



Uludag Bee Journal

Uludag Bee J.

e-ISSN: 2687-5594

ISSN 1303-0248

<https://dergipark.org.tr/en/pub/uluaricilik><https://uadubj.uludag.edu.tr/>

## Heavy metal content, phytochemical profile, and radical scavenging activity of stingless bee (*Heterotrigona itama*) propolis cultivated on post-mining land at East Kalimantan, Indonesia

Mayusef SUKMANA<sup>1</sup> , Swandari PARAMITA<sup>2\*</sup> , Sri WAHYUNINGSIH<sup>3</sup> , Yadi YASIR<sup>4</sup> 

<sup>1</sup>Doctoral Program of Environmental Sciences, Mulawarman University, Jl. Sambaliung, Samarinda, East Kalimantan, 75123, Indonesia

<sup>2</sup>Faculty of Medicine, Mulawarman University, Kampus Gunung Kelua, Jl. Kerayan, Samarinda, East Kalimantan, 75123, Indonesia, Corresponding author E-mail: s.paramita@fk.unmul.ac.id

<sup>3</sup>Faculty of Mathematics and Natural Sciences, Mulawarman University, Kampus Gunung Kelua, Jl. Penajam, Samarinda, East Kalimantan, 75123, Indonesia

<sup>4</sup>Faculty of Medicine, Mulawarman University, Kampus Gunung Kelua, Jl. Kerayan, Samarinda, East Kalimantan, 75123, Indonesia

Received: 09.01.2026

Accepted: 12.03.2026

Published: 04.04.2026

### Abstract

Stingless bees (*Heterotrigona itama*) propolis is a promising natural product with diverse biomedical uses. However, its safety and bioactivity in disturbed environments, such as post-mining lands, are not well understood. This study evaluated heavy metal content, phytochemical, and antioxidant activity of *H. itama* propolis from post-mining land in Kalimantan, Indonesia. Heavy metals (As, Pb, Hg, Cd, and Cr) were analysed using ICP-OES. Phytochemical screening identified secondary metabolites. Antioxidant capacity was measured using the DPPH assay, with ascorbic acid as a standard. All heavy metal concentrations were below FAO/WHO limits, indicating safety. Phytochemical tests showed saponins, flavonoids, phenolics, tannins, terpenoids, and triterpenoids. The DPPH assay showed moderate to strong antioxidant activity (IC<sub>50</sub> 36.52 ppm vs 2.71 ppm for ascorbic acid). *H. itama* propolis from post-mining land is chemically safe and active, demonstrating antioxidant potential linked to its phenolic and flavonoid content. These findings support sustainable stingless beekeeping in post-mining areas for environmental rehabilitation and as a source of valuable natural products.

**Keywords:** Stingless bee propolis, heavy metals, phytochemicals, antioxidant, post-mining land

### INTRODUCTION

Propolis is a resinous substance produced by bees from plant exudates. Bees mix these exudates with wax and salivary enzymes. This natural material is recognized for diverse biological aspects, including antibacterial, anti-inflammatory, antioxidant, and anticancer activities (Salleh et al. 2021a, Al-Hatamleh et al. 2020). In recent years, propolis produced by stingless bees, particularly *Heterotrigona itama*, has received increasing scientific attention. This is due to its high levels of bioactive compounds, including flavonoids, phenolics, terpenoids, and triterpenoids, all of which contribute to its therapeutic potential (Pratami et al. 2024, Goh et al. 2023). The chemical composition of propolis varies with the plant sources available to the bees and environmental conditions, creating distinct regional chemical profiles (Rocha et al. 2023, Zuhendri et al. 2022). Indonesia's tropical ecosystems, especially those in Kalimantan, provide abundant botanical resources for stingless bees.

These resources support the development of stingless bee farming in rural communities (Syafrizal et al. 2020). However, extensive coal mining activities have transformed large forest areas into post-mining landscapes. These environments may retain residues of heavy metals, such as arsenic (As), lead (Pb), mercury (Hg), cadmium (Cd), and chromium (Cr), which can persist in soil and surrounding ecosystems (Shaheen et al. 2023). Stingless bee farming has recently been introduced as a sustainable livelihood option in some rehabilitated mining areas. However, information about the safety and quality of bee products from such environments remains limited (Harianja et al. 2023).

