Original article

Stingless bee honey (Tetragonula laeviceps): Chemical composition and their potential roles as an immunomodulator in malnourished rats

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A B S T R A C T

Honey is rich in bioactive compounds, phenolic acids, and flavonoids and is an antioxidant and an immunomodulator. The objectives of this study were to determine the honey chemical composition of Indonesian stingless bees and their potential roles as an immunomodulator in the malnourished rats. Tetragonula laeviceps honey was used to analyses of chemical composition was obtained from three different geographical origins were Depok Sleman, Bayan Lombok, and Nglipar Gunungkidul. Thirty-two two rats were divided into four groups of 8 rats and placed in individual cages. The experimental design was as follows: T1 = normal rats + without honey (0–7 weeks), T2 = normal rats + with honey of 1.8 g/kg BW/day (0–7 weeks), T3 = malnourished honey of 1.8 g/kg BW/day started from 2 weeks after the malnourished condition (2–7 weeks). The results showed that the chemical composition of Tetragonula laeviceps honey from three different geographical origins were vitamin C content (6.49–13. 58 mg/100 g), total phenolic content (0.65–2.30% GAE/100 g), total flavonoid content (0.28–1.00 mg QE/g), and antioxidant activity DPPH (61.43–90.28%). The application of fresh honey from stingless bee that was offered to either normal or malnourished rats were increased lymphocytes proliferation and decreased the production of both proinflammatory markers, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) from tissue culture supernatant of lymphocytes (p < 0.01). Data from this study clearly indicates the potential role of honey from stingless bee as an immunomodulator in malnourished rats.

1. Introduction

All over the world, stingless bees are composed of about 500 species, of which approximately 100 species have not been studied yet (Michener, 2013). It was reported by Kahono et al. (2018) that 46 species exist in some islands of Indonesia, such as Borneo, Sumatera, Java, Timor, Sulawesi, Maluku, Ambon, and Papua. While in the Yogyakarta province, other researchers reported seven species consisting of Heterotrigona itama, Tetragonula sapiens, T. biroi, T. laeviceps, T. sarawakensis, T. iridipennis, and Lepidotrigona terminata, (Trianto and Purwanto, 2020). Tetragonula laeviceps’ natural habitats include trees trunk or woods, sugar palm stalks, bamboo tree, and in the ground hole (Agus et al., 2019; Agussalim et al., 2021, 2020, 2019b; Supeno et al., 2021). The stingless bee products from T. laeviceps consists of honey, propolis, and bee bake (Agus et al., 2019; Agussalim et al., 2021, 2020, 2019b, 2019a; Erwan et al., 2021, 2020; Sabir et al., 2021).

Several factors may affect the quality and production of the honey such as the availability of nectar from plant flowers and geographical origins (Agus et al., 2019; Agussalim et al., 2021, 2019b, 2019a, 2019b). Nectar is a sweet liquid that is secreted by the nectar glands of the plant flowers (floral nectar) and the leaf or leaf stalks (extrafloral nectar). It is the main material used by bee workers to produce honey. The quality of the honey is depends on the plant types as the nectar source, nectar chemical composition, plant flowers number may be visited by foragers, and climate (temperature, humidity, and seasonal) (Agus et al., 2019; Da Silva et al., 2016). Honey is composed of sugars, protein (enzymes and amino acids), secondary metabolites, carotenoids, organic acids, minerals, and vitamins (Da Silva et al., 2016). Honey's physiochemical composition in several species of stingless bees from various countries have been studied previously.
2. Materials and methods

2.1. Chemical composition

Honey used in this study was collected directly from the hive of T. laeviceps from three different origins: 1. Depok Sleman, Special Region of Yogyakarta Province (Faculty of Animal Science, Universitas Gadjah Mada (UGM)), 2. Nglipar (Katongan Village), Gunungkidul, Special Region of Yogyakarta Province, and 3. Bayan Lombok (North Lombok), West Nusa Tenggara Province, Indonesia. From each origin, three samples of 0.5 L honey were taken, respectively; thus, total honey collected was 4.5 L (3 × 3 × 0.5 L). Honey samples were kept in a room temperature when collecting in the farm for one week prior to the analysis.

2.1.1. Vitamin-C content

The vitamin-C content (i.e., ascorbic acid) was determined using the 2,6-dichlorophenolindophenol titrimetric as described in the study by Verma et al. (1996) with a minor modification. The extraction sorbent of C18 was activated by passing one–two columns of water volumes, and then one–two columns of water volumes via sorbent. The sample solution of 2 mL was added to the column and allowed to drain under suction and was then washed using one–two mL of water. The combination of precipitate and eluate were collected in flask used in the titration and 1 mL of acetic acid of anhydrous was added and mixed. Afterwards, the solution was titrated using a reagent 5 × 10⁻⁴ M 2,6-dichlorophenolindophenol until pink a colour was observed. A very sharp endpoint and blank of indicator was usually 0.05 mL. All analyses were performed in three replicates and each in a duplo.

2.1.2. Total phenolic content (TPC)

The TPC was determined by the spectrophotometry method using the reagent of folin–ciocalteu. To prepare the sample for analysis, two-gram honey was placed into the 10-mL tube, 0.5 mL reagent of folin–ciocalteu and 7.5 mL of aquabidest were added, and all were mixed thoroughly using the vortex. Afterwards, this solution was stored for about 10 min at 18–25 °C before adding 1 mL of sodium carbonate 20% and aquabidest until reaching 10 mL of volume. The solution was read at an absorbance wavelength of 760 nm. For a standard curve, we used 10 mg gallic acid, and it was prepared according to the sample preparation procedure previously. The gallic acid concentrations for the standard curve were 0, 1.563, 3.125, 6.250, 12.500, 25.000, and 50.000 ppm with R² = 0.9994 (Aloiti et al., 2018; Ranneh et al., 2018). All analyses were performed in three replicates and each in a duplo.

