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Physicochemical Properties, Phenolic, Flavonoid Contents and Antioxidant Potential of Stingless Bee (*Heterotrigona Itama*) Honey From Thailand

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Abstract

This research aimed to investigate the impact of heat treatment on stingless bee honey obtained by *Heterotrigona itama*, a commercial stingless bee found in the southern region of Thailand. Three honey samples originating from three different forest types (mangrove forest, swamp forest, and mixed forest) were heated to 37 °C and 45 °C for 24 and 48 h and then analyzed for their physicochemical properties, total phenolic content, the flavonoid content, and antioxidant activity by radical scavenging activity on 2,2-Diphenyl-1-picrylhydrazyl (DPPH). The results showed the raw honey from mixed forest had the highest radical scavenging activity with IC_{50} of 43.996±0.377 mg/ml. In addition, this honey sample also exhibited the highest phenolic and flavonoid contents with 89.916±0.358 mg GAE /100 g of honey and 58.093±0.294 mg QE/ 100 g of honey, respectively. After heat treatment, the honey samples showed little change in physicochemical properties when compared to raw honey samples. After incubation at 45 °C for 48 hours, the moisture content decreased 27.93±0.17 to 20.14±0.34 g/100 g. Interestingly, heat treatment at 37 °C and 45 °C did not affect the

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total phenolic, flavonoid contents, and antioxidant activities ($p > 0.05$) in the honey samples. While heat treatment aids in keeping the physicochemical and bioactive properties of dehydrated honey, it can be concluded that the proposed method can be employed as an alternate method for preserving honey from stingless bees.

Introduction

Honey is a naturally occurring sweet substance produced by honeybees of the genus *Apis* (subfamily Apinae), primarily from the nectar of plants or from the secretions of living parts of plants.[1] Another group of bees, which also produce honey, is well diversified and distributed in tropical and subtropical regions. This group of bees is known as stingless bees and belongs to the subfamily Meliponinae.[2] More than 500 stingless bee species have been reported worldwide.[3] In Thailand, 33 species belonging to 10 genera have been reported.[4-6] Among these, *Heterotrigona itama* is one of the most often managed species for the production of honey and commercial pollination services.[7] Importantly, the selling price of stingless bee honey is approximately 10 times higher than that of honey from Thai-produced *Apis mellifera*.[7]

Similar to honey produced by other stingless bees, honey obtained from *H. itama* is less sweet and has an acidic flavor and a more fluid consistency.[8,9] This type of honey is also recognized for its therapeutic properties, ascribed to phenolic and flavonoid contents and its antioxidant activity.[9,10] Stingless bee honey is cultivated in Southeast Asia,[7,11,12] South America,[2] and Australia.[13] However, its commercialization status is still limited owing to low production (1–5 kg per colony per year)[9] and the lack of honey quality standards.[14] Moreover, stingless bee honey deteriorates more rapidly than *Apis* honey[14] because of its higher water content and acidity than that of *Apis* honey.[15] For instance, the water content of stingless bee honey collected from humid areas is much higher, up to 42% (v/v).[16,17] High moisture content facilitates the loss of chemical stability during storage, as demonstrated[11,18,19]

Additionally, the osmophilic yeast and other fungi are inevitably present in honey during the foraging activity of bees, which must be processed to prolong its shelf life.[17,20] Therefore, the use of suitable processing to prevent fermentation by controlling the moisture content to inhibit the growth of fungi is one of the most fundamental bases for extending the productive system of stingless bee honey.[9] Owing to the particular characteristics of stingless bee honey, thermal treatment could become a useful alternative for conservation.

