

Physicochemical Profiles, Antioxidant and Antibacterial Capacity of Honey from Stingless Bee *Tetragonula laeviceps* Species Complex

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Abstract. Stingless bee (Apidae, Meliponini) is a group of eusocial insects that widely distributed in the neotropical regions of the globe. Similar to honeybees, stingless bees produce honey that is usually valued much higher, likely due to both its unique flavor and properties. In this study, honey samples produced by stingless bee *Tetragonula laeviceps* species complex were collected from different meliponary in Eastern Thailand (Rayong, Chantaburi, and Trat provinces). The honey samples were examined the physicochemical parameters, antioxidant, and antimicrobial activities. The results revealed the physicochemical parameter of honey from *T. laeviceps* species complex to be an average color (75 ± 15 mm Pfund), moisture (27 ± 2 g/100 g), pH (3.70 ± 0.3), total sugar (50 ± 7.80 g/100 g), electrical conductivity (0.62 ± 0.15 ms/cm) and the soluble solids (51.70 ± 4.12 °Brix). Besides, the honey from the *T. laeviceps* species complex showed the highest reducing power ($18 \pm 1.20\%$) and antimicrobial property against four species of bacteria (*Bacillus subtilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium*) and yeast (*Candida albicans*).

1 Introduction

Stingless bee is a highly eusocial insect as well as a honey bee. These bees are inhabitants of the tropics area around the world. Stingless bee belongs to the family Hymenoptera and subfamily Meliponinae, with over 500 species in 32 genera worldwide [1]. In Thailand, the stingless bee is distributed in all areas; about 32 species in 10 genera have been reported [2]. A few species can be cultured in the artificial hive for meliponiculture. The majority of managed stingless bees for meliponiculture is *Tetragonula laeviceps* species complex, which widely distributed across the country. However, the meliponiculture industry in Thailand is still an infant phase but expanding. The Department of agricultural extension (DOAE) estimated that the numbers of meliponiculturists in Thailand is about 700 over 5,000 colonies [3].

Not just a pollinator, stingless bee also produced honey, propolis (cerumen), and pollen. Stingless bees store honey within cerumen pots, which differs from honey bees where honey is stored in comb cells (made from wax) and concentrated by fanning behavior [4]. Stingless bees collect nectar from flowers and save them into honey pots within the hive matrix. Nectar is a viscous liquid substance produced by glands called nectaries comprising most carbohydrate, amino acid, alkaloid, and polyphenols. Stingless bee collected nectar from various flowers and stored in the hive as honey pot

and then seals the pot with cerumen when it is full as a reserved food. This behavior is not found in the honey bee [5]. Honey pot plays a crucial role as a dietary source of carbohydrate, vitamins, and minerals that helps maintain the colony [6, 7].

The characteristics of honey pot shape and size vary between stingless bee species, but food storage pots are always much larger substantial than brood cells. The honey pot varies in flavor, color, and texture depending on botanical origin and microbial communities associated with the pot [8, 9]. The honey pot of the *T. laeviceps* species complex is soft, sticky, and separated from the pollen pot but similar in shape and volume (about 27.7 ± 6.2 mm³), as shown in fig. 1a. [3].

Studies also report that the stingless bee honey was containing phenolic compounds and flavonoids, which are widely valued [5, 8, 10]. Studies showed that the stingless bee honey had significant antioxidant and antimicrobial activities due to the presence of phenolic compounds as well as honey from honey bee [11]. However, few studies reported that the antimicrobial activity of stingless bee honey is slightly stronger. The comparison study of therapeutic properties between stingless bee honey and honey from honey bee found that stingless bee honey has excellent potential to be developed for common medicinal uses due to the varieties of bioactive components as a therapeutic agent over honey from honey bee in various health issue such as anticancer, antidiabetic and wound healing [11].

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Several countries in Central and South America are attempting to establish the standards for stingless bee honey. Thailand still has no international nor national standard of stingless bee honey due to lack of information regarding physicochemical characteristics, antioxidant, and antimicrobial properties of honey from stingless bees, particularly *T. laeviceps* species complex, the most common meliponiculture in Thailand [12].

This novel research will be conducted to characterize physicochemical properties, phenolic contents, flavonoid contents, antimicrobial activity, and antioxidant capacity of the *T. laeviceps* species complex honey. The obtained data of this study will be act as an initiation for future research on Thai stingless bee honey, and so aim to standardize protocols as a resource of bioactive compounds.