2.1.3. Total flavonoid content (TFC)

The TFC was determined by the method of spectrophotometry. Honey of 0.10 g was added into the 10-mL tube followed by 0.3 mL of sodium citrate, and 5 min later 0.6 mL of aluminium chloride 10% was added. After the solution was stored for about 5 min, 2 mL of sodium hydroxide 1 M and aquabidest was added until the volume reached 10 mL. The solution was then mixed and read at an absorbance wavelength of 510 nm. The standard curve was made using the quercetin 10 mg, and it was prepared according to the sample preparation procedure previously. The quercetin concentrations for the standard curve were 0, 1.563, 3.125, 6.250, 12.500, 25.000, and 50.000 ppm with R² = 0.9979 (Khalil et al., 2011). All analyses were performed in three replicates and each in a duplo (Agus et al., 2019).

2.1.4. Antioxidant activity

The antioxidant activity of honey was determined based on free radical-scavenging activity (RSA) of DPPH (2,2-diphenyl-1-picrylhydrazyl). Briefly, 0.10 mL of honey mixed with 0.9 mL of methanolic solution containing DPPH of 0.1 mM. Afterwards, the solution was incubated for about 30 min in a dark condition. DPPH radical reduction was read at an absorbance wavelength of 517 nm. In addition, the reference using butylated hydroxytoluene and RSA was calculated as the change of percentage in the DPPH color by percentage of RSA = ([A₈₅₀nm – A₈₅₀nm]/A₈₅₀nm × 100), where A₈₅₀nm was the solution absorbance when a sample was added at a certain level, and A₈₅₀nm was DPPH solution absorbance (Aloiti et al., 2018; Ferreira et al., 2009). All analyses were performed in three replicates and each in a duplo (Agus et al., 2019).

2.2. Preclinical experiment

2.2.1. Ethical clearance, animals, feed, and honey

This experiment protocol was approved by the Ethical Commission from the Faculty of Veterinary Medicine UGM, Indonesia (003/EC-FKH/Eks.,2019). This experiment was conducted in a laboratory of preclinical study and animal development in the Integrated Research and Testing Laboratory (LPPT) UGM. Thirty-two male rats (Rattus norvegicus) were of strain Sprague Dawley aged 7 weeks, with body weight (BW) ranging from 141.37 to 198.83 g. All rats were acclimatized for 7 days before experiment for 12 h in a controlled light and a dark cycle at 22 °C and 70–75% of humidity. This study had a standard feed of AIN-93 M.
Fig. 1. The DPPH antioxidant (A), TPC, TFC, and vitamin C (B) of honey from *T. laeviceps*. *Superscripts with different letters in the same parameter indicates significant at p < 0.05 (*) and p < 0.01 (**). Abbreviations: TPC: total phenolic content; TFC: total flavonoid content; Depok Sleman: Special Region of Yogyakarta Province (Faculty of Animal Science UGM); Nglipar Gunungkidul, (Katóngan Village) Special Region of Yogyakarta Province; Bayan Lombok: North Lombok, West Nusa Tenggara Province, Indonesia.

| Ingredients | Percent by weight |
|-------------|------------------|
| Corn starch | 46.5692          |
| Dextrin     | 15.5000          |
| Casein (vitamin free) | 14.0000     |
| Sucrose     | 10.0000          |
| Powdered cellulose | 5.0000     |
| Soybean oil | 4.0000           |
| AIN-93 M mineral mix | 3.5000    |
| AIN-93 M vitamin mix | 1.0000    |
| Choline bitartrate | 0.2500      |
| l-Cystine   | 0.1800           |
| t-Butylhydroquinone | 0.0008   |

Source: Reeves et al. (1993).
(PHA) and further incubated into the incubator CO₂ at 37 °C for 2 h and each well were 200 μL. The final concentration of PHA was reached to 5 μg/mL, and then 10 μL/well were added and each treatment was done using three replicates. Afterwards, the plates were incubated in a humid atmosphere with the 5% CO₂ (Biobase) at 37 °C for 72 h. Briefly, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added 30 μL/well (concentration was 5 mg/mL) into each well and then incubated for 4 h. Afterwards, the supernatant was removed and placed to the Eppendorf 1.5 mL (to measure IL-6 and TNF-α levels), and the reaction was stopped with dimethyl sulfoxide (DMSO) 100 μL for each well. Afterwards, the plate was read at an absorbance wavelength of 570 nm using the enzyme-linked immunoabsorbent assay (ELISA) (Spark Tecan Multimode Microplate Reader, Tecan Trading AG, Switzerland). The absorbance or optical density of each sample was calculated for its stimulation index (SI) by using an equation: SI = (sample absorbance/control absorbance). All analyses were performed in eight rats as the replicates.

