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Short Communication

Antioxidant activity of bee pollen ethanolic extracts from Malaysian stingless bee measured using DPPH-HPLC assay

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Abstract

Bee pollen is considered as one of the functional foods due to its complex biochemical properties. Bee pollen which is collected from pollen grains from various botanical sources contains almost a complete nutrition such as carbohydrates, proteins, amino acids, vitamins and minerals. Its beneficial effect on health is thought to be due to the presence of phenolic compounds with its antioxidant activity. Antioxidant activities of ethanolic bee pollen extract (BPE) from three species of Malaysian stingless bee; *Trigona thoracica, Trigona itama* and *Trigona apicalis* in this study were measured using DPPH-HPLC method and gallic acid (GA) as a standard reference. The percentage of DPPH inhibition by *T. apicalis* BPE at 1 mg/mL showed the highest inhibition (39%, GA equivalent to 0.3 mg/mL) compared with *T. itama* (14.3%, GA equivalent to 0.1 mg/mL) and *T. thoracica* (6.7%, GA equivalent to 0.05 mg/mL). Our result was the first in reporting antioxidant activity of BPE measured using DPPH-HPLC method from three different species of Malaysian stingless bee.

Introduction

Bee pollen is a collection of pollen grains from various botanical sources, collected by the bees and mixed with nectar and secretion from the hypopharyngeal glands such as β-glycosidase enzymes (Carpes *et al*., 2009; Graikou *et al*., 2011). It is considered as one of the functional foods due to its complex chemical compositions constituted of carbohydrates, proteins, amino acids, vitamins and minerals (Carpes *et al*., 2009). Its beneficial effect on health is thought to be due to the presence of phenolic compounds with antioxidant activity (Carpes *et al*., 2009; Graikou *et al*., 2011).

Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent (Sies, 1997). This reaction can produce free radicals, which in turn can start chain reactions. Antioxidants prevent chain reactions by removing free radical intermediates and inhibit other oxidation reactions (Sies, 1997). Free radicals that are produced in cells include hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical (•OH) and superoxide anion (O_2-) (Valko *et al*., 2007). These free radicals can damage DNA, thus can cause genetic mutations and changes in the epigenetics such as gene-specific hypermethylation and genomic hypomethylation (Donkena *et al*., 2010). Furthermore, the situation could contribute to the high risk of cancer development (Kamiya, 2003; Donkena *et al.*, 2010).

A study on ethanolic extract of bee pollen from Brazilian stingless bee by Silva *et al.* (2009) showed high antioxidant capacity. However studies of bee pollen of stingless bee in Malaysia to our understanding are still not available. Three species of Malaysian stingless bee were selected in this study; *Trigona thoracica*, *Trigona itama* and *Trigona apicalis*, because they are the main domesticated species used by the industries involved in the commercialization of stingless bee products in Malaysia. This study was aimed at investigating the antioxidant capacity of bee pollen extracts (BPE) from three species of Malaysian stingless bee using DPPH-HPLC antioxidant assay. The samples were extracted using ethanol as a process of separation to obtain crude extracts (BPE) which contain phytochemicals that have similar polarity to the solvent (Chew *et al*., 2011).

Materials and Methods

Bee pollen sampling

The samples of bee-collected pollen from three species of stingless bee were obtained from Syamille Stingless Bee Farm, Kuala Kangsar, Perak, Malaysia. About 10 grams of fresh bee pollen samples were collected from each hive. The samples were then stored

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in 50 mL Falcon tube at +4°C fridge (LG700L) before further analysis. In addition, the samples of preserved bees were sent to the Centre for Insect Systematics, Universiti Kebangsaan Malaysia (UKM) for species identification by an entomologist. Specimen codes were according to the bee hives' number, which were 158 (*Trigona* (Tetragona) *thoracica* Smith/ *T. thoracica*), 175 (*Trigona* (Heterotrigona) *itama* Cockerell/ *T. itama*) and 182 (*Trigona* (Tetragona) *apicalis* var apicalis Smith/ *T. apicalis*).

Bee pollen extract (BPE)

The Crude BPE was prepared by extracting 10 grams of bee pollen in 100 mL of ethanol (1:10) (w:v) and shaken overnight (Memmert shaking incubator). The solutions were then subjected for drying using rotary evaporator (Eyela OSB-2100) before being freeze-dried for 2 days (Martin Christ Alpha freeze dryer). Yields of the extract were 1.3 grams for *T. thoracica,* 2.4 grams for *T. itama* and 1.6 grams for *T. apicalis.* Samples were then stored at +4°C until further analysis.

