Total phenolic, flavonoid contents and Antioxidant Activity of Thai stingless bee honey (*Tetragonula pegdini*) from different botanical regions in Chanthaburi

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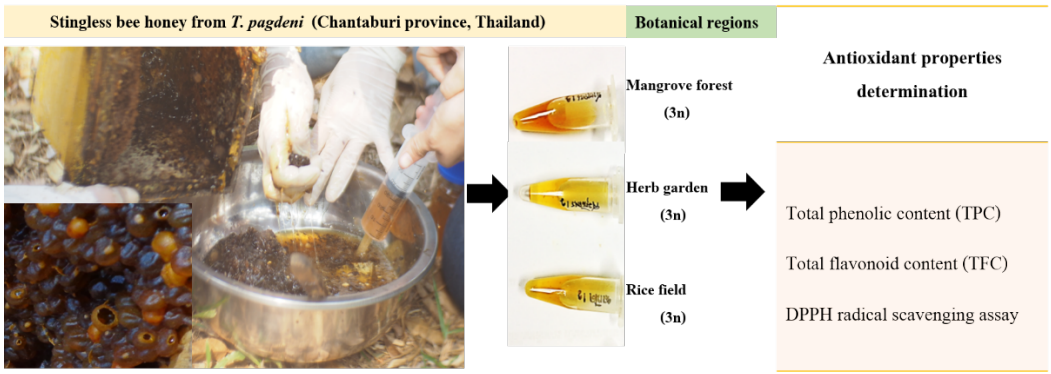
Abstract: It is well established that stingless bee honey (SBH) contains substantial antioxidant compounds that could prevent oxidative stress in humans and has several beneficial properties for health such as anti-inflammatory, antioxidant and antimicrobial activities. In this study, three SBH samples produced by *Tetragonula pegdini* were collected from different three botanical and geographical regions in Chantaburi province, Thailand, including mangrove forest, rice field and herb garden. The total phenolic content of the SBH samples were examined using Folin–Ciocalteu reagent. Total flavonoid content was determined by aluminium chloride method. Antioxidant activity was also characterized by performing reaction with DPPH radical. The results revealed the SBH from mangrove forest had the highest phenolic and flavonoid contents with 2.66±0.13 g GAE /100 g and 0.91±0.01 QE/ 100 g honey, respectively ($p<0.05$). The radical scavenging DPPH assays further demonstrated that significantly higher antioxidant activity (10.02±0.12 mg Trolox/100 g of honey) was detected in SBH sample collected from mangrove forest when compare with the samples collected from the herb garden (9.31±0.15 mg Trolox/ 100 g honey) and rice field (6.95±0.13 mg Trolox/ 100 g honey), respectively ($p<0.05$). Statistical analysis demonstrated positive correlation between the antioxidant activities of honeys and their total phenolic and flavonoid contents. This research might promote the exploitation and the development of alternative natural products from stingless bee honey.

Graphical abstract:



Keywords: Stingless bee honey; Antioxidant; Phenolic acid; Flavonoids

1. Introduction

Stingless bee (subfamily Meliponinae) exists in the tropical regions of the world, such as Thailand, Brazil, Malaysia and Indonesia [1]. In Thailand, the stingless bee is distributed around the country and also have been reported about 32 species in 10 genera [2]. In the eastern part of Thailand especially Chanthaburi and Trat province, stingless bee genus *Tetragonula* being the major cultivated genus in artificial hives in fruit gardens for honey harvesting and crop pollination [3]. The products of stingless bees, such as propolis, pollen, and honey are important sources of commonly used natural products in folk medicine by virtue of their powerful healing properties and bioactive compounds. Many bioactive compounds have already been found in honeys from the *Tetragonula* species in different botanical and geographical regions [4-6]. Previous studies reported that the SBH showed an important role in human health related to antioxidant activity. The growing interest in the SBH proceeds from its composition, which displays antioxidant capacity, such as phenolic acids, flavonoids and the enzymes glucose oxidase and catalase [7-8]. The therapeutic applications of honey product from *T. pagdeni* propolis, describing their antimicrobial, antioxidant, and antitumor properties. The medicinal properties are directly related to the chemical composition in plants contained in honey [9]. However, the knowledge of the health-promoting anti-oxidative effects of honey from *T. pagdeni* species is limited.