The heat treatment application on *Apis*'s honey can be used for prevention or deceleration of crystallization process as well in the devastation of microorganisms that stimulate fermentation during storage.[21] There are a few studies that investigate different heat treatment technique with various temperature ranges and times for decreasing the moisture content in stingless bee honey. However, these findings indicated higher moisture content than the international standard for *Apis mellifera* honey (20 g /100 g of honey).[11,22] Moreover, heat treatment has been proposed to liquify and pasteurize honey at 45 °C and 80 °C.[23] However, high temperatures causes undesirable changes in quality of honey, such as a decrease in enzymes, an increase in hydroxymethylfurfural (HMF) levels, rapid loss of antioxidant activity, darkened honey, and flavor deterioration.[24] Thus, it is important to find the optimum heating condition to reduce the moisture content in stingless bee honey below 20 g/ 100 g of honey for shelf-life prolongation without any negative effects on honey properties.

Considering the necessity for postharvest chemical stability and the understanding of the effects of heat treatment on stingless bee honey, this study aimed to apply thermal treatment under short-term conditions, and evaluate the physical characteristics, phenolic and flavonoid contents, and antioxidant capacity. In this manner, the acquired results aim to enhance the production chain of honey from stingless bees by utilizing an efficient approach for honey product maintenance and subsequent commercialization.

Materials and Methods

Honey Samples

In this study, 10 honey samples were taken from 10 different apiaries of *H. itama* located at three forest types in Narathiwat Province, southern Thailand (Table 1). All honey samples were directly collected from the colonies of *H. itama* by the owner in 2022. The honey samples were stored in sterile glass bottles at 0–4 °C until further use.

Table 1: Heterotrigona itama's honey samples collected from three forest types of Narathiwat province, southern Thailand.

Forest type	Sample no.	Sample location	Coordinate	Harvest date	Main vegetation
Mangrove forest	1	Tak Bai	06° 17' 09" N, 102° 00' 31" E	04-Apr-22	*Melastoma saigonense* (Kuntse) Merr.; *Scolopia macrophylla* (Wight and Arn.) Clos; *Pandanus odoratissimus* L.f.; *Peltophorum pterocarpum* Linn.; *Hibiscus tiliaceu*s L.; *Lumnitzera littorea* Voigt; *Elaeis guineensis* Jacq.
	2	Tak Bai	06° 16' 28" N, 102° 01' 47" E	04-Apr-22 08-Apr-22	
	3	Muang	06° 17' 09" N, 102° 00' 31" E		
Swamp forest	4	Bacho	06° 31' 37" N, 101° 41' 19" E	05-Apr-22 18-Oct-22	*Mangifera pentandra* Hook; *Calophyllum calaba* L. var. bracteatum (Wight) Stevens; *Melaleuca quinquenervia* (Cav.) S.T. Blake; *Metroxylon sagus* Rottb; *Eleiodoxa conferta* (Griff.) Burr.
	5	Su-ngai Kolok	06° 04' 16" N, 101° 57' 59" E	18-Oct-22	
	6	Sukhirin	05° 56' 33" N, 101° 46' 60" E		
Mixed forest	7	Yi-ngo	06° 23' 31" N, 101° 41' 46" E	08-Apr-22	*Nephelium lappaceum* Linn.; *Durio zibethinus* L.; *Pterocarpus macrocarpus* Kurz.; *Coffea robusta* Pierre ex Froehner L.; *Syzygium cumini* L.
	8	Yi-ngo	06° 23' 59" N, 101° 41' 35" E	08-Apr-22	
	9	Sukhirin	05° 56' 25" N, 101° 46' 50" E	18-Oct-22	
	10	Waeng	05° 56' 58" N, 101° 53' 24" E	18-Oct-22	

Thermal Treatment Application

Thermal treatment was conducted according to the protocol developed[25] with minor modifications. Each honey sample was incubated at room temperature for two hours[26] before thermal processing. Honey samples were heated using thermal processing in an incubator (Memmert UF110; Memmert GmbH+ Co., KG, Germany). The honey samples were weighed out to 200 g and placed in 250-ml glass beakers. After weighing, the samples were processed by heat treatment. The honey was then heated thermally at 37 °C (for 24 and 48 hours) and 45 °C (for 24 and 48 hours). This resulted in 5 heat treatment levels (including the untreated control). After the treatment, the honey samples were allowed to cool to room temperature. Untreated honey was used as the control.

