UNIVERSITI PUTRA MALAYSIA

PHYSICOCHEMICAL AND ANTIOXIDANT PROPERTIES OF STINGLESS BEE HONEY AND ITS EFFECT ON COGNITIVE FUNCTION IN MICE

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By

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Chair : Mariatulqabtiah Binti Abdul Razak, PhD
Faculty : Biotechnology and Biomolecular Sciences

Kelulut honey is a local stingless bee honey in Malaysia and has been consumed due to its therapeutic value and high antioxidant content. The antioxidant in honey will helps in giving memory-enhancing effects and improving brain function. However, reports on nootropic effect of this stingless bee honey are still unclear. Therefore, this study aims to investigate the physicochemical and antioxidant constituents of local-harvested stingless bee honey and their effect to spatial learning and memory performance after supplementation to mice. In this study, the physicochemical and antioxidant content of seven stingless bee honey samples (Pasir Mas, Kelantan) were analysed using various spectrophotometric assay and high performance liquid chromatography method. For behaviour study, female Swiss albino mice (N=35) were divided into five groups that consist of four honey-treated groups (n=28) and one untreated control group (n=7). The acute (7 days) and semi-chronic (35 days) honey-treated group mice were supplemented with two dosages of honey (750 mg/kg and 2000 mg/kg) daily via oral gavage. After the treatment period (7 days and 35 days), the mice were then tested with open field test to measure their locomotor activity. Then, they were tested with Morris water maze (MWM) behavioural task to evaluate for their spatial learning and memory performance. Results for physicochemical and antioxidant analysis showed that the highest antioxidant content was detected from Ita-7-TS sample with 57.38 ± 0.24% anti-radical activity; 853.958 ± 150.04 mg GAE/kg honey of total phenolic content; and 1245.00 ± 204.63 mg of quercetin equivalent/kg honey of total flavonoid content. In the MWM test results, the honey-treated group mice showed a decreased in escape latency during acquisition testing. In probe trial test, results showed that all the honey-fed mice group had significant visit (p =.008) and spent more time (p =.000) in the previous platform quadrant as compared to control group, indicating a significant improvement in reference memory. Both semi-chronic treated mice also shown better performance than acute treated mice in dose dependent manner. The high antioxidant potential of the analysed stingless bee honey samples are due to its polyphenols content such as phenolic acid and

flavonoid. This polyphenols are phytochemical compounds that give scavenging activities and can activate antioxidant defend system in the brain, hence providing nootropic and neuroprotective effects against brain oxidative stress and tissue damage. This will results in improvements of cognitive function including promoting learning and memory by modulating synaptic plasticity through synaptogenesis. Therefore, the positively significant results from this study, especially on reference memory may suggest that this stingless bee honey supplementation can increase the learning and memory performance in mice, possibly due to its high antioxidant content.

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KANDUNGAN FIZIKOKIMIA DAN ANTIOKSIDAN MADU LEBAH KELULUT DAN KESANNYA TERHADAP FUNGSI KOGNITIF PADA TIKUS

