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# *Heterotrigona Itama* Bee Bread Extracts: Effect of Solvent Polarity on Extraction Yield, Chemical Characteristics and Antioxidant Activity

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## *ABSTRACT*

The stingless bee bread formation in the beehives occurs via lactic acid fermentation of the pollen mixed with nectar and bee salivary enzymes. The lack of research and studies about the effect of different extraction solvents in retaining the chemicals and the antioxidant activity of stingless bee bread were noticed. Hence, the objectives of this study were to determine the chemical contents and DPPH scavenging activity of different extracts of *Heterotrigona itama* stingless bee bread. The bee bread sample was macerated with four different extraction solvents including 95% ethanol (95EE), 70% ethanol (70EE), dichloromethane (DE) and hexane (HE). The chemicals analyses were proximate analysis, resorcinol-sulfuric acid assay and fourier transform infrared (FTIR) spectroscopy, while antioxidant activity was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. As a result, the bee bread of *H. itama* was found to contain 46.88% carbohydrates, 24.72% moisture content, 21.10% crude proteins, 3.41% crude fats, 2.32% ash and 1.75% crude fibers. The highest yield of extract was recorded by 70EE (41.1%, w/w). Resorcinol-sulfuric acid assay revealed that 70EE contained more total carbohydrates (842.585 mg/mL) compared to 95EE (738.178 mg/mL). In addition, intense FTIR signals at 3100–3600 $cm^{-1}$ were found in 95EE and 70EE, indicating the presence of hydroxyl group, while strong 2800–3000 $cm^{-1}$ signals found in HE revealed the presence of aliphatic group, and an intense carbonyl group signal at 1550–1750 $cm^{-1}$ were detected in DE. Furthermore, 70EE also showed the strongest antioxidant activity compared to other extracts with $IC_{50}$ value of DPPH radicals scavenging was 0.5173 mg/mL. Thus, these findings would provide the information about chemical composition and antioxidant activity of bee bread, as well as help in promoting the bee bread as a healthy functional food.

**Keywords:** *Heterotrigona itama* stingless bee bread, solvent polarity, FTIR, proximate composition, DPPH free radical scavenging activity

## INTRODUCTION

Stingless bees (Meliponines) belong to the Apidae, a social bees genus under the superfamily Apoidea (Tuksitha et al., 2018). There are more than 50 genera and 600 species of stingless bee worldwide. Stingless bees are widely scattered in the Indo-Malay/Australasian region, covering China to Australia and from India to Solomon Island

(Rasmussen & Cameron, 2010). It has been implied that the great number of stingless bees in Southeast Asian regions including Thailand and Malaysia is because of the abundance of the Dipterocarpaceae, resin-secreted trees and humid tropical climate (Schwarz, 1937; Rasmussen & Cameron, 2007). *Heterotrigona itama* is a common stingless bee species and functions as an important pollinator for crops (Kek et al., 2014). *H. itama* belongs to Apidae family and has no sting (Roubik, 2006). In the recent years, the bee products consumption becomes a lifestyle due to the nutritional values and therapeutic properties (Othman et al., 2019). Stingless bee products include honey, propolis and bee bread. Bee bread which is naturally produced by stingless bee inside beehive, refers to the collected pollen added with nectar and bee salivary enzymes and then undergoes lactic acid fermentation (Wan-Ismail, 2016). Bee bread is rich in antioxidants (phenolic compounds, coenzyme Q10, and α-tocopherol), and other chemicals (free sugars, fatty acid, organic acids, and polyphenols) and nutrients (fats, proteins, vitamins, and minerals) (Zuluaga et al., 2015; Urcan et al., 2018). The chemical content in bee bread depends on the pollen plant sources, geographic origin, climatic condition, soil types, and beekeepers' activities (Feas et al., 2012).