The phenolic compounds display in honey are causally related to botanical resources, such as pollens, nectars, resins, and oils that are supplied to the bees, and consequently, honey from different floral origins possess distinct bioactive properties [4-6]. Many studies have explored the total phenolic content (TPC) and the total flavonoid content (TFC), calculated in mg gallic acid equivalent (GAE)/g sample. The characterization of the phenolic compounds has been performed by different methods, especially the high-performance liquid chromatography (HPLC), Folin–Ciocalteu and aluminium chloride colorimetric method [10-11]. While the most common assays to detect the antioxidant activity were DPPH -free radical scavenging activity, ABTS radical cation scavenging activity and ferric reducing antioxidant power (FRAP) [12]. These assays considered relatively affordable spectrophotometric-based tests based on color quenching or gaining of synthetic organic radicals. The colors of DPPH and ABTS radicals are reducible upon exposure to antioxidants, and this decrease is negatively correlated with the measured wavelength, which, thus, refers to the antioxidant capacity. Antioxidant activity of honey mainly associated with the nectar source used by bees to make honey. In addition, chemical constituents are derived from pollen and propolis constituents present in honey [11, 13-14].

Since honey is produced from the nectar or the secretions of plants, various bioactive substances are transferred from these productions and accumulated in honey. Consequently, the composition of honey, including its physical, chemical, organoleptic, and nutraceutical properties are relative to the geographical, climatic, and environmental characteristics of the areas where it is produced [2-3]. These differences represent a useful discriminatory tool for the classification and identification of honey [5] The botanical and geographical sources of honey have been reported as the main factor that affect constituents and antioxidant activity of honey. Honey from different botanical origins has different antioxidant activity. Therefore, the present study was undertaken with the purpose of determining the phenolic, flavonoid compounds of honey produced by stingless bee *T. pagdeni* from different botanical regions in Chanthaburi province, mangrove forest, rice paddy field, fruit garden and herbal garden. In addition, we evaluated the honeys for antioxidant activities.

2. Materials and Methods

2.1 Stingless Bee Honey (SBH) samples

Three SBH (*Tetragonula pagdeni* Schwarz) were collected from different area in Chantaburi province, including mangrove forest (Kung Krabaen Bay Royal Development

Study Centre, 3n), rice paddy field (Burapha university, Chanthaburi campus, 3n), and herbal garden (Makham district, 3n), during the period of March – December 2018. The samples were refrigerated at 4 °C until further processing within 3 months. The stingless bee voucher specimens (Tetragonula pagdeni No.1214003) were identified and deposited at the Faculty of Pharmaceutical Sciences, Burapha University, Thailand [15]. The other reagents and solvents were analytical grade.

2.2 Total Phenol Contents (TPC

Total phenolic content (TPC) was performed in 96-well plate in a reaction with Folin–Ciocalteu reagent [16]. 1 g of each sample of honey was treated with distilled water, mixed and filtered using a qualitative filter. 50 µl of this solution was mixed with 20 µl Folin–Ciocalteu reagent for 5 min and then 80 µl of a Na_2CO_3 solution was added (7.5%). All samples were incubated at room temperature in darkness for 30 min, and their absorbance was read at 765 nm using microplate reader. Total phenolic content was expressed as mg gallic acid equivalents (GAE)/ 100 g of honey from a calibration curve (R^2 = 0.999). All samples were analyzed in triplicate.

2.3 Total flavonoids contents (TFC)

Total flavonoid content (TFC) was determined in a reaction with aluminum chloride in 96-well plate according to Ávila et al. [17]. 1 g of honey was treated with distilled water, mixed, and filtered using a qualitative filter. Then 100 µl of the SBH solution was mixed with 100 µl of 10% $AlCl_3.6H_2O$. After it was incubated at room temperature for 30 minutes, its absorbance was immediately measured at 415 nm using microplate reader. The total flavonoids contents are expressed in mg quercetin equivalent (QE)/100 g of the SBH sample. A standard curve (R^2= 0.999) was constructed using a standard solution of quercetin. All samples were analyzed in triplicate.