Oleh

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Madu kelulut merupakan madu dari lebah kelulut tempatan di Malaysia dan telah digunakan kerana nilai terapeutiknya dan kandungan antioksidan yang tinggi. Antioksidan di dalam madu akan membantu dalam memberikan kesan peningkatan daya ingatan dan menambah baik fungsi otak. Walau bagaimanapun, laporan mengenai kesan nootropik madu kelulut ini adalah masih tidak jelas. Oleh itu, kajian ini bertujuan untuk mengkaji kandungan fizikokimia dan antioksidan madu lebah kelulut tempatan dan kesannya kepada prestasi kognitif dan ingatan pada tikus. Dalam kajian ini, kandungan fizikokimia dan antioksidan tujuh sampel madu lebah kelulut (Pasir Mas, Kelantan) telah dianalisis menggunakan pelbagai kaedah asai spektrofotometrik dan kromatografi cecair prestasi tinggi. Untuk kajian tingkah laku, tikus Swiss albino betina (N=35) telah dibahagikan kepada lima kumpulan yang terdiri daripada empat kumpulan yang dirawat dengan madu (n=28) dan satu kumpulan kawalan yang tidak dirawat (n=7). Kumpulan tikus yang dirawat dengan madu secara akut (7 hari) dan separa-kronik (35 hari) telah diberikan dua dos madu (750 mg/kg dan 2000 mg/kg) setiap hari melalui gavage oral. Selepas tempoh rawatan (7 hari dan 35 hari), tikus kemudiannya telah diuji dengan ujian lapangan terbuka untuk mengukur aktiviti lokomotor mereka. Kemudian, tikus telah diuji dengan ujian Morris water maze (MWM) bagi menilai prestasi kognitif dan ingatan mereka. Hasil dari analisis fizikokimia menunjukkan bahawa kandungan antioksidan yang paling tinggi telah dikesan dari sampel Ita-7-TS dengan 57.38 ± 0.24% aktiviti anti-radikal, jumlah kandungan fenolik sebanyak 853.958 ± 150.04 mg GAE/kg madu, dan jumlah kandungan flavonoid sebanyak 1245.00 ± 204.63 mg kuarsetin setara/kg madu. Dalam hasil ujian MWM, kumpulan tikus yang dirawat dengan madu menunjukkan penurunan dalam masa menyelamatkan diri semasa ujian pengambilalihan. Dalam ujian percubaan prob, hasil menunjukkan bahawa kesemua kumpulan tikus yang diberi makan madu mempunyai lawatan ketara (p =.008) dan menghabiskan lebih banyak masa (p =.000) dalam kuadran platform sebelumnya berbanding dengan kumpulan kawalan, justeru menunjukkan peningkatan yang ketara dalam memori rujukan pada tikus. Kedua-

dua kumpulan tikus yang dirawat secara separa-kronik juga menunjukkan prestasi yang lebih baik berbanding tikus yang dirawat secara akut dalam cara berkeperluan dos. Potensi antioksidan yang tinggi bagi sampel madu lebah kelulut yang dianalisis adalah disebabkan oleh kandungan polifenolnya seperti asid fenolik dan flavonoid. Polifenol ini adalah sebatian fitokimia yang memberikan aktiviti skavengan dan boleh mengaktifkan sistem pertahanan antioksidan pada otak, dengan itu memberikan kesan nootropik dan pelindungan neuro terhadap tekanan oksidatif otak dan kerosakan tisu. Ini akan menyebabkan penambahbaikan fungsi kognitif termasuk menggalakkan pembelajaran dan ingatan dengan memodulasi keplastikan sinapsis melalui sinaptogenesis. Oleh itu, keputusan positif ketara dari kajian ini, terutamanya pada memori rujukan menunjukkan bahawa pemberian makanan tambahan madu kelulut ini boleh meningkatkan prestasi kognitif dan ingatan pada tikus, berkemungkinan disebabkan oleh kandungan antioksidannya yang tinggi.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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ABS	Absorbance
ACh	Acetylcholine
AChE	Acetylcholinesterase
$AlCl_3$	Aluminium chloride
ANOVA	One-way analysis of variance
Apis sp.	*Apis* species
ARASC	Animal Research and Service Centre
BDNF	Brain-derived neurotrophic factor
CNS	Central nervous system
DPPH	2, 2 – diphenyl – 1 – picrylhydrazyl
G. thoracica	*Geniotrigona thoracica*
GAEs	Gallic acid equivalents
H. itama	*Heterotrigona itama*
H_2O_2	Hydrogen peroxide
HMF	5-hydroxymethyl-2-furfural
HPLC	High performance liquid chromatography
IHC	International Honey Commission
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
mAU	milli-absorbance unit
mPFC	Medial prefrontal cortex
MTL	Medial temporal lobe
MWM	Morris water maze
Na_2CO_3	Sodium carbonate

$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
OFT	Open field test
QEQ	Quercetin equivalent
SD	Standard deviations
SEM	Standard error of means
SPSS	Statistical Packages for Social Science
STM	Short-term memory
Trigona sp.	*Trigona* species

CHAPTER 1

INTRODUCTION

Honey is an insect-derived natural product which has been consumed due to its high nutritional and therapeutic value. It has been widely used by many cultures in various parts of the world as natural sweetener and supplementation or alternative medicine through apitheraphy (El-Soud, 2012; Alvarez-Suarez *et al.,* 2014; and Rahman *et al.,* 2014). Apitheraphy has been practiced since ancient times and is still actively used in modern times for various medicinal purposes (Ajibola *et al.,* 2012). Honey is a viscous solution made up from various molecules, including sugar mainly fructose and glucose, water, ash, protein, amino acids and also trace amounts of enzymes, vitamins and other substances, such as phenolic compounds (Rao *et al.,* 2016).