In Malaysia, bee farming industry generates profits mainly from honey. For example, bee bread is still underutilized and not collected during the harvest by many beekeepers due to the partial destruction of the hive (Urcan et al., 2018). Nevertheless, bee bread has high potential to be explored for commercialization with an expected high return (Kelly et al., 2014). In recent years, bee bread has received much interest and has been used as a healthy dietary supplement. The price for bee bread in local could be up to 95 USD/kg. Even though Malaysia stingless bee keeping industry is progressively developing, studies on the by-product such as bee bread of Malaysian stingless bees are still limited (Wan-Ismail, 2016). Therefore, this study focused on the Northeast Peninsular Malaysia bee bread and the aims were to evaluate the proximate compositions, chemical characteristics and to determine the DPPH scavenging activity of different extracts of stingless bee bread. The findings could vary from the previous data involving other localities. The data could also help in promoting the commercialisation of the local bee bread.

## MATERIALS AND METHODS

### Sample collection and preparation

The bee bread of *Heterotrigona itama* was collected from the supplier Honeygold Enterprise, Tumpat, Kelantan. The dried stingless bee bread was packed in polypropylene bags and stored at 4 °C before being used for proximate analysis and extraction process. The bee bread was air dried overnight at room temperature before extraction.

### Proximate composition analysis

The dried bee bread was analysed for proximate composition including crude fibre, fat, moisture content, protein, and ash content according to AOAC Method (2011).

### Extraction and percentage of extract yield

Dried bee bread was successively extracted using solvents of different polarity, starting with hexane, dichloromethane, 95% ethanol, and lastly 70% ethanol. The samples were extracted according to previous method by Markiewicz-Zukowska et al. (2013) with some modifications. For every 20 g of sample, 200 mL of solvent was used for extraction using Soxhlet apparatus for 2 hours. Then, the extract was filtered through a Whatmann No. 1 filter paper. The bee bread residue was extracted for another two times with the same solvent for another 30 minutes (min). Then, the residue and similar procedures were used for the next extraction using different solvents. Filtrates of the similar solvents were pooled in a flask. Dichloromethane and hexane filtrates were evaporated using ae rotary evaporator to obtain concentrated crude extracts. 70% ethanol extract was dried using freeze dryer to remove excess water, while for 95% ethanol extracts, nitrogen gas was used to remove

excess ethanol. All crude extracts were stored at 4 ºC until further analysis. The weight of each crude extract was recorded. The total extraction yield was calculated and expressed as percentage by using Eqn. 1.

$$\% \text{ Yield} = \left[\frac{W1}{W2}\right] \times 100 \quad \textbf{Eqn. 1}$$

Where; W1 = Weight of extract after evaporation of solvent; W2 = Weight of dried raw material

**Preparation of stock solutions and working solutions**

The stock solutions (10 mg/mL) were prepared by diluting the extracts with methanol/dichloromethane mixture (1:10). The stock solutions were vortexed and sonicated to thoroughly dissolve the extract. They were stored at 4 °C before used. Then, the solutions were diluted to obtain a series of working solutions at concentrations of 1000.0, 500.0, 250.0, 125.0, 62.5, 31.3, 15.6, and 7.8 µg/mL.

**Fourier transform infrared (FTIR) spectroscopy**

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) was used to determine the characteristics of chemical functional groups present in the bee bread extracts. The result of infrared spectrum revealed absorption peaks which represent chemical fingerprints that correspond to the frequencies of vibrations between the bonds and the atoms that make up the compound. Spectra of extracts were generated using ATR-FTIR Shimadzu Prestige-21 Spectrophotometer (Shimadzu, Nakagyo-ku, Kyoto, Japan) connected to a DTGS KBr detector and a Golden Gate Single Reflection Diamond ATR accessory (incident angle of 45°). A small quantity of stingless bee bread crude extract was used to cover ATR diamond plate. The pressure clump was swung assembly and screwed down between the opposing base and the tip till the sample was firmly trap. Three spectra were recorded for each sample at mid infrared region (4000–400 $cm^{-1}$) by using 16 scans and 4 $cm^{-1}$ resolution. After measurement, the sample was removed, and the plate was cleaned using nonabrasive tissue (Rashid et al., 2020).

