoracica* that recorded the lowest concentration of that metabolite.

Correlation between metabolites and antioxidant activity in stingless bee honey

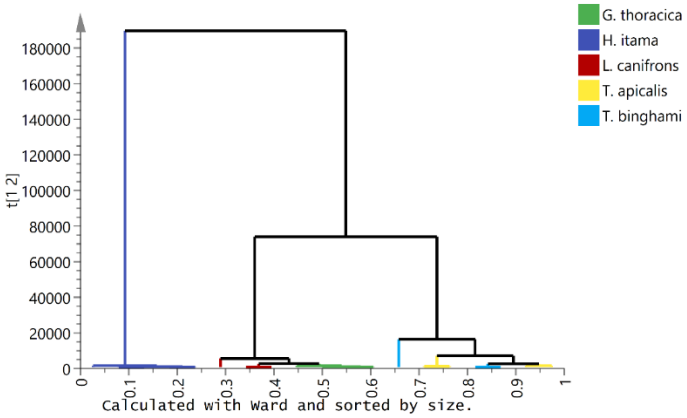
In addition to the study of metabolite profiling, the correlation between the identified metabolites in stingless bee honey samples and their antioxidant activities (DPPH and TPC) was conducted using PLS regression. The identification of metabolites via the ^{1}H-NMR analysis required confirmation regarding their relative contribution to antioxidant activity. Therefore, the combined score and loading plots, known as biplot based on the PLS model, are presented in **Figure 3**. In this study, the PLS model exhibited excellent goodness of fit with the R2Y value of 0.976 and reasonable predictability (Q2 = 0.641).

The observation at the left quadrant of the PLS biplot found that metabolites, such as amino acids (glutamic acid, leucine, alanine, valine, isoleucine and threonine) and organic acids (methylmalonic acid [MMA], citric and acetic acids) were the compounds, which exhibited a positive relationship with antioxidant activity (**Figure 3**). Those metabolites were found abundantly in *T. binghami* and *T. apicalis* honey species. On the other hand, the PLS biplot (**Figure 3**) showed prominent signals of carbohydrates (glucose, sucrose and maltose) in *H. itama* at the upper right quadrant of the biplot, which served the least affiliation with antioxidant activity.

(a)



(b)



(c)

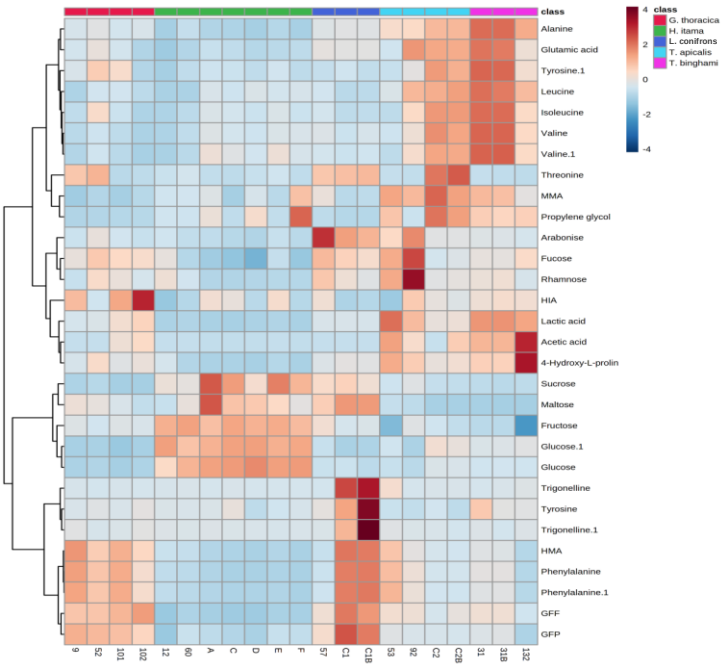


Figure 2. (a) Principal component analysis (PCA) score plot; (b) hierarchical cluster analysis (HCA); (c) and heat map of stingless bee honey samples from different species