Physicochemical Properties

Physicochemical properties (pH, moisture content, and °Brix) were investigated.[27,28] The soluble solids (°Brix) of *H. itama* honey were quantified using a refractometer. The mean of three readings was used. The pH of the honey was measured using a pH meter. Before measuring the pH of the samples, a two-point calibration employing two standard buffer solutions was performed. The probe of the pH meter was immersed directly into the honey samples, and

then the values were recorded. The moisture level of honey was determined using a digital refractometer. First, a digital refractometer was calibrated using a drop of distilled water. The honey sample was then placed onto the prism of the refractometer. The refractive index is recorded as R1. Moisture content was then measured as described[28]

Estimation of Total Phenolic Content

Total phenolic content was evaluated using the Folin-Ciocalteu method.[29] Briefly, each A sample of honey (0.5 g) was diluted with distilled water to make 5 ml, and then it was filtered through Whatman No.1 filter paper. Then, the honey solution (25 µL) was mixed with 125 µl of 0.2 N Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) for 5 min. Subsequently, 100 µl of 75 g/L g/L sodium carbonate (Na_2CO_3) (Kemaus, N.S.W., Australia) was added. The absorbance of the solution was measured at 760 nm (Tecan Microplate Reader Infinite 200 Pro; Zurich, Switzerland) after incubation at room temperature for 2 h against a methanol blank. Gallic acid (Merck KGaA, Darmstadt, Germany) (0 – 200 mg/ml) was used as the reference for the calibration curve. The mean of three readings was used, and the total contents are expressed as mg of gallic acid equivalents (GAE)/g of honey.

Estimation of Total Flavonoid Contents

The Dowd method[30] was used to evaluate total flavonoid content. Twenty-five microliters of honey solution (0.01 mg/ml) were mixed with 225 µL of 2% aluminum trichloride ($AlCl_3$) (Sigma-Aldrich, St. Louis, USA) in methanol (Merck KGaA, Darmstadt, Germany). After being incubated at room temperature for 10 minutes, the absorbance of the reaction mixture was measured at 415 nm compared to a blank sample composed of 125 µL of methanol and 125 µL of honey solution without $AlCl_3$ The total flavonoid content was assessed using a standard curve with quercetin (0–50 mg/l) (Merck KGaA, Darmstadt, Germany) as the standard. The total flavonoid content was expressed as mg of quercetin equivalents (QE)/g of honey. The experiment was conducted in triplicate.

Radical Scavenging Activity and Antioxidant Content

The radical-scavenging activity of *H. itama* honey samples for 2,2-diphenyl-1-picrylhydrazyl (DPPH) was determined[31] with slightly modifications. Each honey sample was dissolved in distilled water at a concentration ranging from 1–120 mg/ml, and 50 µL of each sample was mixed with 150 µL DPPH (Merck KGaA, Darmstadt, Germany), which was dissolved in methanol at a concentration of 0.02 mg/ml. Methanol served as the blank sample. After 15 minutes of incubation at room temperature, the absorbance was measured at 517 nm. Ascorbic acid (0–50 mg/l) (Kemaus, N.S.W., Australia) and quercetin (0–50 mg/l) were used as positive controls. Radical scavenging activity was determined as follows.

% Inhibition = [(blank absorbance − sample absorbance)/blank absorbance] × 100.

The graphic representation of the mean of three IC_{50} values (concentration producing 50% inhibition) for each honey sample was illustrated.

