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Research paper

Physicochemical analysis, free radical scavenging activity and anti-acetylcholinesterase activity of stingless bee honey

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Abstract

Alzheimer's disease (AD) is characterized by memory loss due to the deficiency of acetylcholine (ACh) concentration in the brain. It can be improved by inhibiting the Acetylcholinesterase (AChE) enzyme through cholinesterase inhibitors. This neurodegenerative disorder also can be caused by neuronal loss through oxidative stress which can be reduced by natural antioxidant. Honey appears to possess high scavenging properties. However, not much study of stingless bee honey focus on AChE inhibition. The objective of this project is to determine the scavenging properties of stingless bee (*Heterotrigona itama*) honey and its potential as natural AChE inhibitor. Physicochemical analysis of the honey was conducted to test its quality and purity through moisture, ash and pH test. The results show that the purity test is in the standard range of stingless bee honey. Different concentrations of honey were tested for its scavenging properties through DPPH assay. The honey samples were also tested for its AChE inhibitory effect. Results show that the stingless bee honey possesses high scavenging properties at 100% concentration. The inhibition of AChE enzyme showed through the white spot produced on the TLC plate. This finding proves that stingless bee honey able to combat oxidative stress and act as AChE inhibitors.

Keywords: *Heterotrigona itama Honey; Acetylcholinesterase Inhibitor; Free Radical; Scavenging Activity.*

1. Introduction

Alzheimer's disease (AD) is a type of neurodegenerative disorder which normally attacks the brain nerve cell [1]. This disease is categorized as a "killer disease" by Alzheimer's Association [2] because there is still no Alzheimer's survivor. Acetylcholine (ACh) is an organic molecule released at the nerve endings and act as a neurotransmitter [3]. Excessive breaks down of ACh by acetylcholinesterase (AChE) enzyme plays a vital role in learning and memory deterioration found in AD patients [4], [5]. ACh concentration in the brain can be improved by inhibiting the AChE enzyme through cholinesterase inhibitor. This inhibitor blocks the enzyme activity and automatically increases the ACh concentration at synapses. This helps to improve memory of individual who suffered dementia [6].

Oxidative stress is one of the common manifestations of biochemical insults to the structural and functional integrity of neural cells. It contributes to the key factor of AD [7], [8]. Exposure to harsh environment in daily life will increase the production of free radical which leads to the accumulation of oxidative stress in human body [9]. The presence of antioxidant in the body helps to balance and reduce the damage caused by oxidative stress [10]. Nutraceutical supplement from natural sources appear to possess high antioxidant properties which may contribute to memory improvement and prevention of AD [11].

To date, there is still no cure for this disease and current treatment can only slow down the progression of the symptoms [4]. Thus, there is a dire need to prevent this disease by searching an alternative supplement and treatment from natural sources. Honey is one of natural products that have been reported to possess high nutritional properties and provides wide range of benefits to human health. Compared to other honey, stingless bee honeys have a wide range of nutrients and provide higher nutritional value [12]. It has been reported to possess not only high antibacterial, anti-fungal and antiviral properties, but it has also been reported to have high antioxidant activity [13]. Thus, this honey has a high potential as a nutraceutical product in combating neurodegenerative disease. The most popular species of stingless bees is *Hetrotrigona itama*. It produces multifloral honey which comes from both higher and lower level of plant flowers [14]. *Heterotrigona itama* bee also commonly known as "Kelulut" is one of the species of stingless bees in Malaysia. It produces honey and propolis that contain high level of phenolic bioactive molecules that serves as natural antioxidants [15]. Due to its high medicinal properties, present study was conducted to test the potential of *Heterotrigona itama* honey as natural radical scavenger and AChE inhibitor. High antioxidant properties of this honey might contribute to memory improvement and reduces memory impairment by combating the free radical and oxidative stress in the brain.

