Terra Alimentaria **2026**, Vol. 1 (1), e2026003
Original Article

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Metabolite Profiling and Bioactive Characterization of Stingless Bee Propolis Extracts: Implications for Functional Food Valorization

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ARTICLE INFO

Keywords:
agrifood by-products
food chemistry
functional foods
metabolomics
propolis
stingless bee

Article history:
Received 6 September 2026
Revised 8 September 2026
Accepted 8 September 2026
Published 30 November 2026

ABSTRACT

Propolis is a resinous matrix generated as a by-product of stingless bee apiculture. Its biochemical complexity makes it a candidate for agrifood waste valorization into functional food ingredients. This study evaluated the metabolite composition and in vitro biological activity of methanolic propolis extracts from four stingless bee species (*Trigona clypearis*, *T. drescheri*, *T. sarawakensis*, and *T. terminata*). Metabolomic profiling was conducted via Ultra High-Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS). The functional health properties were quantified using the MTT cytotoxicity assay on the WiDr colorectal carcinoma cell line. Extracts from *T. clypearis*, *T. drescheri*, and *T. terminata* demonstrated bioactive suppression of cell viability with IC_{50} values of 230.8, 290.7, and 388.8 µg/mL, respectively. *T. sarawakensis* exhibited an IC_{50} > 1,000 µg/mL. Variable Importance in Projection (VIP) analysis identified 2,6-Dimethyl-y-pyrone, 5-OxoETE, 1,3,7-Trihydroxy-6-methoxy-4,5-diisoprenylxanthone, and ethyl oleate as primary differentiating metabolites across the extracts. The bioactivity of *T. clypearis* correlates with higher relative concentrations of ethyl oleate, betaine, and glycitein. The entomological origin of propolis determines its metabolic profile. Methanolic extracts from specific *Trigona* species exhibit measurable in vitro bioactivity, providing a biochemical basis for their valorization as functional food components.

1. INTRODUCTION

The valorization of agricultural by-products is a core component of sustainable agrifood systems. In apiculture, propolis is a resinous matrix collected and enzymatically modified by bees, frequently generated as a secondary by-product during honey extraction. From the perspective of food biochemistry, propolis contains diverse secondary metabolites, including phenolic acids, flavonoids, and terpenes, which possess established bioactive properties (Abduh et al., 2024; Pratami et al., 2024).

The utilization of such agrifood by-products in the development of functional foods requires rigorous chemical characterization. The biochemical composition of propolis is highly heterogeneous, determined by botanical origin and the specific digestive and metabolic interactions of the bee species (Zullkiflee et al., 2022). This metabolic variability yields distinct phytochemical profiles, which subsequently dictate the functional efficacy of the extracts, including their capacity to modulate oxidative stress or inhibit aberrant cellular proliferation (Elumalai et al., 2022; Hermansyah et al., 2022).

To evaluate its viability as a functional food ingredient, this study characterized the metabolite profiles of methanolic propolis extracts from four distinct Indonesian *Trigona* species using UHPLC-HRMS. Additionally, the in vitro bioactivity of these extracts was quantified against the WiDr colorectal

carcinoma cell line to establish the relationship between the specific food chemistry constituents and their functional health properties.

2. MATERIALS AND METHODS

2.1. Biological Materials and Reagents

Raw propolis samples were harvested from the nests of four distinct stingless bee species (*Trigona clypearis*, *T. drescheri*, *T. sarawakensis*, and *T. terminata*). All samples were directly sourced from established stingless bee aquaculture facilities in Bangunjiwo, Bantul, Special Region of Yogyakarta, Indonesia. The WiDr human colorectal carcinoma cell line, utilized for all in vitro bioactivity assays, was obtained from the cell culture repository at the Faculty of Medicine and Public Health, Universitas Muhammadiyah Yogyakarta. All analytical grade solvents, including methanol and dimethyl sulfoxide (DMSO), as well as assay reagents including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS), were purchased from Sigma-Aldrich.

2.2. Preparation and Extraction of Propolis

To valorize the raw propolis matrix into testable functional fractions, a sequential solvent extraction protocol was employed. Approximately 80 g of desiccated raw propolis from each *Trigona* species was subjected to maceration in analytical-grade methanol in a temperature-controlled water bath for 1 hour at ambient room temperature. Following maceration, the suspension was subjected to ultrasonic extraction using a bath sonicator for 20 minutes to maximize the dissolution of bioactive phytochemicals.