For the antioxidant activity, each honey sample was dissolved in distilled water at a concentration of 20 mg/ml. The honey solution (75 µL) was then mixed with 150 µL of a 0.02 mg/ml DPPH solution in methanol. After incubation at room temperature for 15 min, the absorbance of the solution was measured at 517 nm wavelength. The blank sample consisted of 75 µL of honey mixture solution with 150 µL of methanol. The antioxidant activity was examined using a standard curve with quercetin (0 – 10 µg/ml) and ascorbic acid (0 – 10 µg/ml) as standards. The antioxidant content was expressed as mg of quercetin equivalent per gram of honey and mg of ascorbic acid equivalent per gram of honey. All measurements were performed in triplicate.

Statistical Analysis

All experiments were performed in triplicate, and the data are represented as mean ± standard deviation (SD). Analysis of variance (ANOVA) and Tukey's test at the 95% confidence level were performed using the R program.[32] The correlation coefficients were used to examine the relationship between the two variables were also determined using the R-program.[32]

Results and Discussion

Physicochemical Properties

The physicochemical properties of *H. itama* honey collected from three different forest types (mangrove: three samples; swamp: three samples; and mixed

forests: four samples; see Table 1 for the main vegetation information) in Narathiwat Province, south of Thailand, were similar (Table 2). The moisture content of raw stingless bee honey collected from mangrove, swamp, and mixed forests were 27.89 ± 0.25, 27.78 ± 0.58, and 28.12 ± 0.28 g/100 g of honey, respectively. We found no significant difference in the moisture content of *H. itama* honey collected from three different floral origins ($p>0.05$). However, the moisture contents of fresh honey of *H. itama* collected from southern Thailand in this study were considerably high with those reported for *H. itama* honey from Malaysia where the values ranged between 25.49 ± 0.45 g/100 g[33] and 25.82 ± 0.03 g/100 g.[34] In comparison with honey from another Thai' stingless bee species, Chuttong . 11 showed that the mean moisture content for 28 honey samples collected from[11] stingless bee species was 31.0 ± 5.4 g/100 g, ranged from 25 g/100 g to 47 g/100 g. Additionally, Suntiparapop . 22 reported similar moisture content of fresh honey of *Tetragonula leaviceps,* which ranged from 26.50 ± 0.24 g/100 g to 27.46 ± 0.23 g/100 g. In contrast, the moisture content of *H. itama* honey was much higher than that of honey from *Apis* species.[35,36] All *Apis* species have evolved behavioral mechanisms to reduce the moisture levels of their honey, whereas stingless bees do not.[3]

After heat treatment at 37 °C for 24 and 48 hours in this study, the moisture contents of *H. itama* honey were decreased to 26.77 ± 0.49 g/100 g (ranged from 26.22 ± 0.10 to 27.17 ± 0.33 g/100 g) and 23.79 ± 0.27 g/100 g (ranged from 23.50 ± 0.44 to 24.04 ± 0.37 g/100 g), respectively (Table 2). Whereas, after incubated at 45 °C for 24 and 48 hours, the moisture contents were decreased to 22.97 ± 0.25 g/100 g (ranged from 22.71 ± 0.22 to 23.22 ± 0.58 g/100 g) and 20.14 ± 0.34 g/100 g (ranged from 19.77 ± 0.25 to 20.44 ± 0.25 g/100 g), respectively (Table 2). This result indicates that thermal treatment at a medium temperature level can reduced the moisture content in stingless bee honey close to the 20% maximum allowed, according to the international standard for *Apis mellifera* honey. Research conducted17 demonstrated that using a low temperature of 30 °C combined with vacuum conditions could decrease the moisture content of stingless bee honey from 26 g/100 g to 20 g/100 g in 60 min. Whereas, Braghini.[26] showed that the moisture content of stingless bee honey decreased from 30.8±0.70 g/100 g to 29.50±0.20 g/100 g after incubating at 95 °C for 60 sec. However, honey is considered a heat-sensitive material. High temperatures can affect the physicochemical properties of honey during processing.[37] Fauzi and Farid[38] stated that heating honey at high temperatures may decrease antioxidant and antimicrobial activities and may also cause crystallization, which will decrease honey quality.[39] Additionally, Kretavičius.[40] reported that heating at temperatures lower than 50 °C had no effect on enzyme activity in honey samples. Thus, heating treatment at low temperatures might be suitable to apply as a method for preservation of stingless bee honey.

