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RESEARCH NOTE

Characterization and Antioxidant Activities of Different Solvent Extracts of Philippine Stingless Bee (*Tetragonula biroi* Friese) Pollen Samples

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Bee pollen is among the bee products that are considered functional food due to its components exhibiting various biological activities. This study aimed to characterize the volatile compounds present in stingless bee pollen extracted by different solvents using gas chromatography–mass spectrometry (GC-MS). Antioxidant activities and total phenolic content of bee pollen solvent extracts were also determined by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Folin-Ciocalteu assays, respectively. Fatty acids and esters were detected in ethanolic and methanolic extracts – namely, n-hexadecanoic acid, hexadecanoic acid ethyl ester, and 9,12,15-octadecatienal acid ethyl ester (Z,Z,Z). Long-chain hydrocarbons – namely, heptacosane and pentacosane – were detected in dicholoromethane, chloroform, and hexane extracts. Total phenolic content was in the range of 20.43 ± 2.78 to 371.66 ± 11.26 mg GAE/g, with ethanolic and methanolic extracts having the highest amounts detected. Antioxidant activities IC_{50} values ranged from 5.69 ± 0.74 to 153.90 ± 13.43 mg/mL, with ethanol and methanol exhibiting the highest activities. Hence, bee pollen is a potential source of antioxidant and phenolic compounds.

Keywords: antioxidant activity, bee pollen, Folin-Ciocalteu assay, GC-MS, total phenolic content

Bee pollen is becoming popular in the market as a functional food because of its high nutritive content and wide range of beneficial biological activities. A study conducted by Belina-Aldemita and co-authors (2019) on bee pollen from the Philippines showed that it is mainly composed of carbohydrates, lipids, proteins, amino acids, vitamins, minerals, sterols, carotenoids, and polyphenols. The polyphenolic content of bee pollen consists mainly of flavonoids and phenolic compounds and is responsible for its antimicrobial, antimutagenic, antioxidative, anti-inflammatory, antifungal, and other pharmacological properties (Denisow *et al.* 2016; Belina-Aldemita *et al.* 2019).

Propagation of stingless bees in the Philippines is feasible due to the abundant populations of this species,

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resistance to pests and diseases, and ability to forage more floral resources. Bee pollen production is also favorable because of the diversity of foraging locations for the bees (Locsin *et al*. 2021). The composition of bee pollen is usually dictated by the geographical location, vegetation, botanical origin, and bee species. Variations in the composition of bee pollen may affect its biological and pharmacological activities (Belina-Aldemita *et al.* 2019). Evaluation of the functional properties of bee pollen could provide useful information as it is marketed as a functional food.

The aim of this study was to determine the chemical composition of the various solvent extracts of *Tetragonula biroi* Friese pollen using GC-MS and quantify its antioxidant activities and total phenolic content.

Pollen samples were collected from cultivated colonies of *T. biroi* located at Calamba, Laguna in the Philippines (geographic coordinates in decimal degree: 14.203340, 121.106319). Samples were composite from three different colonies and were collected using forceps by manually separating the pollen from the honeycombs. These were ground as fine as possible using mortar and pestle and stored in glass containers at 4 °C until further processing.

Solvent-soluble compounds in bee pollen were extracted using five different liquids: methanol, ethanol, dichloromethane, hexane, and chloroform. The pollen-solvent mixtures (1:10 w/v) were sonicated for 30 min using an 80 W 40 kHz ultrasonic bath (Fischer Scientific FS20H, Pittsburg, USA). The resulting mixtures were then clarified by filtration through a Whatman® UNIFLO® syringe filter with 0.45-µm pore size. The clarified bee pollen solvent extracts (BPSEs) were stored in a 4-mL dram vial and stored in the dark below 0 °C until further use in GC-MS and antioxidant assays.

BPSEs were centrifuged, and 20-µL of each extract was diluted to 1 mL of its respective solvent. The electronic absorbance spectra of various BPSEs were measured using a JASCO v-570 spectrophotometer (Easton, MD, USA). The samples were scanned at 200–500 nm using quartz cuvettes (1 cm^2). Measurement of the spectra was done between 200–500 nm since most phenolic compounds generally exhibit an absorption peak in the ultraviolet light range of 250–350 nm (de Lima *et al.* 2015).