Major constituents of honey are sugar and water. Sugar or carbohydrate is the main component of honey and represents 80-85% of honey dry matter with nearly 30% of it is fructose (Bogdanov *et al.,* 2008). Other than sugar, various phenolic compounds such as phenolic acid and flavonoid can also be found in honey and these compounds are known to have antioxidant properties (Nurul *et al.,* 2013). These high carbohydrate content and antioxidant potential of honey have contribute to its main nutritional values which lead to various therapeutic promises of honey consumption (Rahman *et al.,* 2014). Traditionally, honey has been used as a natural remedy to treat burns, wounds, and ulcers (Alvarez-Suarez *et al.,* 2014). It has also been actively used as cold and sore throat medicine in certain culture (El-Soud, 2012). Nowadays, various research studies of honey have been conducted to determine its biological properties and medicinal values in a more scientific approach. Among established therapeutic effects of honey are its anti-microbial and anti-inflammatory activity; anti-diabetic and anti-cancer properties; high antioxidant potential; and also its ability to treat cardiovascular and gastrointestinal tract diseases, and also eye and neurological disorder (de Queiroz Pimentel *et al.,* 2013; Cooke *et al.,* 2015; AL-Waili *et al.,* 2013; Erejuwa *et al.,* 2012; Kustiawan *et al.,* 2014; Rao *et al.,* 2016; Kamarulzaidi *et al.,* 2016; Al-Rahbi *et al.,* 2014; and Akanmu *et al.,* 2011). However, differences in the flora sources, geographical and climate conditions and type of honey bee influenced the physical and chemical properties of honey resulting in different nutritional benefits and therapeutic values (Othman *et al.,* 2015).

Honey can be a natural production of either honey bee from *Apis* species (*Apis* sp.) or stingless bee from *Trigona* species (*Trigona* sp.) and different types of honey bee may produce different compositions of honey (Rao *et al.,* 2016). Honey bees are bees with sting from *Apis* sp. and they produce honey by collecting nectar from flowers into a hexagonal hive. Honey products that are available in the market are mostly from this species, such as Manuka honey from New Zealand and Tualang honey from Malaysia. Meanwhile, stingless bees are usually from *Trigona* sp. As compared to *Apis* sp. bee, they are smaller in size and they collect nectar into honey pot instead of hexagonal hive. This bee can be widely found in tropical area such as Malaysian temperate rainforest and

is locally known as *Kelulut* (Kek *et al.,* 2014). Stingless bee honey is known as meliponini, pot honey, or locally as *Kelulut* honey. This honey is different from honey from *Apis* sp. in terms of its colour, taste, and viscosity. It is usually more acidic, darker in colour, less sweet, and less viscous (Souza *et al.,* 2006). Recently, stingless bee has been actively reared and cultivated as rural agro-industry in Malaysia as its honey was believed to give better therapeutic performance than honey from *Apis* sp. due to its higher phenolic content (Kelly *et al.,* 2014; Kek *et al.,* 2014). However, as compared to *Apis* sp., there is still lack of investigation reported on therapeutic effects of this stingless bee honey. In the past decade and until now, research have been actively done to investigate the potential of this stingless bee honey and among established reports are on its anti-cancer, anti-inflammatory, and anti-microbial activity (Kustiawan *et al.,* 2014; Boorn *et al.,* 2010; and de Queiroz Pimentel *et al.,* 2013). Therefore, more research should be conducted to further investigate the potential of this valuable bee product.