2. Methodology

2.1. Physicochemical analysis

Heterotrigona itama honey was collected from Kuala Kangsar, Perak. The quality and purity of stingless bee honey were evaluated through physicochemical analysis by measuring the moisture, ash and pH of the sample. Moisture test of honey was prepared according to Gebremariam and Brhane [16]. 5 g of 100% pure stingless bee honey was weighed and placed in a small beaker. The sample was then placed in a vacuum oven to dry at 105 °C for 4 hours. Percentage of moisture content was calculated as follow:

$$\text{Moisture (\%)} = \frac{w1-w2}{w1-w0} \times 100$$

Where;
w0 = weight of empty beaker
w1 = weight of fresh sample + beaker
w2 = weight of dried sample + beaker
Ash test was performed according to Gebremariam and Brhane [16]. 5 g of pure honey sample was weighed in a crucible. The sample was then charred on a Bunsen burner until it became dry and smokeless. The sample was transferred into a muffle furnace at 600 °C for 4 hours. The dried sample was then placed in a desiccator. After cooling down, the content was weighed. The percentage for ash content was calculated as follow:

$$\text{Ash content (\%)} = \frac{w2-w1}{m} \times 100$$

Where;
w1 = weight of empty crucible
w2 = weight of ash + crucible
m = mass of sample
The preparation for pH test was adapted from Nascimento, Marchini [18]. To determine the pH, 10 g of pure honey was diluted in 75 ml of distilled water. The solution was then mixed homogenously. The pH was read directly using pH meter. The pH meter was calibrated before being used with standard buffer solution of pH 7 and pH 4.

2.2. DPPH Free-radical scavenging assay

The scavenging activity of honey sample was evaluated using DPPH solution as a free radical model and the method was adapted from Philip and Mohd Fadzelly [19]. Five different concentrations (0.01%, 0.1%, 1%, 10% and 100%) of honey were prepared through serial dilution. An aliquot of 100 µl of each sample was mixed with 1 ml of 500 µM ethanolic DPPH solution. The mixture was shaken vigorously and incubated in a dark at room temperature for 30 minutes. The absorbance was measured spectrophotometrically at 517nm. The percentage of anti-radical activity was calculated as follows:

$$\text{Antiradical activity (\%)} = \frac{Ac-As}{Ac} \times 100$$

Where;
Ac = Absorbance of control DPPH
As = Absorbance of sample
The decolourization of DPPH solution from purple to yellow indicates the presence of antioxidant properties in the honey sample and able to scavenge the free radical of the solution.

2.3. AChE inhibitory test

The anticholinesterase inhibition assay was performed based on previous method described by [4, 20]. To prepare the AChE solution, AChE (500 U) powder were dissolved in 500 ml of Tris-hydrochloric acid buffer (0.05 mol/L, pH 7.8). 500 mg Bovine Serum Albumin were added to stabilize the enzyme during the bioassay. This solution can be used up to six months when stored at 4°C. 1-Naphtyl acetate (150 mg) was dissolved in 40 ml ethanol. The solution was then diluted with 60 ml of distilled water. Fast Blue B solution was prepared by dissolving 50 mg of Fast Blue B salt in 100 ml of distilled water. Stingless bee honey sample was applied to silica gel TLC plate and migrated by a proper solvent. The plate was then sprayed with the enzyme and 1-Naphtyl solution and was allowed to dry. Then it was placed in a closed vessel containing water for humidity. The plate should not be in contact with water directly. The plate was kept in the closed vessel at 37°C for 20 minutes to allow the enzyme reacted with 1-Naphthyl acetate. After the incubation, the plate was sprayed with Fast Blue B solution, where the inhibited acetylcholinesterase spot would appear as white and other parts as purple.

2.4. Statistical analysis

All tests were done in triplicate and all data were expressed as mean ± standard deviation that was analysed by Scientific Package of Social Science (SPSS) of 20.0 version software for Windows 10. The one-way analysis of variance (one-way ANOVA) was applied. P value less than 0.05 was considered to have significant statistical difference.

3. Results and discussion

3.1. Physicochemical analysis of stingless bee honey

Physicochemical analysis can determine the purity and quality of the honey based on several parameters such as moisture content, ash content, and pH value [21].