The resulting methanolic mixture was filtered to separate the liquid extract from the insoluble dregs. The methanolic filtrate was subsequently evaporated to dryness using a decoction pan and a rotary evaporator under reduced pressure to yield a concentrated, crude methanolic extract. Concurrently, the remaining filtered dregs were re-suspended in an aqueous solvent, sonicated for an additional 20 minutes, filtered, and evaporated under identical conditions to ensure comprehensive extraction of polar constituents. All concentrated extracts were hermetically sealed and stored under refrigeration (4 °C) prior to chemical profiling and biological evaluation.

2.3. Cell Culture and In Vitro Cytotoxicity Assay

The functional bioactivity of the methanolic propolis extracts was quantified using a standardized MTT colorimetric assay based on published studies (Barboza et al., 2020; Mustika et al., 2023; Peters et al., 2024). WiDr colon cancer cells were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS). The cells were maintained in a humidified incubator at 37 °C with an atmosphere of 5% CO2.

For the cytotoxicity evaluation, cells were harvested and seeded into sterile, flat-bottom 96-well microplates (Iwaki) at a standardized density of 5×10^3 cells/well in a total volume of 100 µL. The plates were incubated overnight to facilitate optimal cell adherence. Following the adhesion period, the culture medium was aspirated, and the cells were treated with 100 µL of the prepared propolis extract solutions. The extracts were dissolved in 0.1% DMSO and administered at serial concentrations ranging from 31.25 to 1,000 µg/mL. Doxorubicin was utilized as the positive control, and culture media devoid of the extract served as the negative control. All treatments were performed in independent triplicates.

Following a 24-hour incubation period with the respective treatments, the liquid medium was carefully aspirated from each well. Subsequently, 100 µL of MTT solution (5 mg/mL in PBS) was added to each well, and the microplates were returned to the CO2 incubator for 4 hours at 37 °C to allow the viable cells to reduce the tetrazolium salt into insoluble formazan crystals. To solubilize the resulting formazan, 100 µL of SDS solution was added to each well. The absorbance, corresponding to the quantity of viable cells, was measured spectrophotometrically at 595 nm using a microplate ELISA reader (Bio-Rad, Hercules, CA, USA).

The percentage of cell growth inhibition was calculated using the following formula: Percentage Inhibition (%) = [(Absorbance of Control - Absorbance of Treatment) / Absorbance of Control] × 100

2.4. Metabolomic Profiling via UHPLC–HRMS

The food chemistry profile and bioactive constituents of the methanolic extracts were characterized using Ultra High-Performance Liquid Chromatography-High Resolution Mass Spectrometry (UHPLC-HRMS), adapting protocols previously established in the literature (Stavropoulou et al., 2021). Extract aliquots were reconstituted in LCMS-grade methanol prior to injection. The chromatographic separation was achieved using a binary mobile phase system consisting of water and methanol. Spectral data acquisition and preliminary compound identification were executed using X-Calibur software, with mass spectra cross-referenced against the ChemSpider structural database to identify the predominant secondary metabolites.

2.5. Statistical and Chemometric Analysis

The half-maximal inhibitory concentration (IC_{50}) for each propolis extract was determined via linear regression analysis, plotting the log10 of the extract concentration against the calculated percentage of cellular inhibition. For the metabolomic data, the maximum peak area (intensity) of the identified compounds was exported for multivariate statistical evaluation. Chemometric analysis was conducted using the web-based MetaboAnalyst 6.0 platform (accessed August 1, 2024). Principal Component Analysis (PCA) and Sparse Partial Least Squares Discriminant Analysis (sPLSDA) were utilized to visualize clustering and assess the biochemical divergence among the four *Trigona* species. Variable Importance in Projection (VIP) scores were calculated to isolate and identify the specific marker metabolites driving the variance in functional bioactivity across the sample groups. Hierarchical

clustering and heatmap generation were employed to map the relative abundance of the dominant compounds.

3. RESULTS AND DISCUSSION

3.1. Propolis Recovery and Extraction

The successful recovery and extraction of propolis from the *Trigona* nests illustrate a viable pathway for agrifood waste valorization. The physical architecture of the nests (Figure 1A) and the raw collected honey pots (Figure 1B) highlight the natural matrix variations prior to processing. Following the sequential extraction protocol, the final methanolic crude extracts exhibited distinct visual characteristics (Figure 1C), indicating divergent preliminary phytochemical compositions.