The various BPSEs were initially filtered prior to sample loading. For each scan, a 5-µL extract volume was injected into an HP 6890 Series GC system (California, USA) with a 5973 mass selective detector using the following parameters: HP5-MS column (30 µm x 250 µm x 0.28 µm); injector temperature of 110 °C; initial column temperature of 110 °C for 2 min, then ramped to 200 °C, and further increase to 280 °C for the next 15 min; total analysis run of 36 min; and helium flow rate at 1.5 mL/min. After the run, the peak identities from the various MS profiles were established using the NIST Mass Spectral Library.

For the determination of total phenolic content, a 50-µL sample of the BPSEs was mixed with 125 µL of 0.1 N Folin-Ciocalteu reagent (Sigma-Aldrich, Missouri, USA) for 4 min in a microplate, then 100 µL of 1.0 M Na_2CO_3 solution was added onto the mixture. The mixture was then allowed to stand for 2 h, then the absorbance was read at 745 nm using a Biotek Cytation 5 Cell Imaging Multi-Mode Reader (Vermont, USA). Gallic acid, with concentrations ranging from 2.5–100 µg/mL, served as standard. Four replicates were done for the assay. Ethanol was used as the blank solution.

For the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, various dilutions of the BPSEs (1:10 w/v) were prepared by adding the required amount of solvent to give a final concentration that ranges from 0.05–5.0 x 10^{-6} g/mL. A 50-µL sample of the diluted extract was mixed with 200 µL of 0.2 mM DPPH solution (dissolved in methanol) in a microtiter plate and allowed to stand for 30 min. The absorbance of the mixture was then read at 519 nm using a Biotek Cytation 5 Cell Imaging Multi-Mode Reader (Vermont, USA). The percent scavenging activity was calculated using the equation below. Then, the antioxidant activity was ultimately expressed in terms of IC_{50}, which was calculated from the plot of percent scavenging activity against concentration.

$$\text{Percent scavenging activity} = \frac{\text{Blank absorbance} - \text{Sample absorbance}}{\text{Blank absorbance}} \; x \; 100$$

Assay results were expressed as mean ± standard deviation. Significance testing (at a 95% confidence level) was performed using Tukey's HSD (honestly significant difference) test. Correlation analysis between phenolic content and the IC_{50} from DPPH was also performed using Microsoft Excel.

The absorbance spectra of the samples show the number and characteristics of absorption peaks which can be used to identify the classes of bioactive compounds present in the sample. As shown in Figure 1, methanol and ethanol BPSEs showed similar peaks or shoulders at 230–290 nm and 300–390 nm, which can be attributed to the presence of flavonoids and phenolic compounds. Chloroform, dichloromethane, and hexane BPSEs showed similar peaks at around 250 nm, which is not present in methanol and ethanol BPSEs, which indicates another group of compounds. Furthermore, the shoulder at around 350–390 nm observed in methanol and ethanol BPSEs was not present in the BPSEs of the other solvents. The

Figure 1. Stingless bee pollen samples.

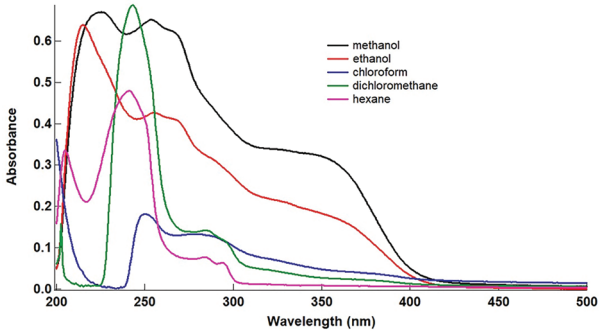


Figure 2. Absorbance spectra of different BPSEs.

absorbance profiles of the BPSEs are comparable with the study by Adaskeviciute (2019).

Various solvents were utilized to extract the components from bee pollen, particularly those that may contribute to its antioxidative potential. Initial characterization was done by looking at the ion chromatogram profiles of the BPSEs (Table 1; Figure 3). Ethanol and methanol extracts were found to contain n-hexadecanoic acid, hexadecanoic acid ethyl ester, and 9,12,15-octadecatienal acid ethyl ester (Z,Z,Z). Both heptacosane and pentacosane were extracted in the less polar dichloromethane, chloroform, and hexane solvents. The fractionation of the various compounds in the different solvents was expected due to the varying degrees of solute-solvent polarities. Slightly polar components like carboxylic acids and esters partition into the slightly polar ethanol and methanol solvents, whereas the nonpolar hydrocarbons partition into the less polar chloroform, dichloromethane, and hexane solvents.

Table 1. Bee pollen components as resolved and identified using GC-MS.