Heavy metals can enter bee products through environmental pathways, such as plant resins, soil particles, and atmospheric deposition. These contaminants may accumulate in honey, bee pollen, and propolis (Glevitzky et al. 2025, Hodel et al. 2020). Bees forage widely and interact closely with

their environment. As a result, bee products are seen as useful bioindicators of contamination, especially in mining-affected regions (Végh et al. 2023, Balderrama-Carmona et al. 2024). Measuring concentrations of heavy metals like As, Pb, Hg, Cd, and Cr in propolis is important for evaluating environmental quality and the safety of bee products for consumption or medicinal use (Paramita et al. 2024b).

Beyond toxicological considerations, propolis is also recognized as a rich source of natural antioxidants. These compounds play an essential role in neutralizing ROS (reactive oxygen species). They help reduce oxidative stress and prevent chronic degenerative diseases (Alsarayrah et al. 2025, Rozman et al. 2022). The antioxidant capacity of propolis is mainly linked to its phenolic and flavonoid content, which act as radical scavengers and metal chelators (Salleh et al. 2022). As these compounds come from plant resins collected by bees, the antioxidant profile of propolis may mirror the botanical diversity and ecological conditions of the surrounding environment, including rehabilitated ecosystems (Sanches et al. 2017).

Despite growing global interest in stingless bee products, few studies evaluate the chemical safety, phytochemical composition, and antioxidant properties of propolis from post-mining areas in Indonesia. Understanding these aspects is important for assessing the environmental implications of stingless beekeeping and the potential biomedical value of propolis from such ecosystems. This study aims to determine concentrations of selected heavy metals in stingless bee propolis from post-mining land in East Kalimantan, identify its phytochemical constituents via qualitative screening, and evaluate antioxidant activity using the DPPH radical scavenging assay. The results provide new insights into environmental safety and bioactive potential of propolis from rehabilitated mining landscapes. They also contribute to the development of sustainable stingless bee farming and to the use of natural products in post-mining ecosystems.

## MATERIALS AND METHODS

### Research Location

Stingless bee (*H. itama*) propolis was collected at a stingless bee farm in post-mining land at Tanah Merah, Samarinda, East Kalimantan, Indonesia (0° 27' 10.8" S 117° 12' 25.2" E). Mining in this area began around 1980 and continued until the early 1990s, with an open-pit system. Operations declined and ceased completely in 1997. Since 2000, Mulawarman University has led rehabilitation programs focused on ecological restoration and revegetation. Due to these efforts, the site has undergone more than 25 years of recovery. The current vegetation is dominated by fast-growing pioneer species, particularly *Calliandra calothyrsus*,

often used in revegetation programs in East Kalimantan (Edi et al. 2024, Junaidi et al. 2023).

### Stingless Bee Identification and Propolis Collection

Propolis samples (Figure 1) were collected from managed colonies of stingless bees identified as *Heterotrigona itama*. The bees were identified at the Forest Cultivation Laboratory, Faculty of Forestry and Tropical Environment, Mulawarman University, Indonesia. Species identification used morphological traits and standard meliponine bee taxonomic keys. Propolis was scraped from hive walls with sterile stainless-steel tools to avoid contamination. Samples were placed in sterile containers, transported to the laboratory, and stored at 4°C until extraction and analysis.



Figure 1. Propolis samples of the stingless bee (*H. itama*)

### Preparation and Ethanolic Extraction of Propolis

Raw propolis samples were cleaned to remove visible debris such as wood fragments and hive impurities. The samples were ground into small particles to increase the extraction surface area. Ethanolic extraction was done by maceration. Briefly, 100 g of powdered propolis was mixed with 1 L of 96% ethanol (1:10 w/v) in a glass container. The mixture was left at room temperature for 72 hours and shaken occasionally. After maceration, the extract was filtered with Whatman filter paper to remove insoluble residues. The filtrate was concentrated using a rotary evaporator under reduced pressure at 40°C to remove the solvent. This yielded the crude ethanolic propolis extract. The concentrated extract was stored at 4°C until further analysis (Bankova et al. 2021; Zin et al. 2018).