2.2.6. TNF-α and IL-6 levels

The TNF-α and IL-6 levels were analyzed using a rat TNF-α kit (catalog number BMS622) and rat IL-6 kit (catalog number BMS625) (Invitrogen, Thermo Fisher Scientific Inc., Vienna, Austria) by following the manufacturer’s instruction. Briefly, microwell strips washed twice using a wash buffer of 400 μL/well. A sample diluent for TNF-α and assay buffer for IL-6 were each 100 μL added to all standard wells (duplicate), and then standard TNF-α and IL-6 levels were each 100 μL (concentration = 5,000.0 pg/mL for TNF-α and 4,000.0 pg/mL for IL-6) were added in A1 and A2 wells. The wells’ contents were mixed by repeated aspiration (standard 1 concentration, S1 = 2,500.0 pg/mL for TNF-α and S1 = 2,000.0 pg/mL for IL-6) and 100 μL were transferred to B1 and B2 wells (S2). Furthermore, this procedure was continued for about five times (S3, S4, S5, S6, and S7), creating two rows of rat TNF-α (standard dilutions of 2500.0, 1250.0, 625.0, 312.5, 156.3, 78.1, and 39.1 pg/mL with R² = 0.9558) and IL-6 (standard dilutions were 2500.0, 1250.0, 625.0, 312.5, 125.0, 62.5, and 31.3 pg/mL with R² = 0.9904), respectively. Afterwards, the contents (100 μL) from G1 and G2 (last microwells) were discarded. Sample diluent for TNF-α and assay buffer for IL-6 each 100 μL were added to blank wells (duplicate) and each 50 μL were added to sample wells. Furthermore, sample (supernatant) of 50 μL was added to sample wells (duplicate) and biotin conjugate of 50 μL/well. A sample (supernatant) of 50 μL was added to sample wells (duplicate) and biotin conjugate of 50 μL was added to all wells. Adhesive film was used to cover plate wells and incubated at 18–25 °C for 2 h on the microplate shaker. Afterwards, all wells were emptied and microwell strips were washed four times for TNF-α and six times for IL-6 using a wash buffer of 400 μL/well. Furthermore, diluted streptavidin HRP of 100 μL of was added to all wells and then incubated at 18–25 °C for 1 h on the microplate shaker. Afterwards, all wells were emptied, and microwell strips were washed four times for TNF-α and six times for IL-6 using a wash buffer of 400 μL/well. Afterwards, the substrate solution of TMB 100 μL was added to all wells and incubated at 18–25 °C for 10 min in a dark condition. Reaction of enzyme was stopped adding the stop solution 100 μL into each well. The results were read immediately using the microliter ELISA reader (Spark Tecan Multimode Microplate Reader, Tecan Trading AG, Switzerland) at wavelength of 450 nm. All analyses were performed in eight rats as the replicates.

2.3. Statistical analysis

Data of honey chemical composition were analyzed using the one-way variance analysis followed by the Tukey test. Body weight, lymphocytes proliferation, TNF-α, and IL-6 levels were analyzed using a covariate analysis, and the feed consumption and hematocrit levels were analyzed using Kruskal Wallis based on the completed randomized design followed by Tukey test using the help of SAS Studio version 3.8. Values of p < 0.05 and p < 0.01 were considered statistically significant. All analyses All data were presented as the means ± standard deviation.

3. Results

3.1. Chemical composition

3.1.1. Antioxidant activity

Antioxidant activity of honey from T. laeviceps was related to TPC, TFC, and vitamin-C. The results showed that the DPPH antioxidant of honey from T. laeviceps was influenced by geographical origins (p < 0.01). The honey DPPH antioxidant from Depok Sleman was 90.28% (at concentration 0.1 mM), which did not differ with honey from Bayan Lombok, which was 89.79%, but both were significantly higher than honey from Nglipar Gunungkidul, which was 61.43% (p < 0.01) (Fig. 1A).

3.1.2. Vitamin-C content

The present results showed that honey from T. laeviceps (p < 0.05) was influenced by geographical origins. Vitamin-C of honey from Depok Sleman was 7.45 mg/100 g honey, which did not differ with the honey from the Nglipar Gunungkidul which was 6.49 mg/100 g honey. However, both were lower than the vitamin-C content of honey from the Bayan Lombok, which was 13.58 mg/100 g honey (Fig. 1B).

3.1.3. Total phenolic content (TPC) and total flavonoid contents (TFC)

The results showed that honey TPC (p < 0.01) and TFC (p < 0.05) from T. laeviceps were influenced by geographical origins. The TPC of honey from Depok Sleman was 1.67% GAE/100 g which did not differ with the honey from Bayan Lombok which was 2.30% GAE/100 g, but significantly different from the lowest of 0.65% GAE/100 g for honey from Nglipar Gunungkidul (p < 0.01) (Fig. 1B). The TFC of honey from Depok Sleman was 0.75 mg QE/g, honey which did not differ with the honey from Bayan Lombok, which was 1.00 mg QE/g; however, both were higher than honey from Nglipar Gunungkidul, which was 0.28 mg QE/g (Fig. 1B).

3.2. Honey as an immunomodulator

3.2.1. Feed consumption and BW

The results showed that the feed consumption and BW for each treatment group was influenced by the different in status of both normal and malnourished rats (p < 0.01), but not on feed consumption at first week (Figs. 2 and 3). The BW of normal rats without treated honey ranged from 213 to 275 g/rat/week (increment mean was 90.1 g/rat), 216–281 g for normal rats treated with honey (increment mean was 91.0 g/rat), 208–230 g for malnourished rats without treated honey (increment mean was 41.0 g/rat), and 203–218 g/rat for malnourished rats treated with honey (increment mean was 35.6 g/rat) (Fig. 3). Generally, the BW increment and BW from normal rats were higher compared to malnourished rats because their feed consumption was higher than the malnourished rats (Fig. 2).

3.2.2. Hematocrit profiles

The present results showed that hematocrit profiles for all the rats prior treated with honey did not differ, but after the treatment with honey had significant differences HGB, MCHC, LYM, and RDW (p < 0.05), but not on WBC, RBC, HCT, MCV, MCH, PLT, NEUT, PDW, MPV, and P-LCR (Table 3 and 4). The lymphocytes percentage from the normal rats treated with honey was 72.2%, which

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did not differ with the malnourished rats treated with honey (71.6%), and normal rats without treated honey was 75.2%, while it was higher from the malnourished rats without treated (62.1%, $p < 0.05$) (Table 4).