DPPH-HPLC antioxidant assay

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich) was solubilised in methanol. Gallic acid (GA) was used as the standard reference in order to generate a standard curve. GA was prepared in methanol at a concentration of 10 mM/mL and stored at +4°C. 10 mM/mL of DPPH and 2 mg/mL of BPE stock solution were freshly prepared on the day of analysis. All BPE samples were diluted in methanol. Different concentrations of GA (ranged between 0.0625 mM to 0.125 mM) or BPE were added to 200 µL of DPPH in 1 mL solution. The mixture was vortexed and left in the dark for 30 minutes at room temperature (+24°C).

HPLC analysis

DPPH-HPLC analysis was carried out according to Chandrasekar *et al*. (2006) with slight modification. Briefly, all samples were filtered through 0.2 µm nylon membrane filter. Methanol was used as blank/ control solution and included in every run. The effect of BPE on DPPH radical was determined by adding 500 µL of BPE to 200 µL of DPPH in the final volume of 1 mL (final concentration of BPE 1 mg/mL). All samples were run in duplicate. About 10 µL of the sample was injected into HPLC machine (Varian, Germany). Separation analyses were carried out using an Eclipse XDB-C18 4.6 mm x 250 mm, 5u C18 column (250 mm × 4 mm, 5 µM) (Agilent, Germany). Isocratic elution was carried out with methanol/water (80:20, v/v) at a flow rate of 1.3 mL/min. The DPPH peaks were monitored at 517 nm. Data analysis and processing were done using Galaxie Workstation software. The difference in the reduction of DPPH peak area (PA) between blank and BPE sample was used to determine the percentage of radical scavenging activity of the samples.

% DPPH inhibition (radical scavenging capacity) = $(Peak_{control} - Peak_{sample}) / (Peak_{control}) \times 100$.

Results of % DPPH inhibition of each stingless bee species were presented as mean + standard deviation. Student's t-test was used to compare significant difference between means of % DPPH inhibition.

Results and Discussion

This is the first study that compared DPPH antioxidant activity of three species of Malaysian stingless bee that are being commonly reared in Malaysia. The objective measurement reflected by the changes of DPPH peak areas in HPLC appeared to be a reliable and consistent method in determining the antioxidant activity of BPE. Few studies (Chandrasekar *et al*., 2006; Tang *et al*., 2008; Yan *et al*., 2014) have shown that HPLC method was specific for DPPH assay with an acceptable reproducibility. This method was successfully applied for the determination of antioxidant activity of polyherbal formulations (Chandrasekar *et al*., 2006). Our result showed a linear graph derived from the standard antioxidant (GA) at concentrations in the range between 0.0156-0.1250 mM, with good correlation coefficient, r^2=0.9924 (Figure 1).

The percentage of DPPH inhibition by *T. apicalis* crude BPE at 1 mg/mL showed the highest inhibition when compared with *T. thoracica* (39% + 0.7%, vs 6.7% + 0.3%, $p<0.05$), followed by T. itama (14.3% + 0.3%, $p<0.05$) (Table 1). This is the first result to be reported on DPPH-HPLC antioxidant activity comparing the three species of Malaysian stingless bee. In colorimetric analysis of DPPH for determination of antioxidant activity, Silva *et al.* (2009) had reported that the crude concentration of BPE which caused 50% inhibition of DPPH (IC_{50}) for Brazilian stingless bee, *Melipona rufiventris* was 0.1 mg/mL. Compared to the *T. apicalis* BPE, we only observed 39% of DPPH inhibition using 1 mg/mL. The difference in antioxidant activity could be due to specific pollen foraging activities and different diets of stingless bee itself (Nagamitsu and Inoue, 2002; Silva *et al*., 2009), and these factors may contribute to the different compounds found in BPE.