(A)

(B)

(C)

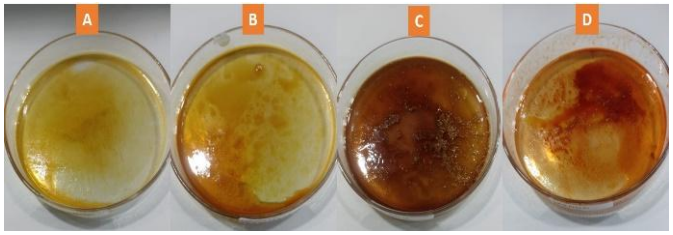

Figure 1. Sequential stages of propolis recovery and methanolic extraction. The panels illustrate the physical progression of the agrifood waste valorization process: (A) intact stingless bee nests in situ, (B) raw harvested propolis matrices prior to processing, and (C) the resulting concentrated methanolic extracts. Across all panels, letters denote the specific entomological origin: (A) *Trigona drescheri*, (B) *T. terminata*, (C) *T. clypearis*, and (D) *T. sarawakensis*.

Propolis is a matrix primarily composed of resin, yet it possesses highly distinctive characteristics due to variability that depends on the environment and the producing species (Rocha et al., 2023). From scientific and pharmaceutical-chemical perspectives, raw propolis and propolis methanol extract (PME) differ fundamentally in terms of purity, concentration, and the bioavailability of their active components (Pratami, Sahlan, et al., 2024). Raw propolis generally still contains high levels of inactive components: ~30% beeswax, plant tissue residues, wood fibers, soil, and pollen. Meanwhile, EMP is free from beeswax, coarse impurities, and water, leaving behind a pure matrix of bioactive resin (Pratami, Sahlan, et al., 2024).

3.2. Metabolite Composition and Food Chemistry Profile

The valorization of apiculture by-products into functional food ingredients requires a comprehensive understanding of their biochemical matrices. UHPLC-HRMS was employed to characterize the phytochemical complexity of the methanolic propolis extracts from the four *Trigona* species. As depicted in the chromatograms (Figure 2), the distribution of secondary metabolites exhibited substantial interspecies variability. Generally, non-polar compounds, eluting at later retention times, predominated across the samples.

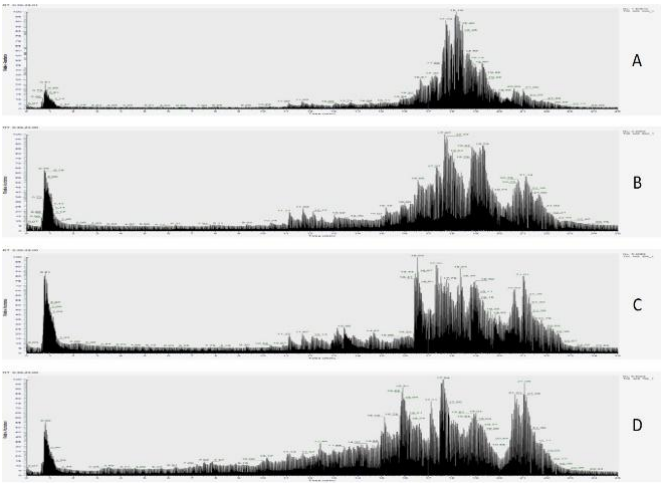

Figure 2. Results of compound analysis of methanol extract of propolis using LC-HRMS. A). *T. clypearis*, B). *T. dressery*, C). *T. sarawakensis*, and D). *T. terminata*.

The chromatographic profiles of *T. clypearis* and *T. terminata* were highly distinct, whereas *T. drescheri* and *T. sarawakensis* shared closer structural similarities. Further quantification of the mass spectra identified the 20 most abundant compounds across all samples based on maximum peak area (Table 2). *T. clypearis* was notably enriched in specific functional metabolites compared to the other species, primarily ethyl oleate, betaine, and glycitein. In the context of functional nutrition, betaine functions as a critical osmotic regulator (Buonaiuto et al., 2025) and methyl donor (Singh et al., 2026),

while glycitein is a recognized isoflavone with documented antioxidant capacity (Wang et al., 2025).

The presence of these bioactive constituents provides a preliminary chemical basis for the biological activity of the extracts. Stingless bee propolis is extremely rich in phenolic compounds, flavonoids, and terpenoids, which are successfully dissolved due to the properties of methanol as a universal polar solvent (Pratami, Sahlan, et al., 2024). Due to the presence of these active compounds, numerous key studies have described the anticancer potential of propolis from various stingless bee species, covering their chemical constituents, mechanisms of action, and cell death profiles (Campos et al., 2021).

3.3. Chemometric Discrimination of Propolis Origins

To identify the biochemical differentiators among the four stingless bee species, multivariate chemometric analyses were performed on the LC-HRMS dataset. The Principal Component Analysis (PCA) and Sparse Partial Least Squares Discriminant Analysis (sPLSDA) score plots (Figure 6A-B) confirmed the distinct metabolic signatures of the extracts. *T. clypearis* and *T. terminata* occupied isolated spatial coordinates, indicating divergent phytochemical compositions, while *T. drescheri* and *T. sarawakensis* clustered more closely.