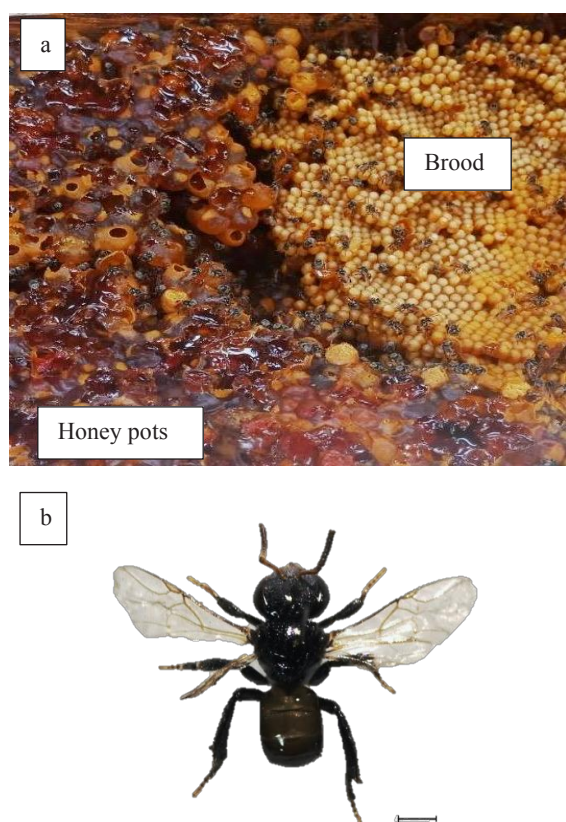


Fig. 1. *T. laeviceps* species complex: a) workers were gathering around honey pots and brood in their colony; b) adult worker. (scale bar in b = 1 cm). Photo credit: Kanokwan Khamyotchai.

2 Materials and methods

2.1 Stingless bee *T. laeviceps* species complex and honey samples

Thirty samples of stingless bees and honey were collected from different meliponary in the Eastern part of Thailand (Rayong, Chantaburi, and Trat provinces) during the summer of 2019. Honey and adult stingless bees were collected from the beehives. Honey samples were taken by using a 10 mL pipette (Eppendorf, Germany) penetrated the honey pots and kept in 50 mL

centrifuge tubes (Biologix, China). Adult stingless bees were collected from the hive by using aspirator and stored in a plastic tube. All samples were stored at -20°C in a zip lock bag until use.

2.2 Physicochemical parameters

The honey samples solution in deionized water at different concentration were prepared, respectively. All assays were measured in parallel for three times.

2.2.1 Color

Stingless bee honey color was determined, according to Al-Farsi et al [13]. Honey samples were diluted to 50% with deionized water and filtrated through the Whatman no.1 filter. The A_{635} was measured using a NanoDrop™ UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). The stingless bee honey color was determined according to the Pfund scale using the following equation.

$$\text{Pfund} = -38.70 + 371.39 \times \text{Abs} \quad [14]$$

2.2.2 Moisture and total soluble solid content

The moisture and total soluble solid content in stingless bee honey were determined by refractometer [15] using the digital refractometer model PR-201 α (Atago, Japan).

2.2.3 Electrical conductivity

Honey electrical conductivity was determined according to the international honey commission (IHC) [16]. The stingless bee honey 20% (w/v) in deionized water was measured electrical conductivity using electrical conductivity tester model EC-3 (HM Digital, Inc., USA).

2.2.4 pH

Honey pH was determined according to the IHC. A pH pen meter model ST20 (Ohaus, USA) was used to take the pH measurement.

2.2.3 Total sugar

Total carbohydrate content (soluble sugar) was determined according to the Wusiman *et al.* [17], and the results were compared against a standard glucose curve.

2.3 Determination of total phenolic content

The total phenolic compounds in honey samples were determined by the Foiln-Ciocalteu colorimetric method, according to Ávila *et al.* [8]. The total phenolic content was performed in 96-well plate by added 80 μL of honey samples (1-0.062 $\mu\text{g}/\text{mL}$) to 100 μL of Foiln-Ciocalteu following by 80 μL of 14% sodium carbonate (Na_2CO_3) solution and incubated at room temperature in the dark for 2 h. A_{760} reading was performed by a NanoDrop™ UV-Vis Spectrophotometer (Thermo Fisher Scientific

Inc., USA). Gallic acid (0.4-16 µg/mL) was used as a standard. The total phenolic contents in honey were expressed in mg of GAE/g of the honey sample.

