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ORIGINAL ARTICLE

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

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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 µg/mL, 290.7 µg/mL, and 388.8 µg/mL, respectively. *T. sarawakensis* exhibited an $IC_{50} > 1000$ µg/mL. Variable Importance in Projection (VIP) analysis identified 2,6-dimethyl-γ-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. 2024a,b).

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

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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 h at ambient room temperature. Following maceration, the suspension was subjected to ultrasonic extraction using a bath sonicator for 20 min 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 min, 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 % CO_2.

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 31.25–1000 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 h 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 CO_2 incubator for 4 h 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 Equation (1):

$$\text{Inhibition } (\%) = \frac{\text{Abs}_{\text{Control}} - \text{Abs}_{\text{Treatment}}}{\text{Abs}_{\text{Control}}} \times 100 \quad (1)$$

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 $\log_{10}$ 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

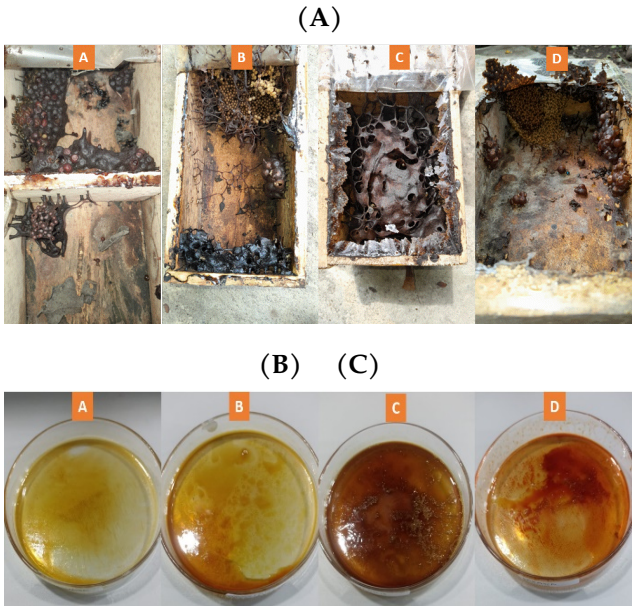


Figure 1. Sequential stages of propolis recovery and methanolic extraction. The panels illustrate the physical progression of the agrifood waste valorization process: (A) intact stingless bee nests in situ, (B) raw harvested propolis matrices prior to processing, and (C) the resulting concentrated methanolic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

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.

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 et al. 2024b). Raw propolis generally still contains high levels of inactive components: ~30 % beeswax, plant tissue residues, wood fibers, soil, and pollen. Meanwhile, PME is free from beeswax, coarse impurities, and water, leaving behind a pure matrix of bioactive resin (Pratami et al. 2024b).

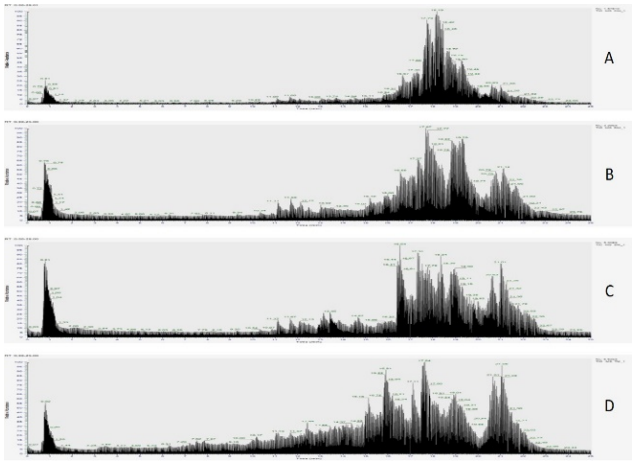


Figure 2. Results of compound analysis of methanol extract of propolis using LC-HRMS: (A) *T. clypearis*, (B) *T. drescheri*, (C) *T. sarawakensis*, and (D) *T. terminata*.

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.

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 dominant compounds across all samples based on maximum peak area (Table 1). *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 et al. 2024b). 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 3) 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.

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 4): 2,6-dimethyl-γ-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-γ-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 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 5).