Table 1: Physicochemical Analysis of *Heterogona itama* Honey

Parameter	Present study	Previous study[12], [14], [17]
Moisture	24.10 %	21.40 – 31.59 %
Ash	0.28 %	0.22-0.41 %
pH	2.54	3.24-3.71

Average moisture content of *Heterotrigona itama* honey in this study is 24.10% which is within the range of standard stingless bee honey found in Malaysia (Table 1). Previous study has conducted a moisture test on numerous stingless bee honeys including Trigona spp. They have revealed that the range of moisture content of stingless bee honey in Malaysia is within 21.40-31.59% [12], [14], [17]. Moisture content of honey is one of the most important criteria to determine the quality, stability, shelf-life as well as spoilage resistance against yeast fermentation. Low moisture content of honey helps to promote longer shelf life. However, moisture content are depends on the temperature and relative humidity in the geographical origin during honey producing colonies [22]. Even in Malaysia, different region of nectar collected by the stingless bee may produce different moisture content of honey[17].
Ash content of honey that has been tested in this study is 0.28% (Table 1). This result is linked with the previous study that record the ash content of Malaysian stingless bee honey are within 0.22-0.41% [12], [14], [17]. The ash content of honey are influenced by the plant nectar and it correlates with its mineral content which includes specific inorganic matter such as calcium, sodium, potassium, iron and other important minerals for human diet [23], [24]. The minerals were absorbed from the soil and later end up in the nectar [24]. Ash content can determine the quality of the sample as it can influence flavor, form, texture and stability of the honey [25]. Due to the foraging activity of honey are different among region, the ash content will be varied [17], [22].
The pH value of honey sample measured in this study is 2.54 (Table 1), which is acidic. As compare to previous study, the pH value is slightly lower and is not in the range between 3.24-3.71 [12], [14], [17]. Different of pH value may be attributed to the different acid found in different floral type. The high acidity of honey correlates with the fermentation of its sugar into organic acid which is responsible for flavour and stability of the honey against microbial spoilage [22]. It might also indicate that honey samples have high content of minerals [22]. According to Gebremariam and Brhane [16], honey is considered as a buffer which means its pH will not change by the addition of small quantity of acids and bases due to the content of phosphate, carbonates and others mineral salts.

3.2. Free radicals scavenging properties of stingless bee honey

DPPH assay is based on the reduction of DPPH, stable free radical. According to Shekhar and Anju [26], the free radical of DPPH with an odd electron gives maximum absorption value at 517 nm. Antioxidant molecules are able to quench DPPH free radicals, resulting in discoloration of the purple colour of DPPH [19]. As referred to Figure 1, 100% and 10% concentration of stingless bee honey caused decolourization of DPPH solution. The purple colour of DPPH solution turns into yellow. As for the 1% concentration, the colour changes slightly from purple to pale purple. The other two concentrations which are 0.1% and 0.01% only show slight colour changes.

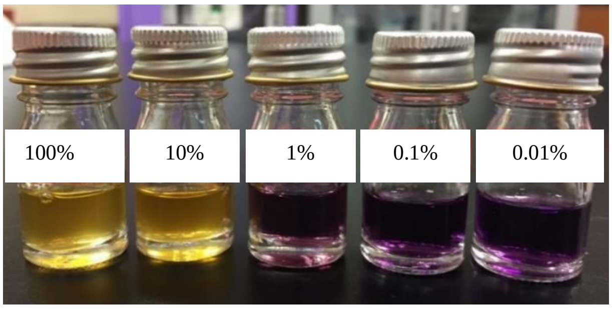

Fig. 1: Decolourization of DPPH Solution from Purple to Yellow by Different Concentration of Stingless Bee Honey.

Result shown in Table 2 tabulated the anti radical properties of stingless bee honey ranging from 0.01% to 100% concentration. There is a significant difference ($p<0.05$) of scavenging activity between different concentration of stingless bee honey. At 100% concentration, it shows the highest antiradical activity with 77.29% DPPH scavenging effect (Figure 2). This data supported by Chan, Haron [17] where their report shows that the stingless bee honey from east coast region of Malaysia possess the highest scavenging activity against DPPH solution with 79.99% of inhibition at 60mg/ml of honey concentration.

