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Research Article

Chemical Profile, Physicochemical Properties and Antioxidant Activity of Malaysian Propolis: Insights from Honeybee and Stingless Bee

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ABSTRACT

This study investigated the chemical composition, physicochemical properties and antioxidant activities of propolis from two distinct bee species, *Apis dorsata* (honeybees) and *Heterotrigona itama* (stingless bees), collected from different regions in Malaysia. The chemical profile of propolis samples was examined using nuclear magnetic resonance (NMR) spectroscopy while metabolite identification was performed using AssureNMR™ 2.0. Additionally, a range of physicochemical analyses was conducted to determine pH, moisture, lipid, resin and wax contents. Determination of total phenolic content (TPC) and total flavonoid content (TFC) was carried out using Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively. Antioxidant activities were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. The NMR analysis revealed distinct chemical compositions between *A. dorsata* and *H. itama* propolis. Notably, α-D-glucose was prominent in *A. dorsata* propolis, whereas *H. itama* propolis exhibited higher levels of L-lactic acid and phenolic compounds. *H. itama* propolis also demonstrated significantly higher levels of resin, lipid, and wax contents, coupled with lower moisture content compared to *A. dorsata* propolis. Additionally, *H. itama* propolis extract exhibited significantly higher TFC and antioxidant activities compared to *A. dorsata*. A significant and strong correlation between TPC, TFC and the antioxidant properties of propolis was observed. These findings suggest that *H. itama* propolis exhibits promising antioxidant efficacy, warranting further research to identify its specific compounds and potential health applications.

Keywords: propolis, *Heterotrigona itama*, *Apis dorsata*, antioxidant, phytochemical, physicochemical

1. INTRODUCTION

Propolis is a complex mixture of plant resin, beeswax, pollen and enzymes produced by bees. It serves a multifaceted role within the beehive, acting as a sealant, structural support and safeguarding the hive against external threats. Bees manufacture propolis by combining insect saliva, rich in enzymes, with resins obtained from buds, exudates and various plant components (Portal and de Cordova, 2024). Both honeybees and stingless bees are capable of producing propolis (Bonamigo et al., 2017). Stingless bees produce a higher amount of propolis compared to its honeybee counterpart to compensate for their lack of functional stingers, as a protective measure against predatory and microbial invasions (Al-Hatamleh et al., 2020; Tirtasari et al., 2024). Honeybees construct their hives with the combination of waxes, plant exudates and saliva, whereas stingless bees use propolis or plant resin as the key components for the construction of their nests (Bonamigo et al., 2017; Al-Hatamleh et al., 2020).

*A*pis *dorsata* Fabricius, the giant honeybee, is notable for its large size and significant role in honey production (Robinson, 2012). Native to Asia, *A. dorsata* is predominantly found in South and Southeast Asia, inhabiting diverse environments ranging from tropical lowlands to mountainous forest regions (Roy et al., 2011; Huang et al., 2022). This well-regarded species in apicultural practice constructs striking single-comb nests, typically hanging from tree branches or elevated structures, characterized by a series of vertically aligned combs. The nests are typically exposed, which allows for effective thermoregulation and defense but also makes them susceptible to environmental stressors and predators (Koeniger et al., 2017). *A. dorsata* is well known for its aggressive stinging attacks, effectively deterring a wide array of predators (Srinivasan et al., 2024).

Heterotrigona itama, one of the most common stingless bee species in Malaysian meliponiculture, is known to produce significant amounts of propolis in comparison to honeybees (Ibrahim et al., 2016). *H. itama* builds its nests in protected cavities such as hollow tree trunks, underground burrows, or man-made structures like wall crevices. This bee species constructs a unique hive structure featuring a horizontal comb formation, with each comb distinctly separated from the preceding one by support pillars (Purwanto et al., 2022). This architectural design, enriched with propolis, serves as a foundation for hive defence mechanisms and environmental adaptability (Agussalim et al., 2015). *H. itama*, commonly known as the "black jet" owing to its distinctive black colour and grey wings, is widely distributed across the Malay Archipelago, encompassing Peninsular Malaysia, Malaysian Borneo, Southern Thailand, Singapore, and the islands of Java, Kalimantan and Sumatra (Azmi et al., 2022).