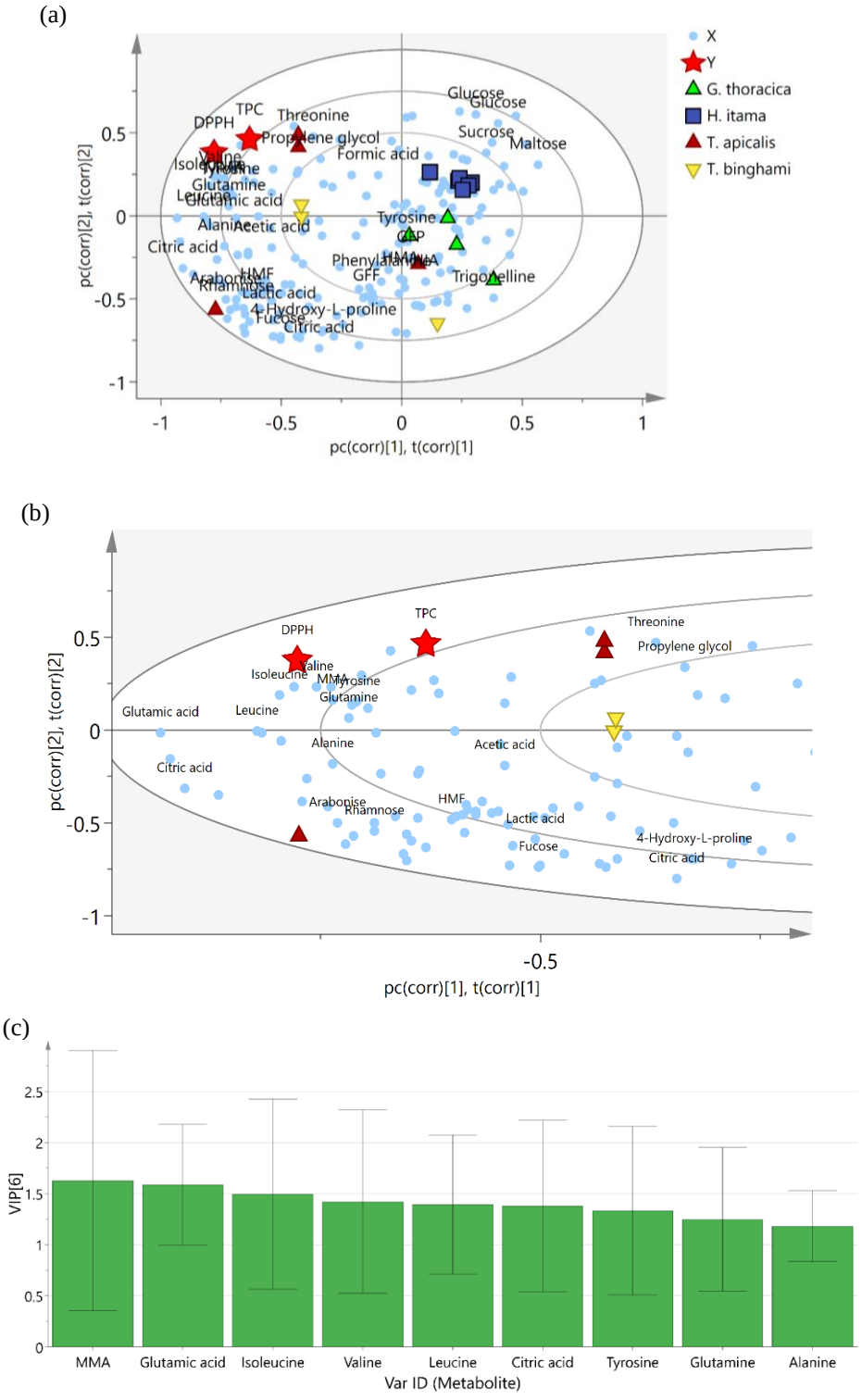


Figure 3. Relationship of the metabolites and antioxidant activity in (a) PLS biplot; (b) its expansion region at the point location of DPPH and TPC; and (c) variable importance in projection (VIP) of the PLS model

Likewise, some of the metabolites in *G. thoracica* that are located at the lower right quadrant of the biplot also had minimum correlation with antioxidant activity. Furthermore, the variable importance in projection (VIP) of the PLS model showed nine metabolites, including amino acids (alanine, leucine,

valine, glutamic acid, glutamine, isoleucine and tyrosine) and organic acids (methylmalonic acid [MMA] and citric acid), exhibiting a strong correlation with antioxidant activity (**Figure 3**). A VIP value greater than 1, along with confidence intervals derived from jack-knifing, is considered indicative of important variables in the PLS model. Jack-knifing is used to calculate standard errors, which are displayed as error bars at the end of each VIP column plot.

One-way ANOVA followed by Tukey's HSD post hoc-test were further carried out to determine the significant differences in the antioxidant metabolites amongst the stingless bee honey species as determined by the VIP values. Box plots of nine antioxidant metabolites in various stingless bee honey species are presented in **Figure 4**. A target analysis was conducted to compare the different species on honey metabolite more intuitively. According to the statistical analysis, *T. apicalis* and *T. binghami* consisted significant concentrations of leucine, isoleucine, valine, glutamic acid, glutamine and citric acid compared to other species. Alanine was found to be significantly higher in *T. binghami*, while MMA was the significant metabolite of *T. apicalis*. Therefore, the results suggested that amino acids, along with their polyphenols, significantly contributed to the antioxidant activity of stingless bee honey.

Previous studies have reported that phenolics and aromatic compounds are primarily responsible for the antioxidant activity of honey [41–43]. Specific phenolic compounds found in honey, such as gallic acid, chlorogenic acid, coumaric acid and ferulic acid, as well as flavonoids like apigenin, quercetin, kaempferol, luteolin and hesperetin, exhibited antioxidant properties. According to Smetanska et al. [43], the antioxidant activity of honey was attributed not only to its phenolic and flavonoid content but also to its carotenoids and vitamin content. Moreover, the most common essential amino acids, such as glutamine, alanine, valine, isoleucine and threonine, played important roles as precursors for aromatic compounds and flavours. It has also been reported that these amino acids had a strong correlation to the antioxidant properties of honey [44].

Molecular networking (MN) aided the annotation of metabolites in *T. apicalis* stingless bee honey

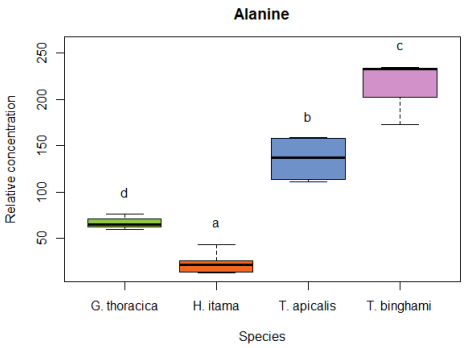
Based on the observation in the PLS biplot, it was found that TPC also had a positive contribution to antioxidant activity, with *T. apicalis* exhibiting the highest TPC amongst the species. Nevertheless, due to its low concentration in the ^{1}H-NMR spectrum, the present authors were unable to identify the phenolics and flavonoids. Therefore, LC-ESI-MS/MS and molecular networking (MN) analyses were conducted to elucidate the polyphenols of *T. apicalis* stingless bee honey. Metabolite identification was done based on accurate molecular mass and its MS/MS fragment ions as shown in TIC-MS (**Figure 5**). Further confirmation was performed using available literature and online databases, such as mzCloud (https://www.mzcloud.org/), Metabolomic work -bench (https://www.metabolomicsworkbench.org/) and Human Metabolome Database (HMDB) (https://hmdb.ca/).

