PHYSICOCHEMICAL PROPERTIES, ANTIOXIDANT PROFILE AND QUALITY STANDARD ADHERENCE OF RAW STINGLESS BEE HONEY FROM SELECTED REGIONS IN PENINSULAR MALAYSIA

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Stingless bee honey (SBH) is a highly nutritious food that can be influenced by various factors, such as climate and soil composition, depending on the geographical area where it is produced. Honey quality standards must encompass a broad range of characteristics to ensure that the final product meets the intended standards for high-quality and pure honey. In this study, forty-three SBH (*Heterotrigona itama*) samples were collected and labelled as <20 West Coast (<20WC), <40 West Coast (<40WC), <40 East Coast (<40EC), <20 East Coast (<20EC) and inland, according to their distance from the coasts. Firstly, the optimization of sugar profile methodology for trehalulose quantification was carried out using high-performance liquid chromatography (HPLC) with an evaporative light scattering detector (ELSD). Then, the SBHs were analyzed for their physiochemical properties, sugar profile, organic acid, minerals composition, bioactive compounds, and antioxidant capacities; total flavonoid content (TFC), total phenolic content (TPC), ferric reducing antioxidant power (FRAP), ABTS and DPPH radical scavenging activity assay, and oxygen radical absorbance capacity (ORAC) assay. The classification of SBH according to the geographical areas was performed using Principal Component Analysis (PCA). An HPLC-ELSD methodology was established, whereas using 88% ACN:12% DH2O and ELSD setting at 60 psi proved highly effective for separating sugars. Terhalulose was the most abundant sugar in *H. itama* SBH, with a mean of 39.0 ± 13.06 g/100g, and the optimized procedure was adequate and reproducible for identifying and quantifying trehalulose in SBH. Moisture, pH, electrical conductivity (EC), hydroxymethylfurfural (HMF), and sugar composition of all SBH samples adhered to the Malaysian Kelulut Honey Standard (MS2683:2017). SBH from <40EC had a darker colour with low L* and significant ($p<0.05$) lower colour parameters (a*, b*) compared to the other four geographical areas. Potassium (K) was found to be significantly ($p<0.05$) higher in SBH from the <40EC group compared to the Inland and <20EC groups.

Gluconic acid (GA) was significantly ($p<0.05$) higher in the SBH from the <40WC group than the <20WC group. Lactic, acetic, 3-phenyllactic acid (3-PLA), and phenylalanine were not significant ($p>0.05$) in SBH in all five geographical areas. TFC, TPC, FRAP, and ABTS scavenging activities were significantly higher in the <40EC samples. DPPH scavenging activity increased significantly in SBH from <20WC, while SBH from <20EC had higher ORAC activity. The PCA shows that the discrimination of SBH according to the five different areas was not feasible, indicating sample homogeneity. Pearson correlation shows that all antioxidant capacities positively correlate with EC, FA, Mg, K, P, acetic, and lactic acid. TFC and TPC are also moderately to strongly associated with FRAP and ABTS. 3-PLA also have a positive correlation with phenylalanine. In contrast, L* and b* showed a moderate to strong negative association with antioxidant capacities. For further research, conducting a more thorough investigation of both phenolic and volatile chemical fingerprinting can improve the capacity to discriminate between SBHs depending on their geographical origins and also include more samples from the East of Malaysia (Sabah and Sarawak) to see the fingerprint of those SBH based on geographical areas.

Keywords: Geographical area; *Heterotrigona itama*; Quality standard; Stingless bee honey; Trehalulose

SDG: GOAL 1: No Poverty; GOAL 2; Zero Hunger; GOAL 3: Good Health and Well-being

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

SIFAT FIZIKOKIMIA, PROFIL ANTIOKSIDAN DAN PEMATUHAN STANDARD KUALITI MADU KELULUT MENTAH DARI KAWASAN TERPILIH DI SEMENANJUNG MALAYSIA