Table 2: Percentage of Radical Scavenging Effect of *Heterotrigona itama* Honey

Concentration of honey (%)	Percentage of inhibition of honey in mean ± SD (I %)	P value
Control	0±0	
100	77.29±0.84	
10	72.39±10.38	<0.0001
1	57.12±1.28	
0.1	35.59±1.45	
0.01	26±0.82	

The capability of stingless bee honey to scavenge free radical might be contributed by the presence of various antioxidant compounds in the honey. It is rich of beneficial bioactive compound such as flavanoid and phenolic acid which exhibit wide range of biological effect including antioxidants [12], [23], [27], [28]. Vitamin C also known as ascorbic acid in honey were also found to contribute to its antioxidants properties [23]. A good quality of stingless bee honey is indicated by the presence of plant phenolic in the honey and its capability to possess scavenging properties [24]. However, the antioxidant content and its antioxidative activities will be greatly varied with the geographic origin of the honey [17].

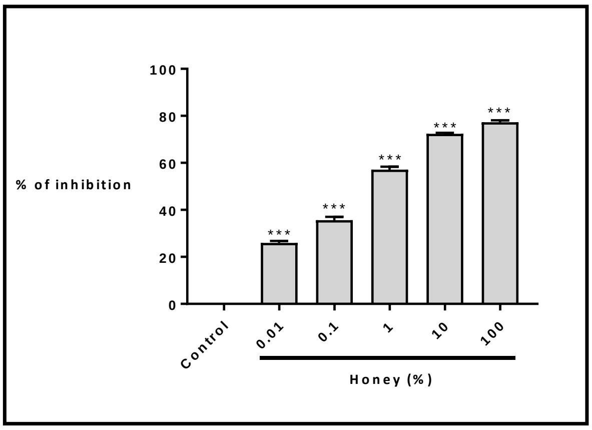

Fig. 2: Graph of Percentage of Inhibition against Concentration of the Stingless Bee Honey.

3.3. Acetylcholinesterase (AChE) inhibitory effect of stingless bee honey

AChE test result shows that there is a positive inhibition of *Heterotrigona itama* honey towards the AChE enzyme. Figure 3 shows the inhibition of AChE on TLC plate through the appearance of white spot. The enzyme converts 1-napthyl acetate into 1-naphtol that reacts with fast blue B salt which caused the purple coloured background on the TLC plates. Enzyme inhibitors block the cleavage of 1-naphtyl acetate. Thus no purple colour is produced and white spots will appear [20], [29]. This indicates the ability of stingless bee honey to inhibit acetylcholinesterase enzyme activity [4], [20].

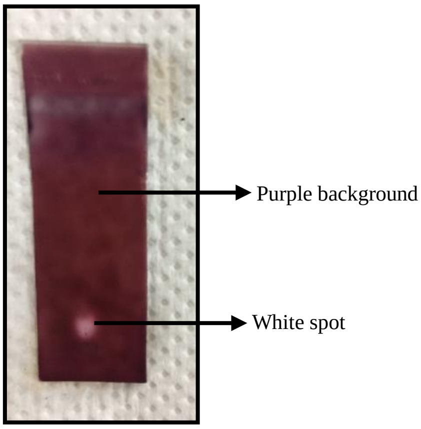

Fig. 3: Inhibition of Ache on TLC Plate by Stingless Bee Honey.

The AChE inhibitory effect of honey might be contributed by its antioxidant properties. It is due to the presence of aromatic ring found in antioxidant compound such as flavanoid and has been reported to be the main inhibitory effect of AChE [30], [31]. A strong positive correlation of antioxidant properties with acetylcholinesterase inhibitory effect were also supported by Philip and Mohd Fadzelly [19].

Stingless bee honey used in this study has shown a positive result on antioxidant and AChE inhibition activity. These will give an insight on its beneficial effects towards human. The natural alternative of AChE inhibitor will contribute to AD theraphy [32].

4. Conclusion

In conclusion, *Heterotrigona itama* honey is proven to possess scavenging activity towards DPPH solution and was found to inhibit AChE activity.

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