2.4 Determination of total flavonoids content

The total flavonoids in honey samples were performed in 96-well plate according to Ávila et al. [8] by added 20 µL of honey samples (1-0.062 µg/mL) to 180 µL of methanolic solution of 2% aluminum chloride hexahydrate (AlCl₃.6H₂O) then incubated at room temperature for 30 min in the dark. A₄₁₅ reading was performed by a NanoDrop™ UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., USA). Quercetin (0.4-16 µg/mL) was used as a standard and standard curve. The flavonoid contents in honey samples were reported in mg of QE/g of the honey sample.

2.5 Antioxidant activities (DPPH assay)

For antioxidant activities, the DPPH assay was performed according to Jantakee and Tragoolpua [18] by added 20 µL of honey samples at different concentrations (1-0.062 µg/mL) was mixed with 180 µL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical solution in methanol. The mixtures were incubated at room temperature in the dark for 30 min. A₅₁₇ were observed by a microplate reader. Ascorbic acid was used as a reference antioxidant. The corresponding blank readings were also taken, and percent of radical scavenging activity (RSA) was then calculated as follows:

$$\%RSA = (\text{Abs. DPPH} - \text{Abs. Sample}) / \text{Abs. DPPH} \times 100$$

The EC₅₀ value, the concentration of sample required for 50% scavenging of the DPPH free radical, was determined from the curve of percent scavenging plotted against the concentration using GraphPad Prism V.7.0. Each determination was done in triplicate, and the average EC₅₀ value was calculated.

2.6 Antibacterial activity

2.6.1 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) determination

Four bacteria; *Bacillus cereus* TISTR 2372, *Pseudomonas aeruginosa* TISTR 1287, *Staphylococcus aureus* TISTR 1840, and *Salmonella typhimurium* TISTR 1469 were sub-cultured on TSB at 37°C, 120 rpm for 24 h. The MIC of honey samples was measured following the method described by Tuksitha et al. [19].

2.7 Statistical analysis

The results were presented as a mean ± standard deviation from the triplicate analysis. Pearson's correlation coefficient was measured to find the association between two variables of color, phenolic, flavonoids, and antioxidants. The data were analyzed

using NCSS 12 Statistical Software (2018). NCSS, LLC. Kaysville, Utah, <http://ncss.com/software/ncss>.

3 Results and discussion

The physicochemical parameters of the honey from stingless bee *T. laeviceps* species complex, including color, moisture, pH, total sugar, electrical conductivity, and the soluble solid, are presented in Table 1.

Table 1. Physicochemical parameters of stingless bee *T. laeviceps* species complex honey.

| | |
|--------------------------------|--------------------|
| Moisture | 27.20 ±2.5 % (w/w) |
| Color | 75.00 ±15 mm Pfund |
| Total soluble solid | 51.00 ±4.12°Brix |
| Electrical conductivity | 0.62 ±0.5 ms/cm |
| pH | 3.70 ±0.3 |
| Carbohydrate | 50 ±7.80 % (w/w) |

3.1 Physicochemical properties

3.1.1 Color

The Pfund unit is a scale of honey color analysis described by USDA in 1925 [20] with a scale ranges from 1-140 mm. The Pfund unit can be used to classify honey color from the honey bee. The color of honey from the *T. laeviceps* species complex in this analysis ranges from 32-99 mm Pfund with an average value of 75 ± 17 mm Pfund. Based on the Pfund scale results, honey from the *T. laeviceps* species complex would grade in the color range of light amber. Similar values are reported by Chuttong *et al.* [12], ranging from 50-70 mm Pfund. However, the results observed from the same season may affect the color of the honey due to the high temperature in summer (TEMP) [21].