### Heavy Metal Analysis Using ICP-OES

#### Sample Digestion and Preparation

Heavy metal concentrations in propolis samples were determined by Inductively Coupled Plasma–Optical Emission Spectrometry (ICP-OES) at an accredited analytical laboratory. Propolis samples were first homogenized and prepared as powdered matrices prior to analysis. Approximately 0.5 g of powdered propolis was subjected to acid digestion using concentrated nitric acid (HNO<sub>3</sub>, 65%) to

decompose the organic matrix and release trace elements. Digestion was performed using a closed-vessel microwave system. The digestion program consisted of a temperature ramp to 180–200 °C under controlled pressure conditions, reaching approximately 30–40 bar for 20 min, followed by a cooling stage to room temperature. After digestion, the resulting clear solution was diluted to a defined volume with ultrapure deionized water, filtered when necessary, and transferred to a polypropylene tube prior to instrumental analysis. The analyzed elements included As, Pb, Hg, Cd, and Cr. The laboratory determined analytical detection and quantification limits during method validation and instrument calibration. The limits of detection (LOD) were 0.0948 mg/kg for As, 0.0467 mg/kg for Pb, 0.0244 mg/kg for Hg, 0.0133 mg/kg for Cd, and 0.0174 mg/kg for Cr. The limit of quantification (LOQ) for the analyzed metals was 0.1 mg/kg (Douvris et al. 2023, Chen et al. 2022).

### ICP-OES Instrumental Conditions

Heavy metal analysis was performed using a PlasmaQuant 9100 ICP-OES spectrometer (Analytik Jena, Germany). Calibration curves for each element were established using multi-element standard solutions across a concentration range of 0–14 mg/L, depending on the element analyzed. The regression equations obtained from the calibration curves were As ( $y = 17.3278x + 59.134$ ), Hg ( $y = 77.732x + 31.7835$ ), Pb ( $y = 121.200x + 112.000$ ), Cd ( $y = 1993.996x + 579.351$ ), and Cr ( $y = 413.7175x + 956.0103$ ). The calibration curves demonstrated linearity, with correlation coefficients (R) of 0.9977 for As, 0.9989 for Hg, 0.9978 for Pb, 0.9990 for Cd, and 0.9992 for Cr. Quality control procedures included analyzing reagent blanks, calibration standards, and replicate measurements. Instrument performance and analytical validity were evaluated using internal quality control (IQC) parameters, including calibration linearity and relative percent difference (RPD) between replicate measurements. Acceptance criteria followed the laboratory quality assurance protocol, requiring correlation coefficients within 0.990–1.000 and replicate variation within acceptable ranges depending on concentration levels. The concentration of each metal in the propolis samples was calculated from the calibration curve obtained by ICP-OES measurements and expressed as mg/kg of dry sample weight (dos Reis et al. 2025, Matuszewska et al. 2021).

### Qualitative Phytochemical Screening

Qualitative phytochemical screening was performed to identify the major classes of secondary metabolites present in the ethanolic propolis extract. The analyses were conducted using standard qualitative assays commonly applied for natural product screening. Saponins were detected using the foam test, in which the formation of a stable froth indicated their presence (El Aziz et al. 2019). Alkaloids were identified using Wagner's and

Mayer's reagents, where the formation of a brown or cream precipitate indicated a positive reaction (Oscar et al. 2020). Flavonoids were detected using the Shinoda test, which involves adding magnesium powder and concentrated hydrochloric acid to produce a reddish or pink color (Sankhalkar and Vernekar 2016). Phenolic compounds were determined using the ferric chloride ( $\text{FeCl}_3$ ) test, where the appearance of a dark blue or green coloration indicated the presence of phenolic substances (Shaikh and Patil 2020). Tannins were identified by hot-water extraction followed by a ferric chloride reaction, which produces a characteristic dark color (Rao et al. 2023). Terpenoids and steroids were detected using the Liebermann–Burchard reaction, which yields a green or blue color upon the addition of acetic anhydride and concentrated sulfuric acid (Malik et al. 2017). In addition, triterpenoids were identified using the Salkowski test, which produces a reddish-brown coloration at the interface of the reaction mixture (Rajkumar et al. 2022). The presence of each phytochemical group was determined based on the appearance of characteristic color changes or precipitate formation during the respective reactions.