**Resorcinol-sulfuric acid assay**

The total carbohydrate content assay was modified from high to microscale in a 96-well plate. 20 µL of sample (1 mg/mL) was mixed with 20 µL of resorcinol (6 mg/mL in water) solution and 100 µL concentrated $H_2SO_4$ and heated at 90°C for 30 min in an oven. The mixture was subsequently cooled at room temperature for 30 min, under dark condition. The absorbance was read after 5 sec shaking at 490 nm as reference using microplate reader (Multiskan GO, Thermo Scientific, Vantaa, Finland). Anhydrous D-glucose, L-mannose and D-galactose (2-fold dilution, concentration range; 500 to 3.9 µg/mL in water) were used for calibration and standard linear range was determined.

**2,2-Diphenyl-1-Picrylhydrazyl (DPPH) scavenging assay**

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was conducted according to Rashid et al. (2020). The assay was performed in 96-well plate. Quercetin was used as positive control. Aliquots of 50 µL of samples and working solutions (concentration: 1000 - 7.8 µg/mL) were placed in each well. 100 µL of DPPH methanolic solution (0.004% w/v) was mixed with the aliquots and incubated for 30 min (room temperature, dark condition). DPPH inhibition was read at 517 nm using a microplate reader (Multiskan GO, Thermo Scientific, Vantaa, Finland). The percentage of DPPH radical scavenging was calculated using Eqn. 2.

$$\% \text{ Inhibition} = \left[\frac{A0-(A1-A2)}{A0}\right] \times 100 \quad \textbf{Eqn. 2}$$

Where; A0 = the absorbance of the reagent blank; A1 = the absorbance of the test sample; A2 = the absorbance of the blank sample

### Statistical analysis

Values of data are mean ± standard deviation (SD) of triplicates experiments. Statistical analysis was conducted by using Statistical Package for the Social Science software (SPSS version 20.0.). Analysis of t-test was used to compare each test sample with standard or control, and one-way analysis of variance (ANOVA) was used to compare between samples. The data with *p*-value of less than 0.05 was considered as statistically significant.

## RESULTS AND DISCUSSION

### Proximate composition

In this study, five nutritional contents (moisture, ash, proteins, fats, crude fibers and carbohydrate) were measured. Based on data in Table 1, the local stingless bee bread consisted of 46.88% carbohydrates, 24.72% moisture, 21.10% crude proteins, 3.41% fats, 2.32% ash, and 1.75% crude fibers.

The carbohydrate content found in this study corresponded to previous studies. The previous reports recorded content in range of 10.85% to 59.94% (Feas et al., 2012; Mohammad et al., 2020) which mostly composed of fructose, glucose, sucrose, sucrose and mannitol (Belina-Aldemita et al., 2019). The carbohydrate main source was the nectar that was added during formation of the bee bread (Mohammad et al., 2020). Meanwhile, the present protein level was in agreement with previous study reporting 21.8% crude protein (Feas et al., 2012) in which the predominant amino acids were proline and serine (da Silva et al., 2014). The proteins is the main source for bee development, which differ across species and geographical locations. The protein percentage could decreased from fresh pollen to bee bread, while the amino acids were commonly unchanged in fresh pollen, bee pollen, and bee bread (Nicolson & Human, 2013). All the essential amino acids that cannot be synthesised by human body have been found in the bee bread (Kostić et al., 2015). In addition, the fats content result of the current study was also in agreement with Kieliszek et al. (2018) recording a range of 6–13% fats and the main fatty acids were palmitic, α-linolenic and linoleic acids (Belina-Aldemita et al., 2019). The diverse fatty acid profile provides nutrition to the bee and also benefits for human health. These lipids could originated from the outer pollen wall (pollenkitt) (Roulston & Cane, 2000). The ash content are contributed by nutrients such as minerals (Mohammad et al., 2020). The most abundant mineral reported in *H. itama* bread was potassium, followed by phosphorus and magnesium. However, toxic metals such as lead, arsenic, mercury, and cadmium were also found but within the permitted safety limit (Mohammad et al., 2020). Only a few *H. itama* samples exceeded the proposed heavy metal limit (Campos et al., 2008).

