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Research Article

Botanical Origin and Extraction Methods of Philippine Stingless Bee (*Tetragonula biroi* Friese) Pollen and its Food Functionality: Phenolic and Flavonoid Content and Antioxidant Activity

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ABSTRACT

With its high nutritional and bioactive content, bee pollen is recognized as a functional food. However, its composition is highly dependent on a range of factors, such as geographical location. In this research, we used DPPH to determine the antioxidant activity of several stingless bee (*Tetragonula biroi*) pollen samples sourced from five different areas in the Philippines and compare their phenolic and flavonoid content. Additionally, we compared two extraction methods: maceration (or soaking of samples) for three days and sonication for 30 minutes. Interestingly, our findings show that the phenolic content of bee pollen is weakly associated with antioxidant activity, indicating that other bioactive chemicals may play a more significant part in the antioxidant characteristics of bee pollen. Antioxidant properties based on the DPPH assay of pollen harvested from Calamba are higher compared to other samples from different geographic areas, i.e., Quezon Province, Sorsogon, Laguna, and Albay. When compared to the maceration approach, extracts generated from the sonication process had lower antioxidant activity. These findings suggest that Philippine stingless bee pollen is a potential source of bioactive compounds, and the choice of extraction method and geographic source are significant factors affecting its antioxidant activity.

Keywords: Antioxidant activity, Extraction Methods, Flavonoids, Natural products, Phenolic compounds, Philippine stingless bee, Pollen, Tetragonula biroi

Introduction

Bee products, such as pollen, honey, and propolis, have been utilized for a variety of purposes in many regions of the world throughout history [1]. Bee pollen is a hive product that honeybees collect from flowers, place in the honeycomb, incorporate with salivary gland secretion, and change into bee bread. Just like honey and propolis, bee pollen is also noted for its bioactive and nutritional content. Its medicinal benefits are ascribed to its roughly 250 distinct constituents, which include sugars, proteins, minerals, vitamins, lipids, and flavonoids. The nutritional composition of bee pollen comprises an average of 25.7% fructose and glucose, 22.7% protein, 10.4% indispensable amino acids, 30.8% digestible carbohydrates, and 5.1% essential fatty acids. The residual 5.3% encompasses amino acids, other macronutrients, as well as flavonoids and phenolic compounds [2].

Bee pollen has been recognized as a functional food due to its potential health benefits [3]. Abundant in bioactive compounds, including flavonoids, phenolic acids, vitamins, and minerals, it exhibits pharmacological effects encompassing antioxidant, anti-inflammatory, anti-cancer, anti-

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microbial, and anti-diabetic properties. Bee pollen has significant antioxidant activity due to its phenolics and flavonoids, which neutralize free radicals and reduce oxidative stress [4, 5]. Furthermore, bee pollen has been shown to have immunomodulatory effects, improving the immune system's response to infections and lowering the risk of chronic diseases by decreasing IL-1, IL-6, TNF-α, and nitric oxide (NO) levels [6]. Bee pollen exhibits noteworthy wound-healing attributes, encompassing its capacity to inhibit bacterial proliferation, reduce wound pH, mitigate pain and inflammation, enhance fibroblast migration and keratinocyte closure, and promote the deposition of collagen. [7]. As a food supplement, bee pollen is known to improve glycemic control for persons with metabolic illnesses such as diabetes as it is a rich source of natural α-glucosidase inhibitors, making it a suitable dietary supplement [8]. Overall, bee pollen's functional dietary features make it a potential natural product for the prevention and management of a variety of health disorders.

The foraging behavior of bees is characterized by a marked preference for the collection of pollen from plant species within their immediate geographic proximity. Consequently, the intricate composition of bee pollen is subject to the botanical composition of the region in which it is harvested, comprising factors such as the proportional distribution, abundance, and accessibility of flowers from distinct plant species [2]. It is imperative to underscore that the nutritive content of bee products is notably influenced by an interplay of various environmental determinants, including but not limited to soil type, temperature, humidity, and other pertinent conditions. These environmental variables exert a discernible influence on plant development and metabolic processes, thereby contributing to the divergence in nutritional profiles observed across bee products [9]. While bee pollen from different botanical sources in Alagoas, Brazil, had different physicochemical compositions and antioxidant potential [10], phenolics and free amino acid profiles may also vary in bee bread and bee pollen even with the same botanical origin [11]. Additionally, from our previous paper, we found that natural product composition varies with different extraction procedures used, and, notably, there were substantial disparities in the antioxidant activities attributed to bee propolis [12]. Therefore, in this study, we elucidate the effect of geographic location and extraction methodologies on bee pollen composition on the phenolic and flavonoid content and antioxidant activity of Philippine stingless bee pollen. Our investigation revealed that sonication extraction exhibited superior antioxidant activity in comparison to the soaking approach. The phenolic content of bee pollen was weakly associated with its antioxidant activity, indicating the potential presence of other bioactive chemicals. Thus, it is essential to carefully evaluate the botanical origin of bee pollen and its processing when studying its properties and food functionality potential.