### 3.2.3. Lymphocyte Proliferation

The lymphocytes proliferation from normal and malnourished rats in our study was described by the stimulation index. The results showed that the administration of Indonesian stingless bee honey (1.8 g/kg BW) on the normal and malnourished rat groups significantly increased the stimulation index of lymphocytes than in rats that were not treated with honey ($p < 0.01$). Stimulation index normal rats treated honey was 1.37, which did not differ with stimulation index normal rats not treated honey (1.06) and that of malnourished rats treated with honey (0.97). However, the stimulation index for normal rats treated with honey was higher than the malnourished rats which were not treated with honey (0.82) ($p < 0.01$) (Fig. 4A).

### 3.2.4. TNF-α and IL-6 levels

The results showed that the administration of Indonesian stingless bee honey (1.8 g/kg BW) in normal and malnourished rats' groups decreased the proinflammation of cytokines TNF-α and IL-6 levels from supernatant lymphocytes culture ($p < 0.01$). The TNF-α level from normal rats not treated with honey was 68.86 pg/mL, which was higher than normal rats treated with honey and malnourished rats not treated with honey, which were 55.63 pg/mL and 51.51 pg/mL, respectively. Furthermore, the low-
The honey vitamin-C from calli oxidation by several factors, such as heat, oxygen, and light, has negative a relationship with the antioxidant activity, because it quickly undergoes enzymatic and chemical oxidation by several factors, such as heat, oxygen, and light. Vitamin-C is not stably used as an indicator of the antioxidant effect. Vitamin-C is related to the honey vitamin-C, which is low in our study (Fig. 1B). However, in several studies, vitamin-C has an antioxidant activity in normal and malnourished rats treated with honey (Fig. 4B).

4. Discussion

4.1. Chemical composition

4.1.1. Vitamin C

The vitamin-C in honey is not used as the criteria to determine the honey quality, but its content has a beneficial effect on human health. Honey from Bayan Lombok was higher in vitamin-C because it has a sweet flavor mixed with sour than the honey from Depok Sleman (predominantly sweet flavor) and Nglijar Gunungkidul (sweet flavor mixed with bitterness). In addition, the honey pH from Bayan Lombok was lower, which did not differ with the honey pH from Depok Sleman, but the honey pH from Nglijar Gunungkidul was higher (Agussalim et al., 2021). This might have been influenced by different plant types as the nectar source for honey produce (Da Silva et al., 2016; Agussalim et al., 2021), bee species, and acidity of the honey (Da Silva et al., 2016).

The Pearson correlation showed that vitamin-C of honey from T. laeviceps has negative a relationship with the antioxidant activity (vitamin-C/DPPH with \( r = 0.482 \) at \( p > 0.05 \)) (Table 2). This finding is related to the honey vitamin-C, which is low in our study (Fig. 1B). However, in several studies, vitamin-C has an antioxidant effect. Vitamin-C is not stably used as an indicator of the antioxidant activity, because it quickly undergoes enzymatic and chemical oxidation by several factors, such as heat, oxygen, and light (Da Silva et al., 2016). The honey vitamin-C from T. laeviceps (Fig. 1B) was lower than that of Kelulut honey (79.5–87.19 mg/100 g), whereas plant types as a source of nectar include Acacia mangium, rambutan, longan, and star fruit (Ranneh et al., 2018). Plant types predominant from Depok Sleman are banana, rambutan, canarium, and tamarind, while in Bayan Lombok are coconut, mango, kapok, and cashew, and in Nglijar Gunungkidul are calliandra, Mexican creeper, banana, mango, and white albizia (Agussalim et al., 2021). The different plant types as the nectar source to produce honey is impacted on the different chemical composition of nectar which is influencing the honey chemical composition (Agus et al., 2019; Da Silva et al., 2016; Nordin et al., 2018), however in our study was not studied the nectar chemical composition.

4.1.2. Total phenolic content

Flavonoid and phenolic are the bioactive compounds in honey that may be used as functional food to improve the human health. The flavonoid compounds are divided into phenolic acid and flavonoid (flavanols, flavones, anthocyanins, flavonones, isoflavones, and chalcones) (Da Silva et al., 2016). The flavonoid compounds present in honey consists of vanillic acid, caffeic acid, quercetin, syringic acid, ferulic acid, kaempferol, pinobanksin, myricetin, p-coumaric acid, gallic acid, pinocembrin, ellagic acid, galangin, chlorogenic acid, 3- and 4-hydroxybenzoic acid, rosmarinic acid, and benzoic acid (Álvarez-Suárez et al., 2012; Da Silva et al., 2016; Scopanchova et al., 2017; Trautvetter et al., 2009).

The different honey TPC for each origin was influenced by the different plant types (Agus et al., 2019; Da Silva et al., 2016). The different geographical origins were impacted on the plant types which can grow, the soil nutrients are related to fertility, flowering from plants (time and duration of flowering, flowering cycle, and flowers number, and nectar number of plant flowers). All factors are contributing to the different chemical composition of nectar from plant flowers which have impacts on the chemical composition of honey including phenolic compounds. Furthermore, Agus et al. (2019) was reported that the different geographical origins from Sleman, Gunungkidul, and Klaten Indonesia are significant influencing of TPC, TFC, and antioxidant of T. laeviceps honey.

The plant types predominant for the source of nectar from Depok Sleman are banana, rambutan, canarium, and tamarind, while in Bayan Lombok are coconut, mango, kapok, and cashew and in Nglijar Gunungkidul are calliandra, Mexican creeper, banana, mango, and white albizia (Agussalim et al., 2021). Additionally, honey from stingless bee was stored in the honey pots made from propolis, so indirectly, the TFC from honey was also influenced by the phenolic content of propolis from the honey pots. Generally, honey with a darker color has higher TPC, TFC, and DPPH levels.