### Effect of solvent on the extraction yield

Extraction was an important step in isolating, purifying, and recovering bioactive compounds from natural sources. In this study, the stingless bee bread samples were extracted with different solvents which were 95% of ethanol, 70% of ethanol, dichloromethane (DCM) and hexane. The collected extracts were concentrated and weighed yielding respective dried crude extracts of 95EE, 70EE, DE and HE. Throughout sample preparation steps, the temperature used was not exceeding 40°C to retain the thermo unstable phytochemical in bee bread extracts.

Table 2 shows the decreasing percentages of the extracts yield in following order; 70EE, 95EE, DE and HE with 41.1%, 10.9%, 5.65% and 2.32%, respectively. Methanol, ethanol, acetone, propanol and ethyl acetate are the common solvents for extraction of fresh product (Michiels et al., 2012). The high yield of 70EE was suggested to be due to the addition of water to ethanol, which previously reported to improves extraction rate, in which the high-water content could resulted in an increase of simultaneous extraction of various hydrophilic compounds (Spigno et al., 2007).

**Table 1.** Proximate composition (%) of dried powdered *Heterotrigona itama* stingless bee bread.

| Parameters | Composition (%) |
|---|---|
| Carbohydrate | 24.72 ± 0.04 |
| Moisture Contents | 46.88 ± 0.48 |
| Crude Proteins | 21.10 ± 0.24 |
| Crude Fats | 3.41 ± 0.37 |
| Ash | 1.75 ± 0.25 |
| Crude Fibers | 2.32 ± 0.05 |

Notes: Values are mean ± standard deviation (SD), n=3.

**Table 2.** Percentage yield of different crude extracts from *Heterotrigona itama* stingless bee bread.

| Extracts | Code | Dried sample, ($W_1$) (g) | Extract, ($W_2$) (g) | Yield, ($W_2/W_1$) (%) |
|---|---|---|---|---|
| 95% Ethanol | 95EE | 20 | 2.19 | 10.9 |
| 70% Ethanol | 70EE | 20 | 8.25 | 41.1 |
| Dichloromethane | DE | 20 | 1.13 | 5.65 |
| Hexane | HE | 20 | 0.47 | 2.32 |

**Fourier transform infrared (FTIR) spectroscopy analysis**

The solvent used during the extraction process was important because of the type and chemicals composition recovered from the sample would be different. To identify the functional groups in each sample, FTIR was used to evaluate stingless bee bread of various extracts. Six important areas (A1-A6) with different chemicals fingerprint were detected in four different extracts of stingless bee bread (Fig. 1 and Table 3). The spectra shows that 95EE and 70EE extracts contained the broad peak in area 1 (3600-3100 $cm^{-1}$) which represent the characteristic of hydroxyl (O-H bond) functional group stretching vibration. The signals in this range belong to hydroxyl group in water molecules, alcohols, phenolics and carbohydrates (Silverstein et al., 2005). N-H bonds of proteins compounds which typically present as a moderate sharp peak signal could also overlapped under the broad O-H group signal. The hydroxyl group presence in 95EE and 70EE revealed the possible presence of phenolic compounds in the extracts. Meanwhile, area 2 (3000-2800 $cm^{-1}$) indicates the presence of stretching vibrations of the aliphatic C-H bonds of $-CH_3$ and $-CH_2$ groups in lipids and methoxy derivatives (Rashid et al., 2018). Sample HE facilitates the most intense and sharp peak at 2922.16 $cm^{-1}$ and 2852.72 $cm^{-1}$, while other extracts displayed weak signals. Area 3 (1750-1550 $cm^{-1}$) signals were assigned to the vibrations of C=O stretching of aldehydes, ketones and esters, carboxyl groups of free acids and C=C stretching of the aromatic compounds which could belong to the phenolics, as well as C-N stretching and N-H bending of the proteins amino acids (Silverstein et al., 2005). In this area, all spectra of the extracts contained chemical fingerprint with DE extract facilitated the sharpest peak at 1712.79 $cm^{-1}$. In addition, all the extracts contained the chemical fingerprints in area 4 (1550-1220 $cm^{-1}$). This region corresponds to the vibrations of C-O and C-C stretching of the phenyl groups, C-N stretching and N-N bending of amide, and O-H bending of alcoholic group (Md-Zin et al., 2019). Meanwhile, all of bee bread extracts except HE contained chemical fingerprints in area 5 (1150-950 $cm^{-1}$). The extracts of 95EE and 70EE had strong signals at 1039.63 $cm^{-1}$ and 1020.34 $cm^{-1}$, while DE facilitated a weak signal at 1043.49 $cm^{-1}$. This region indicates the presence of C-O stretching vibrations of the

