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Physicochemical and Antioxidant Properties of *Heterotrigona itama* and *Trigona laeviceps* from Narathiwas Province, Thailand

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Abstract:
Stingless bee honey (SBH) is a local and natural bee product produced by domestic stingless bee honey pots. The physicochemical and bioactivity contents can affect the types and botanical origin of stingless bee honey. This study aims to compare the physiochemical profiles and bioactive compounds of *Heterotrigona itama* (*H. itama*) and *Trigona laeviceps* (*T. laeviceps*) including pH, electrical conductivity (EC), colour intensity, and antioxidant properties. We analysed the physicochemical properties (pH, colour and EC) of SBH in different methods. Further, we quantified the antioxidant activities using ABTS assay and DPPH (IC_{50}) assay. These findings showed that the pH, EC, and colour intensity values of *H. itama* were higher than *T. laeviceps* whereas the total phenolic content (TPC), total flavonoids content (TFC), IC_{50} and ABTS assay values of *T. laeviceps* were higher than *H. itama*. The empirical findings in this study provide new light and strong evidence supporting the potential use of SBH as an alternative therapeutic potential as pharmacological use.

Keywords: Bioactivity; Stingless bee honey; Antioxidant; Physicochemical

1. Introduction

A domestic stingless bees-Meliponini, known as Channarong (Thai language) have been discovered to include approximately 500 species that may be found in the tropical and subtropical areas around the globe. Recently, for Thailand, it was found that there were more

than 26 species of stingless bee. A survey and classification of stingless bees in 14 southern provinces (except for the three southern border provinces) found 22 species. Stingless bees are small pollinating insects and it is classified as a bee but does not have a stinger like bees. Channarong is more evolved than wild bees and buzzing bees. In addition, it also produces honey and pollen are more expensive than regular honey because they are believed to have higher nutritional value because Channarong's nests are hard to find and has a small amount of honey. Most stingless bees were found spreading and living in humid tropical forests, hot dry forests, swamp forests, cloud forests, and others. The production of SBH has been growing and brought stingless bee products into the limelight, particularly in Thailand and Malaysia, which attracts progressive commercial development in many countries [1-2]. Some of the stingless bee species, *H itama* and *T. laeviceps* are commercially bred by farmers [3]. Because of its highly nutritional and therapeutic properties, SBH is also regarded as a superfood [4].

Generally, the pH, moisture content, free acidity, organic acids, and 5-hydroxymethylfurfural (HMF) are the quality indicators used to assess SBH [5] Physicochemical properties show that SBH has low moisture content, low pH and low ash [6-7]. The SBH components include proteins, amino acids, fructose, glucose, sucrose, trace enzymes, vitamins, minerals and other substances, including phenolics and other secondary metabolic compounds [8-13]. The antioxidant properties of SBH vary from type to type due to different geographical regions [14]. The antioxidant capacity of honey is not only influenced by the total phenolics in honey but also by its flavonoid content, which can significantly reduce oxidative stress [15]. However, antioxidant activity can also be influenced by protein content, which can be expressed in terms of phenolic and flavonoid compounds, total phenolic content, total flavonoid content and antioxidant capacity [14]. Phenolic compounds are a heterogeneous group of compounds formed during the secondary metabolism of plants, and they can be divided into two groups: flavonoids and non-flavonoids. Flavonoids are also known as phenolic acids and their derivatives are flavanols, flavanones and flavones. However, non-flavonoids are stilbenes, tannins and lignin [16]. Flavonoids and many other phenolic compounds have been reported to be effective as antioxidants, anticancer, antibacterial, boost the immune system, anti-inflammatory and cardioprotective [17]. SBH has a rich phenolic content, which is because the stingless bees are smaller and can collect nectar from different flowers [18].

Furthermore, previous research has established that when building and sealing honeycombs, stingless bees combine their saliva secreted from abdominal glands and beeswax [19]. Therefore, the phytochemical composition of honey may be due to plant substances contained in earwax. However, the phenolic composition of SBH varies depending on the flower species, geographical origin as well as the foraging preferences of each bee species [12,20]. Furthermore, SBH is considered a natural antioxidant because it can help prevent cell damage. These components likely play an important role in some of the biological activities of SBH. Thus, SBH quality should have fructose, glucose, sucrose, moisture, free acidity, ash, diastase activity, and HMF [21].