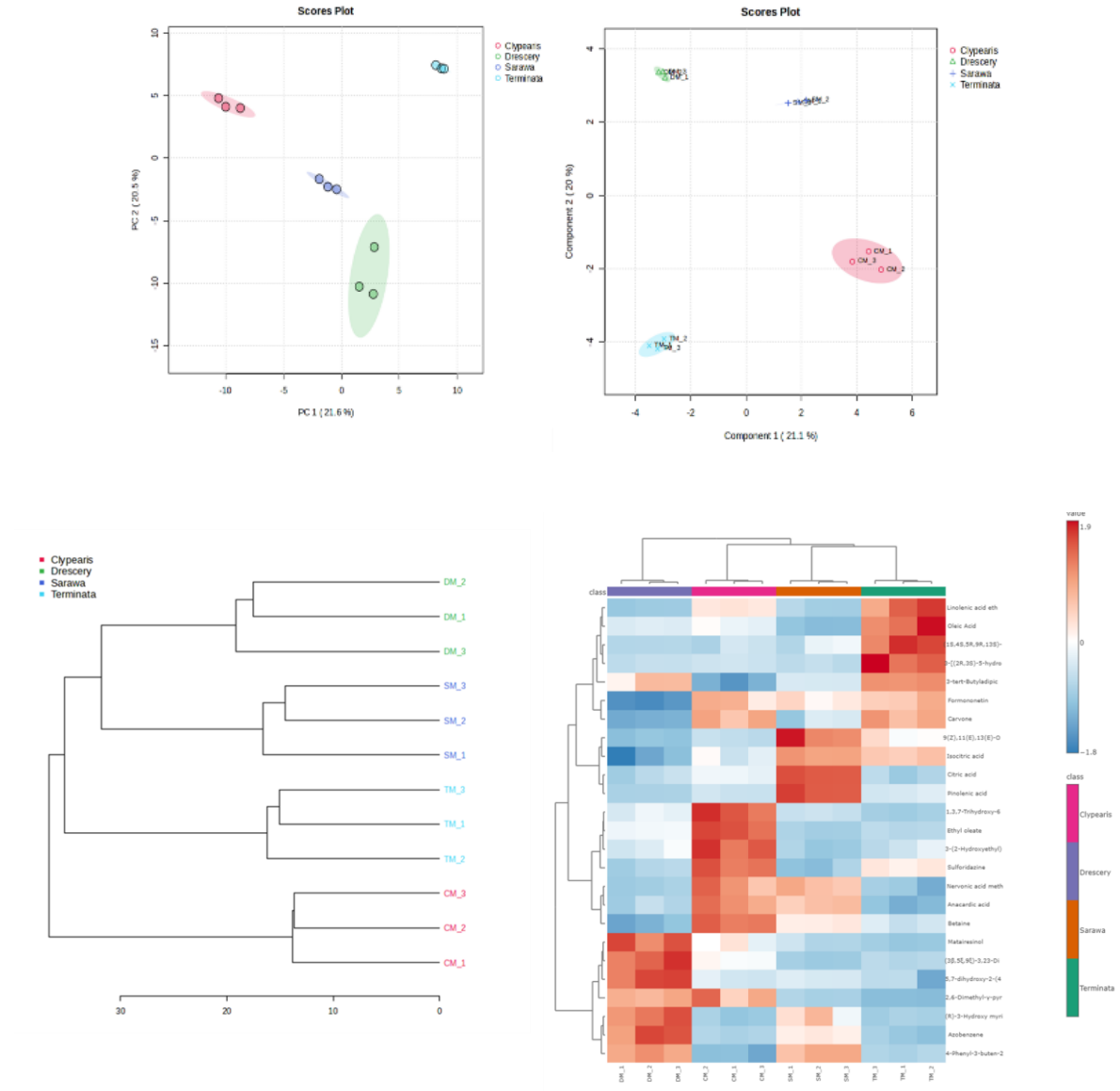

Figure 6. Principal Component Analysis (PCA) and Sparse Partial Least Squares Discriminant Analysis (sPLSDA) score plots.