Table 2

The Pearson correlation among TPC, TFC, vitamin C, and DPPH antioxidant.

| Vitamin C  | TPC   | TFC   | DPPH |
|-----------|-------|-------|------|
| Vitamin C | 1     | 0.701*| 0.668*|
| TPC       |       | 0.915*| 0.853*|
| TFC       |       |       | 0.903*|

* Significant at \( p < 0.05 \) (2-tailed).
** Significant at \( p < 0.01 \) (2-tailed).
antioxidant activity than bright honey (Scepkanova et al., 2017). Honey from Depok Sleman and Bayan Lombok have a darker color than honey from NgliparGunungkidul, which has a bright color and have impact on the higher of honey TPC from Depok Sleman and Bayan Lombok.

The Pearson correlation showed that honey TPC from *T. laeviceps* has a positive relationship with antioxidant activity (TPC/DPPH with \( r = 0.903 \) and \( p < 0.01 \)) (Table 2). This finding in our study is similar to that of a previous study by Gül and Pehlivan (2018) in that the TPC of monofloral honey has a positive relationship with antioxidant activity, which was also observed by Rodica et al. (2021). The TPC of honey from *T. laeviceps* (Fig. 1B) was comparatively higher to those previously done studies for honey from the several stingless bee species (Biluca et al., 2016; da S. Sant’ana et al., 2020; Kek et al., 2014; Ranneh et al., 2018) and also, the difference was reported by Gül and Pehlivan (2018) for *Geniotrigona thoracica, H. itama,* and *Tetrigona binghami,* and Mokaya et al. (2022) that honey TFC from Afrotropical stingless bee (57–214 mg GAE/100 g). The difference in honey TPC was influenced by a different bee species (Da Silva et al., 2016; Gül and Pehlivan, 2018) and geographical origins (Agus et al., 2019; Da Silva et al., 2016).

4.1.3. Total flavonoid content

The difference in TFC of honey is influenced by different plant types as the nectar source (Agus et al., 2019; Da Silva et al., 2016). The phenolic acids are the secondary metabolites which are found fruits and vegetables are produced by the plants contributing to their color and flavor which is required for normal operation of naturally occurring plants (Da Silva et al., 2016; Yahfoufi et al., 2018). All plants have different secondary metabolites content including in the nectar of plant flowers which is collected by the foragers to produce honey which is impact on the different flavonoid content in honey (Da Silva et al., 2016). Additionally, stingless bee honey was stored in the honey pots made from propolis, so indirectly, the TFC of honey is also influenced by a flavonoid content of propolis from honey pots. Honey pots from stingless bees were made by resin as the main raw material, whereas the plants resin is known rich in phenolic compounds (phenolic acid and flavonoids) (Agus et al., 2019; Biluca et al., 2020; Da Silva et al., 2013, 2016). The TFC of honey is used as the floral marker to identify the plant types from different origins (Da Silva et al., 2016). The Pearson correlation showed that honey TFC from *T. laeviceps* has a positive relationship with an antioxidant activity (TFC/DPPH with \( r = 0.853 \) and \( p < 0.01 \)) (Table 2). This finding in our study is similar to those of previous studies (Da Silva et al., 2013; Rodica et al., 2021). The TFC of honey in our study differed from those of previous studies (da S. Sant’ana et al., 2020; Gül and Pehlivan, 2018; Ranneh et al., 2018) and Mokaya et al. (2022) that studied honey TFC from Afrotropical stingless bee (28.7–73.0 mg QE/100 g). The different honey TFC is affected by a different bee species (Da Silva et al., 2016; Gül and Pehlivan, 2018) and geographical origins (Agus et al., 2019; Da Silva et al., 2016).

4.1.4. Antioxidant activity

The high of honey DPPH antioxidant from Depok Sleman and Bayan Lombok is influenced by the TPC and TFC were higher than honey from Nglipar Gunungkidul (Fig. 1A), whereas the bioactive compounds are known as the stronger antioxidant source (Da Silva et al., 2016). The Pearson correlation showed that the DPPH antioxidant of the honey from *T. laeviceps* had positive relationship with honey TPC and TFC (DPPH/TFC with \( r = 0.903 \) and \( p < 0.01 \)) (Table 2). The honey DPPH antioxidant was affected by the honey TFC and TPC (Da Silva et al., 2016; Gül and Pehlivan, 2018; Rodica et al., 2021). Phenolic acid is a stronger antioxidant to eliminate and decrease the free radicals like reactive oxygen species (ROS) and inhibits the lipid oxidation process. The DPPH is measured based on their ability to donate hydrogen atom on the electrons of unpaired free radical, so it may inhibit the free radical formation (Da Silva et al., 2016; Rodica et al., 2021).

Generally, honey with a darker color has higher TPC and TFC than bright honey (Scepkanova et al., 2017), resulting in a higher antioxidant activity. Furthermore, Ferreira et al. (2009) reported that darker honey has a higher of antioxidant concentration of flavonoid, ascorbic acid, and β-carotene than those in bright honey. The honey DPPH antioxidant from Depok Sleman and Bayan Lombok were similar to the study by Ranneh et al. (2018), which had the highest antioxidant character of 90% at the concentration of 40 mg/mL for Kelulut honey, whereas honey from stingless bee *T. laeviceps* from Klaten, Indonesia was 91.2% at the concentration 0.1 mM (Agus et al., 2019). Additionally, the antioxidant activity was higher than that reported by Mokaya et al. (2022) from Afrotropical stingless bee ranging from 30.0% to 76.2%, which differed to that reported by Gül and Pehlivan (2018) for monofloral honey with the DPPH ranging from 12.01 to 65.52 mg/mL.

4.2. Honey as an immunomodulator

4.2.1. Feed consumption and BW

The trend that feed consumption in each treatment group every week was decreased showed that they take feed based on their daily requirement. Overall feed consumption in normal rat groups was higher than the malnourished rat groups because of the given feed and their status (normal and malnourished). Chepulis (2007) reported that Sprague Dawley rats treated with honey for 365 days had no effect on feed consumption. This finding was in line with our study in that the feed consumption among rats in the same group did not differ.