All raw honey samples of *H. itama* were acidic, with low pH values ranging from 3.06 ± 0.01 to 3.32 ± 0.01, which is in close agreement with a report.[41] The pH values of honey collected from swamp forests were slightly lower than those of honey collected from mangrove and mixed forests (Table 2). After heat treatment, pH values of all honey samples were slightly increased compared with fresh honey (Table 2). Our results are similar to *H. itama* stingless bee honey collected from Malaysia, which had a mean pH value of 3.26 ± 0.17.[42] As reported[43] the lower pH of stingless bee honey is owing to the presence of citric acid, acetic acid, gluconic acid, and benzoic acid. The acidity of stingless bee honey increases as fermentation occurs during storage. Chuttong.[11] reported that the mean pH value of honey samples obtained from various stingless bee species in Thailand was 3.6 ± 0.198. A study conducted by Nascimento . 44 on 30 stingless bee honey samples from Brazil also showed that the pH value varied from 2.93 − 4.08. Another study by Boorn.[45] demonstrated a similar average pH value of Australian stingless bee honey of 3.85 ± 2.6. Although the low pH value of stingless bee honey could prevent the growth of bacteria and thus enhance its antibacterial properties, this property might affect consumers' preferences for stingless bee honey.[42]

In this study, the soluble solids (ºBrix) of raw *H. itama* honey varied from 61.11±0.51 69.55±0.35. Similar to the pH value, the ºBrix values increased slightly after heat treatment (Table 2). Our results are in keeping with a report[26] which showed slightly

Table 2: A compilation of data from Heterotrigona itama's honey samples collected from three habitats of Narathiwat province, Southern Thailand. Phenolic, flavonoid, radicle scavenging activity (IC_{50}), and antioxidant contents (AEAC and QEAC) were examined. SE, standard error of the mean; IC_{50}, 50% inhibitory concentration; GAE, gallic acid equivalent; QE, quercetin equivalent; AEAC, ascorbic acid equivalent antioxidant content; QEAC, quercetin equivalent antioxidant content.