2.4 DPPH Radical-scavenging Activity

Radical scavenging activity of methanolic extracts was determined spectrophometrically at 517 nm against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. The method was developed and modified from Blois (1958) [17, 18]. The assay based on the color change of the DPPH solution from purple to yellow as the radical is neutralized by the antioxidants. Briefly, 20 µl of SBH sample were mixed with 180 µl of a 10mM of DPPH in methanol. The measurement of radical scavenging activity was done using Trolox as standard and the results were expressed as mg Trolox/ 100 g honey.

2.5 Statistical Analysis

The results were presented as a mean ± standard deviation from the triplicate analysis. Statistical analysis was performed using SPSS version 18.0. One-way ANOVA was used to compare mean in each analysis. If the ANOVA test indicated the significant result ($p < 0.05$), then the significant means were separated using Tukey's test. The relationships between parameters of antioxidant activity, phenolic acids, and flavonoids content in SBH were determined by calculating Pearson's correlation coefficients (r) in bivariate linear correlations ($p < 0.05$ and $p < 0.001$).

3. Results

3.1. Total Phenolic Acids and Total Flavonoid contents

Stingless bee honey (SBH) from different botanical regions can be classified by color for light amber (herb garden and rice paddy SBH) and dark amber (mangrove forest SBH). Mangrove forest honey and herb garden honey are multiflora honey, whereas honey from rice field is uniflora honey. The obtained results for three tested

SBH samples showed that total phenolics content of mangrove forest SBH (2.66±0.13 g GAE/ 100 g honey) was significantly higher ($p < 0.05$) in comparison with herb and rice field SBH samples (1.19 ±0.05 and 1.13±0.06 g GAE/ 100 g honey), respectively. (Table 1)

Table 1. Total phenolic content, flavonoid content obtained from SBH samples of different botanical regions.

Stingless bee honey	Total phenolic content (g GAE/ 100 g Honey)	Total flavonoid content (g QE/ 100 g Honey)
Mangrove forest SBH	2.66±0.13[a]	0.91±0.01[a]
Herb garden SBH	1.19 ±0.05[b]	0.46±0.01[b]
Rice field SBH	1.13±0.06[b]	0.34±0.01[c]

Note: Results are expressed as mean values±SD (n=3). SD: Standard deviation. [a-c] value within each collumm followed different letters indicate significant differences between samples ($p < 0.05$) according to the Turkey test.

The results of total flavonoids content can be concluded that mangrove forest SBH contained the highest concentration ($p < 0.05$) of flavonoids (0.91±0.01 g QE/ 100 g honey) compared with other tested samples (Table 1).

3.2 Antioxidant activity

The DPPH radical scavenging analysis was used to investigate the overall hydrogen or electron donating activity of single antioxidants. The values for all of the different types of stingless bee honey in DPPH radical scavenging activity with the average values being 6.95±0.13 to 10.02±0.12 mg Trolox/ 100 g honey. The DPPH levels in mangrove forest SBH showed the highest radical scavenging activities ($p < 0.05$), compared with antioxidant activities of herb and rice field SBH (Table 2).

Table 2. Radical scavenging activities (DPPH assay) of tested SBH.

Stingless bee honey	mg Trolox/ 100 g honey
Mangrove forest SBH	10.02±0.12[a]
Herb garden SBH	9.31±0.15[b]
Rice field SBH	6.95±0.13[c]

Note: mean±standard deviation (n:3), [a-c] value within each collumm followed different letters indicate significant differences between samples ($p < 0.05$) according to the Turkey test.

Furthermore, correlation analysis indicated that TPC is in moderate positive correlation with antioxidant activities DPPH ($r = 0.677$, $p < 0.05$) (Table 3). The TFC and antioxidant activity usually show a strong positive correlation ($r = 0.808$, $p<0.001$) as showed in Table 3.

Table 3 Pearson's correlation coefficient between phenolics, flavonoids, and antioxidant value.

	DPPH	TFC	TPC
DPPH	1.000	-	-
TFC	0.808**	1.000	-
TPC	0.677*	0.971**	1.000

Note: * Correlation is significant at $p <0.05$, ** Correlation is significant at $p < 0.01$

From table 3, the correlation coefficients of the antioxidant activity from the SBH presented positively related to phenolic and flavonoid contents of the sample.

