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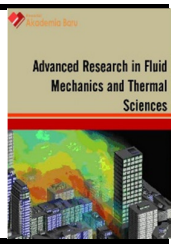

Antioxidant and Antimicrobial Potential of Stingless Bee (*Heterotrigona itama*) By-Products

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ABSTRACT

Bee bread is the pollen that has been stored in the cells of the honeycomb with various enzymes and honey, which undergoes lactic acid fermentation, while propolis is a resinous material collected by bees from buds of the plants and transform it using bee enzymes. The main objective of this research was to investigate the antioxidant and antimicrobial activity of the stingless bee bread and propolis extracted using 70% ethanol and n-hexane. The antioxidant activity of sample extracts were determined by 2,2,-di-phenyl-2-picryl-hydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and Ferric Reducing Antioxidant Power (FRAP) assays while for the antimicrobial activity, the sample extracts were analyzed using disc diffusion and broth macrodilution assay. For the DPPH and ABTS assays, the results showed that ethanolic extract of bee bread showed the highest percentage of free radical scavenging as compared to other samples with the values of 93.60 ± 0.03% and 97.95±0.01%, respectively. However, FRAP value for both hexanic extract samples, bee bread and propolis have the values of 0.85±0.0 mM FE/g and 0.87±0.00 mM FE/g respectively which were higher than ethanolic extract samples, 2.41±0.02 mM FE/g (bee bread) and 6.64±0.04 mM FE/g (propolis). For disc diffusion assay, the results showed that ethanolic extract of bee bread and propolis as well as hexanic extract of propolis were able to inhibit all tested bacteria in varying diameter of the inhibition zone. Broth macrodilution assay showed minimum inhibition zone (MIC) ranging from <6.67 to 33.33 µL/mL. As the conclusion, all of the samples in this study displayed antioxidant and antimicrobial effect but there are different in the degree of antioxidant and antimicrobial activity exhibited between each of the samples.

Keywords:
Stingless bee, bee bread, propolis, antioxidant activity, antimicrobial activity

1. Introduction

Stingless bees are a vast monophyletic class of highly eusocial bees commonly found in abundance in warm humid forests around the world. They belong to the family Apidae, subfamily

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Apinae and tribe Meliponini. *Heterotrigona itama* is the most abundant species found in Southern part of Malaysia [1]. Bee bread and propolis are among the stingless bee products that have high potential to be explored for their benefits and uses, which also have high commercial value in Malaysia [2, 3]. There are numerous factors that can greatly affect the bioactivities of the stingless bee products i.e., the bee species, chemical composition, geographical zones, type of source plant, season and harvesting time as well as extraction methods [4–7]. In spite of this variability, primarily all samples have antioxidant and antimicrobial properties, since this is the function of bee bread and propolis in beehives. Many studies revealed that bee bread and propolis possesses some biological roles, including antioxidant and antimicrobial activity [8–15]. Basically, their bioactivities were mainly as a result of substances belonging to the phenolic group, particularly flavonoids, which make bee bread and propolis a vital object of study for the most diverse food and pharmaceutical applications.9 However, there are very little information available in the literatures regarding the antioxidant and antimicrobial activity of the stingless bee products in Malaysia especially for the bee bread. Therefore, the present study was conducted to investigate the antioxidant and antimicrobial activity in the stingless bee bread and propolis extracts.

2. Materials and Methods

2.1 Samples Collection

Samples of the stingless bee products (bee bread and propolis) from *Heterotrigona itama* species were harvested at National Apiary Centre, Malaysian Department of Agriculture, Parit Botak, Johor, Malaysia in May, 2016. Harvesting process was done by removing the samples from the hive, separating between the bee bread and propolis and then grounding the samples into small pieces. The samples were stored at 4ºC.

2.2 Samples Extraction

Both samples were extracted using solvent extraction method, and the solvents used were hexane and 70% ethanol. The samples were extracted according to the method described previously with some modifications. 16 For every 60g of sample, 300 ml of solvent was used for the extraction which last for about 2 hours using Soxhlet apparatus. Thereafter, it was filtered through a Whatmann No. 1 filter paper, then the filtrate was evaporated using the Rotary Evaporator for about 3 to 4 hours and was left evaporated in water bath with 50ºC until the solvent totally evaporated. All final extracts were stored at 4ºC condition until used.

2.3 Determination of Free Radical Scavenging Activity

2.3.2 DPPH assay

The DPPH free radical of the stingless bee bread and propolis was measured according to the previous method with some modifications.17 DPPH solution was prepared by adding the 5.9mg of the DPPH into the 100ml methanol. Then, 150µL of sample extract was added into the 3.0ml of DPPH solution each in the test tube. It was then shaken vigorously and left in the dark at room temperature for 15 minutes. By using the spectrophotometer, the absorbance of the mixture was determined at 517nm. All of the tests were done triplicate. The Ascorbic Acid was used as standard for positive control while the distilled water as the negative control. The ability of extract to scavenge DPPH free radical was calculated using the following equation.