Based on logarithmic regression analysis, extracts from three of the four species demonstrated significant in vitro bioactivity ($IC_{50} < 1000\ \mu g/mL$). The highest efficacy was observed in *T. clypearis* ($IC_{50} = 230.8\ \mu g/mL$), followed by *T. drescheri* ($IC_{50} = 290.7\ \mu g/mL$) and *T. terminata* ($IC_{50} = 388.8\ \mu g/mL$). The extract from *T. sarawakensis* lacked meaningful functional activity, failing to reach an IC_{50} threshold below $1000\ \mu g/mL$. 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 et al. 2024a). 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 ($IC_{50} = 0.3\ \mu g/mL$), 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 ethyl oleate and related constituents as 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 anticancer 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

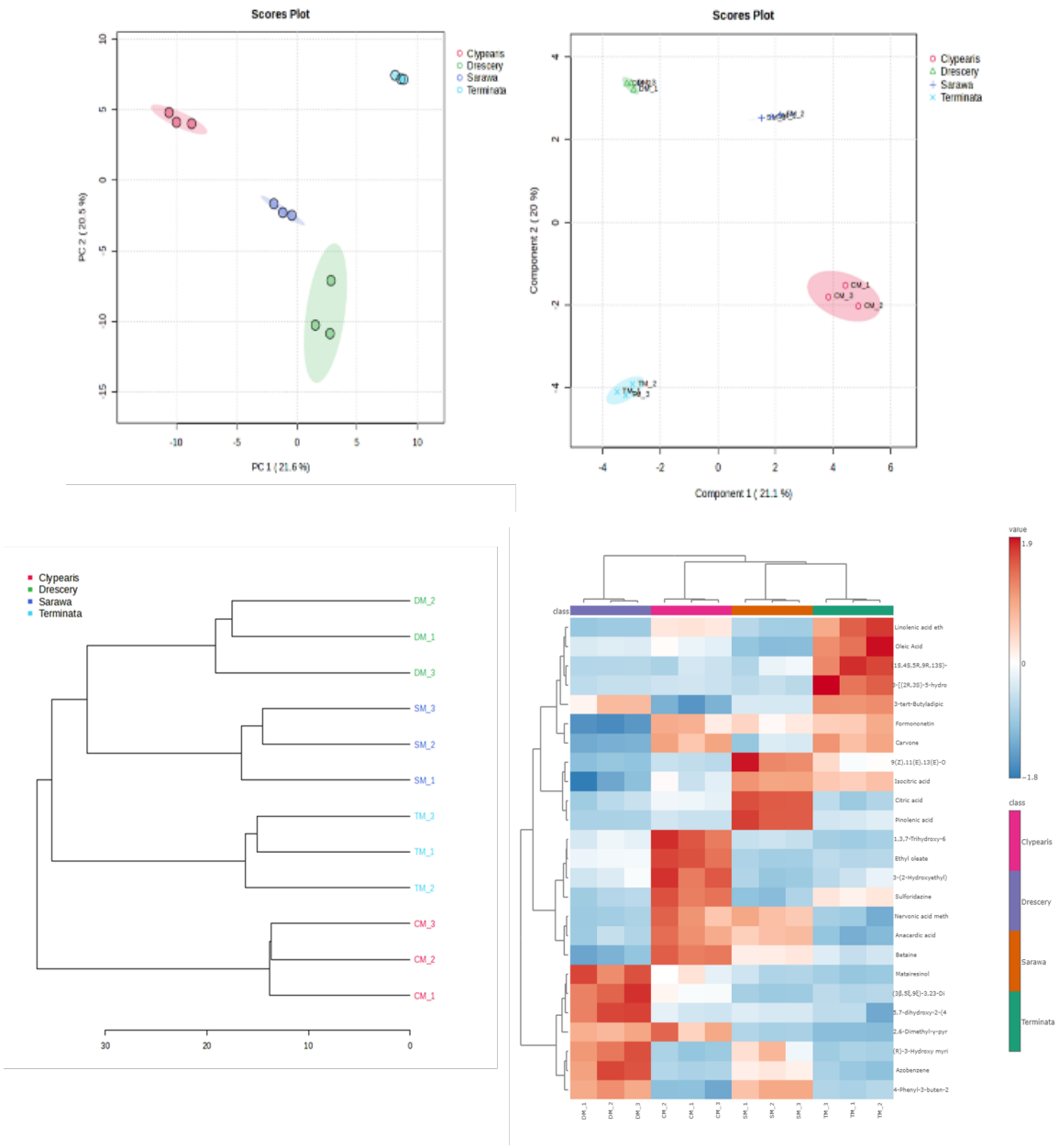


Figure 3. Principal Component Analysis (PCA) and Sparse Partial Least Squares Discriminant Analysis (sPLSDA) score plots.