Material and Methods

Sample preparation

The Philippine stingless bee pollen samples were collected from various beehives in different geographic locations from January to June 2018 (Figure 1). The collection sites include Calamba City (14.187671°N, 121.125084°E), Tayabas, Quezon (14.0364°N, 121.6533°E), Los Banos, Laguna (14.169912°N, 121.244063°E), Bulusan, Sorsogon (12.4528°N, 124.0860°E), Guinobatan, Albay (13.1541°N, 123.5504°E), and San Jose, Batangas (13.5238°N, 121.0618°E). The collected samples were transported to the University of the Philippines- Los Banos (UPLB) Bee Program and subsequently to Pace University in New York while being stored at 0°C during transport and stored at -4°C refrigerator until utilized.

Each bee pollen sample was divided into two sets. For each set, 0.5 g of bee pollen was mixed with 10 mL of extraction solution containing 90% ethanol, 5% methanol, and 5% isopropyl alcohol. The solvents were procured from Fisher Chemical (Waltham, MA). One set was subjected to a maceration process, allowing it to undergo immersion for a duration of three days. Concurrently, the second set underwent sonication for a duration of 30 minutes, utilizing an 80 W ultrasonic bath (Fischer Scientific FS20H), thereby constituting the sonication set. After sonication or maceration, the resulting extract was centrifuged for two minutes, and the supernatant was isolated using 2 mL Norm-Ject® syringes and filtered with 13 mm syringe filters with 0.45 µm polytetrafluoroethylene (PTFE) membranes. The collected extracts were then used to determine the phenolic content, flavonoid content, and antioxidant activity using DPPH.

Figure 1. Philippine stingless bees. Photograph of (A) nest and (B) internal structure showing storing pots of honey and pollen.

Determination of phenolic and flavonoid content

The methods used to determine the phenolic content and flavonoid content of the bee pollen extracts are described as follows. The Folin-Ciocalteu assay was used to determine the phenolic content of the bee pollen extracts obtained by maceration and sonication. A 10% concentration of the Folin-Ciocalteu reagent (Sigma Aldrich, St. Louis, MO) and 1.0 M of Na_2CO_3 (Sigma Aldrich) were prepared. On a 96-microwell plate, 20 µL of each bee pollen extract was mixed with 100 µL of the Folin-Ciocalteu reagent, followed by incubation for 4 minutes. Then, 100 µL of 1 M Na_2CO_3 was added, and the mixture was incubated for 2 hours in the dark. Absorbance was measured at 645 nm using a spectrophotometer. Distilled water was used as a negative control, while gallic acid (Acros Organics, Belgium) was used as the positive control and the standard to generate a calibration curve for expressing the total phenolic content (TPC) in mg/mL gallic acid equivalents (GAE).

An aluminum chloride assay was used to determine flavonoid content. To prepare the reagent, 10% aluminum chloride (Sigma Aldrich), 95% ethanol (Fisher Chemical), and 1 M sodium acetate (Sigma Aldrich) were mixed in a 1:15:1 ratio. On a 96-microwell plate, 20 µL of each bee pollen extract was mixed with 200 µL of the reagent solution, followed by incubation for 40 minutes in the dark. Absorbance was measured at 420 nm using a spectrophotometer. Ethanol was used as a negative control, while quercetin (Sigma Aldrich) was used as the positive control and the standard to generate a calibration curve for expressing the total flavonoid content (TFC) in mg/mL quercetin equivalents (QE).