The increased and decreased BW of the rats are influenced by the feed amount given, and their consumption that had an impact on the amount of the calories and other nutrients that may be absorbed per BW unit (Hubert et al., 2000). Aliyu et al. (2012) explained that daily honey consumption has a positive effect on the increased BW and negative effect on losing BW. Furthermore, Kalantari et al. (2016) reported that the administration of 200 μL from 50% honey suspension significantly increased the BW of mice NMR group before they were infected by an acute *Toxoplasma gondii*. The control did not, and infection may, reduce mice BW.

4.2.2. Hematology profiles

The high lymphocytes percentage from normal and malnourished rats that were treated with the honey occurred because their nutrients had protein, amino acids, phenolic, and flavonoid compounds are, which known as the good sources of the antioxidant to eliminate ROS and inhibit lipid oxidation (Da Silva et al., 2016; Meo et al., 2017; Rodica et al., 2021; Scepkanova et al., 2017). Furthermore, honey is highly nutritional and has an effective and potential properties, such as an anti-inflammatory, antitumor activity, immunomodulatory, wound healing, and antibacterial (Meo et al., 2017; Ooi et al., 2021; Scepkanova et al., 2017).

Lymphocytes control the adaptive immunity in the both B and T cells that act on the humoral and cellular immune responses, respectively (Actor, 2011). The lymphocyte percentage in our study was in line with the reports by Aliyu et al. (2012) that administrated honey of 20% (v/v) which increased the lymphocytes level from Wistar rats blood. Furthermore, Chepulis (2007) reported that the administrated honey in Sprague Dawley rats significantly increased lymphocytes percentage (53%) than feed without sugar (29.5%) and sucrose (40%), however their lymphocytes percentage was lower than that in our study (Table 4).

In our study, the leucocytes level in normal and malnourished rats did not differ before and after the treatment with honey.
This study was in line with study by Ranneh et al. (2019) in that the leucocytes level from Sprague Dawley rats induced by LPS treated with the stingless bee honey and without treated honey were significantly higher than normal rats which were treated with honey. This conditions in both normal and malnourished rats with treated and not treated with honey may not be exposed by bacterial and viral infections; therefore, the leucocyte level was not changed for each group. However, the leucocyte level (Table 4) was lower in the study by Elbakry et al. (2015) in that the administration with the honey of 0.5 mL/kg BW in rats increased the leucocyte level than rat groups (treated propolis and olive oil).

Ranneh et al. (2019) reported that leucocytes, neutrophils, lymphocytes, monocytes, and lymphocytes on neutrophils ratio increase significantly in rat groups induced by LPS (treated stingless bee honey 4.6 g and 9.3 g/kg BW and not treated) than in normal rats (treated and not treated with honey). In addition, the administration of stingless bee honey did not show any significant changes on normal rat groups compared to control groups which did not infect bacteria. This condition also applies to all groups of normal and malnourished rats (treated and not treated with honey 1.8 g/kg BW) which had no effects on hematology profiles, except on HGB, LYM, MCHC, and RDW (Table 4), because may not be challenged by any bacteria or viruses, so it cannot stimulate organs that play a major role in cellular immune system to stimulate the production of leucocytes and lymphocytes. This condition is similar to that reported by Ranneh et al. (2019) on normal rats which was not induced by LPS which did not influence leucocytes, neutrophiles, lymphocytes, monocytes, and lymphocytes on neutrophil ratio than the control group.

4.2.3. Lymphocyte proliferation

Proliferation of lymphocytes on malnourished rats treated with honey was similar to the stimulation index from normal rats did not treat with honey. This condition shows that administration of honey plays as an immunomodulator on malnourished rats by increasing their lymphocyte proliferation. Higher lymphocyte proliferation is related to the nutrients such as protein, amino acids, phenolic, flavonoid, and vitamin-C present in honey, which is known as a good sources of antioxidants to eliminate the free radical ROS and to protect cells membranes from lipid oxidation (Afrin et al., 2018; Da Silva et al., 2016; Gasparrini et al., 2018; Meo et al., 2017; Rodica et al., 2021; Scepankova et al., 2017). Immunity is also regulated by the polyphenols by interfering with the regulation of immune cell, production of proinflammatory cytokines', and gene expression. Polyphenolic compounds inhibit mammalian target of rapamycin complex 1 (mTORC1), inhibitor of kappa kinase/c-Jun amino-terminal kinases (IKK/JNK), phosphatidylinoside-3-kinases/protein kinase B (PI3K/AKT), which is a protein complex that controls synthesis of protein, and JAK/STAT. They can suppress toll-like receptor (TLR) and genes’ expression of pro-inflammatory. They are modulating mitogen-activated protein Kinase (MAPK) and arachidonic acids pathways, and they can inactivate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Furthermore, their antioxidant activity and their ability to inhibit enzymes involved in the production of eicosanoids contribute as well to their anti-inflammation properties. They inhibit certain enzymes involved in ROS production like xanthine oxidase and NADPH oxidase (NOX) while they upregulate other endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase (Px) (Yahfoufi et al., 2018).

Honey from the Indonesian stingless bee rich in TPC was 1.67% GAE/100 g, TFC was 0.75 mg QE/g, and high in antioxidant activity DPPH was 90.28% (at concentration 0.1 mM) (Fig. 1). Therefore, it may protect the spleen from the attack of foreign objects which may enter the body successfully, so it is increasing their ability to proliferate. In addition, Indonesian stingless bee honey was found with 16 amino acids (Agussalim et al., 2021), whereas proline, phenylalanine, tyrosine, leucine, and alanine increased the lymphocytes proliferation (Li et al., 2007). Therefore, these bioactive compounds acts as an immunomodulator in normal and malnourished rats treated with honey (Biluca et al., 2020; Meo et al., 2017; Ooi et al., 2021). Stimulation index (Fig. 4A) was higher than reported by Abuharfeil et al. (1999) in that administrated of honey (Apis mellifera) increased the proliferation both B dan T cells of lymphocytes in vitro.