Solvent extract	Peak #	Chemical compound
Ethanol	1	n-Hexadecanoic acid
	2	Hexadecanoic acid ethyl ester
	3	9, 12, 15-Octadecatrienal acid ethyl ester (Z,Z,Z)
Methanol	1	n-Hexadecanoic acid
	2	Hexadecanoic acid ethyl ester
	3	9,12,15-Octadecatrienal acid ethyl ester (Z,Z,Z)
Dichloromethane	1	Pentacosane
	2	Heptacosane
Chloroform	1	Pentacosane
	2	Heptacosane
Hexane	1	Pentacosane
	2	Heptacosane

Palmitic acid (n-hexadecanoic acid) is one of the most abundant fatty acids found in a variety of bee pollen samples, in addition to other saturated and unsaturated fatty acids like oleic, stearic, linoleic, linoleic, *etc.* (Negri *et al*. 2018). Palmitic acid and other lipid components are essential to the bee's growth, development, diet, and perpetuation. Furthermore, palmitic acid is also one of the predominant fatty acids found in body fats of both larvae and adult bees (Al-Kahtani *et al.* 2021; Negri *et al*. 2018).

Esters such as hexadecanoic acid ethyl ester are also a common component of bee products (Naik *et al.* 2021). These esters may confer antioxidant and therapeutic activities that are beneficial to human health (Xu *et al.* 2012; Negri *et al.* 2018; Kocot *et al*. 2018). In addition, the volatility of esters – together with low molecular weight hydrocarbons – also contribute to the sensory characteristics and aroma compounds of bee pollen and other bee products (Starowicz *et al.* 2021).

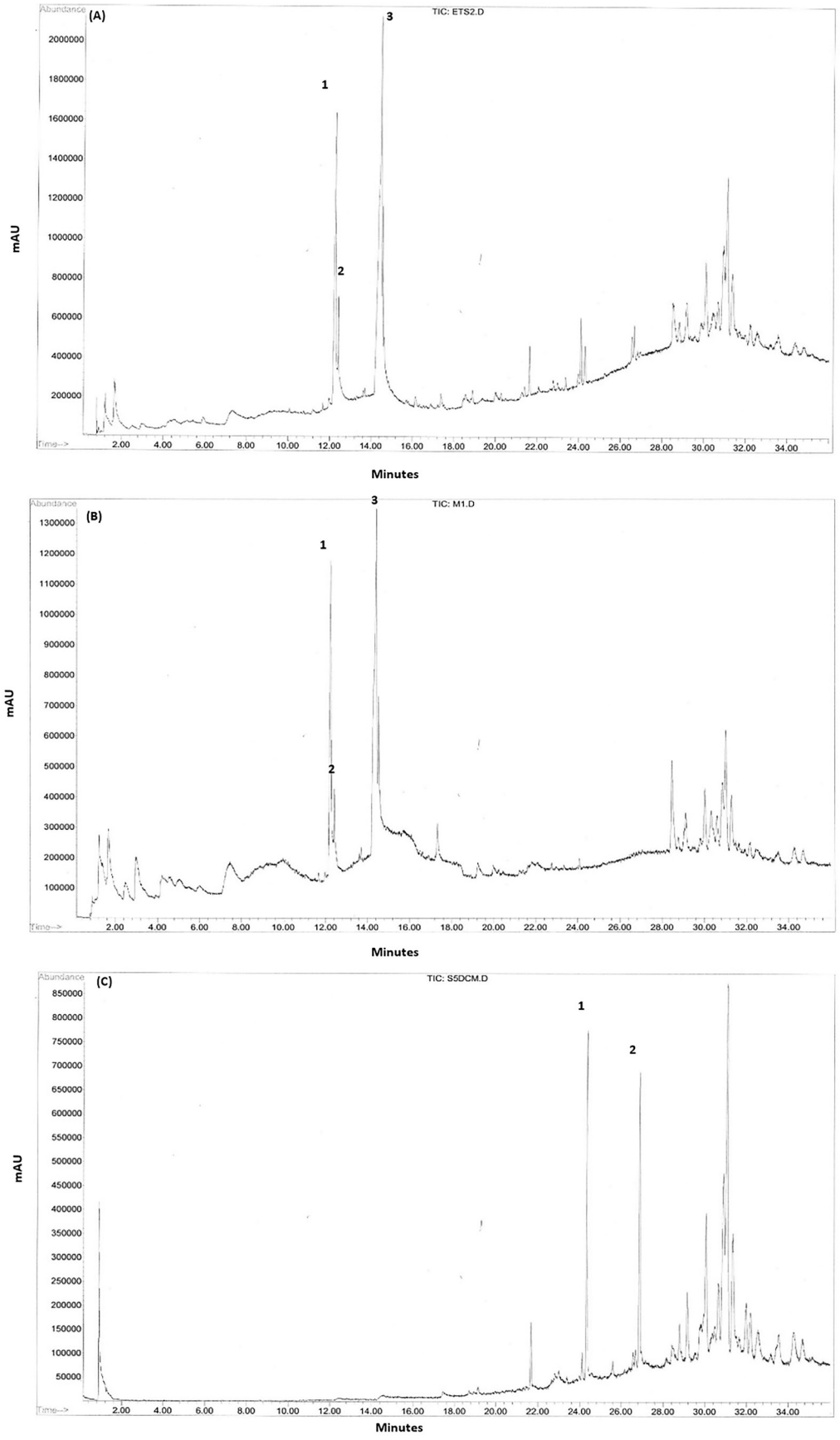
Another bee product, which is beeswax, is composed of esters and long-chain hydrocarbons like pentacosane and heptacosane (Fratini *et al*. 2016). The presence of these substances in bee pollen suggests that during pollen collection and storage, there may have been mixing and transfer of wax traces into pollen by bees.