### Determination of Antioxidant Activity (DPPH Assay)

The antioxidant activity of propolis extracts was evaluated using the DPPH radical scavenging assay. Propolis extracts were prepared in methanol and diluted in a series to obtain concentrations ranging from 0.78 to 500 ppm. This concentration range was chosen based on previous studies evaluating the antioxidant activity of stingless bee propolis. For the assay, an aliquot of the propolis extract solution was mixed with 0.1 mM DPPH solution and incubated in the dark at room temperature for 30 minutes to allow the reaction between antioxidant compounds and DPPH radicals. The decrease in absorbance was measured using a UV–Vis spectrophotometer at 517 nm. The radical scavenging activity was calculated as the percentage inhibition of DPPH radicals relative to the control solution containing DPPH without extract. The percentage inhibition was calculated using the standard formula. The  $\text{IC}_{50}$  value, defined as the concentration of extract required to inhibit 50% of DPPH radicals, was determined from the inhibition curve. Ascorbic acid was used as the positive control to compare antioxidant activity (Gulcin and Alwasel 2023, Tang et al. 2019).

### Statistical Analysis

All measurements were performed in triplicate and expressed as mean  $\pm$  standard deviation (SD). Antioxidant activity data obtained from the DPPH assay. Dose-response curves and  $\text{IC}_{50}$  values were calculated using nonlinear regression analysis in PSPPP version 2.1.1 (GNU Project, Boston, USA). Differences between groups were evaluated using

one-way ANOVA followed by Tukey's post hoc test, with  $p < 0.05$  considered significant.

## RESULTS

### Heavy Metal Content

The concentrations of selected heavy metals in stingless bee propolis collected from post-mining land are presented in Table 1. Three propolis samples were analyzed using ICP-OES. Arsenic (As), mercury (Hg), cadmium (Cd), and chromium

(Cr) were below the limit of quantification (LOQ) in all analyzed samples. Lead (Pb) was detected in all samples, with concentrations ranging from 0.65 to 0.78 mg/kg.

### Phytochemical Assays

The qualitative phytochemical screening of propolis extract revealed the presence of several classes of secondary metabolites. Flavonoids, phenolic compounds, tannins, saponins, terpenoids, and triterpenoids were detected in the extract. In contrast, alkaloids and steroids were not detected under the applied test conditions (Table 2).

Table 1. Heavy Metal Content of Propolis Collected from Post-Mining Land (mg/kg, ICP-OES)

Heavy Metal	Propolis 01	Propolis 02	Propolis 03	Permissible Limits
As	<LOQ	<LOQ	<LOQ	$\leq 1.0$
Pb	0.75	0.65	0.78	$\leq 2.0$
Hg	<LOQ	<LOQ	<LOQ	$\leq 0.5$
Cd	<LOQ	<LOQ	<LOQ	$\leq 0.3$
Cr	<LOQ	<LOQ	<LOQ	$\leq 2.0$

Note: Permissible Limits from Codex Alimentarius (FAO/WHO 2024)

LOQ = Limit of Quantitation

ICP-OES = Inductively Coupled Plasma – Optical Emission Spectroscopy

Table 2. Qualitative Phytochemical Screening of Propolis Extract

Phytochemical class	Test performed	Result
Alkaloids	Wagner's and Mayer's test	–
Flavonoids	Shinoda test	+
Phenolics	FeCl <sub>3</sub> test	+
Tannins	FeCl <sub>3</sub> test	+
Saponins	Froth test	+
Triterpenoids	Salkowski's test	+
Terpenoids	Liebermann–Burchard test	+
Steroids	Liebermann–Burchard test	–

The antioxidant activity of propolis extract was evaluated using the DPPH radical scavenging assay. The extract showed concentration-dependent radical scavenging activity across the tested concentration range. The calculated IC<sub>50</sub> value for the propolis extract was 36.52 ppm, whereas the positive control, ascorbic acid, exhibited an IC<sub>50</sub> of

2.71 ppm. At the highest tested concentration (100 ppm), the propolis extract produced  $72.16 \pm 0.56\%$  inhibition of DPPH radicals. The dose–response relationship between concentration and radical scavenging activity for the propolis extract and ascorbic acid is presented in Figure 2.