Brain is part of our central nervous system (CNS). It contains over billion of neurons and glial cells that inter-connected to form synapses which made-up our information processing and storage system through learning and memory (Nelson and Alkon, 2014). Learning is a process of acquiring knowledge that will modify subsequent behaviour while memory is the ability to process that knowledge and remember past experiences (Squire and Wixted, 2011). They are both closely related neural phenomenon that occur through three stages which are acquisition of knowledge, storage of knowledge into memory, and retrieval of memory (Al-Rahbi *et al.,* 2014). Synaptic plasticity plays a central role in associative learning and memory through synapse formation and neuronal cell communication (Sofroniew and Vinters, 2010). These processes are induced by learning and will lead to memory formation by synaptogenesis (Nelson and Alkon, 2014). The discovery of long-term potentiation (LTP) by Bliss and Lomo in 1973, was the first demonstration of synaptic plasticity and has been accepted as a cellular model system for mechanism of learning and memory (Bliss and Lomo, 1973; Mayadevi *et al.,* 2012). This process of memory formation from learning mostly takes place in the inner part of the brain such as hippocampus and other parts of the medial temporal lobe (MTL) structures.

One of the established therapeutic values of honey is it possesses high antioxidant potential. Antioxidant has been proposed to be good for various memory types as it can reduce and neutralize free radicals, preventing their harmful cell-damaging effects to the brain (da Silva *et al.,* 2016; Spencer, 2010). Honey is one of the highly recommended alternative foods used to enhance memory as it is rich in polyphenols such as phenolic acids and flavonoids that give antioxidant effects (Chepulis *et al.,* 2009). Studies have reported that these honey polyphenols give honey its neuroprotective and nootropic or memory enhancing effects as it improves morphology of memory-related brain areas, reduces brain oxidative stress, increases brain-derived neurotrophic factor (BDNF), and acetylcholine (ACh) concentrations in the brain (Othman *et al.,* 2015; Rahman *et al.,* 2014; and Al-Rahbi *et al.,* 2014). Malaysian honeys have shown good antioxidant potential and several studies have reported that Tualang honey has the highest antioxidant contents among Malaysian honey samples (Khalil *et al.,* 2011; Kishore *et al.,* 2011; and Moniruzzaman *et al.,* 2013).

Although stingless bee honey is multifloral honey with different properties than that of honey from *Apis* sp., it is believed that it still possess relevant amount of phenolic compounds and antioxidant activity. In 2014, a study has reported that this stingless bee honey possess higher levels of phenolic content than honeys from *Apis* sp. in Malaysia (Kek *et al.,* 2014). In 2016, another study has also reported that this stingless bee honey from *Trigona* sp. had the highest number of phenolic compound detected and identified among Malaysian honey samples (Ismail *et al.,* 2016). Hence, these studies show a high antioxidant potential in local stingless bee honey which may contribute to its nootropic and neuroprotective effects. Although they are many studies have been reported on the positive effects of *Apis* sp. honey supplementation on improvements of cognitive function, scientific research showing specific positive effects of stingless bee honey in learning and memory is still unclear (Al-Himyari, 2009; Othman *et al.,* 2011; Chepulis *et al.,* 2009; Akanmu *et al.,* 2011; Al-Rahbi *et al.,* 2014; and Kamarulzaidi *et al.,* 2016). Besides, full reports on both physicochemical and antioxidant composition of this Malaysian stingless bee honey are also still unclear as most research are still on early stages and only focusing on the antioxidant composition (Kek *et al.,* 2014; and Ismail *et al.,* 2016).

Therefore, the aims of this present study is to analyse the physicochemical and antioxidant properties of Malaysian stingless bee honey and to investigate the effects of this honey supplementation on learning and memory performance in mice using Morris water maze (MWM) test. In this study, the physicochemical content and antioxidant levels were analysed from seven local harvested stingless bee honey samples (from Pasir Mas, Kelantan) and the behavioural analysis of honey supplementation were analysed between two dosages of acute and semi-chronic treated mice and also compared with untreated control group mice. The learning and memory performance of the mice were analysed using the MWM test. It is hypothesized that the stingless bee honey supplementation helps in increasing the learning and memory performance in mice and it may be due to its high antioxidant content. Hence, the objectives of the study are:

1. To analyse the physicochemical contents and antioxidant levels of seven local-harvested stingless bee honey samples.

2. To investigate the effect of selected stingless bee honey supplementation on locomotor activity of mice using open field test.

3. To investigate the effect of selected stingless bee honey supplementation on learning and memory function of mice using Morris water maze test.

4. To analyse the escape latency between low (750 mg/kg) and high (2000 mg/kg) dosages of acute (7 days) and semi-chronic (35 days) honey-treated mice and untreated control mice.

5. To evaluate the effect of selected stingless bee honey supplementation on spatial learning and spatial reference memory using probe trial test.

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