(a) **(b)**

Figure 1. The images of stingless bees (a) *H. itama* and (b) *T. laeviceps* along with their propolis.

The importance of obtaining as much information and comparative properties of the SBH as possible. In our research, the physicochemical contents in commercial honey of two stingless bees, *H. itama*, and *T. laeviceps*, found in the same ecological conditions and environment in Narathiwas Province, Thailand, were investigated. The antioxidant activity of each honey was also evaluated.

Figure 2. Map of Narathiwat province of Thailand and the Thai-Malaysian border. This adapted figure appears in color at www.ajtmh.org.

2. Materials and Methods

2.1 Sample collection

The honey of stingless bee species (Fig. 1), *H. itama* and *T. laeviceps* were purchased from HOBEE international farm, Muang District, Narathiwas Province, Thailand (Fig. 2). The honey was obtained from their beehives during May - October 2023 without disrupt any endangered or protected species.

2.2 pH and electrical conductivity

The pH of honey was measured by pH meter (Denver instrument, USA) and the electrical conductivity was measured at 20 °C in solutions of SBH samples (20.0 g dry matter of SBH in volume solution in 100 mL distilled water) using a conductometer Crison (Type Basic 30). Method of measuring is prescribed by International Honey Commission Methods (2009).

2.3 Color intensity

A 50% (w/v) honey solution was prepared with warm water at 45 to 50 °C and filtered to remove any coarse particles. The net absorbance was determined as the difference between the absorbance at 450 nm and 720 nm using the UV-VIS spectrophotometer. The measurements were performed in triplicates for each SBH sample and the results were expressed as mAU.

2.4 Total phenolic contents

The presences of phenolics of SBH were analyzed using colorimetric methods. The

TPC were determined using the Folin-Ciocalteau analysis. A 100 mL of the solutions were mixed with 7.5% of sodium carbonate solution (Na_2CO_3). A 50 mL of Folin-Ciocalteau reagent was added into the mixture, then incubated in the dark for 30 min at room temperature and measured at 760 nm. A similar procedure was carried out for 10 different concentrations of gallic acid (0-100 mg L^{-1}). A linear regression plot of absorbance as a function of the concentration of gallic acid was used as a calibration curve to calculate the TPC contained in the SBH. The results were expressed as mg gallic acid equivalent (GAE) per 100 grams (g) of distilled water. The analysis was carried out in triplicate.

2.5 Total flavonoid contents

The TFC of SBH was investigated using the spectrophotometric method based on flavonoid-$AlCl_3$ complexation. A 100 µL of each solution of SBH species was mixed with 2% of aluminum chloride ($AlCl_3$), then incubated for 30 min at room temperature and measured at 420 nm. The TFC of the raw SBH was expressed as mg quercetin equivalent (QE) per 100 grams (g) of distilled water. The absorbance of quercetin solutions (0-100 mg L^{-1}) was recorded, plotted and used as a calibration curve. The analysis was carried out in triplicate.

2.6 Antioxidant assays

The antioxidant properties of SBH of the different stingless bees was determined using the 2,2-diphenyl1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) free radical scavenging assay. In the DPPH assay, 0.5 mL of each sample was then mixed with 3.5 mL of DPPH solution (50 mg L^{-1}) in ethanol. The mixture was vigorously vortexed and incubated at room temperature for 30 min in the dark. The decrease of DPPH radical was monitored by ELISA microplate reader at 517 nm. The IC_{50} was defined as the concentration of SBH to scavenge 50% initial DPPH radical, as reflected by a 50% reduction of absorbance in mg/mL.

In the ABTS free radical scavenging assay, the stock solution, a 1:1 (v/v) mixture of 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS) (7 mmol/l) and potassium persulfate (4.95 mmol/l), was incubated for 12 hours at room temperature in dark to form radical-cation $ABTS^{\bullet+}$. The final solution was stable for at least one week at 4°C in dark. To give the absorbance values between 1.0 and 1.5 AU at 734 nm (the same absorbance value must be used for the standard and samples), the stock solution was diluted with phosphate buffer solution. The reduction of the absorbance at 734 nm was measured after 30 min. Radical scavenging activity was measured by using Trolox and BHT as standards and the values are expressed as mg/100g. Both assays were analyzed in triplicate.