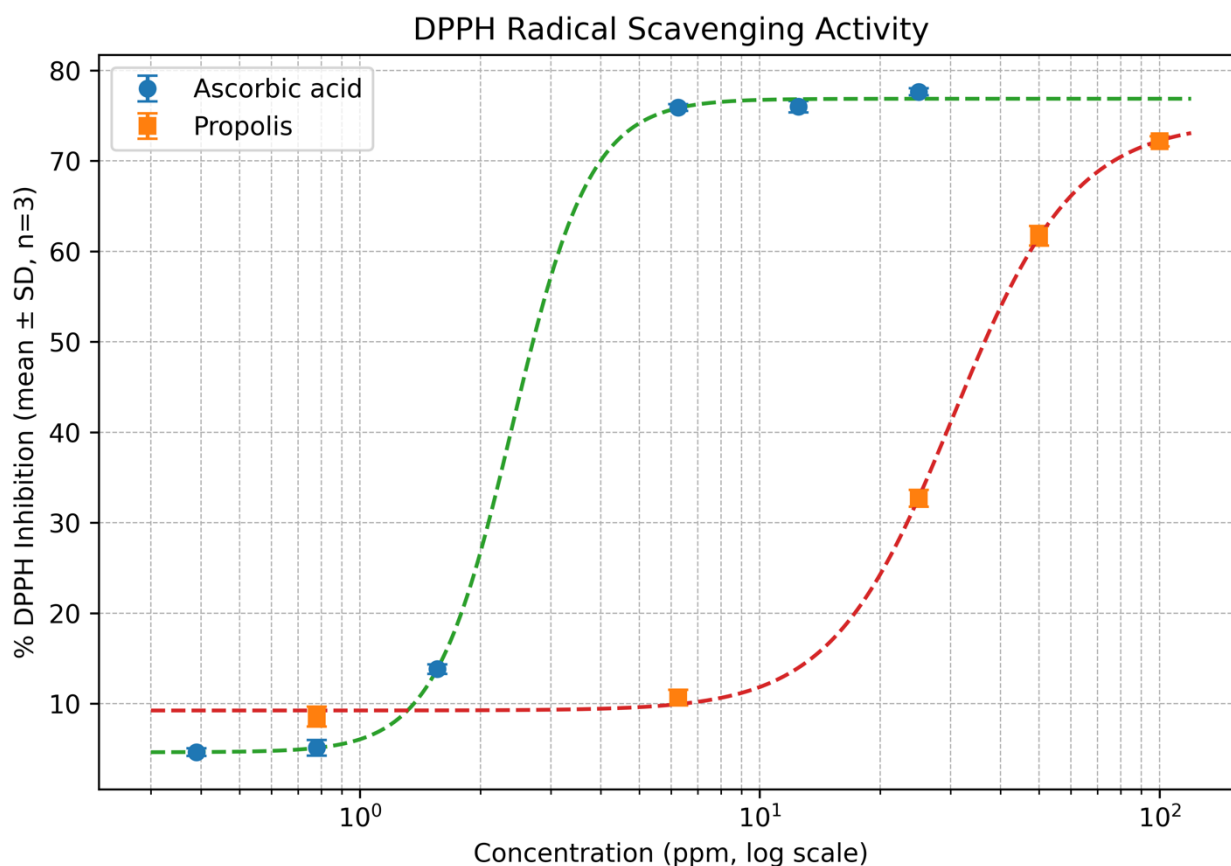


Figure 2. Dose-response curves of DPPH radical scavenging: dose–response curve for propolis and ascorbic acid

## DISCUSSION

This study presents the first integrated determination of the heavy metal content, phytochemical profile, and antioxidant potential of *H. itama* stingless bee propolis cultivated on post-mining land in Kalimantan, Indonesia. The results demonstrate that despite the mining legacy of the sampling site, the analysed propolis samples contained heavy metal concentrations well below international safety limits, while maintaining a diverse phytochemical composition and notable antioxidant activity.

The ICP-OES analysis indicated that As, Hg, Cd, and Cr concentrations in all propolis samples were below the LOQ, while Pb was detected at relatively low concentrations. The measured levels of Pb are below the limits by international guidelines, including those recommended by the Codex Alimentarius Commission for contaminants in food products (FAO/WHO 2024). These findings indicate that the propolis samples did not exhibit elevated contamination levels despite being collected from a post-mining environment. Nevertheless, the detection of Pb suggests that environmental exposure may still contribute to trace metal accumulation.

Bee products are widely recognized as bioindicators of environmental contamination, as bees forage over large areas and may collect plant resins, pollen, and

nectar exposed to environmental pollutants (Mair et al. 2023). However, several studies have reported that propolis often contains lower concentrations of heavy metals than other bee products, such as honey or pollen, likely due to differences in the botanical origin and physicochemical properties of the plant resins used in propolis formation (Zavrtnik et al. 2024). The resinous matrix of propolis, which contains complex mixtures of phenolic and flavonoid compounds, may also influence the mobility and bioavailability of certain metal ions, potentially limiting their accumulation within the propolis matrix (Glevitzky et al. 2021). Comparable findings have been reported in different geographical regions. For example, Véggh et al. (2023) observed that most toxic metals in propolis samples collected in Central Europe were either absent or present only at trace levels. Similarly, Barganska et al. (2016) reported that propolis generally accumulated lower metal concentrations than pollen collected from the same environment. Other studies evaluating mineral composition in bee products have also detected only low levels of heavy metals, supporting the potential use of propolis as a matrix for environmental monitoring (Tutun et al. 2021).