Antioxidant activities

The antioxidant activities for each bee pollen extract were determined using a DPPH assay. Using a microplate, 20 uL of the extract/standard was mixed with 200 uL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich). After 30 minutes, absorbance at 519 nm was obtained. The

antioxidant activity was determined using the given formula below, and IC_{50} was derived to express the antioxidant activity

$$\%\text{Scavenging Activity} = \frac{\text{blank absorbance} - \text{sample absorbance}}{\text{blank absorbanc}} \times 100$$

where IC_{50} value (mg/mL) was the half-maximal effective concentration. The IC_{50} value was generated by plotting the measured absorbance of the serial dilutions of each sample against their specific concentrations (0.5, 0.05, 0.005 mg/mL). This plot generates a sigmoidal curve, for which the point of 50% effectiveness can be determined from the logarithmic equation. All absorbance readings were obtained using Biotek Cytation 5 Image Reader (Agilent Technologies, USA). Both assays were replicated at least three times.

Statistical analysis

Experimental data (phenolic content, flavonoid content, and IC_{50} values using DPPH assay) of extracts obtained from the two extraction methods were evaluated using a student's t-test ($p < 0.05$). Correlation analysis between phenolic content, flavonoid content, and IC_{50} values was performed using Microsoft Excel. Multiple pairwise comparisons between the different bee pollen sources were conducted using One-Way ANOVA with Tukey HSD post-hoc test at an α set at 0.05. Data were presented as mean values in tables and graphs. Multiple linear regression (MLR) was used to describe the linear relationship between various explanatory variables and one response variable. MLR, as opposed to conventional linear regression, accounts for the effects of several explanatory variables on the response variable. Graphs were made with GraphPad Prism 5.

Results and Discussion

Differences in the chemical properties of bee pollen-based geographical source

Table 1 presents the phenolic and flavonoid content of the bee pollen samples collected from six different locations. Based on our findings, it is evident that the concentrations of phenols obtained through sonication were markedly elevated across all sources in comparison to maceration. However, in terms of maceration, the phenolic content was similar between Albay, Batangas, and Laguna. In the case of flavonoids, those extracted

Table 1. Phenolic content and antioxidant activities of the Philippine stingless bee pollen obtained from different geographical locations. Mean values with different superscript letters are significantly different using Tukeys' HSD test at $p < 0.05$.

Source	Extraction Method	Phenols (µg GAE/g)	Flavonoids (µg QE/g)	Antioxidant Activities, IC_{50} (µg/mL)
Calamba	Maceration	$14562.14 \pm 116.49^{A,a}$	$2520.91 \pm 619.29^{A,a}$	$5.54 \pm 0.21^{A,a}$
	Sonication	$15205 \pm 355.80^{B,a}$	$2354.24 \pm 327.45^{A,a}$	$7.64 \pm 0.25^{B,a}$
Quezon	Maceration	$5521.67 \pm 87.84^{A,b}$	$1268.85 \pm 88.19^{A,b}$	$7.16 \pm 0.67^{A,a}$
	Sonication	$6228.81 \pm 103.29^{B,b}$	$1581.12 \pm 64.89^{B,b}$	$7.28 \pm 0.64^{A,a}$
Los Banos	Maceration	$6233.57 \pm 89.61^{A,c}$	$1312.07 \pm 45.95^{A,b}$	$673.67 \pm 6.98^{A,b}$
	Sonication	$5802.62 \pm 300.71^{B,c}$	$1445.14 \pm 29.02^{B,b}$	$2803.09 \pm 107.92^{B,b}$
Sorsogon	Maceration	$7345.48 \pm 61.29^{A,d}$	$1293.08 \pm 77.28^{A,b}$	$24.47 \pm 3.80^{A,a}$
	Sonication	$7645.48 \pm 129.76^{B,d}$	$1704.40 \pm 60.76^{B,b}$	$18.33 \pm 0.34^{B,a}$
Albay	Maceration	$6038.33 \pm 40.61^{A,c}$	$1304.29 \pm 100.26^{A,b}$	$34.63 \pm 9.47^{A,a}$
	Sonication	$7128.81 \pm 108.05^{B,d}$	$1416.52 \pm 72.44^{A,b}$	$58.48 \pm 9.07^{B,a}$
Batangas	Maceration	$6114.52 \pm 67.54^{A,c}$	$1213.08 \pm 35.52^{A,b}$	$4.07 \pm 0.30^{A,a}$
	Sonication	$6016.91 \pm 25.28^{B,b,c}$	$1340.80 \pm 164.73^{A,b}$	$4.55 \pm 0.76^{A,a}$

*Capital letter superscripts represent comparisons between extraction methods.
**Small letter superscripts represent comparisons between bee pollen sources.

