Antioxidant activity and microbiological quality of bee bread collected from three different species honey bee

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Abstract. Honeybee can produce bee bread beside the most famous commercial products i.e. honey, propolis and pollen. This product is widely believed to have medicinal benefits. However, there are very few scientific data available on the honeybee’s bee bread to prove the claims. In this study, bee bread from different species honeybee, namely Apis mellifera, Apis cerana, and Trigona spp were evaluated as antioxidants agents and microbiological quality. Bee bread from Apis cerana exhibited the highest microbiological quality including total microorganism (5.58 ± 0.38 log CFU/g), total yeast (6.23 ± 0.13 log CFU/g), total LAB (3.68 ± 0.27 log CFU/g) pH (4.36 ± 0.10) and Aw (0.7200 ± 0.0008), followed by Apis mellifera and Trigona spp. Meanwhile, bee bread from Apis cerana showed the highest antioxidant activities as radical scavenger was 14.44±0.25 mg/ mL, total flavonoid was 0.73 ±0.05 mg QE/g and total phenolic was 4.01±0.68 mg GAE/g. These findings establish the potential of bee bread from Apis cerana as antioxidants agent and microbiological quality, promising natural food supplements and natural preservatives.

1. Introduction

Bee bread is a product of the hive obtained from pollen collected by bees, to which they added honey and digestive enzymes and subsequently stored in the combs, lead a lactic fermentation [1]. An anaerobic lactic fermentation process give differs from pollen by lower pH (3.8 – 4.3). It contains less proteins and fats, but more carbohydrates and lactic acid. Bee bread has a better bioavailability because the walls of pollen, which cannot be destructed by gastrointestinal liquids, have been partly destructed by fermentation and the functionally and energetically rich content of pollen can be assimilated and used easier [2].

Bee species is one of numerous factors that can greatly affect the bioactivities of bee product, besides chemical composition, geographical zones, type of source plant, season and harvesting time as well as extraction methods [3]. In spite of this variability, primarily all samples have antioxidant and antimicrobial properties, since this is the function of bee bread in beehives. Many studies revealed that bee bread possesses some biological roles, including antioxidant and antimicrobial activity [4, 5].
because genotypic differences among subspecies to acquire nutrients from the pollen they collect and store and this could affect colony growth and survival [6].

Previous studies on the composition and nutritive value of bee bread were done with European honey bees (EHB) [7]. The other studies also compared from Africanized honey bees and European honey bees when feed on the same pollen [3]. However, there may be differences in the composition and consumption of bee bread made by bees of different sub species even when they feed on the same pollen. We addressed this question by presenting the same pollen to *Apis mellifera*, *Apis cerana* and *Trigona sp.* and then comparing antioxidant activity and microbiological quality of the bee bread produced by each sub species.

### 2. Materials and Method

#### 2.1. Materials

Bee bread was obtained from selected region of Songgoriti, East Java, Indonesia by patent technology developed by PT. Kembang Joyo Sriwijaya. Before the measurement samples were crushed to the powder using mortar and store at 4°C in refrigerator.

#### 2.2. Sample preparation

About 0.1 g of bee bread was extracted with 20 mL of 80% ethanol for 2 hours. After centrifugation at 4000 g for 10 min, the supernatant was used for measurement (antioxidant activity, polyphenols, flavonoids).

#### 2.3. Antioxidant activity

##### 2.3.1. DPPH Radical-scavenging activity

The scavenging activity (H/e- transferring ability) against 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical was evaluated according to the method of Brand-Williams [8] with minor modifications. In the presence of an antioxidant the purple color of DPPH is fading; the change of absorbency can be followed spectrophotometrically. The solution of 6.5 x 10⁻⁵ mol/L DPPH in methanol was prepared daily before measurement on a UV/vis Spectrophotometer. Two mL of DPPH solution were mixed with 50 µL of honey or bee bread phenolic extract solution in methanol (10 mg/mL) in the 1 cm path length disposable microcuvette. The final concentration of extract was 0.244 mg/mL. The absorbency of the remaining DPPH was determined after 16 min. at 515 nm. Blank sample contained the same amount of methanol and DPPH.

The measurements were performed in triplicate. The radical scavenging activity was calculated by the formula [8]

\[
I = [(A_B - A_A)/A_B] \times 100;
\]

Where:

- \(I\) = DPPH inhibition, %;
- \(A_B\) = absorption of a blank sample (t = 0 min);
- \(A_A\) = absorption of a tested honey or bee bread extract solution at the end of the reaction (t = 16 min).