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Madu Kelulut (SBH) adalah makanan yang sangat berkhasiat dan kandunganya dapat dipengaruhi oleh pelbagai faktor, seperti iklim, komposisi tanah, dan juga kawasan geografi di mana ia dihasilkan. Standard kualiti madu yang merangkumi pelbagai ciri amat penting untuk memastikan bahawa produk akhir yang dikeluarkan memenuhi standard untuk madu berkualiti tinggi dan tulen. Dalam kajian ini, empat puluh tiga sampel SBH (*Heterotrigona itama*) dikumpulkan dan dilabel sebagai <20 Pantai Barat (<20WC), <40 pantai barat (<40WC), <20 Pantai Timur (<20EC), <40 Pantai Timur (<40EC) dan pedalaman, sesuai dengan jarak mereka dari pantai. Pertama, pengoptimuman metodologi profil gula untuk kuantifikasi trehalulosa dilakukan menggunakan kromatografi cecair berprestasi tinggi (HPLC) dengan pengesan penyerakan cahaya penyejatan (ELSD). Kemudian, SBH dianalisis untuk sifat fisiokimia, profil gula, asid organik, komposisi mineral, sebatian bioaktif, dan kapasiti antioksidan; jumlah kandungan flavonoid (TFC), jumlah kandungan fenolik (TPC), ferik mengurangkan daya antioksidan (FRAP), ujian penghapus radikal bebas ABTS dan DPPH, dan kapasiti penyerapan radikal oksigen (ORAC) ujian. Klasifikasi SBH mengikut kawasan geografi dilakukan menggunakan Analisis Komponen Utama (PCA). Hasil kajian menunjukkan bahawa prosedur HPLC-ELSD untuk analisis gula dioptimumkan menggunakan tetapan 88% ACN: 12% DH_2O dan ELSD pada 60 psi. Terhalulose adalah gula paling banyak terdapat di madu *H. itama*, dengan purata 39.0 ± 13.06 g / 100g, dan prosedur yang dioptimumkan mencukupi dan dapat dihasilkan semula untuk mengenal pasti dan mengukur trehalulosa di dalam madu kelulut. Kandungan air, pH, konduksi elektrik (EC), hidroksimetilfurfural (HMF), dan komposisi gula dari semua sampel madu kelulut mematuhi Piawaian Madu Kelulut Malaysia (MS2683: 2017). SBH dari <40EC mempunyai warna yang lebih gelap dengan L* rendah dan parameter warna (a*, b*) yang signifikan lebih rendah (p<0.05) berbanding dengan SBH dari empat kawasan geografi yang lain. Kalium (K) didapati jauh (p<0.05) lebih tinggi dalam SBH dari kumpulan <40EC berbanding kumpulan pedalaman dan <20EC. Asid glukonat (GA) secara signifikan (*p*<0.05) lebih tinggi pada SBH dari kumpulan <40WC daripada kumpulan <20WC. Asid laktik, asetik, 3-fenilaktik (3-PLA) dan fenilalanin tidak signifikan (p>0.05) di SBH dari semua lima kawasan geografi. Kapasiti antioksidan; TFC, TPC, FRAP, dan ABTS jauh lebih tinggi pada sampel <40EC. Penghapus radikal bebas DPPH meningkat dengan ketara di SBH dari <20WC, sementara SBH dari <20EC mempunyai aktiviti ORAC yang lebih tinggi. PCA menunjukkan bahawa diskriminasi SBH menurut lima kawasan geografi yang berbeza tidak dapat dilaksanakan dan menunjukkan kesamaan antara sampel. Korelasi Pearson menunjukkan bahawa semua kapasiti antioksidan menunjukkan hubungan yang cukup positif dengan EC, acid bebas (FA), magnesium (Mg), K, phosphorus (P), asetik, dan asid laktik. TFC dan TPC juga menunjukkan hubungan sederhana hingga kuat dengan FRAP dan ABTS. 3-PLA juga mempunyai korelasi positif dengan fenilalanin. Sebaliknya, L* dan b* menunjukkan hubungan negatif sederhana hingga kuat dengan kapasiti antioksidan. Untuk penyelidikan lebih lanjut, melakukan penyelidikan yang lebih teliti terhadap kedua-dua bahan kimia fenolik dan mudah meruap dapat meningkatkan keupayaan untuk membeza-bezakan antara SBH bergantung pada asal geografi mereka, dan juga termasuk lebih banyak sampel dari Malaysia Timur (Sabah dan Sarawak) untuk melihat pencapjarian SBH tersebut berdasarkan kawasan geografi.

Kata Kunci: *Heterotrigona itama*; Kawasan geografi; Madu kelulut; Standard kualiti; Trehalulosa

SDG: *GOAL 1: No Poverty; GOAL 2; Zero Hunger; GOAL 3: Good Health and Well-being*

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LIST OF ABBREVIATIONS

ELSD	Evaporative light scattering detector
ORAC	Oxygen radical absorbance capacity
PCA	Principal component analysis
WC	West Coast
EC	East Coast
DPPH	2,2-Diphenyl-1-picrylhydrazyl
ABTS	2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
TPTZ	2,4,6-tri (2-pyridyl)-s-triazine
AAPH	2,2-Azobis(2-methyl-propionamidine) dihydrochloride
$AlCl_3$	Aluminium chloride
Na_2CO_3	Sodium carbonate
$FeCl_3$	Iron (III) chloride
CH_3CO_2Na	Sodium acetate
HCl	Hydrochloric acid
$FeSO_4$	Iron (II) sulphate
$K_2S_2O_8$	Potassium persulfate
SPE	Solid phase extraction
ACN	Acetonitrile
DH_2O	Deionised water
TE	Trolox equivalent
QE	Quercetin equivalent
GAE	Gallic acid equivalent
3-PLA	3-phenyllactic acid