The total phenolic content of the various BPSEs is shown in Table 2. Ethanol and methanol are the most

Table 2. Antioxidant activity and phenolic content of the BPSEs.

Solvent extract	Antioxidant IC50 (mg/mL)	Phenolic content (mg GAE/g)*
Ethanol	6.27 ± 0.87 b	364.43 ± 9.98b
Methanol	5.69 ± 0.74 b	371.66 ± 11.26b
Dichloromethane	133.63 ± 10.92 a	41.43 ± 3.92a
Chloroform	144.53 ± 12.23 a	34.66. ± 3.50a
Hexane	153.90 ± 13.43 a	20.43 ± 2.78a

*Mean ± SD (n = 4) with different letters within the same column are significantly different at $p < 0.05$ using Tukey's HSD test


(A)
TIC: ETS2.D
Abundance
mAU
Minutes
(B)
TIC: M1.D
Abundance
mAU
Minutes
(C)
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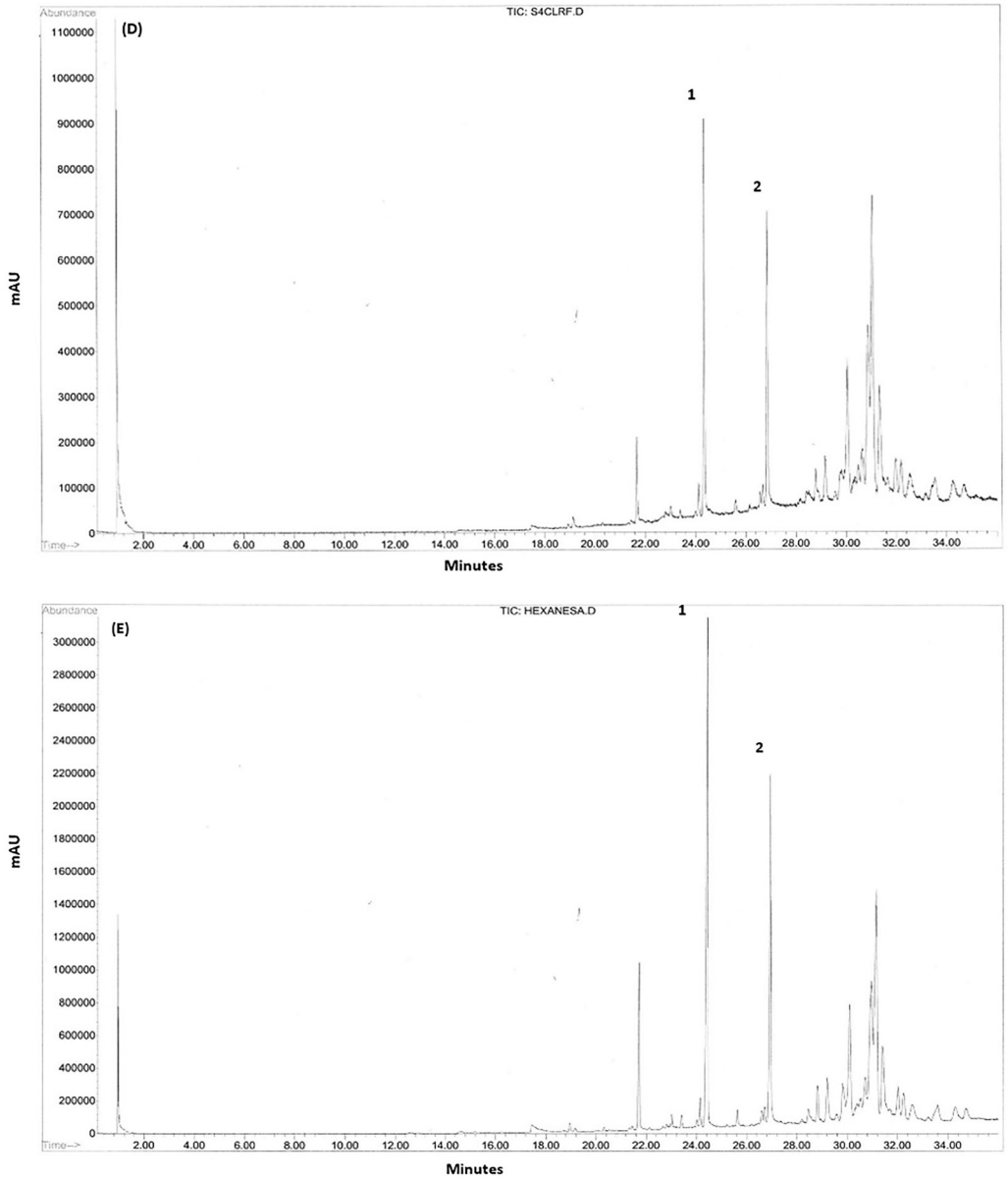