##### 2.3.2. Total polyphenol content

Total polyphenol content of potato extracts was measured by the method using Folin-Ciocalteu reagent [9]. 0.1 mL of each sample extract was mixed with 0.1 mL of the Folin-Ciocalteu reagent, 1 mL of 20% (w/v) sodium carbonate and 8.8 mL of distilled water. After 30 min. in darkness the absorbance at 700 nm was measured using the spectrophotometer. Gallic acid was used as the standard and the results were expressed in mg/g gallic acid equivalents.
2.3.3. Total flavonoid content
Total flavonoids were determined using the modified method of Willet [10]. 0.5 mL of sample extract was mixed with 0.1 mL of 10% (w/v) ethanolic solution of aluminium chloride, 0.1 ml of 1 M sodium acetate and 4.3 mL of distilled water. After 30 min. in darkness the absorbance at 415 nm was measured using the spectrophotometer. Quercetin was used as the standard and the results were expressed in μg·g⁻¹ quercetin equivalents.

2.4. Microbiological analysis
Standard Plate Count (SPC) method used to determine the number of microorganism (log CFU/g) of bee bread from *Apis mellifera*, *Apis cerana* and *Trigona sp.* within hive in natural condition played by bees. The samples were vortexed to suspend bee bread grains in solution and 5 grams of this mixture was placed in 45 mL peptone (10⁻¹). This subsample of bee bread was vortexed on high for 5 minutes. Then diluted in 4 test tubes containing 9 mL peptone (10⁻², 10⁻³, 10⁻⁴, 10⁻⁵).

Plate Count Agar (PCA) is nonselective growth medium commonly used in food microbiology to calculate total or viable heterogeneous microorganisms growth. TPC used sample from dilution 10⁻³, 10⁻⁴, 10⁻⁵ were planted in PCA medium. Colony-forming units were counted after 24 h of incubation at 37°C. Potato Dextrose Agar (PDA) is selective growth medium for yeast (fungi) and de Man Ragosa Sharp agar (MRS) media is selective for lactic acid bacteria. Analysis of total yeast used subsample from 10⁻³, 10⁻⁴, 10⁻⁵ dilution were planted in PDA medium. Colony-forming units were counted after 24 h of incubation at 25°C. Analysis of total LAB (Lactic Acid Bacteria) used subsample from 10⁻², 10⁻³, 10⁻⁴ dilution were planted in PDA medium. Colony-forming units were counted after 48 h of incubation at 37°C. In reporting the absolute number of CFU followed the standard food safety methodology counting only those plates with >25 and <250 colony [11].

2.5. Statistical analysis
Each experiment was repeated four times and the obtained results were expressed as mean ± SD. Statistical data processing (one-way ANOVA; p<0.05) was performed by using Microsoft Office Excel 2013 (Microsoft Corporation, Redmond, WA, USA) on each of the antioxidant and microbiological quality to disclose possible differences among the samples. If there were significant differences the analysis was continued using Duncan Multiple Range Test (DMRT).

3. Results and Discussion
3.1. Antioxidant activity
Bee bread from three species honeybee samples were tested in this study in order to assess their antioxidant activity. The results obtained shows that all tested samples were antioxidatively active varied in a wide range (Table 1).

| Species honeybee | DPPH IC₅₀ (mg/mL) | Total polyphenol content (mg GAE/g) | Total flavonoids (mg QE/g) |
|------------------|-----------------|-------------------------------------|--------------------------|
| *Apis mellifera* | 15.84±0.62      | 5.49abc±0.65                       | 0.70a±0.02               |
| *Apis cerana*    | 5.07±0.54       | 6.50±1.41                          | 1.93b±0.11               |
| *Trigona spp.*   | 14.44±0.25      | 4.01ab±0.68                        | 0.73a±0.05               |

Note: Values are expressed as mean ± standard deviation of four replicate measurements

3.1.1. DPPH IC₅₀
Table 1 showed the results of scavenging activity of inhibition (mg/mL) was shown bee bread from *Apis cerana* 5.07±0.54 was the high antioxidant, followed by *Trigona spp.* (14.44±0.25) and *Apis mellifera*
(15.84±0.62). Molyneux [12] has been explained that IC$_{50}$ is the value of sample concentration to measure the ability of antioxidant activity of sample to reduce free radical by 50%, the lower of the IC$_{50}$ value is the higher to the antioxidant activity.

