eProceedings Chemistry 4 (2019) 123-128

eISSN 2550-1453 http://eproceedings.chemistry.utm.my/

Phytochemicals Study and Biological Activities of *Anacardium Occidentale*

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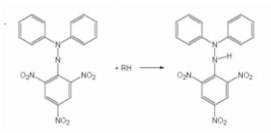
Article history:
Received 20 June 2019
Accepted 25 July 2019

GRAPHICAL ABSTRACT



ABSTRACT

Honey is one of the natural products that are famous with high content of antioxidant. Compounds that associate with antioxidant effect are flavonoids, flavonols, phenolic acid and catalase. This research aimed to determine phenolic compound by using gas chromatography-mass spectrometry (GC-MS) analysis and antioxidant activity by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay in 8 different types of stingless bee honey. A quantitative composition-activity relationship (QCAR) were construct in order to determine the relationship between phenolic compound and antioxidant properties of stingless bee honey. For determination of phenolic compound, samples were determined by using solid phase extraction and followed by GC-MS analysis. For DPPH assay, samples were dissolved in methanol and filtered. Honey extracts were then studied for their antioxidant properties by using DPPH radical scavenging assay. IC50 value are determined by plotting DPPH scavenging percentage of each sample against the concentration. IC50 for Tetragonula Sirindhornae's is the highest which is 108.4 while the lowest IC50 is honeydew honey which is 15.29. Analysis of honey by using GC-MS showed that phenolic content that present abundantly are phenol, hydrocinnamic acid, and benzoic acid. PLS regression methods were utilised, producing the good performance with r2calc 0.729. This is proved that model is reliable. Overall, the model produced is proved to be reliable and can correlate relationship between antioxidant activities and phenolic compounds that present in stingless bee honey.

Keywords: Stingless bee honey, 2,2-diphenyl-1-picrylhydrazyl,phenolic compound, antioxidants

1. INTRODUCTION

Honey has been serving people for a long time for nutrition and medicinal purposes. Honey is a natural sweetener and it is used as food flavouring besides consuming it for healthy purpose. Honey is produced by honeybees and was obtained from the nectar. Honey are composed of supersaturated sugar mainly fructose and glucose. Besides that, it is also contains other compounds such as amino acids, organic acids, vitamins, minerals, lipids, enzymes and phenolic compounds. However, their composition are different depending on types of plants that the bee consume nectar, and other external factors such as climate and environmental conditions [1].

Natural by-products that are formed by oxidation is known as free radicals. Free radicals can be defined as atoms or groups of atoms with and unpaired number of electron and can be formed in the presence of oxygen and were created in the presence of sunlight, environment factors, stress and processed food. Antioxidants are molecules that can inhibit the oxidation of other molecules by donating an electron to unpaired valence electron and for that reason immune system are boost and cell damage can be prevented. Vitamin E, Vitamin C, carotenoids and phenolic compounds are few examples of the antioxidants. Human body can produce highly complex antioxidant system which work together with cells and organs to protect body against radical damage [2].

Main phytochemicals in honey namely phenolic compounds are the one that provide benefits to human. It is proven that phenolic compounds are the main contributor to its antioxidant activity and their pharmacological effect is produced from antioxidant activity of phenolic compounds. Thus, it is significant to explore the relationship between antioxidant activity and phenolic compounds.

2. EXPERIMENTAL

Eight honey samples from stingless bee were obtained locally. Honeys were diluted and filtered by using 0.45 µm membrane filter to remove the possible contaminating microorganisms that present in honey. The concentration of phenolics in honey samples were extracted by using extraction procedure that is commonly used namely solid phase extraction. Initially, raw honey (10g) were weighed. Then it was mixed with deionised water (50 mL) that was adjusted to pH 2 with HCl. Upon mixed, the solution was stirred by using magnetic stirrer for 15 minutes. Next, it was filtered through cotton wool. The filtered solution was then passed through HLB oasis cartridge. Phenolic compounds were retained in the column while sugar and other polar compounds were eluted with aqueous solvent. The column was washed with 50 mL acidified water (pH 2 with HCl). Then, the phenolic fraction was then eluted with methanol (50 mL). The eluted solution was dried until the final volume left is 5 mL under reduced pressure by using rotary evaporator at 40 °C. After that, the

final solution was transferred into a vial and proceed with nitrogen drying. Each sample are carried out in duplicate. Lastly, the sample are analysed by using GC-MS.