Scavenging activity (%) = $\left[\frac{A(-ve)-As}{A(-ve)-A(+ve)}\right] x\ 100$ (1)

where, As is the absorbance of the sample, A(-ve) and A(+ve) are the absorbance values of negative and positive controls, respectively.

2.3.3 ABTS assay

The ABTS scavenging assay was performed based on the previous experiment procedure with some modification [18]. The ABTS stock solution was prepared and left in the dark at room temperature for about 12 to 15 hours. This solution was diluted in the methanol until it reached about 0.700±0.03 unit of the absorbance reading at 734nm to form the ABTS solution. The scavenging activity of each sample was measured by mixing about 150μL of samples extract with 2.0ml of the ABTS solution. The absorbance of the samples at 734nm against the absolute ethanol (blank) was recorded after 20 minutes by using the spectrophotometer. The Ascorbic acid was run with the samples extracts and act as positive control. The percentage of the inhibition was calculated using the similar formula as used in the DPPH assay.

2.3.4 FRAP assay

The reducing capacity of the stingless bee bread and propolis sample were determined using FRAP Assay according to the procedure from the previous experiment with slight modifications [19] Sodium acetate (0.31 g) and glacial acetic acid (1.6 ml) were mixed with 100 ml distilled water to form the acetate buffer. The pH value of the acetate buffer was adjusted to 3.6 and stored at 4ºC condition. After that, in 50ºC of water bath, the TPTZ was prepared by dissolved 0.031g of TPTZ in 10 ml of hydrochloric acid. 0.054 g of FeCl3·6H2O was used to prepare the ferric chloride; by dissolved it in 10 ml distilled water. Prior to use in water bath at 37˚C, the working FRAP reagent was prepared by mixing 25 ml of 300mM acetate buffer at pH 3.6 with 2.5 ml of 10mM TPTZ solution and 2.5 ml 20Mm ferric chloride in a 10:1:1 ratio. Then, to determine the ferric reducing activity, 3ml of FRAP reagent was added to a cuvette and a blank reading was taken at absorbance of 593nm using spectrophotometer. A total of 150 μL of samples extract and 300μL of ethanol were added to the cuvette. After mixing the FRAP reagent with sample extract, a second reading was recorded again at the similar absorbance after 4 minutes. The comparison between the changes in absorbance after 4 minutes from initial blank reading with ferrous sulphate, standard curve was recorded. A standard curve of FRAP values of each standard versus its concentration was plotted and the final result was expressed as mM ferric iron reduction to ferrous iron in 150µg samples extract (mM/µg). The final result was presented as the concentration of antioxidant having a ferric reducing activity.

2.4 Determination of the Antimicrobial Activity
2.4.1 Disc diffusion assay

Disc diffusion method was conducted following the method from previous research with some modifications [20]. 0.5 McFarland standards (1 × 10^8 CFU/mL) overnight bacterial cultures (*B. subtilis, S. aureus, E. coli* and *Salmonella*) were prepared in peptone water. Ten µL of each suspension was spread on the solid Nutrient agar plates by using sterile L-shape glass rod. Six millimetres sterile disc were impregnated with 10µL of each sample extract and allowed to dry in biosafety cabinet at room temperature. Antibiotic, ampicilin was used as positive control while

distilled water as negative control. The dried discs were then placed onto the surface of the inoculated Nutrient agar plates. Then, the agar plates were incubated at 37˚C for 24 hours. The diameter of the inhibition zones was measured in millimetre (mm) using callipers.

2.4.2 Broth Dilution Method

The minimum inhibitory concentration (MIC) of the stingless bee bread and propolis extracts was determined based on the technique used previously [21]. The isolated colonies of the selected bacteria were grown in 10 ml sterile nutrient broth and adjusted to appropriate turbidity. Then, they were transferred into broth that contained different concentrations of samples extract by using a standard loop (10µL) and cultured for 24 hours. The test tube containing the mixture was shaken vigorously. The initial turbidity of culture was measured at wavelength 600nm using the UV-Vis spectrophotometric method which was standardized for the cell growth determination. The MIC of the samples was determined based on the cultures that were incubated aerobically at 37ºC by using the UV-Vis spectrophotometer method at the same wavelength. It was identified from the lowest concentration without visible growth or when the absorbance decline sharply or at zero absorbance.