The identified metabolites' retention time (rt), mass-to-charge ratio (m/z) of the detected molecular ions, ionisation mode, fragment ions, compound classes and identifications are summarised in Table S3. The comprehensive molecular networking analysis via GNPS revealed that the metabolite profile of *T. apicalis* included 731 nodes organised into 49 clusters (each with a minimum of two connected nodes) and self-regarding nodes, as shown in Figure S3. In this study, the main identified secondary metabolites were phenolic compounds and flavonoids. Cluster A consisted of the identified lipids, while clusters B and C contained the identified flavonoids and their derivatives, respectively. Phenolic acids and their derivatives were grouped in cluster D, while clusters E and F included amino acids and organic acids.

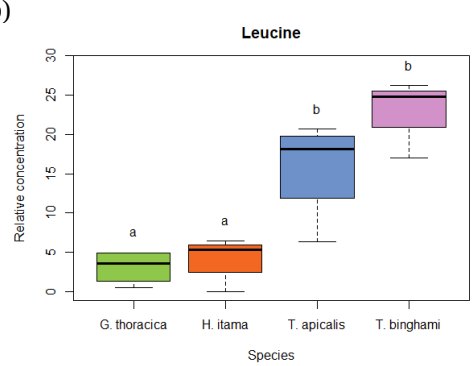
Identification of flavonoids and its derivatives

Several flavonoids, including quercetin, naringenin, kaempferol, genistein, apigenin, hesperetin, isorhamnetin, quercitrin and flavonoid glucosides (kaempferol-5-methyl ether 3-galactoside-4'-glucoside and kaempferol-7-O-glucoside), were detected in *T. apicalis*, as listed in Table S3. The fragmentation pattern of flavonoids can be identified based on the presence of common fragment ions corresponding to specific functional groups or substituents in the parent mass ions, including the flavone base peak at m/z 120, the flavonol base peak at m/z 151 and the anthocyanin base peak at m/z 287. Furthermore, the MN analysis managed to differentiate between the flavonoid isomers in the same cluster, as presented in Figure S3. For example, narigenin, narigenin-7-O-glucoside, naringenin-7-O-beta-D-xylopyranoside and eriodictyol were detected in cluster B in negative ion mode. They shared some similar characteristics and fragment patterns, which were linked with the cosine presented in MN.

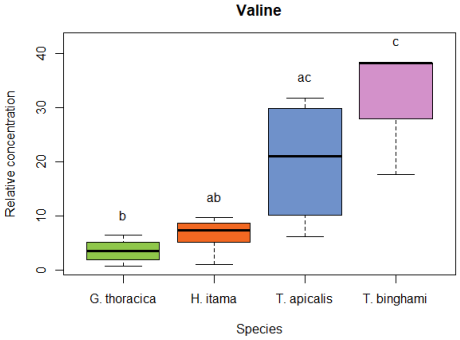
(a)


Alanine
Relative concentration
50
100
150
200
250
d
a
b
c
G. thoracica
H. itama
T. apicalis
T. binghami
Species

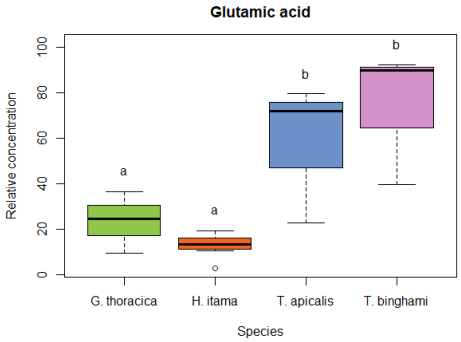
(b)


Leucine
Relative concentration
0
5
10
15
20
25
30
a
a
b
b
G. thoracica
H. itama
T. apicalis
T. binghami
Species

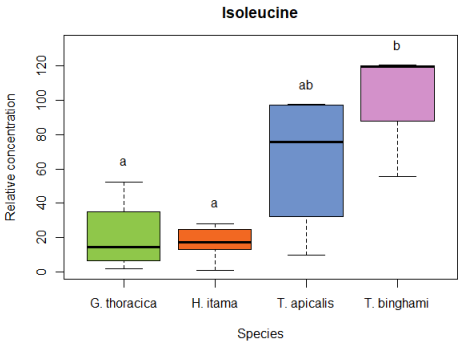
(c)


Valine
Relative concentration
0
10
20
30
40
b
ab
ac
c
G. thoracica
H. itama
T. apicalis
T. binghami
Species

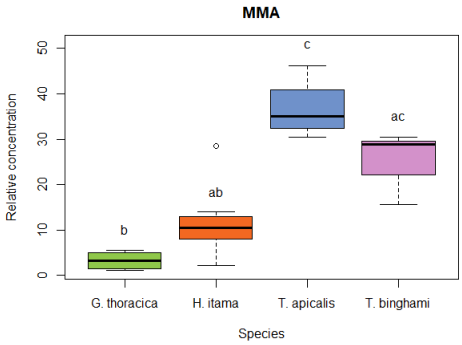
(d)


Glutamic acid
Relative concentration
0
20
40
60
80
100
a
a
b
b
G. thoracica
H. itama
T. apicalis
T. binghami
Species

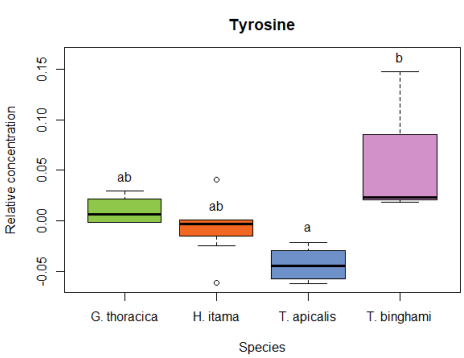
(e)


Isoleucine
Relative concentration
0
20
40
60
80
100
120
a
a
ab
b
G. thoracica
H. itama
T. apicalis
T. binghami
Species