For determination of antioxidant activity,firstly, raw honey (2g) sample was dissolved into methanol (10 mL) and filtered through Whatman No:1. Then, it was diluted into different concentrations ranging from 12.5-200 mg/ml. Diluted honey (100 µL) was added into 96-well microplates followed by methanol (100 µL) and DPPH (100 mM) respectively by using multichannel pipette. Microplates were incubated for 60 minutes at 25℃. The absorbance of the solution was measured at 517 nm. Positive control that were used are BHT and ascorbic acid. Each sample are carried out in triplicate. The free radical scavenging activity (RSA) for each sample was calculated as a percentage of DPPH discolouration by using equation below:

$$\% \text{ RSA} = [(A_{\text{BLANK DPPH}} - A_{\text{SAMPLE)}} / A_{\text{BLANK DPPH}}] \times 100$$

Then, the extract concentration providing 50% of radical scavenging activity (IC_{50}) was obtained by plotting the DPPH scavenging percentage of each sample against the concentration.

Quantitative composition-activity relationship was constructed using software SOLO (Eigenvector_Research_ Inc., 2010). PLS model was developed using multi-linear regression namely PLS. Composition of each phenolic compound was used as independent variable while for antioxidant activity was used as dependent variable. The model was developed to determine its reliability and robustness.

3. RESULTS AND DISCUSSION

3.1. General

8 stingless bee honey were extracted for further analysis. Stingless bee honey was analysed and discussed in terms of their antioxidant activities and phenolic compounds. Antioxidant activity was determined by using DPPH assay while for phenolic compounds, they were analysed by using GC-MS. Varying antioxidant activities and phenolic were expected for each honey sample due to different floral sources of honey. The methods of choice in this study were chosen to provide better study on their biological profiles such phenolic compounds and antioxidant activities QCAR model was constructed to show clearer relationship between antioxidant activity and phenolic compounds.

3.2. Antioxidant Activity in Stingless Bee Honey

The scavenging activity of stingless bee honey samples was measured by a stable nitrogen-based radical which composed of unpaired valence electron at one atom of nitrogen bridge. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a decolourization assay that was observed through reducing colour of DPPH which is from deep purple to pale yellow upon receiving a hydrogen which donated by free radical scavenging activity [3]. It measured the capacity of antioxidants to directly scavenge DPPH radicals by monitoring its absorbance at 517 nm using microplate reader [4]. When dissolving DPPH radical in solvent for instance methanol, dark purple colour solution is formed. Presence of hydrogen or electron donating compound, DPPH radical will be reduced to non-radical form which indicated by change in colour [5].

High activity of antioxidant may show high content of phenolic acids in stingless bee honey. Some of the previous study found that they are significant relationship that correlate between antioxidant capacity, phenolic content and honey's colour. Some of the previous study found that they are significant relationship that correlate between antioxidant capacity, phenolic content and honey's colour [6]. Few of the studies proposed the correlation between antioxidant capacity and honey colour [7,8,9]. This may be due to a reason phytochemicals precisely phenolic compounds present at high level in honey. Honey that are dark in colour revealed to have high number of phytochemicals hence consists higher antioxidant activity. However, the current study does not evaluate the relationship between antioxidant capacity, phenolic content and honey's colour. Graph of determination of the antioxidant activities of extracts of stingless bee honey using DPPH showed in Figure 1.