All statistical analyses were carried out using Prism 5 software. To determine whether there were any differences between activities of samples, variance analysis (one-way ANOVA) was applied to the results. Values of $p \leq 0.05$ were considered as significant different. Values were expressed as mean of three replicates.

3. Results and Discussion

Table 1 showed the results of the scavenging activity of the samples at 150µL/ml concentration in DPPH assay. The highest percentage (%) of inhibition was shown by ethanolic extract of bee bread (EEB) (93.60±0.03), followed by hexane extract of bee bread (HEB) (83.81 ± 0.05). Ethanolic extract of propolis (EEP) and hexane extract of propolis (HEP) were less active with values of 80.07 ± 0.09 and 80.37 ± 0.40, respectively. The high percentage inhibition of bee bread for both ethanol and hexane extract is in an agreement with previous study which have mentioned that in the DPPH reaction system, the antioxidant activity of bee bread was between 90-94% [22]. Both samples showed higher percentage of inhibition when using 70% ethanol as compared to n-hexane. This is because ethanol has the capability to extract a wide range of the component in the samples [23,24]. Principle in DPPH assay explained when there is high in inhibition, means that the radicals was effectively trapped by high antioxidant potential, thereby the propagation was prevented as reported previously [25]. Other study reported that the synergistic effects of multiple components present in the whole extract also strongly contributed to the antioxidant potential of the bee's products extract [26].

Table 1
DPPH radical scavenging activity of both ethanol and n-hexane extract stingless bee bread and propolis

Sample in 150µL/ml	Percentage inhibition (%)
EEB	93.60 ± 0.03
EEP	80.07 ± 0.09
HEB	83.81 ± 0.05
HEP	80.37 ± 0.40

Values are expressed as mean ± standard deviation of three replicate measurements. Antioxidant activity of all samples is expressed as percentage of inhibition (%).

The effect of different solvent extraction used for both bee bread and propolis on free radical scavenging activity in the ABTS assay was presented in the Table 2. Ethanolic extract bee bread (EEB) has the highest percentage inhibition (97.95%) at 150µL/ml concentration of sample, followed by ethanolic extract propolis (EEP) (89.56 %), hexane extract bee bread, HEB (71.23 %) and hexane extract propolis, HEP (82.18%).

Ethanolic extract for both of the samples showed the higher percentage of inhibition (%) than the hexane extract samples in ABTS assay. This pattern of results was in line with previous studies, where they reported that antioxidant activity of ethanolic extract of propolis was higher than that of hexane extract (using ABTS assay) [27,28]. Both DPPH and ABTS assay actually has the same principle, thus, the results for both assays should be the same with similar trend or pattern. However, the results from the ABTS assay were significantly higher than that obtained from DPPH assay. This is in agreement with the previous study; where the following reasons were given, firstly, it might be due to the wavelength used; both ABTS and DPPH assay used different wavelength measurement, 734nm and 517nm respectively. This can cause the interfering of the measurement which may happen when the visible region with colored compound presented in the test samples which may have the spectra overlapped with the DPPH at 517nm [29]. Secondly, this situation might be related to the factor of the reaction mechanism of the ABTS and free radical scavengers in which it can affect the structural conformations of the antioxidants [30].

Table 2
ABTS scavenging activity of both ethanol and n-hexane extract stingless bee bread and propolis.

Sample in 150µL/ml	Percentage Inhibition (%)
EEB	97.95±0.01
EEP	89.56±0.01
HEB	71.23±0.01
HEP	82.18±0.01

Values are expressed as mean ± standard deviation of three replicate measurements. Antioxidant activity of all samples is expressed as percentage inhibition (%). Table 3 showed that the hexane extract of propolis (HEP) had the highest FRAP value of 6.64mM FE/g, followed by hexane extract of bee bread, HEB (2.41mM FE/g), ethanolic extract propolis, EEP (0.87mM FE/g) and the least was ethanolic extract of bee bread (EEB) with the value of 0.85mM FE/g. Propolis sample was found to have higher FRAP value when compared to the bee bread in both extracts of ethanol and n-hexane. These results were proven in the previous study that the propolis is one of the natural powerful antioxidative agents [26]. Unlike in DPPH assay, the results in FRAP assay showed that ethanolic extract sample has very low FRAP value as compared to hexane extract. Even though ethanol has the ability to extract various phenolic constituent from the sample which may contribute to the antioxidant level, [23,24] however, the previous study by Mărghitaş *et al.* [31] showed that low FRAP value is not in any way related to any of the polyphenolic content in the bee bread.

Values are expressed as mean ± standard deviation of three replicate measurements. Antioxidant activity of all samples is expressed as mM ferric ion reduced (mM FE/g).