Honey sample (sample size)	Heat treat -ment (°C)	Incu-bation time (hrs.)	Moisture content	Sample °Brix (SE) (%±SE)	pH (SE)	Total phenolic content (mg GAE/100g of honey ±SE)	Total flavonoid content (mg QE/100g of	Radical scavenger activity: IC_{50} (mg/ml ±SE) honey ±SE)	AEAC (mg/100 g ±SE)	QEAC (mg/100 g ±SE)
Mangrove forest (3)	Raw honey	0	27.89±0.25	61.11±0.51	3.32±0.01	54.483±0.975	27.278±0.201	57.893±0.084	5.280±0.093	3.005±0.231
	37	24	27.17±0.33	71.56±0.35	3.53±0.01	54.889±0.718	27.122±0.467	57.352±0.358	5.343±0.091	2.895±0.050
	37	48	23.50±0.44	74.72±0.35	3.55±0.01	55.097±0.382	28.089±0.302	56.894±0.616	5.256±0.113	3.005±0.127
	45	24	23.22±0.58	74.44±0.63	3.56±0.01	54.416±1.227	27.067±0.067	57.450±0.327	5.317±0.120	2.915±0.031
	45	48	20.44±0.25	77.83±0.17	3.54±0.01	55.409±2.175	27.678±0.568	57.309±0.372	5.236±0.014	2.885±0.156
Mean (SE)			**24.44±3.07**	**71.93±6.44**	**3.50±0.10**	**54.859±0.418**	**27.447±0.431**	**57.369±0.338**	**5.287±0.044**	**2.941±0.059**
Swamp forest (3)	Raw honey	0	27.78±0.58	69.55±0.35	3.06±0.01	49.490±0.785	17.056±0.189	131.384±0.329	4.313±0.050	3.086±0.058
	37	24	26.22±0.10	71.83±0.44	3.24±0.01	49.281±1.049	17.289±0.715	131.346±0.360	4.101±0.359	2.317±0.126
	37	48	23.83±0.17	75.00±0.33	3.23±0.01	50.021±0.477	17.578±0.701	131.223±0.263	4.867±0.106	3.002±0.083
	45	24	22.98±0.42	75.67±0.29	3.26±0.01	50.874±0.319	17.300±0.600	131.426±0.574	4.630±0.211	2.865±0.119
	45	48	19.77±0.25	77.94±0.25	3.33±0.01	50.497±0.548	17.667±0.819	131.447±0.214	4.764±0.129	2.935±0.033
Mean (SE)			**24.12±3.09**	**73.99±3.31**	**3.22±0.10**	**50.032±0.668**	**17.378±0.246**	**131.365±0.088**	**4.536±0.317**	**2.841±0.304**
Mixed forest (4)	Raw honey	0	28.12±0.28	67.42±0.44	3.26±0.02	89.426±1.049	58.119±1.068	43.996±0.377	9.801±0.248	7.192±0.069
	37	24	26.92±0.29	72.87±0.21	3.38±0.01	90.051±0.885	58.467±0.538	43.504±0.530	9.694±0.102	7.043±0.142
	37	48	24.04±0.37	74.67±0.49	3.41±0.01	90.311±0.531	57.713±0.653	43.984±0.438	9.832±0.087	7.256±0.063
	45	24	22.71±0.22	75.21±0.22	3.44±0.01	90.115±0.848	58.258±0.730	44.052±0.648	9.684±0.022	7.191±0.124
	45	48	20.21±0.14	78.04±0.34	3.45±0.01	89.675±0.206	57.907±0.646	44.458±0.875	9.740±0.137	7.247±0.113

Mean (SE)			**24.40±3.19**	**73.64±3.94**	**3.39±0.08**	**89.916±0.358**	**58.093±0.294**	**43.998±0.338**	**9.750±0.065**	**7.186±0.085**
Mean (SE)	Raw honey	0	27.93±0.17	66.23±4.39	3.21±0.14	64.466±21.759	34.151±21.377	77.740±46.970	6.465±2.929	4.427±2.394
		37	26.77±0.49	72.09±0.69	3.38±0.14	64.740±22.098	34.293±21.505	77.401±47.288	6.382±2.933	4.085±2.578
		37	23.79±0.27	74.80±0.18	3.40±0.16	65.143±21.943	34.460±20.812	77.367±47.085	6.652±2.761	4.421±2.455
		45	22.97±0.25	75.11±0.62	3.42±0.15	65.135±21.706	34.208±21.392	77.643±47.057	6.544±2.741	4.324±2.483
		45	20.14±0.34	77.93±0.10	3.44±0.10	65.194±21.343	34.417±20.949	77.738±46.955	6.580±2.747	4.356±2.504

increased ºBrix values of *Melipona* bicolor honey after short-term heat treatment at 90 °C and 95 °C (ranging from 69.0 ± 030 to 69.4 ± 0.20). This result indicates that heat treatment can be applied to honey processing methods without any negative effects on some physicochemical properties.