4.2.4. TNF-α and IL-6 levels

The lower level of TNF-α and IL-6 from lymphocytes culture supernatant in malnourished and normal rats treated with honey than normal and malnourished rats not treated with honey is related to lymphocyte proliferation in each group. The lymphocytes proliferation from malnourished and normal rats treated with honey were higher than normal and malnourished rats not treated with honey. It indicates that more lymphocyte proliferation decreases TNF-α and IL-6 production.

Table 3

| Parameters | Treatment Groups | T1 | T2 | T3 | T4 |
|------------|------------------|----|----|----|----|
| WBC (10^3/μL) | 6.3 ± 0.58 | 7.4 ± 2.11 | 6.8 ± 2.09 | 6.8 ± 1.32 |
| RBC (10^6/μL) | 7.1 ± 0.26 | 7.0 ± 0.38 | 7.0 ± 0.22 | 6.7 ± 0.34 |
| HGB (g/dL) | 14.0 ± 0.78 | 13.9 ± 0.79 | 14.2 ± 0.48 | 13.5 ± 0.51 |
| HCT (%) | 44.1 ± 1.78 | 44.2 ± 2.30 | 44.2 ± 1.20 | 42.5 ± 1.34 |
| MCV (fL) | 62.9 ± 1.35 | 63.4 ± 2.11 | 63.1 ± 1.05 | 63.6 ± 1.54 |
| MCH (pg) | 19.8 ± 0.83 | 20.0 ± 0.95 | 20.0 ± 0.55 | 20.2 ± 0.62 |
| MCHC (g/dL) | 31.6 ± 0.76 | 31.5 ± 0.96 | 32.0 ± 0.67 | 31.8 ± 0.67 |
| PLT (10^3/μL) | 903.3 ± 105.65 | 1034.3 ± 89.18 | 887.8 ± 250.22 | 953.5 ± 92.78 |
| LYM (%) | 74.4 ± 3.51 | 72.9 ± 9.70 | 69.8 ± 7.77 | 75.0 ± 4.78 |
| LYM 10^9/L | 25.7 ± 3.51 | 26.4 ± 9.83 | 30.2 ± 7.77 | 25.1 ± 4.78 |
| NEUT (%) | 4.7 ± 0.48 | 5.3 ± 1.41 | 4.8 ± 1.73 | 5.1 ± 1.26 |
| NEUT 10^9/L | 1.6 ± 0.27 | 2.1 ± 1.23 | 2.0 ± 0.61 | 1.7 ± 0.12 |
| RDW (L) | 29.9 ± 2.22 | 32.0 ± 1.56 | 31.1 ± 1.53 | 31.5 ± 2.58 |
| PDW (L) | 7.3 ± 0.56 | 7.4 ± 0.51 | 7.4 ± 0.53 | 7.6 ± 0.66 |
| MPV (L) | 6.4 ± 0.32 | 6.4 ± 0.26 | 6.5 ± 0.26 | 6.6 ± 0.34 |
| P-LCR (%) | 5.2 ± 1.34 | 5.5 ± 0.97 | 6.0 ± 1.53 | 6.0 ± 1.23 |

Abbreviations: T1 = normal rats + without honey (0–7 weeks), T2 = normal rats + with honey of 1.8 g/kg BW/day (0–7 weeks), T3 = malnourished rats + without honey (0–7 weeks), T4 = malnourished rats + with honey of 1.8 g/kg BW/day started from 2 weeks after malnourished condition (2–7 weeks).
The lowest production of both TNF-α and IL-6 levels in the malnourished rats treated with honey is related to several nutrient contents such as protein, amino acids, vitamin C, phenolic, and flavonoid present in honey, which are good sources of antioxidants to eliminate ROS and protect the cell membrane from lipid oxidation (Afrin et al., 2018; Da Silva et al., 2016; Gasparini et al., 2018; Meo et al., 2017; Rodica et al., 2021; Scepankova et al., 2017). Additionally, honey is highly nutritional and it has effective and potential properties such as an anti-inflammatory, antitumor activity, wound healing, antimutagenic, and antibacterial (Meo et al., 2017; Ooi et al., 2021; Scepankova et al., 2017). Honey from the Indonesian stingless bee is known to be rich in TPC was 1.67% GAE/100 g, TFC was 0.75 mg QE/g, and high in DPPH.

Biluca et al. (2020) explained the effect of antiinflammation of stingless bee honey on macrophages of RAW 264.7 in vitro that induced by LPS showed that it may suppress the production of IL-6, NO, TNF-α, IFN-γ, IL-10, and IL-12p70 from the supernatant of macrophage cell culture. In addition, it is also found from two honey from stingless bee increased the production of MCP-1 and IL-10 levels that has as an anti-inflammatory action, and they are linked with the phenolic content. Furthermore, Gasparini et al. (2018) studied the proinflammation of cytokines inflammatory mediator (iNOS), IL-6, IL-1β, and TNF-α increased after they were induced by the LPS, but after treatment with Manuka honey, it may suppress and inhibit the markers expression from proinflammation of cytokines IL-6, IL-1β, and TNF-α. The similar study also reported by Afrin et al. (2018) that Manuka honey may protect the macrophage cells line (i.e., RAW 264.7) which is induced by LPS (i.e., increasing the cells viability, promoting the proliferation of macrophage cells, reducing apoptosis, and increasing energetic metabolism).