carbohydrates glycosidic bonds (Rashid et al., 2020). Lastly, only DE and HE spectra appeared in area 6 (800-700 $cm^{-1}$) which assigned as the vibration of C=C and C-C compounds. The DE extract facilitated a strong and sharp peak at 736.81 $cm^{-1}$.

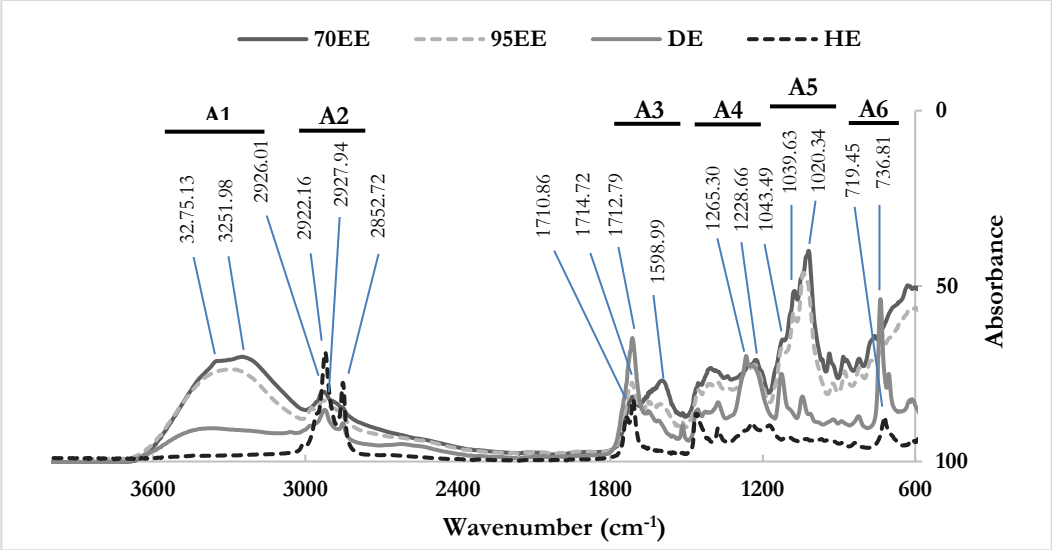


**Fig. 1.** Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy fingerprints of *Heterotrigona itama* stingless bee bread extracts. *1-A6 = Area 1 to area 6; 95EE = 95% ethanol extract; 70EE = 70% ethanol extract; DE = Dichloromethane extract; HE = Hexane extract

**Table 3.** Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra assignment of chemical functional groups of *Heterotrigona itama* stingless bee bread extracts.