Variable Importance in Projection (VIP) analysis was utilized to isolate the specific metabolites driving these metabolic differences. Four primary discriminant compounds yielded VIP scores exceeding 1.90 (Figure 7): 2,6-dimethyl-y-pyrone, 5-OxoETE, 1,3,7-trihydroxy-6-methoxy-4,5-diisoprenylxanthone, and ethyl oleate. The concentrations of

these markers varied strictly by entomological origin. For example, 2,6-dimethyl-y-pyrone and 1,3,7-trihydroxy-6-methoxy-4,5-diisoprenylxanthone were detected at high concentrations in *T. clypearis* but were minimal in *T. terminata*. Conversely, 5-OxoETE was highly abundant in *T. terminata* and negligible in *T. clypearis*.

Hierarchical clustering and heatmap generation (Figure 8) further elucidated these compositional differences. The heatmap visually confirms that the highly bioactive extracts (*T. terminata*, *T. drescheri*, and *T. clypearis*) each possess a high relative abundance of seven distinct dominant compounds. In contrast, the *T. sarawakensis* matrix is chemically narrower, containing only four dominant high-concentration metabolites. Other research on propolis from various stingless bee species indicates that geopropolis is not limited to wax and fatty acids; other compounds—such as fatty acid amides and amines, phenolic lipids, resorcinols, retinoids, abietanoids, diterpenoids, pentacyclic triterpenoids, prostaglandins, and steroids—have also been identified. Multivariate analysis reinforces the hypothesis that stingless bee species and geographical origin are key factors influencing geopropolis composition, driven by species-specific foraging preferences regarding plant types (Fabio et al., 2023).

3.4. In Vitro Cytotoxicity and Functional Bioactivity

The functional properties of the propolis extracts were evaluated in vitro by measuring their capacity to inhibit the proliferation of the WiDr colorectal carcinoma cell line. The MTT cytotoxicity assay revealed a concentration-dependent suppression of cellular viability (Figure 4).

Based on logarithmic regression analysis, extracts from three of the four species demonstrated significant in vitro bioactivity (IC_{50} < 1,000 μg/mL). The highest efficacy was observed in *T. clypearis* (IC_{50} = 230.8 μg/mL), followed by *T. drescheri* (IC_{50} = 290.7 μg/mL) and *T. terminata* (IC_{50} = 388.8 μg/mL). The extract from *T. sarawakensis* lacked meaningful functional activity, failing to reach an IC_{50} threshold below 1,000 μg/mL. Other studies have shown that the phenolic and flavonoid content of *T. clypearis* is higher than that of other stingless bees, such as *T. laeviceps* and *T. biroi* (Pratami, Alfifah, et al., 2024). Consequently, this influences its biological activity, particularly its anticancer properties.

While the crude propolis matrices inherently exhibit lower absolute potency than the purified chemotherapeutic standard doxorubicin (IC_{50} = 0.3 μg/mL), their bioactivity at these concentrations is highly relevant for functional food and dietary applications. The pronounced cytotoxicity of *T. clypearis* is directly attributable to its rich biochemical profile, specifically the synergistic presence of its seven dominant secondary metabolites (including ethyl oleate and betaine). Conversely, the diminished biological efficacy of *T. sarawakensis* correlates strictly with its reduced phytochemical diversity (only four dominant compounds). In another study, GC-MS phytochemical screening identified oleyl acetate as one of the dominant bioactive components of *Onosma bracteatum*, demonstrating the compound's ability to interact effectively to inhibit the activity of BCL-2 (an apoptosis-inhibiting protein) in cancer cells (Saini et al., 2024). Another study also demonstrated that the ethyl oleate compound from *Pancratium maritimum* seeds and flowers possesses antioxidant, antimicrobial, and anticancer properties (Ertuğrul et al., 2025).

Other compounds with anti-cancer properties in propolis include betaine and its derivatives, which functionally suppress tumor development and metastatic migration, as well as block the nutrient supply pathways required for growth (angiogenesis) (Kar et al., 2019; Phattarataratip et al., 2024). Glycitein is the predominant compound found specifically in *T. clypearis* propolis. Comprehensive reviews confirm the pharmacological profile of glycitein as a potent antitumor agent; its mechanisms of action include inhibiting malignant cell proliferation, suppressing tissue invasion capabilities, and blocking angiogenic pathways by modulating the Nrf2-ARE, NF-κB, MAPK, and STAT3 molecular signaling cascades (Wang et al., 2025). Other research indicates that glycitein regulates unique cellular kinetics in human SKBR-3 breast carcinoma cells. At high functional intervention doses (above 30 μg/mL), glycitein acts purely as a cytotoxic agent, disrupting cancer cell membrane permeability and massively halting the *de novo* synthesis of double-stranded DNA (Zhang et al., 2015).