The result of antimicrobial activity of both ethanolic and hexane of propolis and bee bread showed that the zones of inhibitions of gram positive bacteria (*B. cereus* and *S. aureus*) were bigger

than that of gram negative bacteria (*E. coli* and *Salmonella*), thus indicated that the gram positive bacteria were highly sensitive to the samples tested. However, the results were not statistically significant (*p>0.05).* When comparing based on the solvents used, the samples extracted with ethanol showed stronger antimicrobial activity as compared to those extracted with hexane. Gram positive bacteria were more sensitive to all extracts in this study, with ethanol extract showing higher activity than hexane extract. The findings obtained from this study agreed with data from the previous studies [22, 32]. Besides origin of the matrix and method of extraction, other factor such as osmotic effect, pH level as well as the presence of the hydrogen peroxide and phytochemicals in the samples extract might be responsible for the differences in size of inhibition zone [27, 33].

Table 3
FRAP reducing of both ethanol and n-hexane extract stingless bee bread and propolis.

Sample in 150µL/ml	mM ferric ion reduced (mM FE/g)
EEB	0.85±0.01
EEP	0.87±0.00
HEB	2.41±0.02
HEP	6.64±0.04

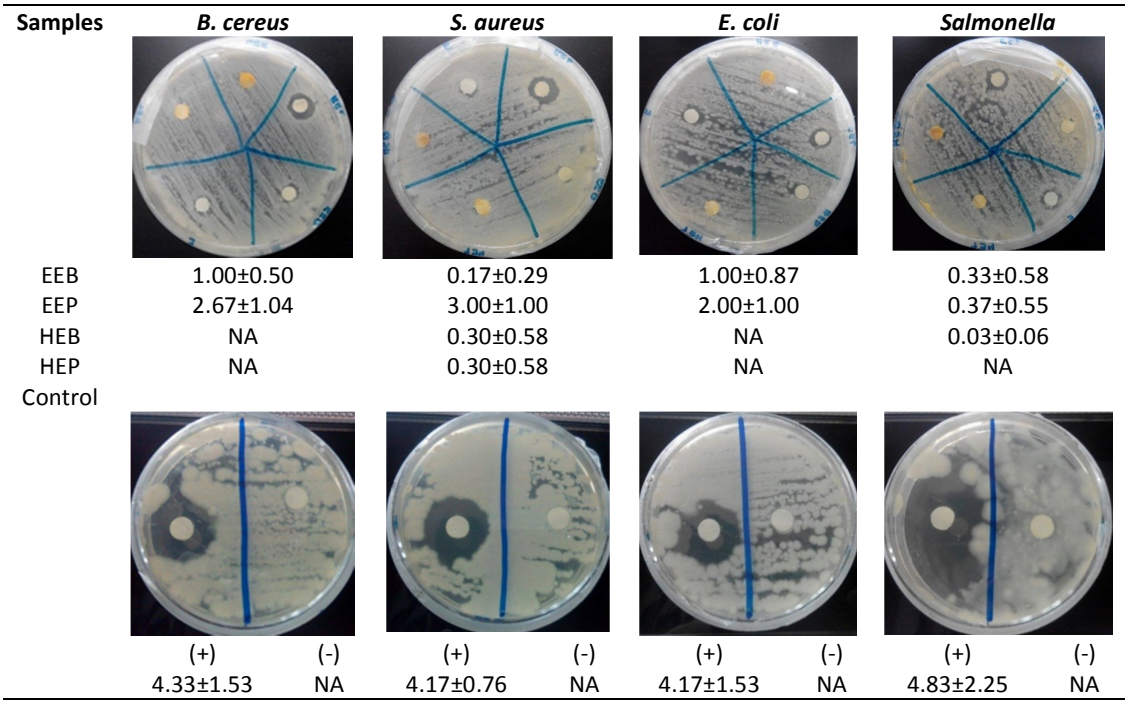

Fig. 4. Inhibition zone of disc diffusion method for both ethanol and n-hexane extract of samples

4. Conclusion

All the samples screened in this study exhibited both antioxidant and antibacterial activity. However, different samples extract exhibited different degree of antioxidant capacity. Likewise, the result for antimicrobial activity also showed that both hexane and ethanol extract of bee bread and

propolis exhibited various degrees of inhibitory effect against the *B. subtilis*, *S. aureus*, *E. coli* and *Salmonella*. Thus, this indicated that bee bread and propolis from stingless bee, *Heterotrigona itama* of Southern Malaysia have broad-spectrum of antimicrobial and antioxidant activity. The study shows that the utilization of honey products (beebread and propolis) in food preparations can be helpful in controlling some food related pathogens; however, there is need for further screening to establish the potential effects of these honey products.

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