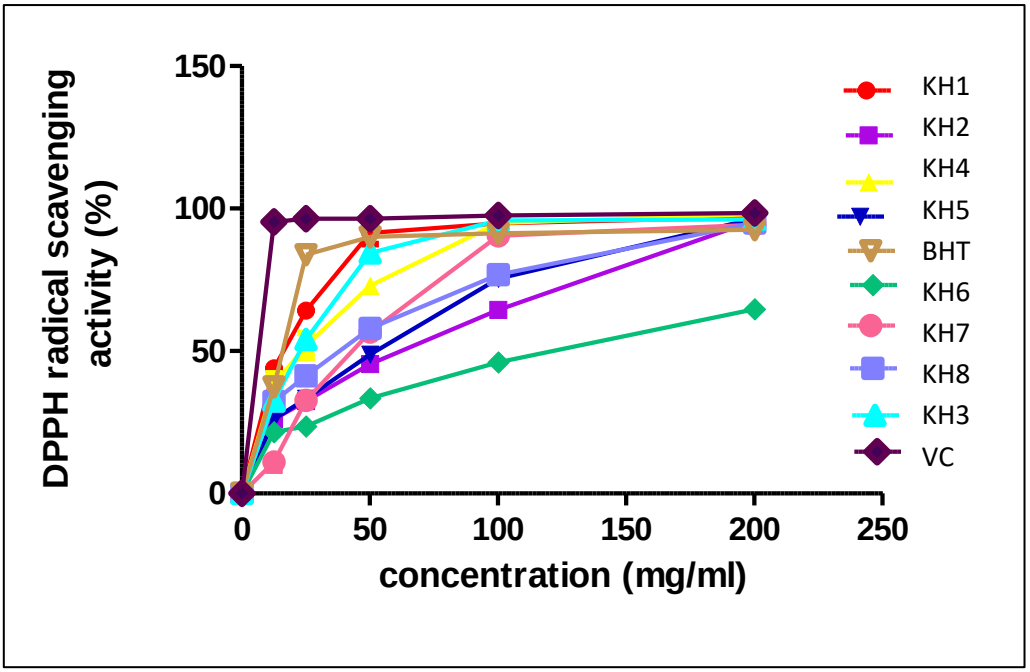


Figure 1. Determination of the antioxidant activities of extracts of stingless bee honey using DPPH

Absorbance of honey sample containing DPPH radical and honey sample without DPPH radical were compared. Result for DPPH assay was expressed by using IC_{50} parameter. IC_{50} can be defined as concentration needed in order to scavenge 50% of DPPH radicals. Lower IC_{50} value demonstrate that the higher radical scavenging activity. All 8 honey extracts as well as positive control exhibited dose-dependent activity. In this research, widely used positive control are used namely ascorbic acid and BHT. There is some other research done by few researchers that also used the same positive control for DPPH radical scavenging assay [10]. IC_{50} was established as concentration required to scavenge 50% of radicals for each sample and indicated by using GraphPad Prism software.

Table 1. Value for IC_{50}

Samples	**IC_{50} (mg/ml)**
Honeydew Honey (KH1)	15.29±4.49
Heterotrigona itama 1 (KH2)	47.06±3.919
Heterotrigona itama 2 (KH3)	20.57±6.252
Geniotrigona Thoracica 1 (KH4)	41.18±5.499
Geniotrigona Thoracica 2 (KH5)	20.63±4.791
Tetragonula Sirindhornae (KH6)	108.4±6.781
Tetrigona Binghami (KH7)	38.99±8.149
Acacia Honey (KH8)	31.39±3.786
Ascorbic Acid (VC)	0.001929±0.4619
Butylated hydroxytoluene	14.83±6.333