These empirical findings indicate that the metabolic processing by specific stingless bee species dictates the phytochemical yield and subsequent functional quality of the propolis, validating the selective upcycling of *T. clypearis*, *T. drescheri*, and *T. terminata* by-products for agrifood applications.

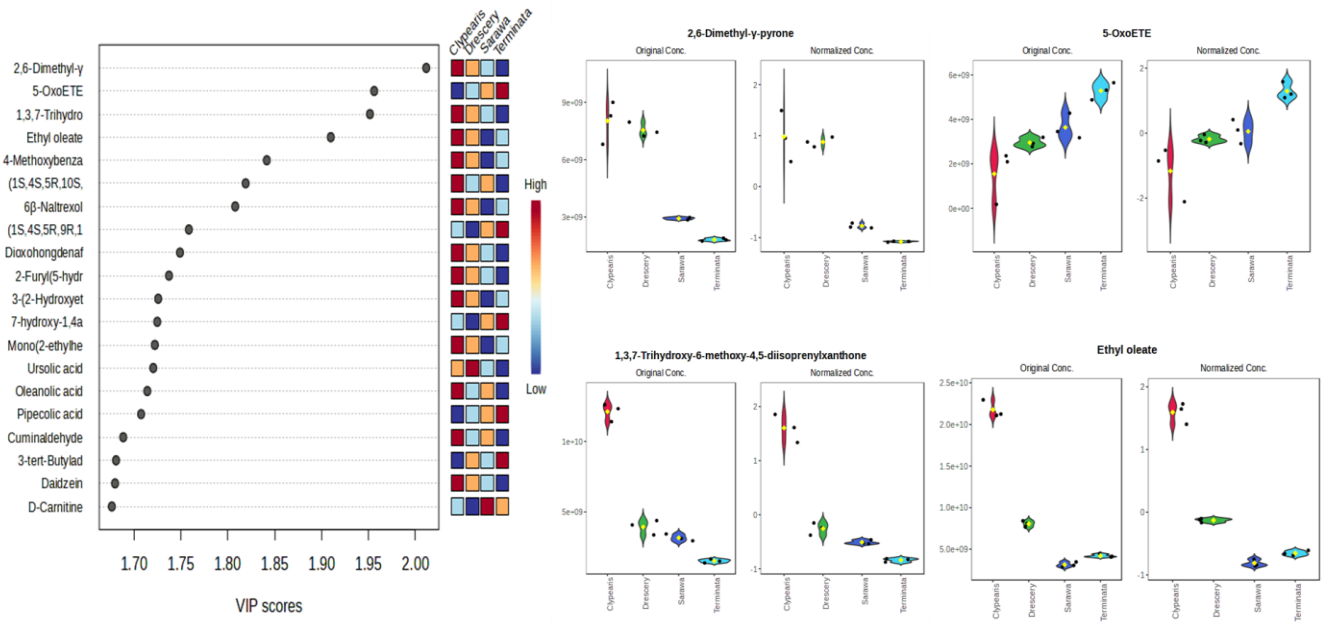

Figure 7. Variable Importance in Projection (VIP) analysis for isolating the specific metabolites of stingless bee.