Table 1. Percentage of DPPH inhibition by BPE samples from three different species of Malaysian stingless bee and their equivalent gallic acid concentrations based on equation y = 127.73x + 0.9436

BPE sample at 1 mg/ml	% DPPH Inhibition	Standard deviation	GA equivalent (mg/mL) following the equation y = 127.73x + 0.9436
T. thoracica	6.7	0.3	0.05
T. itama	14.3	0.3	0.10
T. apicalis	39.0	0.7	0.30

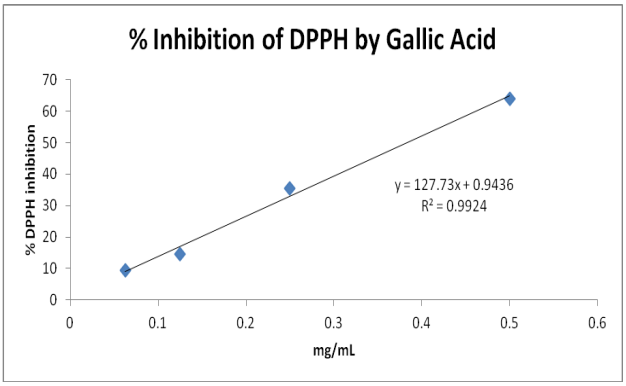

Figure 1. Standard curve for antioxidant activity using gallic acid. Percentage of DPPH inhibition by gallic acid was shown at concentration of 0.0625-0.5000 mg/mL

Conclusion

Different antioxidant activities exhibited by three species of Malaysian stingless bee with highest antioxidant activity were seen with ethanolic extract of bee pollen from *T. apicalis*, followed by *T. itama* and *T. thoracica*. These differences may be due to the different pollen foraging activities from each species.

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References

Carpes, S. T., Mourão, G. B. and Masson, M. L. 2009. Chemical composition and free radical scavenging activity of *Apis mellifera* bee pollen from Southern Brazil. Brazilian Journal of Food Technology 12(3): 220-229.

Chandrasekar, D., Madhusudhana, K., Ramakrishna, S. and Diwan, P. V. 2006. Determination of DPPH free radical scavenging activity by reversed-phase HPLC: a sensitive screening method for polyherbal formulations. Journal of Pharmaceutical and Biomedical Analysis 40: 460-464.

Chew, K. K., Ng, S. Y., Thoo, Y. Y., Khoo, M. Z., Wan Aida, W. M. and Ho, C. W. 2011. Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compunds and antioxidant capacity of *Centella asiatica* extracts. International Food Research Journal 18: 571-578.

Donkena, K. V., Young, C. Y. F. and Tindall, D. J. 2010. Oxidative stress and DNA methylation in prostate cancer. Obstetrics and Gynecology International 2010: 1-14.

Graikou, K., Kapeta, S., Aligiannis, N., Sotiroudis, G., Chondrogianni, N., Gonos, E. and Chinou, I. 2011. Chemical analysis of Greek pollen - antioxidant, antimicrobial and proteasome activation properties. Chemistry Central Journal 5(33): 1-9.

Kamiya, H. 2003. Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/ nitrogen species: approaches using synthetic oligonucleotides and nucleotides. Nucleic Acids Research 31(2): 517-531.

Nagamitsu, T. and Inoue, T. 2002. Foraging activity and pollen diets of subterranean stingless bee colonies in response to general flowering in Sarawak, Malaysia. Apidologie 33: 303-314.

Sies, H. 1997. Physiological society symposium: impaired endothelial and smooth muscle cell function in oxidative stress, Oxidative stress: oxidants and antioxidants. Experimental Physiology 82: 291-295.

Silva, T. M. S., Camara, C. A., Lins, A. C. S., Agra, M. D. F., Silva, E. M. S., Reis, I. T. and Freitas, B. M. 2009. Chemical composition, botanical evaluation and screening of radical scavenging activity of collected pollen by the stingless bees *Melipona rufiventris (Uruçu-amarela)*. Annals of the Brazilian Academy of Sciences 81(2): 173-178.

Tang, D., Li, H. J., Chen, J., Guo, C. W. and Li, P. 2008. Rapid and simple method for screening of natural antioxidants from Chinese herb Flos Lonicerae Japonicae by DPPH-HPLC-DAD-TOF/MS. Journal of Separation Science 31: 3519-3526.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M. and Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology 39: 44-84.

Yan, R., Cao, Y. and Yang, B. 2014. HPLC-DPPH Screening method for evaluation of antioxidant compounds extracted from Semen Oroxyli. Molecules 19: 4409-4417.