The IC_{50} value for ascorbic acid was 0.001929 with percentage of inhibition 98.43% at 200mg/ml while for BHT is 14.83 mg/ml with percentage of inhibition 92.261 mg/ml .Concentration of standard and honey extracts that were used in this assay were 12.5, 25, 50, 100, and 200 mg/ml. 8 extracts showed higher IC_{50} values than VC and BHT significantly ($P< 0.0001$). DPPH radical scavenging activity or percentage inhibition of free radical for all samples increased when the concentrations of honey extracts increased. Tetragonula Sirindhornae showed the highest value of IC_{50} with 108.4 mg/ml. However, percentage of inhibition for Tetragonula Sirindhornae at concentration 200mg/ml is lower compared to other samples with only 64.70%. Conversely, honeydew honey exhibits the lowest IC_{50} value which is 15.29 and highest percentage of inhibitor which is 96.948%. The higher the IC_{50} values, the lower the ability to scavenge the free radicals. Figures 2 showed the abilities of honey extracts to scavenge the DPPH radical in ascending order.

VC > BHT > KH1 > KH3 > KH5 > KH8 > KH7 > KH4 >KH2 > KH6

3.3 Composition of Phenolic Compound in Stingless Bee Honey

The components of phenolic compounds were identified by analysing the chromatogram. The tested honey contain combination of 14 phenolic compounds namely, benzoic acid, phenol, caffeic acid, ferulic acid, dihydrocinnamic acid, cinnamic acid gentisic acid, protocatechuic acid, quercetin, myricetin catechin, vanillic acid, 2,3-dihydroxybenzoic acid, and ellagic acid. Overall, there are 2 phenolic compounds that presence in all samples which is phenol and cinnamic acid. KH7 possessed the highest number of phenolic compounds and KH5 exhibit the lowest number of phenolic compounds. Total of 6 phenolic compounds were detected in KH7 namely benzoic acid, caffeic acid, ferulic acid, dihydrocinnamic acid. KH3 and KH4 both showed presence of 5 phenolic compounds which is vanillic acid, 2,3-dihydroxybenzoic acid and ellagic acid, as for KH4 contain quercetin, myricetin and catechin. Four phenolic compounds were observed in KH1 which is caffeic acid and genistic acid. All other samples exhibit only 3 phenolic compounds with different combinations; KH2 contain dihydrocinnamic acid; KH8 contain ferulic acid; KH6 contain protocatechuic acid.

Honey is well-known with containing a wide range of phenolic compounds started from relatively simple hydroxybenzoic acid to more complex compounds like flavonoids. Besides terpenoids that pumped honey with plenty bioactivity through its nectar, phenolic compounds are also one of the two vital medicinal substances that present in plants. According to research done few researchers, phenolic acids might help in inhibiting the cancer and cardiovascular diseases where their antioxidant activity are high in health relates field. Table 2 showed phenolic compound that detected in stingless bee honey.

Table 2. Phenolic acids detected in stingless bee

Sample	**Phenolic Compound**														
	BA	**PH**	**CFA**	**FA**	**HCA**	**CA**	**GA**	**PA**	**QC**	**MC**	**CC**	**VA**	**DBA**	**EA**	**IC_{50}**
KH1			✓			✓	✓								15.29±4.490
KH2					✓	✓									47.06±8.149
KH3						✓						✓	✓	✓	20.57±3.919
KH4						✓			✓	✓	✓				41.18±6.781
KH5						✓									20.63±6.252
KH6						✓		✓							108.4±3.786
KH7	✓		✓	✓	✓	✓									38.99±4.791
KH8						✓									31.39±5.499

BA: Benzoic Acid, PH: Phenol, CFA: Caffeic Acid, FA: Ferulic Acid, HCA: Hydrocinnamic Acid, CA: Cinnamic Acid, GA: Gentistic Acid, PA: Protocatechuic Acid, QC: Quercetin, MC:Myricetin, CC: Catechin, VA:Vanillic Acid, DBA: 2,3-DihydroxyBenzoic acid, EA: Ellagic Acid

3.4. Development of QCAR Model

The model was developed using multi-linear regression namely PLS by using the SOLO (Eigenvector Research Inc., 2010). The model was expressed in the form of mathematical equation which involving qualified variables together with their regression coefficients. The performance of developed model was analysed based on their reliability.