utilizing the sonication method exhibited statistically significant higher levels from bee pollen sources in Quezon, Laguna, and Sorsogon. Furthermore, a noteworthy observation is the substantial elevation in flavonoid levels present in Calamba bee pollen, which consistently surpassed those from the other five sources, irrespective of the extraction method. As for antioxidant activities, the bee pollen originating from Laguna manifested the most pronounced antioxidant properties in contrast to counterparts from the remaining five sources. This finding underscores the superior antioxidant potential inherent in Laguna-sourced bee pollen. Moreover, bee pollen from Calamba, Laguna, Sorsogon, and Albay displayed significantly elevated antioxidant levels upon sonication compared to maceration. Overall, these findings suggest that sonication is a more effective method of extracting phenols, flavonoids, and antioxidants from bee pollen and that the source of the bee pollen can impact the levels of these compounds.

Notably, the bee pollen samples from Calamba City exhibited a significantly higher phenolic and flavonoid content compared to the other locations. These findings are consistent with a study conducted by Belina-Aldemita *et al.* [13], which

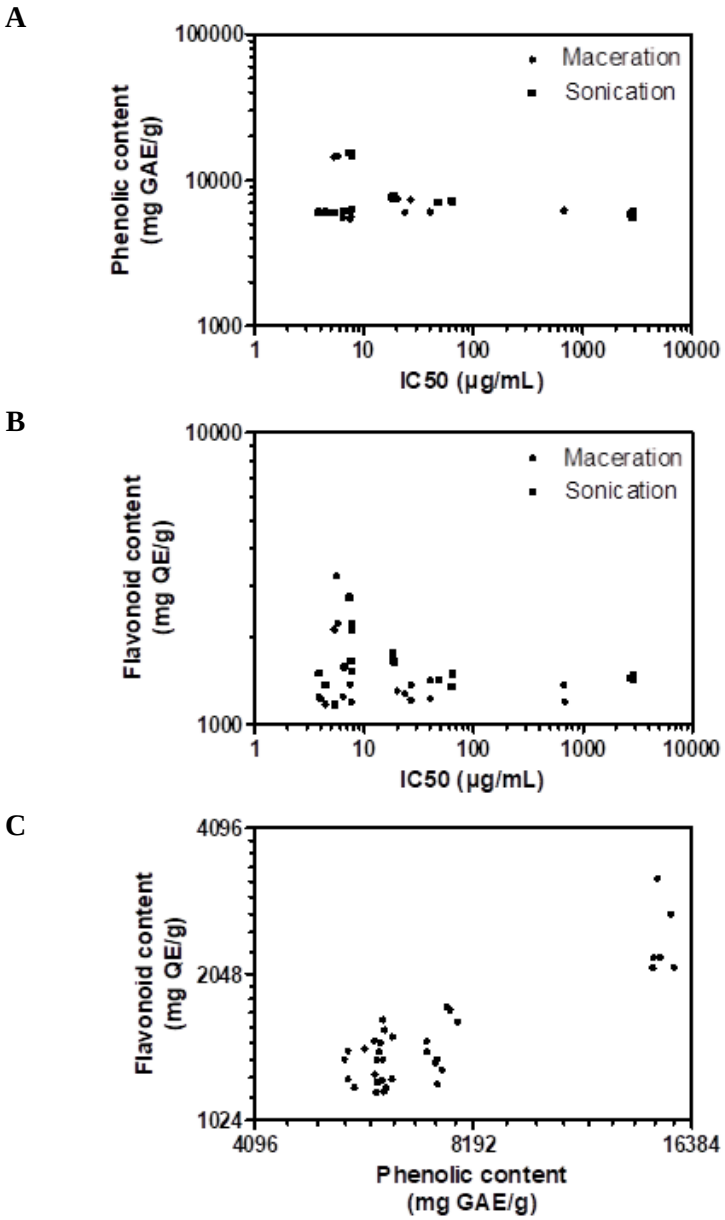

Figure 2. Scatter plot of antioxidant activity (IC50, g/ml) against (a) phenolic (ug GAE/g) and (b) flavonoid content (mg QE/g), and (C) flavonoid with phenolic content.

analyzed pot-pollen samples from Philippine stingless bees in various locations, including Calamba City, Sta. Maria (Laguna), Los Baños (Laguna), Buenavista (Marinduque), Bulusan (Sorsogon), Carles (Ilo-ilo), and Bacolod City (Negros Occidental). They also reported a variation in the phenolic and flavonoid content of bee pollen-based on geographic origin, with Calamba City consistently having higher phenolic (range: 10-18.12 mg GAE/g) and flavonoid content (23.63-25.78 mg QE/g) compared to other locations. The difference in phenolic and flavonoid content between Calamba City and Los Baños, Laguna, is particularly pronounced when compared to the samples collected in our study.