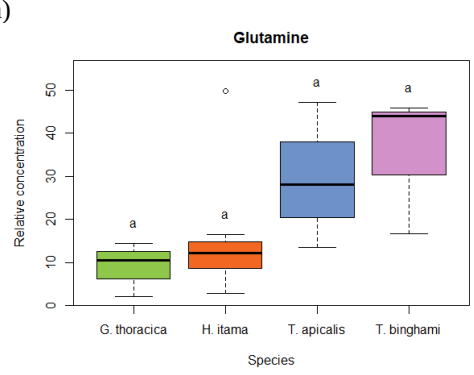
(f)


MMA
Relative concentration
0
10
20
30
40
50
b
ab
c
ac
G. thoracica
H. itama
T. apicalis
T. binghami
Species

(g)


Tyrosine
Relative concentration
-0.05
0.00
0.05
0.10
0.15
ab
ab
a
b
G. thoracica
H. itama
T. apicalis
T. binghami
Species

(h)


Glutamine
Relative concentration
0
10
20
30
40
50
a
a
a
a
G. thoracica
H. itama
T. apicalis
T. binghami
Species

(i)

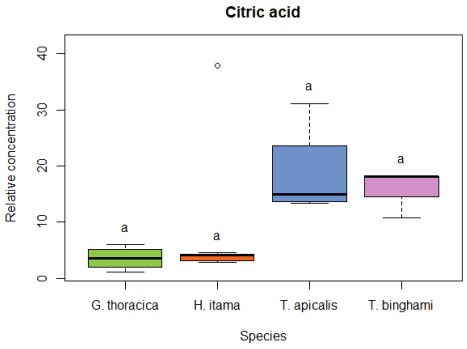


Figure 4. Box plots of the relative concentrations for metabolites in stingless bee honey samples from different species. Plots a, b, c and ab show significant differences between the honey samples at p <0.05. The same plots illustrate the relative concentrations that are not significantly different from each other.

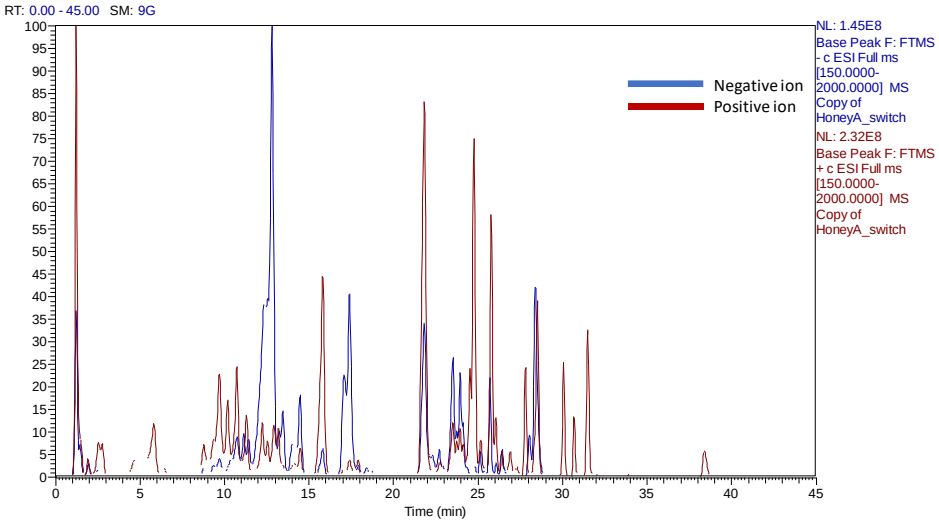


Figure 5. LCMS chromatogram of the polyphenol extract of *T. apicalis* in positive and negative ion modes.

Naringenin was identified based on the molecular ion at m/z 271 in $[M-H]^-$, together with its fragment ions at m/z 151, 119 and 107, which corresponded to the loss of glucoside moiety, carbonyl group and phloroglucinol moiety from its structure, respectively (Figure S4). Eriodictyol was identified based on the presence of base peak at m/z 287, and it consisted similar fragment ions with naringenin (Figure S5). Naringenin-7-O-glucoside (m/z 433) (Figure S6) and narigenin-7-O-beta-D-xylopyranoside (m/z 403) (Figure S7) were both glycosylated derivatives of naringenin. In these compounds, a glucose or a xylopyranosyl group was attached to the 7-hydroxyl group of the naringenin molecule, respectively. The m/z value of each compound in LC-MS corresponded to the mass of the parent molecule and the mass of the attached sugar moiety. The fragmentation pattern of these compounds might include product ions that were similar to those of naringenin, such as the prominent ions at m/z 151, 119 and 107. Additionally, fragment ion at m/z 271 was detected in both compounds, which defined the loss of the attached sugar moiety (glucose or xylopyranosyl group).

Besides, quercitrin was identified based on the molecular ion at m/z 447.0944 and its fragment ions at m/z 301, 271 and 151, respectively (Figure S6). The characteristic product ion at m/z 301 corresponded to quercetin aglycone with the loss of the attached rhamnose group. Quercetin aglycone was also identified based on the molecular ion at m/z 301 [M-H], along with its fragment ion at m/z 179,151, 121 and 107, which corresponded to glucoside moiety, carbonyl group, hydroxyl moiety and phloroglucinol moiety, respectively (Figure S9). The major mass fragmentation of other flavonoids, such as luteolin (Figure S10) and isorhamnetin (Figure S12) included the prominent product ions at m/z 151 and 107, which were similar to those of quercetin. Moreover, kaempferol was identified according to the present molecular ion at m/z 285 $[M-H]^-$, together with characteristic fragment ions at nominal m/z 151, 121,

and 109. The fragment ion at m/z 109 corresponded to the loss of a formaldehyde moiety from the molecular ion at m/z 151 (Figure S11). Flavonoids were the major components that contributed to the antioxidant properties as reported by previous studies [45, 46]. By understanding the unique MS/MS patterns of flavonoids in honey-based products, their chemical compositions associated with quality and potential health benefits could be comprehensively identified.