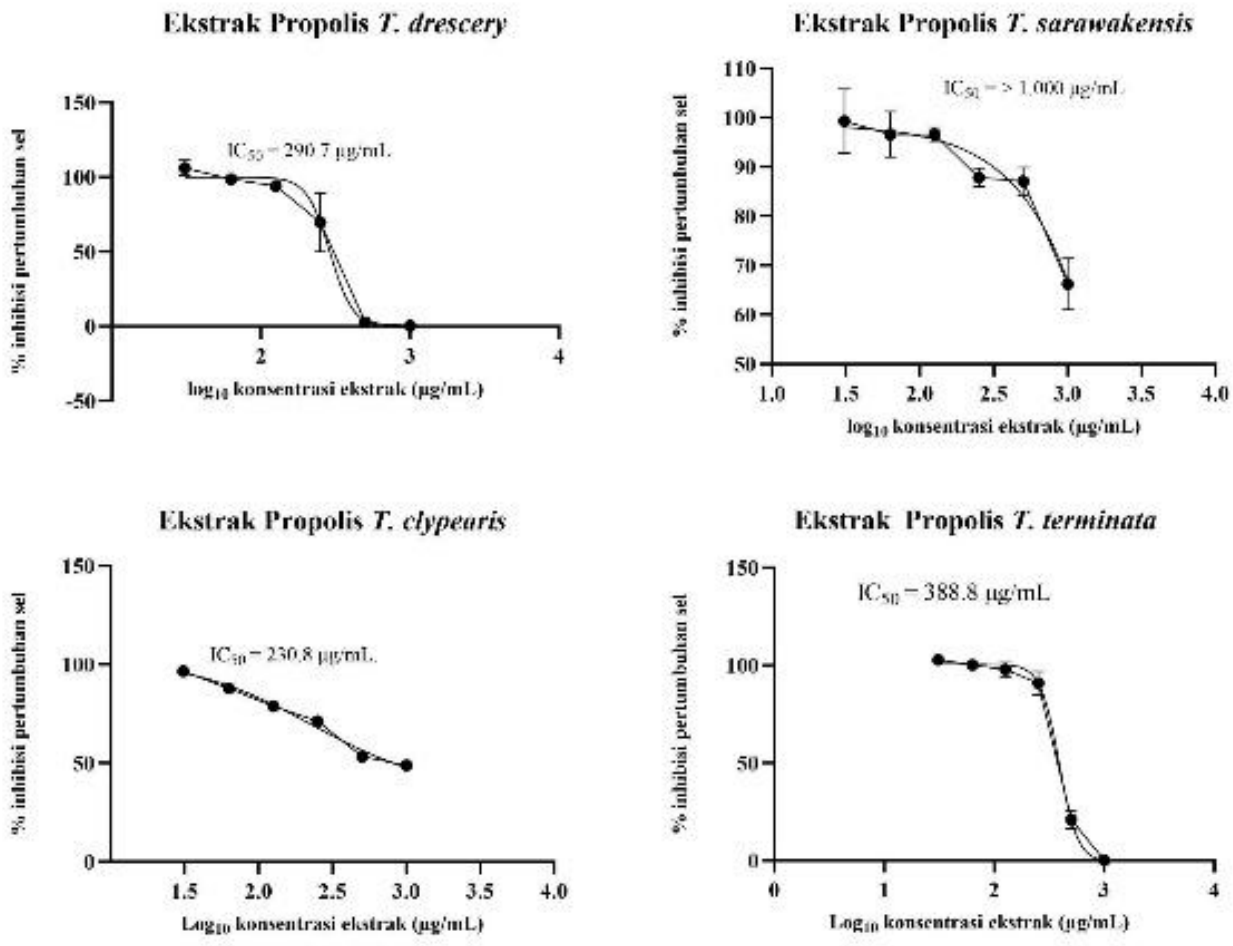

Figure 4. Graph of the concentration of extracts of four types of propolis on the growth of colon cancer cells.

Table 2. The 19 dominant compounds with the highest area results from LC-HRMS analysis of 4 stingless bee propolis extracts.

No.	Name	Formula	Calc. MW	RT [min]	Area (Max.) (10^6)	% relative area	mzCloud Best Match	Area (Max.) (10^6)			
								T. clypearis	*T. drescery*	*T. sarawakensis*	*T. terminata*
1	(1S,4S,5R,9R,13S)-5,9-dimethyl-14-methylidenetetracyclo[11.2.1.0]hexadec-10-ene-5-carboxylic acid	C20 H28 O2	300,20818	17,161	3287,13	2,40	81,5	0,08	0,36	0,02	5,27
2	Ethyl oleate	C20 H38 O2	310,28616	19,357	2295,46	1,67	99,0	3,73	2,12	0,60	0,08
3	Pinolenic acid	C18 H30 O2	278,22370	16,466	2010,84	1,47	86,9	0,06	0,30	3,77	0,08
4	α,α-Trehalose	C12 H22 O11	342,11590	0,846	1772,91	1,29	98,1	2,07	3,77	2,86	2,25
5	Lupeol	C30 H50 O	426,38482	18,413	1648,33	1,20	91,4	0,07	0,37	1,70	0,27
6	(1S,4S,5R,10S,13S,17S,19S,20R)-10-hydroxy-4,5,9,9,13,19,20-heptamethyl-24-oxahexacyclo[15.5.2.0]tetracos-15-en-23-one	C30 H46 O3	454,34373	18,854	1418,61	1,03	86,9	2,39	2,61	2,31	1,45
7	Betaine	C5 H11 N O2	117,07889	0,865	1292,92	0,94	96,1	2,17	0,83	1,70	1,12
8	Bis(2-ethylhexyl) phthalate	C24 H38 O4	390,27600	18,883	1262,85	0,92	99,8	0,02	2,67	0,15	0,16
9	1,3,7-Trihydroxy-6-methoxy-4,5-diisoprenylxanthone	C24 H26 O6	410,17184	17,028	1259,48	0,92	95,7	2,08	1,03	0,60	0,28
10	(3β,5ξ,9ξ)-3,23-Dihydroxy-1-oxoolean-12-en-28-oic acid	C30 H46 O5	486,33361	16,605	1099,00	0,80	71,6	0,80	2,56	0,34	0,44
11	Glycitein	C16 H12 O5	284,06813	13,423	1064,48	0,78	98,3	1,74	0,15	0,02	0,03
12	9(Z),11(E),13(E)-Octadecatrienoic Acid methyl ester	C19 H32 O2	292,23931	18,408	979,42	0,71	99,1	0,04	0,35	1,70	0,09
13	1,4a-dimethyl-9-oxo-7-(propan-2-yl)-1,2,3,4,4a,9,10,10a-octahydrophenanthrene-1-carboxylic acid	C20 H26 O3	314,18752	15,858	823,73	0,60	97,9	0,01	0,18	0,01	1,15
14	Kahweol	C20 H26 O3	314,18746	14,059	781,59	0,57	80,4	0,01	0,07	0,00	1,25
15	Isocitric acid	C6 H8 O7	192,02636	0,941	748,91	0,55	96,0	0,09	0,68	0,17	1,36
16	D-(-)-Mannitol	C6 H14 O6	182,07834	0,813	732,55	0,53	98,8	1,22	1,36	1,21	0,66
17	D-(+)-Galactose	C6 H12 O6	180,06273	0,837	652,57	0,48	98,3	0,53	0,69	0,98	0,77
18	Lupeol	C30 H50 O	426,38536	20,659	629,01	0,46	87,9	0,36	0,55	1,14	0,62
19	5-hydroxy-4-methoxy-5,6-dihydro-2H-pyran-2-one	C6 H8 O4	144,04203	1,075	626,21	0,46	62,8	0,48	0,86	0,99	0,30