The chemical composition of bee pollen can vary greatly depending on the predominant plant species in a certain area. In Laguna, where the bee pollen samples in this study were gathered, Hizon-Fradejas *et al.* identified 19 different plant species present in the samples [14]. The predominant pollen grains in Alaminos (Laguna) were from *Mimosa pudica* L. (touch-me-not plant), with *Aeschynomene* sp. (jointvetches) as a secondary pollen source. *M. pudica* produces head-type inflorescences with exposed stamens that allow for easy access for foraging stingless bees to collect pollen grains, as observed in India [15]. Whereas, in Los Baños, *Fabaceae* (=Leguminosae) and coconut *Cocos nucifera* L. were the predominant and secondary sources, respectively. Notably, *C. nucifera* L. produces pollen profusely, and since several hectares of coconut plantations in Laguna bear flowers and fruits year-round, honeybee colonies are seen thriving in the coconut plantations. In addition to coconut and mangoes, Laguna is also home to several flowers and ethnobotanicals, including *Annona muricata* (soursop), *Garcinia mangostana* (mangosteen), *Antidesma bunius* (currant tree), *Citrofortunella microcarpa* (Philippine lemon), *Hibiscus esculentis* (lady's fingers), *Ananas comosus* (pineapple), *Moringa oleifera* (horse-radish tree), and *Lagerstroemia speciosa* (banaba). These plants are widely used in traditional Filipino herbal medicine and have been reported to possess various biological activities such as anti-inflammatory, anti-diabetic, and anti-hypertensive properties [16]. The presence of these plants in Laguna could contribute to the higher phenol and flavonoid content of bee pollen samples harvested from the area compared to the other locations. However, further research is needed to establish a direct link between the flora of a certain area and the chemical composition of bee pollen.

Relationship of phenolic and flavonoid content with antioxidant activities

The antioxidant activity of pollen, encompassing more than 250 biologically active substances, has been extensively investigated. Numerous studies have consistently elucidated that the pronounced antioxidant efficacy exhibited by pollen primarily emanates from its rich content of phenolic compounds and flavonoids, which possess notable free radical scavenging capabilities [17, 18]. Table 1 exhibits data on the phenolic and flavonoid content of Philippine stingless bee pollen. Results from correlation analysis show a positive correlation between phenol and flavonoid content ($r = 0.877$, $p < 0.001$). However, the correlation for phenol content with antioxidant activity IC_{50} values is lower and insignificant for soaked ($r = -0.19$, $p = 0.4651$) and sonicated ($r = -0.30$, $p = 0.2224$) samples. The same trend was observed between IC_{50} values of antioxidant activity with levels of flavonoid compounds for soaked ($r = -0.16$, $p = 0.5321$) and sonicated ($r = -0.24$, $p = 0.327$) bee pollen (Figure 2).

To model the linear relationship between a combination of the examined parameters as independent variables and antioxidant activity as the dependent variable, we employed Multiple Linear Regression (MLR). Equation 1 represents the MLR form used for our modeling:

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i \cdot X_i \quad \text{Equation 1}$$

where y is the dependent variable, β_0 is the y-intercept or the constant term, and β_i is the slope coefficient of the independent variable X_i. In this study, three models were developed based on our data and that of Belina-Aldemita *et al.* [13] with later-added parameters. The MLR models developed to predict antioxidant activity of Philippine stingless bees using different combinations of parameters are shown in Table 2. That R values from our study are lower compared to the data from Belinda-Aldemita *et al.* [13] which may be attributed to the wider differences in the geographic location of our samples and the differences in extraction procedures. Furthermore, adding more parameters to the models, *i.e.*, anthocyanin, improves the overall results.