Although the detected Pb concentrations were below the maximum limits, the presence of this metal should be interpreted cautiously. Among the analyzed elements, Pb was the most abundant

metal detected in the present study, which may reflect residual contamination from soils affected by past mining activities or atmospheric deposition associated with anthropogenic sources (Salman et al. 2022). Lead is a non-essential, bioaccumulative element that can accumulate in human tissues, particularly bones and internal organs, following prolonged dietary exposure. Even low concentrations of Pb in food products may contribute to cumulative intake over time if consumed regularly (Shaheen et al. 2023). Similar observations have been reported in propolis collected from disturbed environments, where Pb concentrations remained within safety limits despite environmental exposure (Balderrama-Carmona et al. 2024). However, in post-mining landscapes, soil disturbance and historical mining activities can increase the mobility of heavy metals within the surrounding environment, potentially influencing their availability in ecosystems (Tepanosyan et al. 2018).

Overall, the heavy-metal profile observed in this study suggests that propolis produced by stingless bee colonies located in rehabilitated post-mining areas contains low concentrations of heavy metals and remains within safety limits. These findings are consistent with previous studies indicating that propolis collected from disturbed environments may still meet safety standards (Razavi and Khandan 2017). Considering the potential variability of environmental contamination and the possibility of long-term bioaccumulation, continuous environmental monitoring of bee products remains important, particularly in restored mining areas where residual pollutants may persist (Margaoan et al. 2025). Such monitoring can support the development of sustainable bee-keeping and environmentally responsible land-use practices in rehabilitated post-mining areas.

The dominance of polyphenolic and terpenoid compounds in the present study is consistent with previous investigations of stingless bee propolis from Southeast Asia. Salleh et al. (2021) and Miyata et al. (2020) reported that flavonoids, phenolics, and terpenoids are the major bioactive compounds detected in stingless bees' propolis from Indonesia and Malaysia. These compounds are derived from resins collected by bees and reflect the botanical sources from the surrounding environment (Fikri et al. 2019). Polyphenolic compounds such as flavonoids and phenolic acids are known for their antioxidant activity (Awang et al. 2018). These compounds can neutralize reactive oxygen species through free radical scavenging, hydrogen atom donation, and metal chelation mechanisms (Hossain et al. 2022). In addition, the presence of terpenoids and triterpenoids is also associated with antibacterial, anti-inflammatory, and antimicrobial effects (Zulhendri et al. 2021).

The detection of diverse secondary metabolites in the propolis samples suggests that the stingless bees had access to a wide range of plant resins from

the surrounding area (Adli et al. 2022). Plants growing in rehabilitated post-mining areas may recover and produce secondary metabolites as part of their adaptation to environmental conditions (Withaningsih et al. 2023). Previous studies have demonstrated that vegetation in restored mining areas exhibits high levels of phenolic and terpenoid compounds, which support plant defence and ecological resilience (Alsarayrah et al. 2025). Consequently, the chemical composition of propolis can reflect the botanical diversity and ecological recovery of vegetation in post-mining ecosystems (Paramita et al. 2024a).

The DPPH radical-scavenging assay demonstrated that the stingless bee propolis extract exhibited concentration-dependent antioxidant activity, indicating its ability to neutralize free radicals via electron or hydrogen-atom donation. The result in this study is categorized as moderate to strong antioxidant activity according to the classification for natural antioxidant extracts (Conte et al. 2022). However, compared with the positive control (ascorbic acid), the propolis extract showed lower radical-scavenging potency, consistent with the behavior of many natural extracts, which contain complex mixtures of bioactive compounds. The antioxidant capacity observed in the present study is comparable to values previously reported for stingless bee propolis collected from non-mining regions in Southeast Asia. For example, Salleh et al. (2022) reported similar results for Malaysian propolis extracts, whereas Mohan et al. (2024) observed variations in antioxidant activity depending on botanical origin and extraction method. Differences in antioxidant capacity among bee species and geographical regions are often attributed to variations in plant resin sources, climate conditions, and vegetation diversity.