2.7 Statistical analysis

All values reported were mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test was used to determine the significant differences between means at a significance level of $P < 0.05$. Correlation was established using Pearson correlation coefficient. All the analyses were performed using GraphPad Software (GraphPad Prism 10.2.1, Inc., USA).

3. Results and Discussion

The findings of this study demonstrate that SBH derived from *H. itama* and *T. laeviceps* have different physicochemical profiles and bioactive compounds. These variations in pH, EC, color intensity, TPC, TFC, and antioxidant properties suggest that different species of SBH can exhibit unique characteristics and potential health benefits. These findings are consistent with previous research that has identified variations in the physicochemical and bioactivity contents of different types and botanical origins of stingless bee honey [22].

3.1 pH and color

Normally, SBH has sourness in taste due to its acidic nature. The current study revealed the pH values ranged between 3.47 to 3.50 (Table 1). The *T. laeviceps* obtained the lowest pH value (3.47 ± 0.12) while the *H. itama* showed a slightly higher (3.50 ± 0.07). The average pH report of South American stingless bee honeys was 3.9 ± 0.601 (Vit et al., 2004). The SBH pH data in this study is slightly more acidic than the South America SBH. These outcomes agree with previous studies who found that different species of SBH showed difference in pH value between 3.15 to 6.64 [23-24]. Furthermore, the acidity level is determined by the organic acid content in honey, with pH varying based on floral source and bee species [25]. Additionally, honey's pH can be influenced by sugar fermentation into alcohol by microorganisms and subsequent oxidation into carboxylic acids during storage [26].

The color intensity values from *H. itama* had 62.29 ± 0.65 mAU (Table 1) while *T. laeviceps* had 36.88 ± 0.36 mAU. However, the analysis of variance (ANOVA) findings indicated that there is statistically significant difference in color intensity values between the honey produced by *T. laeviceps* and *H. itama*. Likewise, the color intensity of honey varies depending on its mineral contents and pH [27]. Kek et al. (2018) observed variations in the color intensity values of honey across different bee species [28]. Additionally, the differences in the color intensity of honey are influenced by geographical factors and bee species [29]. Nonetheless, several factors such as exposure to light, enzymatic reactions, heat, and storage time also affect the color intensity of honey [25].

3.2 Electrical conductivity

From our samples the average EC ranged between 51.85 to 100.55 S/m (Table 1). The *H. itama* (100.55 ± 1.47 S/m) exhibited extraordinarily high EC compared to *T. laeviceps* (51.85 ± 0.83 S/m) and showed significantly difference ($P \leq 0.05$). This corresponds to the findings of Suntiparapop et al. (2012), who documented electrical conductivity values for *T. laeviceps* from Chanthaburi and Trat provinces as 0.71 and 0.53 ms/cm, respectively [31]. In the literature on stingless bee honey originating from South America, only one study provided information on electrical conductivity. This study reported an electrical conductivity of 0.48 ± 0.06 ms/cm, calculated from three honey samples whose species origins were unspecified [27]. Wanjai et al. (2012) reported an electrical conductivity for *A. mellifera* from Thailand as 0.26 ± 0.04 ms/cm, which is lower than the electrical conductivity observed in our study for SBH [31]. These differences could be attributed to variations in the floral sources of nectar and the enzymatic activities involved in honey production [32-33].