Research studies have highlighted the diverse chemical composition of honeybee propolis, identifying phenolic compounds as the secondary metabolites responsible for its various pharmacological properties (Anjum et al., 2019; Hossain et al., 2022). These properties have paved the way for the development of numerous commercial products derived from honeybee propolis (Abdelrazeg et al., 2020). Nevertheless, there is growing scientific interest in studying stingless bee propolis due to their access to unique flora, potentially resulting in distinct physicochemical and bioactive profiles (Rocha et al., 2023). Claims suggest that stingless bee propolis may exceed honeybee propolis in bioactive complexity due to their diverse botanical sources (Al-Hatamleh et al., 2020). Nevertheless, there is a current lack of scientific evidence supporting this perspective.

Hence, this study aimed to conduct a comparative analysis of the chemical composition, physicochemical characteristics and antioxidant properties of propolis produced by *A. dorsata* and *H. itama*. Additionally, the correlation between the physicochemical properties, phenolic content, flavonoid content and antioxidant activity of the propolis samples was also investigated.

2. MATERIALS AND METHODS

2.1. Collection of propolis

Propolis from *A. dorsata* was obtained from wild sources through Hannah Maryam Legacy, Tanjung Malim, Perak in February 2023. The area is typified by dense forest cover, with the prevalence of tropical hardwood trees such as *Neobalanocarpus heimii*, *Acacia mangium*, *Shorea leprosula* and *Intsia palembanica*. Propolis was produced by *H. itama*, which was collected in October 2022 from Ladang Mini Kelulut of Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor. The apiary site is predominantly surrounded by a diverse range of herbal plants, including *Eurycoma longifolia*, *Labisa pumila*, *Clinacanthus nutans*, *Zingiber zerumbet*, *Andrographis paniculate* and *Orthosiphon stamineus*.

2.2. Preparation of propolis extract

Each sample was finely powdered after cleaning. The ethanolic propolis extract was prepared following the protocol described by Zohdi et al. (2024). The propolis samples were macerated in 70% ethanol (1:10 w/v) for 48 hours at room temperature with continuous agitation. Following filtration, the extract was evaporated under vacuum and concentrated using a rotary evaporator. The concentrated extract underwent centrifugation at 2500 rpm for 5 min to remove wax residues, followed by freeze-drying at -110°C. Samples were then stored at -20°C until further analysis.

2.3. Nuclear Magnetic Resonance (NMR) analysis

The ^{1}H NMR spectra were obtained using Bruker Ascend 500 MHz spectrometer equipped with a 5 mm room temperature probe. Data processing was conducted with Bruker TopSpin 3.6 software. The routine pulse sequence utilized a 90° pulse duration of 9.61 µs, 64 scans, a spectral width of 16 ppm, a relaxation delay of 5 s and an acquisition time of 3.75 s. Spectra were referenced to the dimethyl sulfoxide (DMSO) residual solvent signal at 2.50 ppm. Identification of organic acids was performed by comparing the spectra against an NMR spectral database (SBASE). This database includes data from reference standards acquired under identical experimental conditions to the test samples.

2.4. Physicochemical characterization of propolis samples

The moisture content of the propolis samples was assessed following the procedures outlined by the Association of Official Analytical Chemists (AOAC). Quantification of lipid, resin, wax and pH levels was conducted in accordance with the methods described by Touzani et al. (2019).

2.5. Total phenolic content (TPC)

The Folin-Ciocalteu method was applied as previously described by Pratami et al. (2018) to determine the TPC value in both propolis extracts, with gallic acid utilized as the standard solution. The procedure involved mixing the extracts and standard solutions with Folin-Ciocalteu reagent in a 96-well microplate. After incubation and shaking, sodium carbonate solution was added to the reaction mixture, followed by further incubation. Absorbance readings were then taken at 765 nm using a microplate reader, and TPC values were calculated based on the standard curve regression line, expressed as mg/mL gallic acid equivalent (GAE).

2.6. Total flavonoid content (TFC)

The TFC values of both extracts were determined using the aluminium chloride ($AlCl_3$) colorimetric method, following the procedure outlined by Farasat et al. (2014). Quercetin was used to establish the standard curve. In the assay, the standard solution and each extract were added to a 96-well microplate, followed by the addition of $AlCl_3$ solution, potassium acetate, and distilled water. The plate was shaken and then incubated in the dark before absorbance readings were taken at 415 nm using a microplate reader. TFC values were calculated based on a linear regression line plotted against the standard curve, expressed as mg/mL quercetin equivalent (QE).