PLS regression methods were utilised, it produced the good performance with r^2_{calc} 0.729. Furthermore, PLS model was capable to describe 72.87% variance of antioxidant activity. However, the r^2_{cv} was slightly low with only 0.024. Higher values r^2_{calc} than r^2_{cv} showed that the model were not over-fitting and are reliable. In this study, molecular weight was not chosen as significant variables in the developed models. This is probably due to the stricter procedure for removing the variable based on the criteria. Figure below show the result of the model.

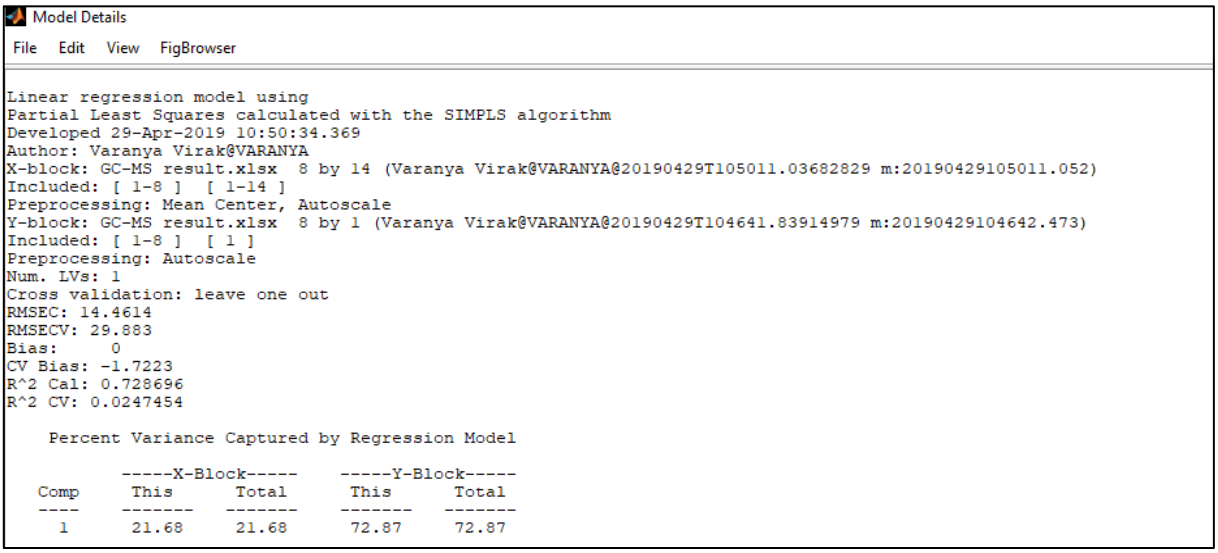


Figure 3. The results of the model

4. CONCLUSION

Honey possesses various biological, biochemical and physiological activities in animals and also humans. The efficacy of these properties depends on the phenolic compounds present in honey. Depending on their source of origin, phenolic compounds are different among the sample. As for phenolic compounds, the components of phenolic compounds were identified by analysing the chromatogram. The tested honey contain combination of 14 phenolic compounds namely; benzoic acid, phenol, caffeic acid, ferulic acid, dihydrocinnamic acid, cinnamic acid gentisic acid, protocatechuic acid, quercetin, myricetin catechin, vanillic acid, 2,3-dihydroxybenzoic acid, ellagic acid. The most abundantly present in stingless bee honey are phenol and cinnamic acid. 8 samples of stingless bee honey were obtained locally and analyzed. DPPH was used to determine their antioxidant activity while GC-MS was used to analyse phenolic compounds that present in honey samples. KH6 show the highest IC_{50} value which is 108.4 mg/mL while KH1 showed the lowest IC_{50} value which is 15.29 mg/mL. For development of QCAR model, r^2_{calc} is found to be 0.729. This proved that the model is reliable. Overall, the model produced is proved to be reliable and can correlate relationship between antioxidant activities and phenolic compounds that present in stingless bee honey.

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