Identification of phenolic acids and their derivatives

Phenolic compounds are common secondary metabolites of plants. Botanical and geographical origins influence their content in honey. In addition, phenolic compounds in honey have attracted significant attention because they can serve as authenticity markers and offer various health benefits, including antioxidant, anti-inflammatory and antimicrobial activities [47–49]. In this study, caffeoyl-, diferuloyl- and chlorogenic acids were defined as the main phenolic components in *T. apicalis* stingless bee honey. They were found mainly conjugated with quinic acid.

According to the observation of the MN analysis, cluster D revealed the presence of caffeoylquinic acid compounds based on the molecular ion at m/z 353.1006 $[M-H]^-$. They were annotated as caffeoylquinic acid and caffeoylquinic acid isomer. Similarly, both compounds revealed product ions at m/z 165, 147 and 119, corresponding to quinic acid, caffeic acid and their decarboxylated ions, respectively. The self-regarding nodes of the molecular ion peak at m/z 439.1873 and the characteristic fragment ions at nominal m/z 289, 247, 149 and 134 led to the identification of diferuloylputrescine. The fragment ions indicated the loss of a putrescine moiety (m/z 289), both ferulic acid and putrescine (m/z 247), ferulic acid alone (m/z 149) and both ferulic acid and carbonyl moieties (m/z 134) from the molecular ion.

Identification of amino acid and organic acid

T. apicalis stingless bee honey consisted of various organic acids, including acetic acid, butyric acid, formic acid, gluconic acid, lactic acid and malic acid (Table S3). In addition, small amounts of amino acids, such as proline, phenylalanine and lysine, were identified in the honey sample. Their corresponding MS/MS spectral data displayed abundant ions due to the loss of CH_2, CO_2 and H_2O groups.

Observation of cluster E in the MN analysis led to the identification of an amino acid, N-acetyl-L-phenylalanine, which could be further confirmed based on its MS/MS data. The molecular ion at m/z 206.081 $[M-H]^-$ of N-acetyl-L-phenylalanine would typically correspond to the characteristic fragment ions of N-acetyl group (m/z 42), the phenylalanine residue (m/z 166) and potentially the phenylalanine side chain (m/z 91 or 120). The presence of these fragments would confirm the identification of N-acetyl-L-phenylalanine. The other two fragment ions at nominal m/z 164 and 147 were also observed. The m/z 164 fragment ion corresponded to the loss of a neutral molecule of acetic acid (CH_3COOH), while the m/z 147 fragment ion corresponded to further fragmentation of the m/z 164 fragment, resulting in the loss of a neutral molecule of carbon dioxide (CO_2).

Identification of fatty acids and terpenoids

Honey is primarily composed of carbohydrates (e.g., glucose, fructose and sucrose), but it also contains a small amount of lipids or fatty acids. It is nonetheless an important component that contributes to the nutritional and sensory properties of this natural sweetener. The lipids in honey, along with its other bioactive compounds, make honey a unique and potentially beneficial food for human consumption. In previous studies, some fatty acids found in honey, such as palmitic acid, oleic acid and linoleic acid, were shown to have anti-inflammatory and antioxidant effects [50]. In this study, some fatty acids identified were 10-hydroxydecanoic acid, ethyl oleic acid and azelaic acid (Table S3).

Based on the MN analysis, cluster A, composed of three nodes, which were assigned as follows: the first node at m/z 219.1736 $[M+H]^+$ was identified as nootkatone, the second node at m/z 221.1895 $[M+H]^+$ was identified as nootkatol and the third node with a molecular ion at m/z 223 remained unidentified (Table S3). Nookatone and nootkatol were structurally similar terpenoid compounds and produced similar fragment ions at m/z 133, 95, 81 and 69. Nootkatol showed fragment ions at m/z 203 and 107, which corresponded to the loss of water molecules and cleavage of a C-C bond, whereas nookatone showed fragment ions at nominal m/z 201, 123 and 83. The m/z 201 fragment could be formed due to the loss of water molecules, while m/z 123 corresponded to the cleavage of a C-C bond. Meanwhile, m/z 83 indicated the loss of CH_3 radical from the cyclopropane ring.

Conclusion

The antioxidant properties of stingless bee honey of different bee species (*T. apicalis*, *H. itama*, *G. thoracica*, *L. canifrons* and *T. binghami*) were evaluated. Amongst the species, *T. apicalis* showed the highest potential contribution to antioxidant properties. This outcome indicated that stingless bee honey from different species remarkably influenced the variation of the antioxidant activity. Additionally, this study successfully discriminated the stingless bee honey from different species by using ^{1}H-NMR

metabolomics. Most amino acids and phenolics were responsible for their antioxidant activity properties according to the PLS analysis. Complementary results on the discrimination of stingless bee honey species were obtained using untargeted analysis (PCA), targeted analysis (PLS-DA) and heatmap analysis. *T. binghami* and *T. apicalis* shared similar chemical characteristics, while the chemical profile of *L. canifrons* was similar to that of *G. thoracica*. *H. itama* showed a distinct chemical profile amongst the species. Moreover, the multivariate data analysis was able to distinguish the stingless bee honey species based on the amino acid profile. The LCMS-MS analysis, in combination with molecular networking, managed to identify and characterise the unknown metabolites present in the *T. apicalis* stingless bee honey, such as phenolic and flavonoids. Molecular networking aided in identifying metabolites with similar structures or biosynthesis origins. The combination of these techniques provided a more comprehensive understanding on the bioactive components of stingless bee honey that are related to its nutritional and therapeutic properties. Therefore, the methods applied in this study may provide new cost-effective tools for the authentication and quality control of Malaysian stingless bee honey. Lastly, the methods are beneficial for identifying stingless bee honey with antioxidant properties, which could have broad applications across various fields. These fields include functional foods and nutraceuticals to combat oxidative stress, pharmaceuticals for disease prevention, natural preservatives in food products and complementary medicine due to its therapeutic and healing properties.