Table 1. Physicochemical contents of SBH samples (mean ± SD; n = 7), * = $P \leq 0.05$.

	pH	**Colour**	**EC**
H. itama	3.50±0.07	62.29±0.65	100.55±1.47
T. laeviceps	3.47±0.12	36.88±0.36*	51.85±0.83*

3.3 Total phenolic and total flavonoid contents

The TPC and TFC contents of SBH samples are showed in Table 2. The mean of TPC and TFC constituent values of *H. itama* (6.73 ± 0.10 mg GAE/100 g DW, and 0.26 ± 0.04 mg QE/100g DW) are significantly different with *T. laeviceps* value of 8.20 mg GAE/100 g DW

and 0.35 ± 0.12 mg QE/100g DW, respectively ($P \leq 0.05$). The variability in total phenolic content (TPC) among stingless bee honey species may be attributed to differences in geographical and botanical origins of the nectar used in honey production and their species. Significant contributions have also been made by recent studies who found TPC values ranging from 1.30 to 66.00 mg GAE/100 g for stingless bee honeys from the Amazon and Paraiba regions of Brazil [34-35]. Biluca et al. (2016) conducted similar research, reporting TPC values ranging from 10.3 to 98.0 mg GAE/100 g for ten different stingless bee honey species from Santa Catarina, Brazil [36]. This study also indicates significant differences in total flavonoid content (TFC) among various stingless bee species. Conversely, Harif Fadzilah et al. (2017) identified TFC values ranging from 15.28 to 31.80 mg/g QE for three different stingless bee species in Malaysia [37].

3.4 Antioxidant activity

The values of DPPH radical scavenging effect are depicted in Table 2. SBH from *T. laeviceps* had a greatest ability and significantly different to scavenge DPPH radicals with the DPPH (IC_{50}) 16.75 ± 0.32 mg/mL. whereas the SBH from *H. itama* showed the lowest IC_{50} (6.58 ± 0.19 mg/mL) ($P \leq 0.05$). The levels of DPPH inhibition in honey exhibit considerable variation due to factors such as botanical and geographical origins, as well as the species of bees involved [38-39]. Discrepancies in DPPH inhibition values among current studies may be attributed to differences in stingless bee species, even when honey is collected from the same geographical location. This notion is supported by Agus et al. (2019), who observed that the antioxidant activity of honey produced by *T. laeviceps* species was influenced by various geographical origins of meliponiculture. Furthermore, the authors noted a significant correlation between the DPPH antioxidant activity of *T. laeviceps* honey and its TPC and TFC. The ABTS assay stands as one of the commonly employed analytical methods for evaluating antioxidant activity. The average values of ABTS assay analysis (Table 2) of SBH from *T. laeviceps* (70.55 ± 2.47 mg/100 g) demonstrated the highest antioxidant potential while the SBH from *H. itama* (67.73 ± 0.53 mg/100 g) obtained the lowest ($P \leq 0.05$). Previous study examined the antioxidant content of both unifloral and multifloral SBH with ABTS inhibition ranging from 15.61% to 65.77%. Results indicated that unifloral honey types possess a higher antioxidant content compared to multifloral honey types. The study concluded that the antioxidant content of honey is influenced by factors such as plant source, geographical origin, climate, and processing methods [40]. SBH was gathered from three distinct locations throughout Malaysia, with antioxidant levels ranging from 216.18 to 2006.87 µg TEAC/g [42].

Table 2. Total phenolic content (mg GAE/100g DW), total flavonoid content (mg QE/100g DW), DPPH (IC_{50}) values (mg/mL), and ABTS assay (mg/100g) obtained from the antioxidant activity of SBH samples (mean ± SD; n = 7), * = $P \leq 0.05$.

	TPC	**TFC**	**DPPH (IC50)**	**ABTS**
H. itama	6.73±0.10*	0.26±0.04*	6.58±0.19*	67.73±0.53*
T. laeviceps	8.37±0.26	0.35±0.12	16.75±0.32	70.55±2.47