| Fingerprints ($cm^{-1}$) | Biochemical Components |
|---|---|
| Area 1 (3600-3100 $cm^{-1}$) | O-H stretching (water molecules, alcohols, phenolics, carbohydrates) vibrations |
| Area 2 (3000-2800 $cm^{-1}$) | C-H stretching vibrations (aliphatics bonds in $-CH_3$ and $CH_2$ groups) |
| Area 3 (1750-1550 $cm^{-1}$) | C=O stretching (aldehydes, ketones, esters and carbonyl), C=C stretching (aromatic skeletal), C-N stretching, N-H bending (amino acids), vibrations |
| Area 4 (1550-1220 $cm^{-1}$) | C-O, C-C stretching (phenyl group), C-N stretching, O-H bending (alcoholic group), and N-N bending (amide) vibrations |
| Area 5 (1100-950 $cm^{-1}$) | C-O stretching vibrations (glucoside bonds) |
| Area 6 (800-700 $cm^{-1}$) | C=C and C-C bending vibrations |

## Resorcinol-sulfuric acid test

Table 4 shows the total carbohydrates content in 95EE and 70EE (5 mg/mL). The findings indicated the carbohydrate content of 738.178 mg/mL and 842.585 mg/mL in 95EE and 70EE, respectively. The presence of carbohydrates was also in conjunction with the detection of hydroxyl functional groups at 3600-3100 $cm^{-1}$ and carbohydrates fingerprints of at 1100-900 $cm^{-1}$ in FTIR spectra analysis. The previous study also reported that the most abundant sugar in bee bread of *H. itama* was glucose (10.270 to 12.397 g/100 g) as compared to fructose, maltose, and sucrose (Mohammad et al., 2020). Meanwhile, Wan-Omar et al. (2018) has identified that the main sugar in bee bread of *Trigona itama* in Malaysia was mannitol (33.05%).

**Table 4.** Total carbohydrate content of 95EE and 70EE stingless bee bread extracts.

| Extracts | Total carbohydrate content (mg/mL) |
|---|---|
| 95EE | 738.178 ± 186.455[a] |
| 70EE | 842.585 ± 201.392[a] |

Notes: Sample concentration is 5 mg/mL. Values are mean ± standard deviation (SD), n=4. The same superscripts indicate no significant different ($p < 0.05$). 95EE = 95% ethanol extract; 70EE = 70% ethanol extract.

## DPPH free radical scavenging activity

In this study, antioxidant activity of four different extracts of stingless bee bread was investigated using DPPH free radicals scavenging assay. The 95EE and 70EE of bee bread sample showed the strongest DPPH scavenging activity. The percentages of DPPH radical scavenging were found to be dose-dependent due to the increase percentages with the increased of extract concentration. The DPPH scavenging activity of ethanol extracts (10 mg/mL) were found to be in the following order: HE < DE < 95EE < 70EE with the value of 72.23 ± 1.53%, 76.66 ± 1.34%, 90.93 ± 0.69% and 92.59 ± 1.01%, respectively. $IC_{50}$ value for DPPH radical scavenging activity was defined as the amount of antioxidant require to decrease 50% of the initial absorbance of DPPH (Mishra et al., 2012). $IC_{50}$ value was calculated from the graph of percentage inhibition against the concentration of extract. 70EE showed the strongest antioxidant activity with $IC_{50}$ value of 0.5173 mg/mL as compared to other samples. Lower $IC_{50}$ value indicates a higher antioxidant capacity of a compound (Olugbami et al., 2014). Antiradical activity was also calculated in this study. It is known as reciprocal of $IC_{50}$ and was used to describe the ability of an antioxidant to fight free radicals (Mishra et al., 2012). The lower the $IC_{50}$ value indicates a stronger antiradical activity of a sample. The highest antiradical capacity was showed by ethanol/water extracts; 70EE (1.9439 ± 0.1696 mg/mL), followed by 95EE (1.165 ± 0.0562 mg/mL). The difference in antioxidant capacity could be attributed to the types of extraction solvent used, the maceration time, as well as solvent concentration (Kasparavičienė et al., 2013). A different solvent solubilises different phytochemical compounds which has different polarity, chemical and biological properties (Othman et al., 2019).