2.7. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The measurements of DPPH free radical scavenging activity of both extracts were carried out following the method outlined by Adli et al. (2024). In brief, DPPH solution was prepared, and various concentrations of the extract and quercetin were serially diluted. Subsequently, the standard and each extract were added to separate wells of a 96-well microplate, followed by the addition of DPPH solution. Following incubation in the dark, the absorbance at 517 nm was assessed, and the scavenging activity was determined using Eq.1. The percentage of DPPH scavenging activity was plotted against the concentration of samples and the IC_{50} values were obtained based on the plot: DPPH scavengng activity (%) = $(A_{blank} - A_{sample}) / (A_{blank}) \times 100$; where A_{blank} is the absorbance of DPPH solution without sample while A_{sample} is the absorbance of DPPH solution with the sample.

2.8. Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted following the protocol outlined by Idris et al. (2023). The FRAP reagent was prepared by combining acetate buffer, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) dissolved in hydrochloric acid and ferric chloride. Standard solutions were prepared at varying concentrations using ferrous sulphate heptahydrate ($FeSO_4.7H_2O$) as the standard. Samples, blanks, positive controls and standards were added to a 96-well plate. FRAP reagent was then added to each well and incubated in the dark at 37°C for 30 min. Absorbance readings were taken at 593 nm using a microplate spectrophotometer and the results were calculated using a linear regression plot.

2.9. Statistical analysis

The experiments were conducted in triplicate and the findings are expressed as mean ±standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 7.0, including one-way analysis of variance (ANOVA) and t-test for comparative analyses. Pearson's correlation test was employed to assess the data correlation. Statistical significance was determined at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. ^{1}H NMR profiling

The chemical composition of propolis samples, as revealed by NMR spectroscopy, exhibited notable variations between the two bee species. The presence of α-D-glucose was abundant in propolis from *A. dorsata*, with peaks observed at δ 4.37 (45.6%), 4.46 (39.9%) and

4.64 (47.1%) (Table 1). Multiple assignments indicated the complexity of glucose-related compounds, with all peaks at δ 4.64 fully identified. Additionally, 2-propanol showed high concentration, with peaks at δ 1.07 (73.3%) and 4.37 (34.9%). 1,2-propanediol exhibited partial assignment, with peaks at δ 1.02 (49.4%), 3.18 (23.7%), 3.28 (25.9%) and 4.42 (27.5%). 4-Aminophenol displayed a high percentage at δ 4.40 (80.3%), indicating its significant presence. 4-Methyl-2-pentanol exhibited notable presence, with all peaks at δ 0.88 fully identified. Glycerol was identified at δ 4.41 (32.1%) and 4.48 (38.9%). L-Lactic acid was detected at δ 4.36 (64.1%), while L-arginine at δ 3.24 (23.7%). L-ascorbic acid was discerned at δ 4.74 (57.5%) and 4.89 (70.5%). These compounds indicate the rich carbohydrate and alcohol content in *A. dorsata* propolis, consistent with previous studies on propolis composition (Hossain et al., 2022; Abdelrazeg et al., 2020; Tran et al., 2022).

Table 1. ^{1}H-NMR chemical shift values associated with metabolites identified in propolis from *A. dorsata*

Metabolites identified	Chemical shift(s) identified in loading plot (ppm)
α-D-Glucose	4.37 (45.6%), 4.46 (39.9%), 4.64 (47.1%)
2-Propanol	1.07 (73.3%), 4.37 (34.9%)
1,2-Propanediol	1.02 (49.4%), 3.18 (23.7%), 3.28 (25.9%), 4.42 (27.5%)
4-Aminophenol	4.40 (80.3%)
4-Methyl-2-pentanol	0.88 (58.3%)
Glycerol	4.41 (32.1%), 4.48 (38.9%)
L-Lactic acid	4.36 (64.1%)
L-Arginine	3.24 (23.7%)
L-Ascorbic acid	4.74 (57.5%)

In contrast, the NMR profile of *H. itama* propolis highlighted the presence of a diverse range of organic and phenolic compounds (Table 2). Notably, L-lactic acid was identified with a peak at δ 1.13 (29.6%) and α-D-glucose showed a peak at δ 4.64 (36.1%). Prominent phenolic compounds such as ferulic acid, *trans*-resveratrol, *m*-coumaric acid and caffeic acid were detected, with ferulic acid manifesting peaks at δ 6.81 (38.0%) and 7.31 (54.5%). These phenolic compounds are known for their antioxidant and anti-inflammatory properties, contributing to the therapeutic potential of propolis (Wieczorek et al., 2022).