4. CONCLUSION

The chemical characterization and biological evaluation of stingless bee propolis validate its viability for upcycling into functional food systems. The biological activity of these matrices is fundamentally dictated by their entomological origin, which drives distinct phytochemical variations. Methanolic extracts from *T. clypearis*, *T. drescheri*, and *T. terminata* demonstrated significant in vitro cytotoxicity against colorectal carcinoma cells, with *T. clypearis* exhibiting the highest functional efficacy (IC_{50} = 230.8 µg/mL). The superior bioactivity of the *T. clypearis* extract correlates with its high relative abundance of specific bioactive metabolites, namely ethyl oleate, betaine, and glycitein. Furthermore, chemometric analysis identified 2,6-dimethyl-y-pyrone, 5-OxoETE, 1,3,7-trihydroxy-6-methoxy-4,5-diisoprenylxanthone, and ethyl oleate as the primary discriminant metabolic markers across the species (VIP > 1.90). Ultimately, these findings establish a robust biochemical foundation for utilizing specific *Trigona* propolis by-products as high-value, bioactive ingredients in sustainable agrifood applications.

ACKNOWLEDGMENTS

All authors thank The Directorate of Laboratory Management, Research Facilities and Science and Technology Park, National Research and Innovation Agency (BRIN) Yogyakarta, Indonesia, for all research instrument facilities. The authors also thank Mr. Setyo Adi Winarno and Mr. Sarmin from Lebah Klanceng Jogjakarta (LKJ) for stingless bees propolis samples. The author also expresses gratitude to Dr. Ema Damayanti and Eko Sugiarto, S.Pd. for their supervision during research and manuscript preparation.

AUTHOR CONTRIBUTIONS

Nail Zufar Arrijal: Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Adi Hermawansyah:** Methodology, Formal analysis, Writing – original draft. **Rifki Febriansyah:** Material, Methodology, Formal analysis, Writing – review & editing. **Lucky Prabowo Miftachul Alam:** Methodology, Formal analysis, Writing – original draft. **Frista Mufti Faisyah Dewanti:** Conceptualization, Writing – original draft, Writing – review & editing. **Wahyu Angga Rizal:** Methodology, Formal analysis, Writing – original draft, Writing – review & editing.

FUNDING INFORMATION

This research was funded by Muhammadiyah Boarding School (MBS) Yogyakarta. This research was presented at the finals of the Indonesian Student Research Olympiad (OPSI), organized by the Indonesian Ministry of Education in Jakarta in 2024.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF AI AND AI–ASSISTED TECHNOLOGIES

During the preparation of this manuscript, the author(s) used Google Gemini Pro and Trinka AI (Enago) solely to improve the readability, grammar, and language clarity of the text. These tools were not used to generate original research ideas, interpret data, or draw scientific conclusions. Following the use of these tools, the author(s) reviewed and edited all suggestions to ensure scientific accuracy and take full responsibility for the content and integrity of the final publication.

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