Ivanišová et al. [4] stated that the antioxidant activity of bee bread ranged from 14.62 to 15.78 mg TEAC/g. According to Markiewicz-Zukowska et al. [13], the antioxidant activity of bee bread taken in the Polish region ranged from 0.56 to 1.11 mmol/L with Randox test on Cintra 3030, and Zuluaga et al. [1] reported that Colombian bee bread has antioxidant activity between 46.1 to 76.3 µmol Trolox/g as measured using ABTS method.

3.1.2. Total polyphenol content
Polyphenols represent a significant group of substances with various bioactive properties including antioxidative properties. In the present work the polyphenol content of bee bread from different species honey bee were quantified using the Folin–Ciocaltau method. The polyphenol of them ranged from 4.01±0.68 mg GAE/g (Trigona spp.) to 6.50±1.41 mg GAE/g (Apis cerana) showed in Table 1.

The content of polyphenols in all species honeybee obtained is in line with the reported by Zuluaga et al. [1], total phenolic of bee bread in Colombia ranged from 2.1 to 13.7 mg GAE/g. These values were lower than several authors, it was reported by Ivanišová et al. [4], the phenolic content ranged between 12.36 to 25.4 mg GAE/g. The higher value was reported by Markiewicz-Zukowska et al. [13], the phenolic content of bee bread of the Polish region ranges from 33.43-36.52 mg GAE/g. Oltica et al. [14] noted that bee bread composition varies widely, being a fermented mixture of floral pollen collected by bees. The major variable in bee bread is the species composition of the pollen, which can be affected by differences in catchment area or season. However, Baltrušaityte et al. [15] reported that there are only a few papers detailing the biochemical composition especially phenolic compounds of bee bread.

3.1.3. Total flavonoids content
Flavonoids are an important group of polyphenol components. The flavonoids of bee bread from different species honey bee were quantified using the aluminium chloride reagent. Table 1 showed the results of total flavonoids content (mg/mL) was shown bee bread from Apis crana (1.93±0.11) was the total flavonoids content, followed by Trigona spp. (0.73±0.05) and Apis mellifera (0.70±0/02).

According to the research by Ivanišová et al. [4], the flavonoid content in bee bread ranged from 0.01356 to 0.01824 mg QE/g lower than present study. However, Zuluaga et al. [1] reported that the total flavonoid of bee bread in Colombia ranged from 1.9 to 4.5 mg QE/g. Sobral et al. [16] reported that the flavonoid content of bee bread in Polandia ranged from 2.5-100 µg/mL. Tavdidishvili et al. [17] also reported that the flavonoid content of bee bread in West Georgia ranged from 6.17-5.03 g/kg using HPLC method. According to Degrandi-Hoffman et al. [3], flavonoid is a secondary component that important in bee bread which can make display visually like colour (pigmentation) and taste (astringency and bitterness).

3.2. Microbiological quality
Microbiology quality is important to find the good quality of bee bread from three species honeybee, such as Apis mellifera, Apis cerana and Trigona sp. The analysis result of bee bread from different species honeybee on total microorganism, total yeast and lactic acid bacteriashowed at Table 2.
Table 2. Microbiological quality of bee bread from three different species honeybee

| Species honeybee | Total microorganism (log CFU/g) | Total yeast (log CFU/g) | Total LAB (log CFU/g) | pH | Aw |
|------------------|---------------------------------|------------------------|----------------------|----|----|
| *Apis mellifera*  | 7.76$^{a}$ ± 0.25               | 8.67$^{b}$ ± 0.03      | 5.17$^{a}$ ± 0.09    | 3.89$^{a}$ ± 0.13 | 0.7293$^{b}$ ± 0.0009 |
| *Apis cerana*     | 5.58$^{a}$ ± 0.38               | 6.23$^{b}$ ± 0.13      | 3.68$^{a}$ ± 0.27    | 4.36$^{a}$ ± 0.10 | 0.7200$^{b}$ ± 0.0008 |
| *Trigona spp.*    | 7.15$^{b}$ ± 0.58               | 5.25$^{a}$ ± 0.06      | 3.86$^{a}$ ± 0.84    | 3.87$^{a}$ ± 0.03 | 0.7990$^{b}$ ± 0.0036 |

Notes: Values are expressed as mean ± standard deviation of four replicate measurements