Table 2. ^{1}H-NMR chemical shift values associated with metabolites of interest in propolis from *H. itama*

Metabolites identified	Chemical shift(s) identified in loading plot (ppm)
L-Lactic acid	1.13 (29.6%)
α-D-Glucose	4.64 (36.1%)
Ferulic acid	6.81 (38.0%), 7.31 (54.5%)
Nicotinic acid	9.10 (71.2%)
Fumaric acid	6.66 (48.9%)
3,4,5-Trihydroxybenzoic acid	6.94 (33.0%), 9.20 (54.8%)
p-Coumaric acid	6.81 (44.6%)
3,4-Dihydroxybenzoic acid	7.31 (39.2%), 7.36 (46.8%)
trans-Resveratrol	6.14 (40.6%), 6.41 (46.3%), 6.84 (18.4%)
L-Ascorbic acid	4.74 (62.1%), 4.89 (70.5%)
m-Coumaric acid	6.43 (18.4%), 6.85 (52.3%), 7.03 (61.6%), 7.13 (43.7%), 7.24 (20.5%)
Caffeic acid	6.19 (17.8%), 6.99 (46.6%), 7.05(49.6%)

3.2. Physicochemical characterization

Table 3 illustrates the physicochemical attributes of each propolis sample. Notably, *H. itama* propolis exhibited significantly lower moisture content (12.44 ± 1.07%) compared to *A. dorsata* (30.00 ± 0.88%). Additionally, *H. itama* propolis displayed significantly higher lipid (8.20 ± 0.42%), wax (43.78 ± 0.43%), and resin (37.00 ± 2.65%) levels compared to *A. dorsata* (3.79 ± 0.60, 27.25 ± 0.10 and 16.33 ± 2.52, respectively). However, the pH values of the propolis samples fell within a narrow range of 5.15-5.48. These components influence the texture, solubility and overall quality of propolis, with higher resin content reflecting botanical diversity, which can contribute to a broader spectrum of bioactive compounds (Dias et al., 2012). The richness in bioactive compounds of the resin can enhance the therapeutic potential of propolis, offering a more potent natural remedy for various ailments (Salatino, 2022).

Table 3. Physicochemical characteristics of propolis from *A. dorsata* and *H. itama*

Propolis	Moisture (%)	Lipid (%)	Wax (%)	Resin (%)	pH
A. dorsata	30.00 ± 0.88^{b}	3.79 ± 0.60^{a}	27.25 ± 0.10^{a}	16.33 ± 2.52^{a}	5.48 ± 0.06^{a}
H. itama	12.44 ± 1.07^{a}	8.20 ± 0.42^{b}	43.78 ± 0.43^{b}	37.00 ± 2.65^{b}	5.15 ± 0.02^{a}

3.3. Total phenolic and flavonoid contents

The TPC and TFC analyses showed that *H. itama* propolis had significantly higher TPC (187.00 ± 2.41 mg/mL GAE) and TFC (71.55 ± 1.55 mg/mL QE) compared to *A. dorsata* propolis (14.24 ± 0.19 mg/mL GAE and 36.55 ± 2.66 mg/mL QE, respectively) (Table 4). The observed differences in phytochemical constituents between propolis samples from different bee species can be attributed to various factors, including the botanical origin, particularly the vegetation surrounding the hive and the foraging activities of stingless bees (Al-Hatamleh et al., 2020; Rocha et al., 2023). Stingless bees have access to a wider variety of plants, which leads to a more diverse range of bioactive compounds in their propolis (Rocha et al., 2023). Furthermore, it has been reported that the diversity of botanical sources is evident in the increased resin content, which positively correlates with TPC and TFC (El Menyiy et al., 2021). The elevated TPC and TFC levels in *H. itama* propolis suggest a richer presence of phenolic and flavonoid compounds, which are known for their potent antioxidant properties. Phenolic compounds, in particular, are effective scavengers of free radicals, preventing oxidative damage to cells and tissues (Salatino, 2022).