The moderate to strong radical-scavenging activity observed in this study is associated with the presence of phenolic and flavonoid compounds, as identified in the qualitative phytochemical screening (Zulhendri et al. 2021). These compounds are known for antioxidant mechanisms, which help stabilize reactive oxygen species and prevent oxidative damage in biological systems. Polyphenols in propolis are also reported as contributors to its antioxidant properties (Hassanpour & Doroudi 2023). The antioxidant potential in propolis collected from post-mining areas provides insights into ecological restoration in these environments. Vegetation in rehabilitated mining areas produces a diverse array of secondary metabolites (Pant et al. 2024). As stingless bees collect plant resins from these vegetation sources, the propolis may reflect the phytochemical diversity of the surrounding flora (Salazar et al. 2024). Similar results have been reported in studies showing that restored landscapes can support plants rich in bioactive metabolites. Pratami et al. (2024) reported correlations between plant diversity and phenolic

content in stingless bee propolis collected from restored tropical landscapes.

Collectively, these results suggest that stingless bee propolis produced in rehabilitated post-mining areas can maintain acceptable environmental safety and significant bioactive potential, reflecting both the resilience of surrounding vegetation and the availability of diverse resin-producing plants (Buchori et al. 2022). The findings highlight the potential of stingless bee-keeping as a sustainable strategy that can support local community income while contributing to ecological restoration in post-mining ecosystems (Harianja et al. 2023). However, because environmental conditions and trace metal availability may vary between places, continuous monitoring of heavy metal contamination in bee products remains important to ensure long-term safety and product quality (Paramita et al. 2024b).

This study provides baseline information on the safety and bioactive potential of *H. itama* propolis collected from post-mining land, several limitations should be known. The current analysis was limited to *in vitro* antioxidant activity and qualitative phytochemical screening. Future studies should include quantitative analysis of total phenolic and flavonoid contents, as well as the identification of bioactive compounds using advanced chromatographic and spectrometric techniques. It provides deeper insights into the chemical composition of propolis and its relationship with botanical sources in rehabilitated ecosystems.

Broader environmental sampling and vegetation assessments would help clarify the relationship between plant diversity, vegetation recovery, and the chemical profile of propolis produced in restored mining areas. Further investigations evaluating biological activities, such as antibacterial and anti-inflammatory properties, together with *in vivo* toxicity assessments, would strengthen the biomedical relevance of this propolis. Long-term environmental monitoring in post-mining stingless beekeeping areas would also be valuable for better understanding temporal and spatial variations in heavy metal accumulation associated with land rehabilitation processes.

### Conclusions

Propolis produced by stingless bee (*H. itama*) colonies cultivated in post-mining land in East Kalimantan contained low concentrations of the heavy metals, with As, Cd, and Hg below the limit of quantification, with Pb and Cr detected at low levels within standard guideline ranges. The propolis extract also contained several bioactive phytochemical groups, including flavonoids, phenolic compounds, tannins, saponins, terpenoids, and triterpenoids, which are commonly associated with propolis's biological properties. Consistent with these phytochemical characteristics, the extract exhibited notable antioxidant activity in the DPPH assay.

These findings suggest that rehabilitated post-mining areas can support stingless bee colonies that produce propolis with low levels of heavy metals and high bioactivity. This highlights the potential role of stingless beekeeping as a strategy for sustainable land use, providing both ecological benefits and economic opportunities for local communities. However, considering the potential of environmental contamination in post-mining ecosystems, regular monitoring of heavy metal levels in bee products remains important to ensure long-term safety and product quality.

**AI Declaration:** AI has not been used to prepare the manuscript, except for grammar and word corrections.

**Author Contribution:** Mayusef Sukmana: investigation, analysis, writing—original draft, writing—review & editing; Swandari Paramita: research planning, funding acquisition, writing—review & editing; Sri Wahyuningsih: research planning, writing—review & editing; Yadi Yasir: research planning, writing—review & editing.

**Data Availability:** Data are available in the manuscript.

**Declaration of interest:** The authors declare that there is no conflict of interest.

**Ethics:** The research was conducted *in vitro* and not with animals or humans.

**Financial support:** This study is funded by the Postgraduate Research Doctoral Dissertation Program, Ministry of Education, Science and Technology, Republic of Indonesia (Grant No. 080/C3/DT.05.00/PL/2025).

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