3.5 Correlation Analysis

Phenolic acids and flavonoids were the primary constituents that contributed significantly to plant antioxidant activity. We employed the Pearson correlation coefficient (PCC), also referred to as Pearson's *r*, to quantify the strength and direction of the linear correlation relationship. Figure 3 illustrates the scatter plots based on the PCC depicting the relationships between antioxidant activity, TPC, and TFC. The variations in matrices among plant species originating from different geographical locations, as well as differences in genetics and cultivation conditions, pose challenges in conducting correlation analyses. The Pearson correlation coefficient (PCC) between antioxidant activity and total phenolic content (TPC) of *H. itama* (Figure 3A and 3B) ranged from 0.8281 to 0.9686 ($P \leq 0.001$). A similar pattern was observed in the PCC between antioxidant activity and TPC of *T. laeviceps*, which ranged from 0.9429 to 0.9928 ($P \leq 0.001$) (Figure 3C and 3D), indicating a strong positive relationship. These findings suggest that the main contributors to plant antioxidant activities are primarily the TPC [28]. Moreover, the PCC between antioxidant activity and TFC of *H. itama* (Figure 4A and 4B) ranged from 0.9163 to 0.9708 ($P \leq 0.001$). Unlike a pattern was observed in the *T. laeviceps*, which ranged between 0.5479 ($P = 0.127$) and 0.7001 ($P = 0.036$) (Figure 4C and 4D), revealing a moderate positive relationship. The stronger correlation between antioxidant activity and TPC in comparison to the correlation between antioxidant activity and TFC suggests that TFC contributes less to antioxidant activity than TPC. This finding aligns with previous research indicating that total flavonoid content plays a less significant role in contributing to antioxidant activity [37]**.**

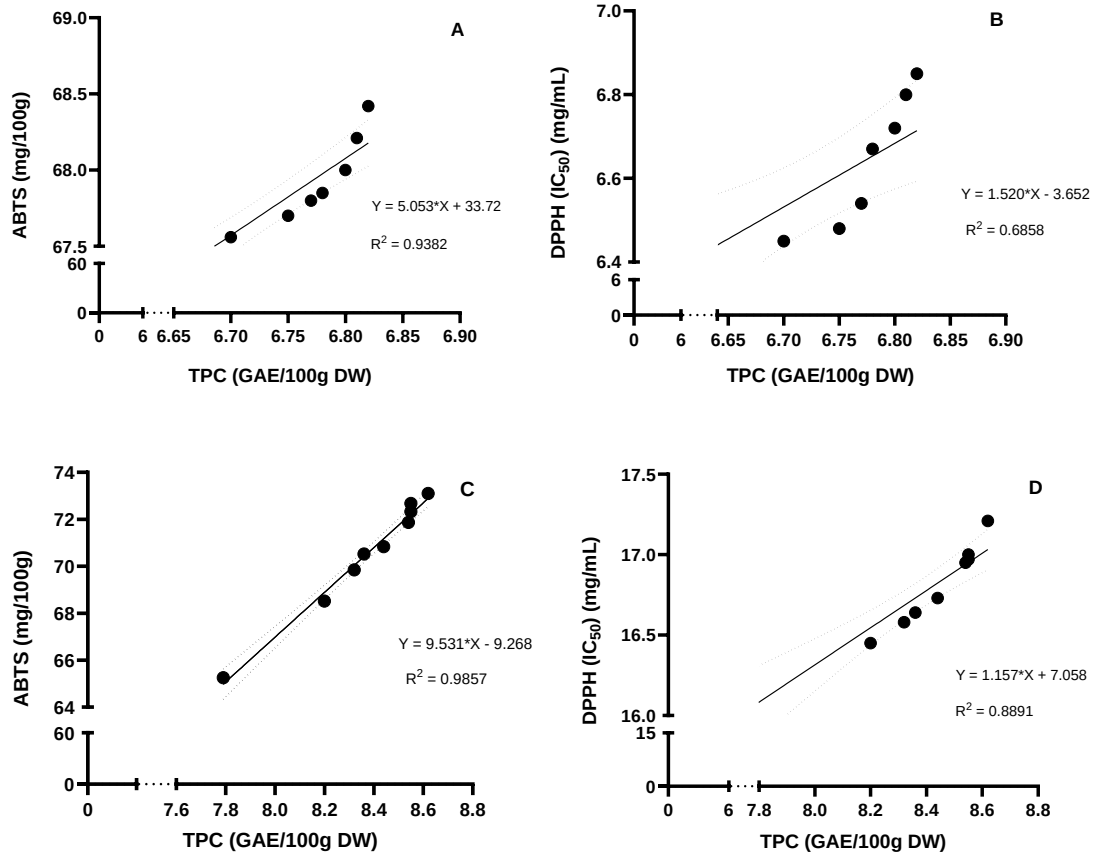

Figure 3. Pearson correlation scatter plots of relationship between (A-B) antioxidant activity with TPC of *H. itama*, and (C-D) antioxidant activity with TPC of *T. laeviceps*.