Acknowledgements

The authors would like to thank Universiti Malaysia Terengganu (UMT) for providing the support through the Talent and Publication Enhancement Research Grant (UMT/TAPE-RG2020/55257). They would also like to thank the Ministry of Higher Education Malaysia for providing the MyBrainSc scholarship to Kok Suet Cheng.

Supplementary data

The supplementary materials of this manuscript are available and can be found at https://doi.org/10.5281/zenodo.11066038.

References

1. Shamsudin, S., Selamat, J., Sanny, M., Abd. Razak, S.-B., Jambari, N.N., Mian, Z. and Khatib, A. (2019). Influence of origins and bee species on physicochemical, antioxidant properties and botanical discrimination of stingless bee honey. *International Journal of Food Properties*, 22(1): 239–264.
2. Rao, P.V., Krishnan, K.T., Salleh, N. and Gan, S.H. (2016). Biological and therapeutic effects of honey produced by honey bees and stingless bees: a comparative review. *Revista Brasileira de Farmacognosia*, 26(5): 657-664.
3. Kek, S.P., Chin, N.L., Yusof, Y.A., Tan, S.W. and Chua, L.S. (2017). Classification of entomological origin of honey based on its physicochemical and antioxidant properties. *International Journal of Food Properties*, 20(sup3): S2723-S2738.
4. Tuksitha, L., Chen, Y.L.S., Chen, Y.L., Wong, K.Y. and Peng, C.C. (2018). Antioxidant and antibacterial capacity of stingless bee honey from Borneo (Sarawak). *Journal of Asia-Pacific Entomology*, 21(2): 563-570.
5. Selvaraju, K., Vikram, P., Soon, J.M., Krishnan, K.T. and Mohammed, A. (2019). Melissopalynological, physicochemical and antioxidant properties of honey from west coast of Malaysia. *Journal of Food Science and Technology*, 56(5): 2508-2521.
6. Shamsudin, S., Selamat, J., Sanny, M., Shamsul Bahari, A.R., Jambari, N.N. and Khatib, A. (2019). A comparative characterization of physicochemical and antioxidants properties of processed Heterotrigona Itama honey from different origins and classification by chemometrics analysis. *Molecules*, 24(21): 3898.
7. Lajis, N., Maulidiani, M., Abas, F. and Ismail, I.S. (2017). Metabolomics approach in pharmacognosy. *Pharmacognosy*, 2017: 597-616.
8. Roberts, L.D., Souza, A.L., Gerszten, R.E. and Clish, C.B. (2012). Targeted metabolomics. *Current Protocols in Molecular Biology*, 98(1): 3002.
9. Szymańska, E., Saccenti, E., Smilde, A.K. and Westerhuis, J.A. (2012). Double-check: validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics*, 8(S1): 3–16.
10. Trygg, J. and Wold, S. (2002). Orthogonal Projections to Latent Structures (O-PLS). *Journal of Chemometrics*, 16(3): 119–128.
11. Yusoff, Y.M., Abbott, G., Young, L. and Edrada-Ebel, R. (2022). Metabolomic profiling of Malaysian and New Zealand honey using concatenated NMR and HRMS datasets. *Metabolites*, 12(1):85.
12. Shamsudin, S., Selamat, J., Sanny, M., Jambari, N.N., Sukor, R., Salleh, N.A., Aziz, M.F.A. and Khatib, A. (2022). Integrated gas chromatography–mass spectrometry and liquid chromatography-quadruple time of flight-mass spectrometry-based untargeted metabolomics reveal possible metabolites related to antioxidant