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.

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-γ-pyrone, 5-OxoETE,

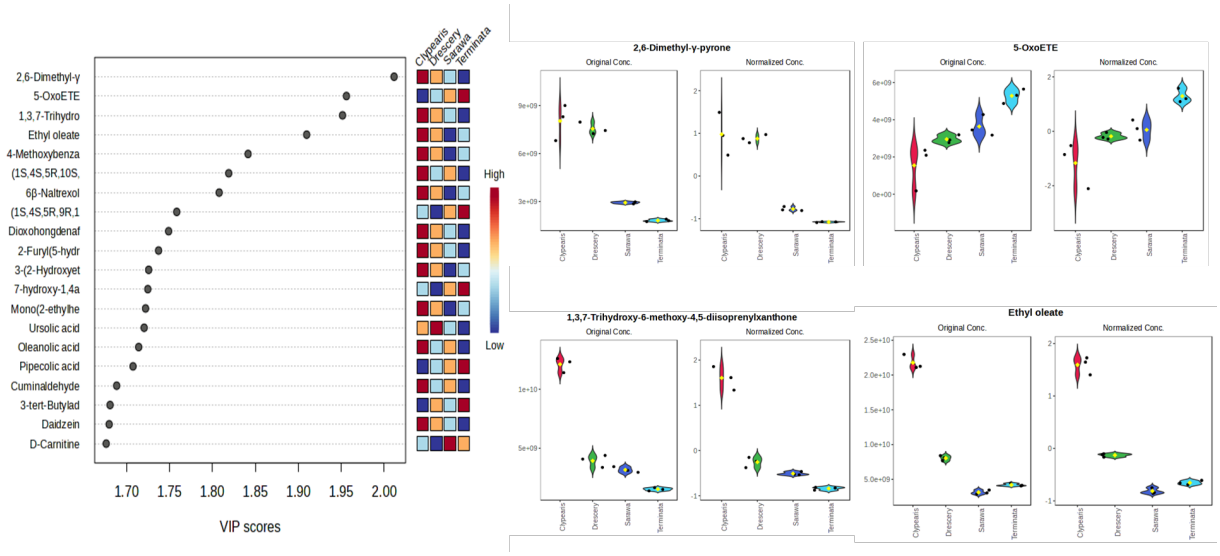


Figure 4. Variable Importance in Projection (VIP) analysis for isolating the specific metabolites of stingless bee propolis.