Total Phenolic and Flavonoid Contents

Several bioactive components have been identified in stingless honey.[10,46-48] Using the standard curve of gallic acid (R^2 = 0.998), the total phenolic content values in raw *H. itama* honey samples collected from mangrove, swamp, and mixed forests were 54.483 ± 0.975, 49.490 ± 0.785, and 89.426 ± 1.049 mg GAE/100 g of honey, respectively (Table 2). In comparison with other studies, Ismail.[47] and Shamsudin.[48] reported similar total phenolic content of *Trigona* sp. and *H. itama* honey collected from Malaysia where the values ranged between 33.2 and 60.2 mg GAE/100 g and 52.64 and 74.62 mg GAE/100g, respectively. In contrast, Imtiazah.[46] reported a higher total phenolic content of Malaysian *H. itama* honey where the values ranged between 435.69 and 516.07 mg GAE/100 g of honey. Abu Bakar.[49] also demonstrated a higher content value in *H. itama* honey collected from Malaysia.

Interestingly, we observed a significant difference ($p < 0.001$) in the total phenolic content of *H. itama* honey from different botanical origins (Table 2). We also observed no significant differences in the total phenolic contents among collecting apiaries within the same botanical origins (p = 0.860). Honey collected from the mixed forest showed the highest total phenolic content (89.916 ± 0.294 mg GAE/100 g of honey), whereas honey samples from the swamp forest showed the lowest (50.032 ± 0.668 mg GAE/100 g of honey). This result is in close agreement with the report,[15] who stated that the phenolic compounds found in honey are derived directly from botanical sources. Honey derived from various floral sources possesses varied biological characteristics.[2] In contrast, Shamsudin.[48] reported no significant differences among *H. itama* honey collected from three botanical origins (gelam: *Melaleuca cajuputi* Powell; acacia: *Acacia penninervis* DC; and starfruit: *Averrhoa carambola* L). However, they found that gelam and starfruit honey collected from *H. itama* colonies shows significantly higher values of total phenolic content than in *Apis mellifera* honey. Different bee

species can explain this difference and how honey is obtained by two different bees.[48,50]

The total flavonoid content (mg QE/100 g of honey) of raw *H. itama* honey samples collected from three different forest types (mangrove, swamp, and mixed forests) varied from 17.056 ± 0.189 to 58.119 ± 1.068 mg with a mean of 34.151 ± 21.377 mg (Table 2), with the highest level observed in mixed forest honey using the standard curve of quercetin (R^2 = 0.997). Like phenolic contents, we found a significant difference ($p < 0.001$) in total flavonoid content among *H. itama* honey from different botanical origins (Table 2). When compare among collecting sites within forest type, no significant difference in total flavonoid content were detected (p = 0.289). We found a low correlation (R = 0.26) between total flavonoid content and total phenolic content. Flavonoids are low molecular weight compounds responsible for honey's aroma properties and antioxidant potential.[49,51] Thus, the different floral sources responsible for honey production might result in different flavonoid types and contents in honey.[52] For instance, Shamsudin.[48] observed significant differences in total flavonoid content values among starfruit honey (25.71 mg QE/100 g honey), gelam honey (20.67 mg QE/100 g honey), and acacia honey (10.70 mg QE/100 g honey), which were obtained from Malaysian' *H. itama* hives. They also reported higher total flavonoid content in *H. itama* honey than in *Apis* honey. Sousa.[53] found high rutin flavonoid values in honey collected from two Brazilian stingless bee species (*Melipona* subnitida and *M. scutellaris*) with plant sources from *Ziziphus juazeiro*. However, Oliveira.[54] found an absence of rutin flavonoids in honey samples obtained from M. *subnitida* and *M. scutellaris*. After heat treatment, we observed no significant differences between the total phenolic (p = 0.566) and flavonoid (p = 0.252) contents in the raw and heat-treated honey samples (Table 2). Comparable results were obtained51 who reported no alteration in the total phenolic content after applying temperatures of 55 °C and 65 °C in Malaysian and Australian stingless bee honey samples. However, Jahan.[55] found an increase in total phenolic and flavonoid contents after incubating honey samples at 50, 70, and 90 °C. Braghini .[9] also reported an increase in total phenolic and flavonoid content in stingless bee (*Tetragonisca angustula*) honeys after heating at high temperatures of 60 °C and 70 °C. They concluded that increasing total phenolic and flavonoid contents might occur due to the loss of moisture level in honey and conversion of some bioactive components during heating application.[26]