activity in stingless bee honey. *Food Analytical Methods*. 15(11): 3209-3224.
13. Montoro, P., D'urso, G., Kowalczyk, A. and Tuberoso, C.I.G. (2021). LC-ESI/LTQ-Orbitrap-MS based metabolomics in evaluation of bitter taste of *Arbutus unedo* honey. *Molecules*, 26(9): 1-14.
14. Bittencourt, M.L.F., Ribeiro, P.R., Franco, R.L.P., Hilhorst, H.W.M., de Castro, R.D. and Fernandez, L.G. (2015). Metabolite profiling, antioxidant and antibacterial activities of brazilian propolis: use of correlation and multivariate analyses to identify potential bioactive compounds. *Food Research International*, 76: 449-457.
15. Zhao, H., Cheng, N., Wang, Q., Zhou, W., Liu, C., Liu, X., Chen, S., Fan, D. and Cao, W. (2019). Effects of honey-extracted polyphenols on serum antioxidant capacity and metabolic phenotype in rats. *Food & Function*, 10(5): 2347-2358.
16. Mannina, L., Sobolev, A.P., Coppo, E., Di Lorenzo, A., Nabavi, S.M., Marchese, A. and Daglia, M. (2016) Antistaphylococcal activity and metabolite profiling of manuka honey (*Leptospermum scoparium L.*) after in vitro simulated digestion. *Food & Function*, 7(3): 1664-1670.
17. Guo, N., Zhao, L., Zhao, Y., Li, Q., Xue, X., Wu, L., Gomez Escalada, M., Wang, K. and Peng, W. (2020). Comparison of the chemical composition and biological activity of mature and immature honey: an HPLC/QTOF/MS-based metabolomic approach. *Journal of Agricultural and Food Chemistry*, 68(13): 4062-4071.
18. Muhammad, A. (2016). Potential biological activity of Acacia honey. *Frontiers in Bioscience*, 8(2): 771.
19. Shamsudin, S., Selamat, J., Abdul Shomad, M., Ab Aziz, M.F. and Haque Akanda, Md. J. (2022). Antioxidant properties and characterization of *Heterotrigona itama* honey from various botanical origins according to their polyphenol compounds. *Journal of Food Quality*, 2022: 1-14.
20. Yong, C.H., Muhammad, S.A., Aziz, F.A., Nasir, F.I., Mustafa, M.Z., Ibrahim, B., Kelly, S.D., Cannavan, A. and Seow, E.K. (2022). Detecting adulteration of stingless bee honey using untargeted ^{1}H NMR metabolomics with chemometrics. *Food Chemistry*, 368:130808.
21. Razali, M.T.A., Zainal, Z.A., Maulidiani, M., Shaari, K., Zamri, Z., Idrus, M.Z.M., Khatib, A., Abas, F., Ling, Y.S., Rui, L.L. and Ismail, I.S. (2018). Classification of raw stingless bee honeys by bee species origins using the NMR- and LC-MS-based metabolomics approach. *Molecules*, 23(9): 2160.
22. Tan, S.H., Pui, L.P., Solihin, M.I., Keat, K.S., Lim, W.H. and Ang, C.K. (2021). Physicochemical analysis and adulteration detection in Malaysia stingless bee honey using a handheld near-infrared spectrometer. *Journal of Food Processing and Preservation*, 45(7): e15576.
23. Se, K.W., Ghoshal, S.K., Wahab, R.A., Ibrahim, R.K.R. and Lani, M.N. (2018). A simple approach for rapid detection and quantification of adulterants in stingless bees (*Heterotrigona itama*) honey. *Food Research International*, 105: 453-460.
24. Sharin, S.N., Sani, M.S.A., Jaafar, M.A., Yuswan, M.H., Kassim, N.K., Manaf, Y.N., Wasoh, H., Zaki, N.N.M. and Hashim, A.M. (2021). Discrimination of Malaysian stingless bee honey from different entomological origins based on physicochemical properties and volatile compound profiles using chemometrics and machine learning. *Food Chemistry*, 346: 128654.
25. Ismail, N.F., Maulidiani, M., Omar, S., Zulkifli, M.F., Mohd Radzi, M.N.F., Ismail, N., Jusoh, A.Z., Roowi, S., Yew, W.M., Rudiyanto, R. and Ismail, W.I.W. (2021). Classification of stingless bee honey based on species, dehumidification process and geographical origins using physicochemical and ATR-FTIR chemometric approach. *Journal of Food Composition and Analysis*, 104: 104126.
26. Hegazi, N.M., Khattab, A.R., Frolov, A., Wessjohann, L.A. and Farag, M.A. (2022). Authentication of saffron spice accessions from its common substitutes via a multiplex approach of UV/VIS fingerprints and UPLC/MS using molecular networking and chemometrics. *Food Chemistry*, 367: 130739.
27. Abd Ghafar, S.Z., Mediani, A., Maulidiani, Ramli, N.S. and Abas, F. (2018). Antioxidant, α-glucosidase, and nitric oxide inhibitory activities of *Phyllanthus acidus* and LC–MS/MS profile of the active extract. *Food Bioscience*, 25:134-140.
28. Xia, J. and Wishart, D.S. (2016). Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis. *Current Protocols in Bioinformatics*, 55(1).
29. Romera-Torres, A., Romero-González, R., Martínez Vidal, J.L. and Garrido Frenich, A. (2020). Comprehensive tropane alkaloids analysis and retrospective screening of contaminants in honey samples using liquid chromatography-high resolution mass spectrometry (Orbitrap). *Food Research International*, 133:109130.
30. Wang, M., Carver, J.J., Phelan, V. V, Sanchez, L.M., Garg, N., Peng, Y., Nguyen, D.D., Watrous, J., Kapono, C.A., Luzzatto-Knaan, T.,

Porto, C., Bouslimani, A., Melnik, A. V, Meehan, M.J., Liu, W.-T., Crüsemann, M., Boudreau, P.D., Esquenazi, E., Sandoval-Calderón, M., Kersten, R.D., Pace, L.A., Quinn, R.A., Duncan, K.R., Hsu, C.-C., Floros, D.J., Gavilan, R.G., Kleigrewe, K., Northen, T., Dutton, R.J., Parrot, D., Carlson, E.E., Aigle, B., Michelsen, C.F., Jelsbak, L., Sohlenkamp, C., Pevzner, P., Edlund, A., McLean, J., Piel, J., Murphy, B.T., Gerwick, L., Liaw, C.-C., Yang, Y.-L., Humpf, H.-U., Maansson, M., Keyzers, R.A., Sims, A.C., Johnson, A.R., Sidebottom, A.M., Sedio, B.E., Klitgaard, A., Larson, C.B., Boya P, C.A., Torres-Mendoza, D., Gonzalez, D.J., Silva, D.B., Marques, L.M., Demarque, D.P., Pociute, E., O'Neill, E.C., Briand, E., Helfrich, E.J.N., Granatosky, E.A., Glukhov, E., Ryffel, F., Houson, H., Mohimani, H., Kharbush, J.J., Zeng, Y., Vorholt, J.A., Kurita, K.L., Charusanti, P., McPhail, K.L., Nielsen, K.F., Vuong, L., Elfeki, M., Traxler, M.F., Engene, N., Koyama, N., Vining, O.B., Baric, R., Silva, R.R., Mascuch, S.J., Tomasi, S., Jenkins, S., Macherla, V., Hoffman, T., Agarwal, V., Williams, P.G., Dai, J., Neupane, R., Gurr, J., Rodríguez, A.M.C., Lamsa, A., Zhang, C., Dorrestein, K., Duggan, B.M., Almaliti, J., et al. (2016). Sharing and community curation of mass spectrometry data with global natural products social molecular networking. *Nature Biotechnology*, 34(8): 828–837.

31. Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B. and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research*, 13(11): 2498-2504.
32. Kek, S.P., Chin, N.L., Yusof, Y.A., Tan, S.W. and Chua, L.S. (2014). Total phenolic contents and colour intensity of Malaysian honeys from the *Apis spp*. and *Trigona spp*. bees. *Agriculture and Agricultural Science Procedia*, 2: 150-155.
33. Maringgal, B., Hashim, N., Tawakkal, I.S.M.A., Mohamed, M.T.M. and Hamzah, M.H. (2021). Phytochemical content, antioxidant activity and mineral elements of honey produced by four different species of Malaysian stingless bees. *Food Research*, 5(S1): 39-46.
34. Samat, S., Kanyan Enchang, F., Nor Hussein, F. and Wan Ismail, W.I. (2017). Four-week consumption of Malaysian honey reduces excess weight gain and improves obesity-related parameters in high fat diet induced obese rats. *Evidence-based Complementary and Alternative Medicine*, 2017: 1-9.
35. Kancheva, V., Taskova, R., Totseva, I. and Handjieva, N. (2007). Antioxidant activity of extracts, fractions and flavonoid constituents from *Carthamus lanatus L*. *Rivista Italiana delle Sostanze Grasse*, 84(2): 77-86.
36. Abdullah, N.A., Zullkiflee, N., Zaini, S.N.Z., Taha, H., Hashim, F. and Usman, A. (2020). Phytochemicals, mineral contents, antioxidants, and antimicrobial activities of propolis produced by Brunei stingless bees *Geniotrigona thoracica, Heterotrigona itama*, and *Tetrigona binghami*. *Saudi Journal of Biological Sciences*, 27(11): 2902-2929.
37. Maringgal, B., Hashim, N., Tawakkal, I.S.M.A., Hamzah, M.H. and Mohamed, M.T.M. (2020). Biosynthesis of CaO nanoparticles using *Trigona sp*. honey: physicochemical characterization, antifungal activity, and cytotoxicity properties. *Journal of Materials Research and Technology*, 9(5): 11756-11768.
38. Fadzilah, N.H., Jaapar, M.F., Jajuli, R. and Wan Omar, W.A. (2017). Total phenolic content, total flavonoid and antioxidant activity of ethanolic bee pollen extracts from three species of Malaysian stingless bee. *Journal of Apicultural Research*, 56(2): 130–135.
39. Wong, P., Hii, S.L., Koh, C.C., Moh, T.S.Y. and Gindi, S.R.A. (2019). Chemical analysis on the honey of *Heterotrigona itam*a and *Tetrigona binghami* from Sarawak, Malaysia. *Sains Malaysiana*, 48(8): 1635-1642.
40. Maulidiani, Abas, F., Khatib, A., Shitan, M., Shaari, K. and Lajis, N.H. (2013). Comparison of partial least squares and artificial neural network for the prediction of antioxidant activity in extract of pegaga (centella) varieties from ^{1}H nuclear magnetic resonance spectroscopy. *Food Research International*, 54(1): 852-860.
41. Guldas, M., Gurbuz, O., Cakmak, I., Yildiz, E. and Sen, H. (2022). Effects of honey enrichment with *Spirulina platensis* on phenolics, bioaccessibility, antioxidant capacity and fatty acids. *LWT*, 153: 112461.
42. Guldas, M., Demircan, H., Cakmak, I., Oral, R.A., Yildiz, E., Gurbuz, O., Tosunoglu, H., Cavus, F. and Sen, H. (2022). Antioxidant and bioaccessibility characteristics of functional fruit and vegetable honeys produced by innovative method. *Food Bioscience*, 48: 101732.
43. Smetanska, I., Alharthi, S.S. and Selim, K.A. (2021). Physicochemical, antioxidant capacity and color analysis of six honeys from different origin. *Journal of King Saud University - Science*, 33(5): 101447.
44. Jonathan Chessum, K., Chen, T., Hamid, N. and Kam, R. (2022). A comprehensive chemical analysis of New Zealand honeydew honey. *Food Research International*, 157: 111436.
45. Al-Farsi, M., Al-Amri, A., Al-Hadhrami, A. and Al-Belushi, S. (2018). Color, flavonoids,

phenolics and antioxidants of omani honey. *Heliyon*, 4(10): e00874.

46. Ullah, A., Munir, S., Badshah, S.L., Khan, N., Ghani, L., Poulson, B.G., Emwas, A.-H. and Jaremko, M. (2020). Important flavonoids and their role as a therapeutic agent. *Molecules*, 25(22): 5243.
47. Koulis, G.A., Tsagkaris, A.S., Aalizadeh, R., Dasenaki, M.E., Panagopoulou, E.I., Drivelos, S., Halagarda, M., Georgiou, C.A., Proestos, C. and Thomaidis, N.S. (2021) Honey phenolic compound profiling and authenticity assessment using HRMS targeted and untargeted metabolomics. *Molecules*, 26(9): 2769.
48. Cianciosi, D., Forbes-Hernández, T., Afrin, S., Gasparrini, M., Reboredo-Rodriguez, P., Manna, P., Zhang, J., Bravo Lamas, L., Martínez Flórez, S., Agudo Toyos, P., Quiles, J., Giampieri, F. and Battino, M. (2018). Phenolic compounds in honey and their associated health benefits: a review. *Molecules*, 23(9): 2322.
49. Gašić, U.M., Milojković-Opsenica, D.M. and Tešić, Ž.L. (2017) Polyphenols as possible markers of botanical origin of honey. *Journal of AOAC International*, 100(4): 852-861.
50. Naik, R.R., Shakya, A.K., Oriquat, G.A., Katekhaye, S., Paradkar, A., Fearnley, H. and Fearnley, J. (2021) Fatty acid analysis, chemical constituents, biological activity and pesticide residues screening in Jordanian propolis. *Molecules*, 26(16): 5076.