Table 4. Total phenolic and flavonoid contents of *A. dorsata* and *H. itama* propolis extracts

Propolis	Total phenolic content (mg/mL GAE)	Total flavonoid content (mg/mL QE)
A. dorsata	14.24 ± 0.19^{a}	36.55 ± 2.66^{a}
H. itama	187 ± 2.41^{b}	71.55 ± 1.55^{b}

3.4. Antioxidant activities

The antioxidant activities of the propolis extracts, as determined by DPPH and FRAP assays, revealed that *H. itama* propolis exhibited significantly higher ($p<0.05$) DPPH free radical scavenging activity (82.76 ± 1.23%) and FRAP value (829.7 ± 6.26 μM Fe^{2+}) compared to *A. dorsata* (64.12 ± 0.27% and 141.9 ± 1.12 μM Fe2+, respectively) (Table 5). These results are further supported by the IC_{50} DPPH values, where *H. itama* (61.22 ± 1.48 μg/mL) demonstrated a lower IC_{50} value than *A. dorsata* (85.02 ± 1.59 μg/mL). The current study indicated that *H. itama* propolis exhibited stronger antioxidant activity compared to *A. dorsata*, likely due to its higher radical scavenging activity and antioxidant-reducing power.

Table 5. Antioxidant activities of *H. itama* and *A. dorsata* propolis extracts expressed as percentage inhibition of DPPH, IC_{50} DPPH and FRAP values

Propolis	DPPH Inhibition (%)	IC_{50} DPPH (µg/mL)	FRAP (µM Fe^{2+})
A. dorsata	64.12 ± 0.27^{a}	85.02 ± 1.59^{c}	141.9 ± 1.12^{a}
H. itama	82.76 ± 1.23^{b}	61.22 ± 1.48^{b}	829.7 ± 6.26^{b}
Quercetin	98.68 ± 0.34^{c}	17.45 ± 0.51^{a}	-
Gallic acid	-	-	2145.43 ± 36.60^{c}

3.5. Correlation between TPC, TFC and antioxidant activities

Table 6 shows the Pearson's correlation coefficients, indicating the strength and association between the TPC and TFC values and the antioxidant activities of propolis samples. The TPC value showed a strong positive correlation (r: 0.9934) with TFC. Additionally, both TPC and TFC exhibited strong negative correlations with the IC_{50} value, with r values of -0.9941 and -0.9877, respectively. These findings suggest that TPC and TFC significantly contribute to the radical scavenging activity of the propolis extracts. In addition, both TPC and TFC demonstrated strong positive correlations with FRAP values, with r values of 0.9996 and 0.9938, respectively. These correlations emphasize the critical role of TPC and TFC in contributing to the antioxidant potential of propolis extracts. The higher antioxidant activity observed in *H. itama* propolis is likely due to its elevated TPC and TFC. Phenolic compounds and flavonoids are well known for their potent antioxidant properties, which can neutralize free radicals and prevent oxidative stress (Abdelrazeg et al., 2020).

Table 6. Pearson's correlation coefficient of TPC, TFC and antioxidant activities (IC_{50} of DPPH and FRAP values) of *A. dorsata* and *H. itama* propolis extracts

Assays	Correlation (r)			
	TPC	TFC	IC_{50}	FRAP
TPC	1	0.9934*	-0.9941*	0.9996*
TFC	0.9934*	1	-0.9877*	0.9938*
IC_{50}	-0.9941*	-0.9877*	1	-0.9949*
FRAP	0.9996*	0.9938*	-0.9949*	1

4. CONCLUSION

This study provides insights into the complex and diverse chemical makeup of propolis, influenced by the bee species, their foraging behaviors, and the surrounding flora. *H. itama* propolis, with its higher phenolic and flavonoid contents, exhibited stronger antioxidant activity compared to *A. dorsata* propolis. These findings underscore the therapeutic potential of stingless bee propolis, particularly due to its rich phenolic content and strong antioxidant properties, which may contribute to its application in various health-related fields.

Conflict of Interest
The authors declare no conflicts of interest.

Author Contribution Statement
Rozaini Mohd Zohdi.: Conceptualization, Methodology, Writing - Original Draft, Writing - Reviewing and Editing. Muhammad Amirul Adli.: Investigation, Writing - Review & Editing. Ahmad Muqriz Miskan: Investigation, Formal Analysis. Zolkapli Eshak: Resources. Richard Johari James: Resources, Writing - Reviewing and Editing. Syahrul Imran Abu Bakar: Investigation, Formal Analysis. Monporn Payaban: Investigation.

Data Availability Statement
The authors confirm that the data supporting the findings of this study are available within the article.

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