3.1.2 Moisture

The moisture content in honey samples from stingless bee *T. laeviceps* species complex ranged from 17.1 – 29.5% (w/w) with an average value of 24.2 ±4.7 (w/w). Similar moisture contents in stingless bee honey are cited in stingless bee from Southeast Asia [12], which showed average moisture content of 31 ± 5.4 (w/w) from *T. laeviceps-pagdeni* complex, and had comparable moisture content from 31 species of stingless bees in South America [22], which showed mean moisture content of 26 ± 4.8% (w/w). The occurrence of humidity in stingless bee honey may be related to geographical origins as Eastern Thailand that has a humid tropical environment. Unlike the honey bee, the stingless bee has no fanning behavior that keeps high water content in the honey.

3.1.3 Total soluble solid

The values of total soluble solid in *T. laeviceps* species complex honey ranged from 39-58 °Brix with an average

value of 51 ± 5.3 °Brix. The total soluble solid data in this study were lower than stingless bee honey from Brazil [23], which ranges from 55.2-76.1 °Brix. The total soluble solid reflected the amount of substantial solution in the honey, such as sugars, minerals, and organic acids. This result indicated the honey from the *T. laeviceps* species complex in Eastern Thailand contained high level of water but lowers sugar content.

3.1.4 Electrical conductivity

The electrical conductivity results provided an average electrical conductivity in *T. laeviceps* species complex honey at 0.62 ± 0.50 ms/cm. Electrical conductivity has been used as a honey quality indicator and also related to the ash content, organic acids, and proteins. Our results were comparable with the electrical conductivity of stingless bee honey from Southeast Asia [12, 24] and Brazil [23] ranging from 0.53-0.71 ms/cm and 0.15-1.34 ms/cm, respectively.

3.1.5 pH

The pH of *T. laeviceps* species complex honey ranged from 3.50-4.30 with an average value of 3.70 ± 0.30 . The pH of *T. laeviceps* species complex honey was similar to other reports of stingless bee honey from South America (ranges from 2.90-5.30) [22] and honey from Brazil (ranging from 3.33-4.75) [24].

3.1.6 Carbohydrate

The amount of carbohydrate, including mono-, di-, oligo-, and polysaccharides in *T. laeviceps* species complex honey ranges from 28.00-59.00% (w/w) with an average value of $50.00 \pm 7.80\%$ (w/w). The carbohydrate of *T. laeviceps* species complex honey was similar to other reports of stingless bee honey from 11 species of stingless bee in Thailand [12] and reducing sugar in stingless bee honey from Brazil (ranges from 48.6-70.5% w/w) [23]. The amount of carbohydrate in honey revealed the sweet taste in honey. Stingless bee honey contained lower sugar and pH but higher moisture compared to honey from honey bee resulting in less lightly sweet and sour taste.

3.2 Total phenolic and flavonoids content

Honey is considered to be a natural source of phenolic and flavonoids depended on the botanical and geographical origins. Our finding regarding the phenolic content of the honey samples from *T. laeviceps* species complex ranged from 100-550 mg GAE /kg of honey with an average value of 480 ± 13.59 mg GAE /kg of honey. The flavonoids content of the honey samples from *T. laeviceps* species complex ranged from 20-150 mg quercetin /kg of honey with an average value of 110 ± 32.20 mg quercetin /kg of honey. When the phenolic and flavonoids contents of honey samples from *T. laeviceps* species complex were compared to different

botanical origin of Thai honey from honey bee (ranges from 234.66-674.81 mg GAE /kg of honey and 29.86-178.31 mg quercetin /kg of honey, respectively) [19], *T. laeviceps* species complex had a higher phenolic and flavonoids contents than most of Thai honey, excepted poly flora and coffee honey. The lower phenolics and flavonoids in our samples compared to honey from honey bee could be the environmental factor, particularly humidity that diluted the honey.

3.3 Antioxidant capacity

The antioxidant activity of the honey relies on the amount and types of phenolic and flavonoid compounds along with other components, such as proteins and minerals. The honey from *T. laeviceps* species complex showed antioxidants in terms of EC₅₀ value ranges from 15.70-52.07 mg/mL with an average value of 20.10 ± 0.60 mg/mL. Based on our results, the antioxidant activity of honey samples related to the amount of phenolic and flavonoid compounds suggested that bioactive compounds in honey samples play a critical role in antioxidant activity.