Figure 3. GC-MS profiles of bee pollen extracted using the following solvents: [A] ethanol, [B] methanol, [C] dichloromethane, [D] chloroform, and [E] hexane. Note: number labels refer to distinct peaks.

effective solvents for extracting bee pollen phenolics with values equal to 364.43 ± 9.98 to 371.66 ± 11.26 mg GAE/g, respectively. On the other hand, the less polar dichloromethane, chloroform, and hexane contained the least amount of phenolics extracted, with values ranging from 20.43 ± 2.78 to 41.43 ± 3.92 mg GAE/g. The alcoholic extracts' values are higher in comparison to those reported in the literature such as the one reported by Freire *et al.* (2012), whose phenolic content ranged from 41.5–213.2 mg GAE/g.

Phenolic compounds are considered the most widely distributed class of secondary metabolites produced by plants in response to biotic and abiotic stresses. They play key roles in plant defense, protection, and signaling or communication (Lattanzio 2013). Extraction of a particular class of phenolic compounds depends on many factors such as time, temperature, solvent type, and combinations thereof, and plant source (Khoddami *et al*. 2013).

In terms of antioxidant activities, the extracts from both ethanol and methanol yielded the highest activities, with IC_{50} values ranging from 5.69 ± 0.74 - 6.27 ± 0.87 mg/mL. Lower IC_{50} values signify higher antioxidant activities. Extracts from dichloromethane, chloroform, and hexane

were less effective antioxidants, as indicated by their higher IC_{50} values ranging from 133.63 ± 10.92 to 153.90 ± 13.43 mg/mL. The results may be attributed to the higher phenolic content extracted by ethanol and methanol.

Results between phenolic content and DPPH IC_{50} values showed a strong correlation with a value of –0.9984. A high phenolic content means a lower IC_{50} value, which agrees with the results reported by Ulusoy and Kolayli (2013), who noted a correlation between the total phenolic content of bee pollen and antioxidant activities. They noted that bee pollen extracts with higher phenolic content, as determined by Folin-Ciocalteu, also conferred greater antioxidant activities. Kocot *et al.* (2018) also confirmed this correlation with their studies on the antioxidant potential of various bee products. They observed that the total phenols and flavonoid content were highest for propolis, followed in decreasing order by bee pollen, and royal jelly, whereas the antioxidant activities of the corresponding bee products also followed the same trend.

CONCLUSION

Fatty acids and esters were the predominant substances extracted by ethanol and methanol, while long-chain hydrocarbons were the components extracted by the less polar dichloromethane, chloroform, and hexane solvents. These substances are important in the growth and development of bees, and some may also confer antioxidant activities. Ethanol and methanol were found to contain the highest phenolic content among the different extracting solvents. Consequently, both solvents also yielded the highest antioxidant activities as signified by their low IC_{50} values. Hence, bee pollen is a good source of antioxidant compounds, with a high potential of being considered a nutraceutical and a key component for the pharmaceutical industries.

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