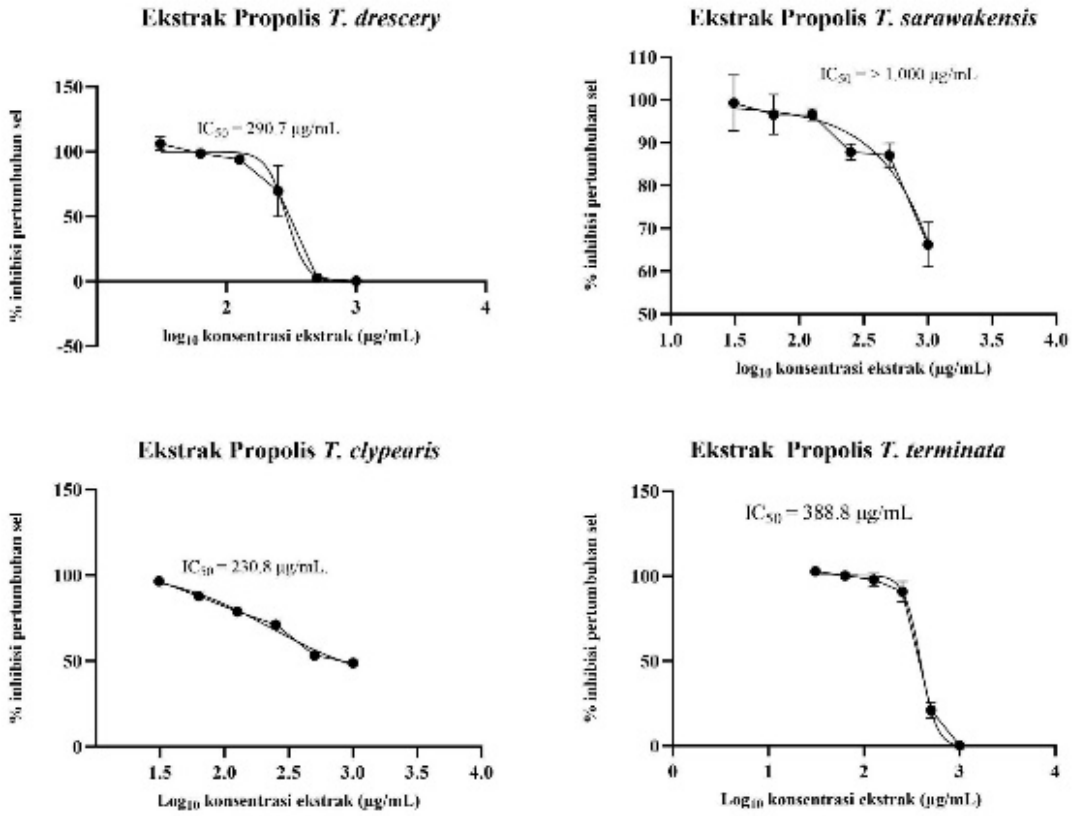


Figure 5. Graph of the concentration of extracts of four types of propolis on the growth of colon cancer cells.

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.

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Table 1. The 19 dominant compounds with the highest area results from LC-HRMS analysis of four stingless bee propolis extracts.

No.	Name	Formula	Calc. MW	RT (min)	Area Max. (10^6)	Rel. Area (%)	mzCloud (%)	Area (Max.) (10^6)			
								T. clyp.	*T. dres.*	*T. sara.*	*T. term.*
1	(1S,4S,5R,9R,13S)-5,9-Dimethyl-14-methylidenetetracyclo[11.2.1.0]hexadec-10-ene-5-carboxylic acid	$C_{20}H_{28}O_2$	300.20818	17.161	3287.13	2.40	81.5	0.08	0.36	0.02	5.27
2	Ethyl oleate	$C_{20}H_{38}O_2$	310.28616	19.357	2295.46	1.67	99.0	3.73	2.12	0.60	0.08
3	Pinolenic acid	$C_{18}H_{30}O_2$	278.22370	16.466	2010.84	1.47	86.9	0.06	0.30	3.77	0.08
4	α,α-Trehalose	$C_{12}H_{22}O_{11}$	342.11590	0.846	1772.91	1.29	98.1	2.07	3.77	2.86	2.25
5	Lupeol	$C_{30}H_{50}O$	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	$C_{30}H_{46}O_3$	454.34373	18.854	1418.61	1.03	86.9	2.39	2.61	2.31	1.45
7	Betaine	$C_5H_{11}NO_2$	117.07889	0.865	1292.92	0.94	96.1	2.17	0.83	1.70	1.12
8	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	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-diisoprenyl xanthone	$C_{24}H_{26}O_6$	410.17184	17.028	1259.48	0.92	95.7	2.08	1.03	0.60	0.28
10	(3β,5ζ,9ξ)-3,23-Dihydroxy-1-oxoolean-12-en-28-oic acid	$C_{30}H_{46}O_5$	486.33361	16.605	1099.00	0.80	71.6	0.80	2.56	0.34	0.44
11	Glycitein	$C_{16}H_{12}O_5$	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	$C_{19}H_{32}O_2$	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	$C_{20}H_{26}O_3$	314.18752	15.858	823.73	0.60	97.9	0.01	0.18	0.01	1.15
14	Kahweol	$C_{20}H_{26}O_3$	314.18746	14.059	781.59	0.57	80.4	0.01	0.07	0.00	1.25
15	Isocitric acid	$C_6H_8O_7$	192.02636	0.941	748.91	0.55	96.0	0.09	0.68	0.17	1.36
16	D-(−)-Mannitol	$C_6H_{14}O_6$	182.07834	0.813	732.55	0.53	98.8	1.22	1.36	1.21	0.66
17	D-(+)-Galactose	$C_6H_{12}O_6$	180.06273	0.837	652.57	0.48	98.3	0.53	0.69	0.98	0.77
18	Lupeol	$C_{30}H_{50}O$	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	$C_6H_8O_4$	144.04203	1.075	626.21	0.46	62.8	0.48	0.86	0.99	0.30

Note: *T. clyp.* = *Trigona clypearis*; *T. dres.* = *Trigona drescheri*; *T. sara.* = *Trigona sarawakensis*; *T. term.* = *Trigona terminata*.

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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:** Resources, 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.

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DATA AVAILABILITY

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 authors 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 authors 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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