3.4 Antibacteria capacity

The honey from *T. laeviceps* species complex showed antimicrobial activity against general pathogens, including four bacteria and one yeast. The minimum inhibitory concentration (MIC) (% w/w) of *T. laeviceps* species complex against different microorganisms ranged from 10-30% (w/w) with an average value of 12.5% (w/w). The minimum bactericidal concentration (MBC) (% w/w) of *T. laeviceps* species complex against different microorganisms ranged from 25-50% (w/w). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of honey from the *T. laeviceps* species complex were presented in Fig. 2.

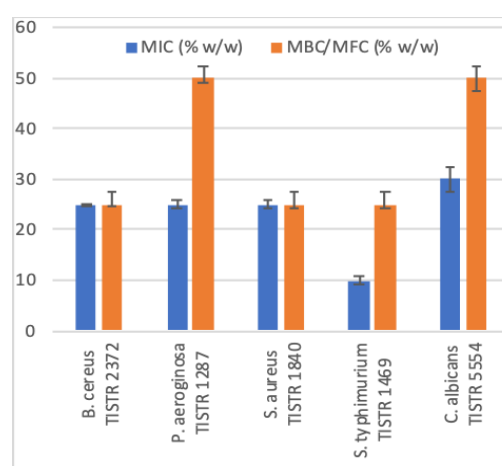


Fig. 2. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of honey (% w/w) from *T. laeviceps* species complex against pathogenic microorganism.

The antibacterial activity of honey samples indicated that the *T. laeviceps* species complex inhibited various

types of microorganisms, including Gram-negative bacteria, Gram-positive bacteria, and yeast. Besides, the antimicrobial activity, based on the MIC and MBC value, of *T. laeviceps* species complex honey was comparable to Thai honey from various botanical origins, particularly *S. aureus* [18] but had a lower MIC and MBC values when compared to stingless bee honey from Borneo [19] and Brazil [25-27].

However, the antimicrobial activity of honey is associated with several factors, such as osmotic properties, phenolics and flavonoids content, hydrogen peroxide content, and pH. Based on the physicochemical results, our honey samples were high in water content. The phenolic and flavonoid compounds in honey play a crucial role in antimicrobial activity. The high amount of water in honey reduced phenolics and flavonoids content in the honey resulting in low antimicrobial activity. The antimicrobial activity of our honey samples could be improved by dehydration and carbohydrate elimination and also isolation and extraction of phenolic and flavonoid compounds.

3.5 Correlation

To investigate the relationships between color, phenolics, flavonoids, and EC₅₀ values, Pearson's correlation coefficient was established (Table 2). There was a positive correlation between color-phenolic (0.729), color-flavonoids (0.050), and flavonoids-EC₅₀ (0.317). Whereas, the negative relationships were found between color-EC₅₀ (-0.429), phenolics-flavonoids (-0.039), and phenolics-EC₅₀ (-0.269). The strong correlation between color-phenolics implied that the dark color (dark amber) in stingless bee honey can be associated with the presence of phenolics and flavonoids that increased the antioxidant capacity of the honey. Several studies have been reported a similar correlation between color-phenolics (0.974), (0.967), 0.816 and color-antioxidants (-0.608), (-0.800) [13, 28, 29].

Table 2. Pearson's correlation coefficient between color, phenolics, flavonoids, and EC₅₀ value.

| | Color | Phenolics | Flavonoids | EC ₅₀ |
|------------------|--------|-----------|------------|------------------|
| Color | 1.000 | | | |
| Phenolics | 0.729 | 1.000 | | |
| Flavonoids | 0.050 | -0.039 | 1.000 | |
| EC ₅₀ | -0.429 | -0.269 | 0.317 | 1.000 |

4 Conclusion

This study innovated new information about the honey from commercial stingless bee in Thailand (*T. laeviceps* species complex). The physicochemical profiles demonstrated that the honey from *T. laeviceps* species complex is different from honey obtained from the honey bee. The antioxidant activity of honey samples was associated with the phenolic and flavonoids contents. The honey samples also had antimicrobial activity against pathogenic bacteria and yeast. Unitedly, our results supported that honey from *T. laeviceps* species complex is a functional food. Also, it provided

some evidences to show its potential for the prevention of health problems, in which free radicals and pathogenic bacteria and yeast are implicated particularly. Further studies about phenolic and flavonoid content obtained from different geographical and botanical origins should be investigated for a better understanding of the bioactive compounds in *T. laeviceps* species complex honey. This study might promote and extend the alternative way concerning stingless bee honey for valuable products.

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