Ooi et al. (2021) reported that the pre-treatment using the stingless bee honey for three days (1% v/v) in RAW 264.7 before they were induced by LPS did not induce the iNOS expression and production of NO and COX-2. After induction by LPS, stingless bee honey did not suppress COX-2 expression, but the expression of iNOS and production of NO were significantly inhibited by stingless bee honey (0.5% v/v). Hussein et al. (2012) studied that the administrated Gelam honey of 1 or 2 g/kg BW for 7 days may decrease the NO level, IL-6, TNF-α, and prostaglandin E2 (PGE2) from plasma rats. In addition, it is interesting that the administration of Gelam honey of 2 g/kg BW for 7 days significantly inhibits TNF-α and IL-6 production than the administration of anti-inflammatory drugs (i.e., NSAID 10 mg/kg BW).

Furthermore, Kassim et al. (2012) studied that the intravenous administration of honey on endotoxemia rats induced by LPS may inhibit the production of NO, TNF-α, IL-10, IL-1, and HMGB1, but it did not have any effect on IL-6 production. In addition, Gannabathula et al. (2017) studied 78 types of honey except Amber honey which stimulate the TNF-α release from monocytes. However, each honey has different bioactive components for immunostimulation such as proteins of arabinogalactan, apisimin, and apalumins, whose levels did not correlate with the activities of immunostimulation. Furthermore, Moreira et al. (2020) reported that polyphenols from honey extracts may decrease the levels of NO in RAW 264.7 macrophage induced by LPS. Furthermore, Liu et al. (2013) studied that Taiwan honey greatly inhibits the proinflammatory cytokine IL-8 secreted by the WiDr cells.

5. Conclusions

Honey from stingless bee T. laeviceps from Depok Sleman and Bayan Lombok have the best quality of honey than that from Ngli-para Gunungkidul based on the TPC, TFC, and antioxidant activity DPPH. In addition, T. laeviceps honey is rich in TCP, TFC, and they were found to be high in the antioxidant activity DPPH. Fresh honey or raw honey from Indonesian stingless bee T. laeviceps acts as an immunomodulator by increasing the percentage and lymphocyte proliferation and inhibits the production of proinflammatory cytokines (i.e., IL-6 and TNF-α) in malnourished rats. This finding indicates that honey from Indonesian stingless bee T. laeviceps may be used as an alternative for functional food therapy and helps in improving the management of the disease in humans, especially in malnourished children. Therefore, it is required to do an advanced study on Indonesian stingless bee honey to study to the impact on improved human health.

Table 4
Hematology profile groups of normal and malnourished rats after treated with Indonesian stingless bee honey.

| Parameters | Treatment Groups |
|------------|------------------|
|            | T1               | T2               | T3               | T4               |
| WBC(10³/µL) | 5.1 ± 0.88       | 7.1 ± 3.01       | 5.3 ± 1.73       | 4.9 ± 0.75       |
| RBC(10⁶/µL) | 8.1 ± 0.26       | 8.1 ± 0.28       | 8.3 ± 0.22       | 8.3 ± 0.42       |
| HGB (g/dL) | 15.2 ± 0.50⁶     | 15.5 ± 0.73      | 16.1 ± 0.51⁸     | 15.9 ± 0.58⁷     |
| HCT (%)    | 490 ± 1.49       | 49.9 ± 1.74      | 50.3 ± 1.32      | 50.4 ± 1.72      |
| MCV (FL)   | 61.0 ± 1.21      | 61.4 ± 1.28      | 60.4 ± 1.06      | 60.7 ± 1.49      |
| MCH (pg)   | 18.9 ± 0.44      | 19.1 ± 0.72      | 19.3 ± 0.63      | 19.3 ± 0.61      |
| MCHC (g/dL)| 31.1 ± 0.28⁵     | 31.0 ± 0.65⁵     | 31.9 ± 0.79⁵     | 31.6 ± 0.54⁴     |
| PLT(10⁹/µL)| 928.5 ± 109.19   | 921.9 ± 127.69   | 1043.9 ± 91.13   | 1005.4 ± 102.31  |
| LYM (%)    | 75.2 ± 7.17⁷     | 72.2 ± 5.51⁶     | 62.1 ± 13.12⁸    | 71.6 ± 8.35⁴     |
| NEUT (%)   | 23.8 ± 7.60      | 25.4 ± 4.36      | 33.2 ± 11.43     | 49.7 ± 7.12      |
| NEUT (10³/µL)| 3.8 ± 0.71     | 5.2 ± 2.39       | 3.4 ± 1.44       | 3.5 ± 0.68       |
| MCV (%)    | 1.2 ± 0.45       | 1.7 ± 0.61       | 1.7 ± 0.67       | 1.2 ± 0.35       |
| RDW (FL)   | 29.6 ± 1.20      | 31.1 ± 1.08⁴     | 30.3 ± 0.91⁶     | 29.9 ± 1.10⁶     |
| PDW (FL)   | 8.0 ± 0.44       | 8.0 ± 0.43       | 7.8 ± 0.30       | 7.9 ± 0.48       |
| MPV (FL)   | 6.8 ± 0.22       | 6.8 ± 0.26       | 6.6 ± 0.20       | 6.7 ± 0.24       |
| P-LCR (%)  | 6.2 ± 1.30       | 6.1 ± 1.29       | 5.3 ± 1.06       | 5.5 ± 1.15       |

Abbreviations: T1 = normal rats + without honey (0–7 weeks), T2 = normal rats + with honey of 1.8 g/kg BW/day (0–7 weeks), T3 = malnourished rats + with honey of 1.8 g/kg BW/day started from 2 weeks after malnourished condition (2–7 weeks), T4 = malnourished rats + with honey of 1.8 g/kg BW/day started from 2 weeks after malnourished condition (2–7 weeks).

⁶⁻⁸ Superscripts with different letters in the same line indicates significant at p < 0.05.
Declaration of Competing 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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