3.2.1. Total microorganism of bee bread
As described in Table 2, the mean total viable count in bee bread from different species honey bee ranged from fresh *Apis cerana* (5.58±0.38 log CFU/g) was significantly (p<0.01) lower than *Trigona sp.* (7.15±0.58 log CFU/g) and *Apis mellifera* (7.76±0.25 log CFU/g). In the present study, we found that bee bread from different honeybee samples had not perfect quality characteristics, especially from view point of microbial quality. This results was higher than standard microbiology in pollen (as the raw material of bee bread) (10$^3$ CFU/g). The contaminants could result from extrinsic factors such as wind, dew, rain, splatter, sprinkler irrigation splash and drip, can also contribute to pollen contamination. Pollen, bacterial, fungal, and several other contaminants all co-exist in the aeroplane [18], thus, bee pollen is more vulnerable to microbial contamination. The sources of feed (pollen) also have strong implications on pollen contamination. Shevtsova et al. [19] reported that pollen grains are highly sterile reproductive gametophytes from flowering plants, therefore any form of contamination is secondary.

The high of total microorganisms of bee bread was in line with Aw and pH value. The Aw is an important factor in food safety and stability [20, 21]. Higher Aw tends to promote the proliferation of microorganisms [22, 20] such as pathogenic bacteria grown at aw>0.85, whereas fungi grown well at aw>0.6 [21]. The Aw values seen in our study were ranged from 0.7200±0.0008 (*Apis cerana*) to 0.7990±0.0036 (*Trigona spp.*), while pH ranged from 3.87±0.003 (<i>Trigona sp.</i>) to 4.36±0.10 (*Apis cerana*). These results were higher than research by Feas et al. [23] who obtained considerable microbial load in bee pollen at aw and pH range of 0.21-0.37 and 4.3-5.2, respectively. Although high Aw affects other aspects of food quality/stability, more aseptic techniques should be employed in the dehydration and handling of pollen since this might reduce the risk of human-induced contaminations. While Jay et al. [24] also stated that pH values suggestive of dynamic microbial activity, such as gram positive bacteria.

3.2.2. Total yeast in bee bread
The yeast colony count in bee bread from different honeybee varied from 5.25±0.06 log CFU/g (<i>Trigona sp.</i>) to 8.67±0.03 log CFU/g (<i>Apis mellifera</i>) with a mean of 6.72 log CFU/g. This results was higher than standard microbiology in pollen (maximum 5 x 10^4 CFU/g) [25]. The results of Petrovic et al. [26] on Serbian bee pollen samples showed presence of <i>Acremonium, Alternaria, Aspergillus, Cladosporium, Epicoccum, Fusarium, Mucor, Penicillium, and Rhizopus</i> ranging from 10$^3$ to 10$^5$ CFU/g which is lower to our results. Several factors affect the number of yeast are storage time and the handling after bee bread harvested. The number of isolates and species decreased during the storage process [27]. The microbiological symbiotic of bee bread will change at temperature ±4°C within one month [19].

3.2.3. Total LAB in bee bread
Lactic acid bacteria (LAB) is one type of bacteria, has a capability to produce lactic acid by homofermentative and heterofermentative. Table 2 showed that total LAB of bee bread from <i>Apis mellifera</i> was significantly different with bee bread from <i>Apis cerana</i> and <i>Trigona sp.</i> However, standard for total LAB in bee bread currently was unknown, therefore the determination quality of bee bread based on total microorganism in bee bread. The previous research from Lozo et al. [28] revealed that
microorganism involves in pollen, bee bread, bee larvae Osmia cornuta were identified strains microbe such as Firmicutes (almost 75%), LAB (23.7%), Bacillus sp. (13.04%) and Enterobacteriaceae (8.15%).

Bee bread contains a lot of carbohydrate with type fructose and glucose [29]. This sugar included a simple sugar (monosaccharides) which is easy to soluble. The amylase enzyme would break down the soluble carbohydrate to be utilized by bacteria. The LAB can ferment carbohydrate became lactic acid 100% by homofermentative and at least 50% by heterofermentative. While, heterofermentative LAB beside produces lactic acid also produces ethanol, acetate acid, and carbon dioxide. Besides carbohydrate content, the highest total LAB related to the pH value. The production lactic acid by LAB lead the acidity content in bee bread, the more lactic acid produced by LAB makes the pH lower. The enhancement lactic acid concentration always followed by the reduction pH value [30].

4. Conclusions

Bee bread from Apis cerana was the highest value of antioxidant activity, phenolic, flavonoid content and microbiological quality compared with bee bread produced from other species. The scavenging activity of inhibition bee bread of Apis cerana was 14.44±0.25 mg/ mL, phenolic content was 4.01±0.68 mg GAE/g and flavonoid content was 0.73±0.05 mg QE/g. Whereas microbiology quality of Apis cerana proved to give the best performance compared with the others.

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