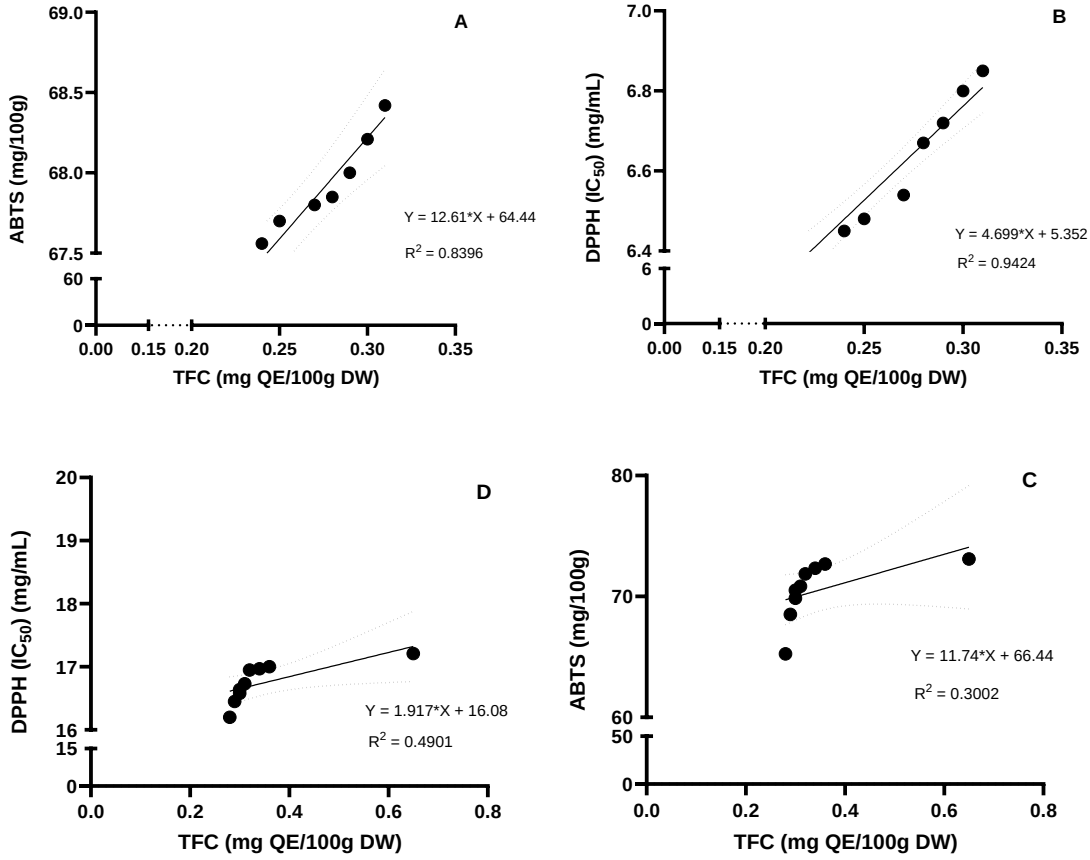

Figure 4. Pearson correlation scatter plots of relationship between (A-B) antioxidant activity with TFC of *H. itama*, and (C-D) antioxidant activity with TFC of *T. laeviceps*.

4. Conclusions

The results suggest that the physicochemical and biological properties of SBH vary according to species and sources. Taken together, these studies support the notion that SBH significantly influences phenolic, flavonoid, and antioxidant activities. SBH from *T. laeviceps* was shown to have significantly higher antioxidant properties compared to *H. itama*. Furthermore, the findings suggest that SBH has a significant impact on the content of phenolic and flavonoid compounds. Thus, SBH can be used as an alternative therapeutic agent against pathogenic diseases and may have therapeutic potential and pharmacological applications. However, the number of samples used in this study is limited. Therefore, further investigation with a larger number of samples and species is required to confirm these results.

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Conflicts of Interest

All authors declare that they have no conflict of interest.

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