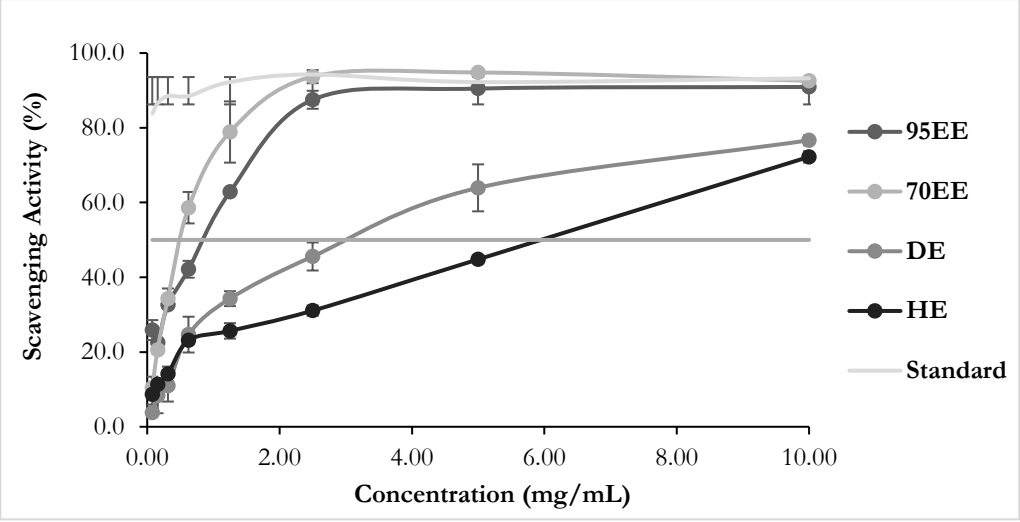


**Fig. 2.** DPPH free radical scavenging activity of different extracts of of *Heterotrigona itama* stingless bee bread extracts at various concentration. Values were given as mean ± SD from replicate determination (n=3).

**Table 5.** DPPH free radical scavenging activity of different extracts of of *Heterotrigona itama* stingless bee bread extracts.

| Extracts | $IC_{50}$ value (mg/mL) | Antiradical Capacity, ($1/IC_{50}$; mg/mL) |
|---|---|---|
| 95EE | $0.8597 \pm 0.0427^{a}$ | $1.1650 \pm 0.0562^{a}$ |
| 70EE | $0.5173 \pm 0.0456^{a}$ | $1.9439 \pm 0.1696^{b}$ |
| DE | $3.3822 \pm 0.475^{b}$ | $0.2999 \pm 0.0453^{c}$ |
| HE | $5.9434 \pm 0.1254^{c}$ | $0.1683 \pm 0.0035^{c}$ |

Notes: Values are mean ± standard deviation (SD), n=3. Different superscripts indicate significant different ($p<0.05$). The same superscripts indicate no significant different. 95EE = 95% ethanol extract; 70EE = 70% ethanol extract; DE = Dichloromethane extract; HE = Hexane extract

## CONCLUSION

In this study, the chemical analysis conducted on stingless bee bread extracts using ATR-FTIR revealed that specific functional groups including hydroxyl, aliphatic, carbonyl, and glucoside functional groups contained in the extracts. These compounds could act as bioactive agent that contribute to antioxidant activity as determined by DPPH scavenging assay. The antiradical activity of 70EE was found to be the strongest compared to other extracts. Determination of total carbohydrate content using resorcinol-sulfuric acid assay indicates that a higher carbohydrate content in 70EE than95EE. Based on this data, further studies such as isolation and purification can be carried out to identify and characterize each bioactive compound that present in the bee bread. This study is important in providing a profile of chemical compounds and antioxidant capacity of bee bread, which can be a base in choosing the right conditions for the isolation of antioxidants of stingless bee bread, and thus promoting the bee bread as functional foods.

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