Inhibition of Free Radicals (DPPH) by Scavenging Activity

Antioxidant activity is described as the potentiality of given compounds or mixtures to reduce pro-oxidants or reactive species, including free radicals. Several methods are available for its determination 56-59. Among them, DPPH seems to be the most commonly used due to its measurement simplicity, short experimental time and the employment of the inexpensive spectrophotometer.[60]

The antioxidant activities of *H. itama* honey samples from southern Thailand were determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The free radical scavenging activity was evaluated as IC_{50}, demonstrating the antioxidant capacity required to reduce the initial concentration of DPPH solution by 50%. Thus, honey with a low IC_{50} value has greater antioxidant activity than honey with a high value.[61,62] The DPPH antioxidant activity of *H. itama* honey samples are documented in Table 2. The IC50 of raw honey from Thai *H. itama* collected from the mixed forest showed the lowest value of 43.996 ± 0.377 mg/ml, followed by mangrove forest (57.893 ± 0.084 mg/ml) and swamp forest (131.384 ± 0.329 mg/ml), respectively. Thus, the mixed forest honey had higher antioxidant activity than the other two honey samples (Table 2). Compared to honey from Malaysia, the IC50 values of raw honey in this study were higher than previously reported. Ismail.[47] reported a range between 10.6 to 19.7 mg/ml. Shamsudin.[48] showed the IC_{50} values of raw honey collected from Malaysian *H. itama* ranged between 11.27 – 24.09 mg/ml, which was significant lower with this study. However, the IC50 values of *H. itama* honey obtained from mixed and mangrove forests of this study (44.299 – 57.754 mg/ml) were consistent with stingless bee honey from Brazil (25.39 – 51.55 mg/ml) and *Apis* honey (53.65 mg/ml).[15]

Using the standard curves of ascorbic acid (R^2 = 0.992) and quercetin (R2 = 0.990), we demonstrated that the highest antioxidant content of raw honey was found in mixed forest honey samples, which were 9.801 ± 0.248 mg AEAC/100 g honey and

7.192 ± 0.069 mg QEAC/100 g honey. The results of this investigation were in keeping with those discovered61 where the antioxidant content in multifloral honey varied from 4.27 to 17.30 mg QEAC/100 g of honey and from 10.20 to 37.87 mg AEAC/100 g of honey.

Within group of honey sample, we found no significant inhibition activity or antioxidant content ($p > 0.05$) between the raw and heat-treated samples (Table 2). Mat Ramlan.[51] also reported that heat treatment had no effect on the DPPH radical scavenging activity and antioxidant content of Malaysian and Australian stingless bee honey. Turkmen . 63 demonstrated that heating honey to 70 °C causes a remarkable increase in the potential of antioxidant activity compared to 50 and 60 °C. They suggested that temperature and time play a crucial role in determining the antioxidant properties of honey. Additionally, Amarowicz 64 suggested that temperature might induce the formation of MRPs pigments in honey, which increases its antioxidant properties. The present study only conducted heat treatment at 37 and 45 °C for 24 and 48 h. Thus, we can detect that the impact of the heat treatment was not significantly different from that of a previous study as they used a higher temperature and longer heating period.

Conclusion

In conclusion, heat treatment at 37 °C and 45 °C for 24 and 48 h had no effects on total phenolic, flavonoid contents, and antioxidant activity determined by DPPH for all *H. itama* honey samples. This study also found that botanical origin substantially affects antioxidant activity. Additionally, floral origin strongly influences the content of phenolic and flavonoid components in raw honey. Stingless bee honey obtained from mixed forests had the highest total phenolic, total flavonoid contents, and free radical scavenging activity, followed by honey from mangrove and swamp forests.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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