Phenols and flavonoids are two classes of an-

antioxidants with different chemical structures and mechanisms of action. Phenols are a diverse group of organic compounds that contain a hydroxyl group attached to an aromatic carbon ring. They act as electron donors and neutralize free radicals by donating an electron. Studies have shown that phenolic compounds can inhibit lipid peroxidation and protect cellular components from oxidative damage. Flavonoids, on the other hand, are a subclass of phenols with a 15-carbon skeleton and two benzene rings linked by a heterocyclic pyran ring [19, 20]. They act as electron donors and chelators of metal ions that catalyze free radical formation. The sugar moieties in flavonoids have a significant effect on antioxidant activity as well. Flavonoids generally exhibit stronger antioxidant activity than phenols due to their greater number of hydroxyl and other functional groups. Moreover, flavonoids possess a broad range of biological activities, including the modulation of signal transduction pathways, regulation of gene expression, and inhibition of enzymes that produce free radicals. However, the actual antioxidant activity of both phenols and flavonoids can vary widely depending on their chemical structure, concentration, and the specific oxidative stress conditions they are exposed to. Similarly, mixtures of different antioxidants may interact synergistically or antagonistically, influencing the system's overall antioxidant activity [21, 22].

Comparison of extraction procedures

Variations in experimental conditions can significantly affect the measurement of antioxidant activities. Therefore, it is crucial to use different extraction methodologies to obtain natural products from bee pollen. The extraction process, including the sample pre-treatment, extraction method, and solvent used, can impact the efficiency of extracting antioxidant constituents and, consequently, the level of antioxidant activity measured. In this study, we evaluated the effectiveness of two commonly used extraction methods (maceration and sonication processes) to extract antioxidant compounds from bee pollen. The results are presented in Table 1. Consistent with the findings of Belina-Aldemita *et al.* [13], our study showed that sonication resulted in lower antioxidant activities than maceration. This may be attributed to the potential breakdown of bioactive compounds due to the formation of free radicals and changes in pH resulting from the use of high-intensity ultrasound during sonication. In contrast, maceration is a gentler extraction method that allows for the release of bioactive compounds without subjecting the bee pollen to harsh physical conditions, leading to extracts with higher antioxidant activity.

To optimize the extraction process of bioactive compounds from bee pollen, the selection of solvent is crucial and should consider factors such as selectivity for the target compounds, the target compound's solubility, and cost and safety. Based on the law of similarity and miscibility, solvents with a polarity value close to that of the solute are likely to perform better. In addition, water, and a range of alcoholic and organic solvents such as methanol, ethanol, acetonitrile, acetone, hexane, and diethyl ether are commonly used in the extraction of bioactive compounds [23]. In the present study, an alcohol solution of 90% ethanol, 5% methanol, and 5% isopropyl alcohol was used as a solvent for the extraction of bioactive from bee pollen. The findings of the study suggest that the selection of the extraction solvent had a stronger influence on the extraction efficiency of bioactive compounds compared to physical disruption techniques, such as sonication.

Ultrasonic-assisted extraction (UAE), also known as sonication, is a technique that utilizes ultrasonic wave energy to enhance the extraction process. The use of ultrasound waves in the

Table 2. The regression equations and R values for the three MLR models created compare this paper with the results from Belina-Aldemita, Schreiner, and D'Amico [13].

Model	Experimental Parameters	Equation	R^2	Reference
A	Flavonoid, Phenolics	$Antioxidant_{DPPH} = \alpha Flavonoid + \beta Phenolics + K$	0.2334	This study
B	Flavonoid, Phenolics	$Antioxidant_{DPPH} = \alpha Flavonoid + \beta Phenolics + K$	0.7727	[13]
C	Flavonoid, Phenolics, Anthocyanins	$Antioxidant_{DPPH} = \alpha Flavonoid + \beta Phenolics + \mu Anthocyanins + K$	0.8416	

solvent creates cavitation, which accelerates the dissolution and diffusion of the solute, resulting in improved extraction efficiency. UAE has several advantages, including low solvent and energy consumption, reduction in extraction time and temperature, and the ability to extract thermolabile and unstable compounds. However, our previous work [12] and other studies [24] have shown that sonication has lower extraction yields and lower phenol and flavonoid contents from propolis compared to other methods. Additionally, sonication may lead to the breakdown of certain compounds and a decrease in antioxidant activity in bee pollen due to the formation of free radicals and changes in pH conditions [25]. In contrast maceration may be a more effective extraction method for obtaining bee pollen extracts with higher antioxidant activity compared to sonication.

Conclusion

Our results showed that the phenolic content of bee pollen was weakly correlated with its antioxidant activity, suggesting the presence of other bioactive compounds. The extracts generated from the sonication method exhibited lower antioxidant activity compared to the maceration method. The bee pollen samples from Calamba City (Laguna) exhibited the highest antioxidant activity. Overall, these findings highlight the potential of Philippine stingless bee pollen as a source of bioactive compounds and the importance of considering extraction methods and geographic sources when evaluating its antioxidant activity.

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