4. Discussion

The constituents of the floral source are influenced by the type of nectar and pollen used by bees, the geographical origins and the species of the honey-producing bee [19]. In this study, the SBH from different botanical origins including multiflora mangrove forest, rice field, and herb garden were evaluated phenolic and flavonoid content. SBH mangrove forest showed the highest phenolic and flavonoid content than honey from herbal, and rice field. In Brazilian multiflora and uniflora citrus honey from *Apis mellifera* were exhibited a similar range of quercetin composition (1.96 ± 1.53 and 0.17 ± 0.15 mg/ 100g) to our study. Similar finding, *T. lavicep* honey from different geographical origins (Indonesia) showed TPC content with 1.08-3.38 mg GAE/ 100 g honey, while TFC content from *T. lavicep* showed low content [20]. High phenolic and flavonoid content were presented in multiflora honey of *T. carbonaria* (Austtralia) and *Trigona* spp. (Malaysia), the flavonoid content (7.83 RE/ 100 g - 10.02 mg QE/100 g) and polyphenol contents (55.74 mg GAE/100 g) higher than SBH in this study (Table 1) [21-22]. The lower phenolics and flavonoids in our samples compared to kelulut honey from *Trigona* spp. With 78.29 mg rutin equivalent/ 100 g honey from Malaysia and Brazilian stingless *Melipona subnitida* honey (0.60 mg GAE/100 g of honey) could be the environmental factor, particularly humidity that diluted the honey [23]. Many studies have explored the correlation of bee color have been related to total phenolic and flavonoids content, it can be related to the color of STB honey that amber dark honey (mangrove forest) contained the highest concentration of phenolic and flavonoids compared with other tested samples [24].

The antioxidant activities in honey originate from nectar, pollen, or propolis, and substances that contain organic acids, vitamins, and enzymes are known to occur in honey [4, 13-14]. The differences in the antioxidant activities of honey depend on the floral sources, the sources of collection, seasonal factors, and environmental factors [5-6], processing, handling, and storage of honey [5-6]. In this study, the highest radical scavenging activity levels was presented in mangrove forest SBH, compared with antioxidant activities of herb and rice field SBH (Table 2). Majid et al [25] observed SBH samples from honey from the mangrove plant (*Rhizophora mucronate*) possessed the highest antioxidant activity (IC_{50} 23.89 mg/ ml) [26].

Data in the literature have shown the higher of TPC and TFC in honey could be increase the radical scavenger DPPH of *T. lavicep* honey [27]. Many reports explained that phenolic acids and flavonoids present in honey have been used as floral markers and they are compounds that act as the antioxidants, eliminating or reduce the formation of free radicals, and inhibiting lipid oxidation. The DPPH antioxidant activity of mangrove forest honey was similar reported by Ranneh et al (2018) [28] for Kelulut honey and was higher to those previously reported [22]. There is usually a correlation between TPC, TFC, and antioxidant activity, but similar TPC and TFC content does not always correspond to similar antioxidant capacity [11, 13-14]. This is because the overall antioxidant capacity of each sample results from the combined activity of other nonphenolic compounds, although phenols do remain the largest class of antioxidants found in nature [13, 28]. However, some authors point out that while phenolic compounds can play an important role in antioxidant activity, other non-phenolic antioxidants (e.g., proteins, ascorbic acid and catalase) may contribute to the whole pattern of antioxidant activity [28].

5. Conclusions

The phenolic, flavonoid contents and antioxidant activity of the stingless bee (*T. pagdeni* species) honey in different botanical regions such as mangrove forest, herb garden and rice field were studied in this work. The content of phenolic and flavonoids were

detected varied significant with the floral type and the botanical regions from which these samples were obtained. When evaluating the antioxidant effect, the highest value of radical scavenging activity (DPPH) was observed for the honey from mangrove forest. This research established a positive correlation between the phenolic, flavonoids contents, and antioxidant capacity of the stingless bee honey in Thailand. This study revealed that the plant sources can influence honey's phytochemical composition and antioxidant activity. Additionally, the information can be used to support the utilization of the honey from *T. pagdeni* for functional food and also provided some evidence to show the potential prevention for health problems especially free radical scavenger. Further studies about physicochemical parameters, phenolic and flavonoid profiles obtained from different geographical and botanical origins should be investigated for a better understanding of the bioactive compounds in *T. pegdini* honey. This study might be promoted and extended the value of the stingless bee honey to increase valuable products.

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