CHAPTER 1

INTRODUCTION

1.1 Background

A stingless bee known as "kelulut" in Malaysia is from the tribe Meliponini, a eusocial insect living in the colony (Trianto & Purwanto, 2022). They produced a golden liquid known as stingless bee honey (SBH), which is rich in quality and suitable for human consumption. SBH is known as a sweet natural substance that is produced by stingless bees from the nectar of the plant from the secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect and transform by combining with the specific substances of their own, deposit, dehydrate, store and leave in the honeypots to ripen and mature (Ávila et al., 2018; CODEX, 2019). SBH consist of a primary composition of sugar and water with other minor constituents such as organic acids, phenolic acids, flavonoids, protein, amino acids, and minerals (Braghini et al., 2021; Gomes et al., 2022; Shamsudin et al., 2022).

The absence of international CODEX standards for SBH is a significant issue. The current standard for honey quality reference, the Codex Alimentarius Commission (CODEX) Standard, last revised in 2019 (CODEX, 2019), was established for honey produced by bee species, not *meliponine* (stingless bee). The lack of a CODEX standard for stingless bees can be attributed to several factors, including the diverse nature of SBH, produced in many countries in subtropical and tropical regions (Grüter, 2020), leading to variations in SBH composition. Another contributing factor could be the lower production of SBH, which has not yet been commercialized on a large scale like *Apis* honey. Limited regional studies on SBH may also be a factor, as each region has unique characteristics. In Malaysia, a specific SBH standard, the Malaysian Standard for Kelulut (Stingless bee) honey-Specification (MS2683:2017), was established in 2017 and revised in 2019 to address the non-adherence to the CODEX Standard issue of SBH produced and sold by Malaysian beekeepers.

SBH generally differs from *Apis* honey regarding taste, colour, and viscosity. SBH possesses a unique sweet taste with a sour and acidic taste due to high acidity and low pH (Souza et al., 2021; Esa et al., 2022). It also has a wide range of colours depending on the mineral, botanical and geographical origin (Fatima et al., 2018). The viscosity of SBH is lower due to high moisture content with a watery texture (Ávila et al., 2018). SBH is especially special compared to *Apis* honey due to the presence of trehalulose sugar. Recent discoveries by Fletcher et al. (2020) have reported that SBH contains trehalulose as one of the primary sugars, other than fructose and glucose. Moreover, *Geniotrigona thoracica*, a stingless bee native to Malaysia, produced honey that contained 84% (w/w) trehalulose out of the total sugar. Other SBH species reported to contain high trehalulose are *Heterotrigona itama, Tetragonula carbonaria* and *Tetragonula hockingsi* species (Fletcher et al., 2020; Zawawi et al., 2022).

SBH is not just a unique culinary delight, but it also offers significant health benefits. It is known for its antioxidant properties, which are attributed to bioactive compounds such as flavonoids, phenolic acids, organic acids, vitamins, and other phytochemicals (Al-Hatamleh et al., 2020; Braghini et al., 2021; Zulkifli et al., 2023). However, the quantity of these compounds can vary due to factors such as floral sources, geographical region, and the processing, handling, and storage of honey (Raja Nurfatin et al., 2021; Souza et al., 2021; Sujanto et al., 2021). The unique composition of SBH, influenced by factors such as the type of nectar chosen and the distance travelled during foraging, can result in distinct SBH compositions in terms of flavonoid and phenolic acid profiles (Majid et al., 2019, 2020).

The problem among meliponiculturists is the need for a standard for referencing the SBH quality (Ng et al., 2021). Although there are Codex Standard for honey and MS2683:2017, both standards do not fully serve the SBH as the Codex only specifies Apis honey, and MS2683:2017 was limited to few characteristics and lacking in specific crucial parameters such as free acidity, total soluble solid (TSS) and minerals (Zawawi et al., 2022). Other than that, many factors affect the composition of each SBH, such as species, geographical and botanical origin, climate and seasons (Braghini et al., 2021) which a large number of SBH samples were needed to serve as data for the revision of current MS2683:2017 and to adopt the new international standard for SBH.

1.2 Problem Statement

Stingless bee honey (SBH) has gained significant popularity among Malaysians, who have embraced it as a daily diet due to its potential health benefits (Saludin et al., 2019). This surge in demand for safe and high-quality SBH has presented a challenge for bee farmers, as the raw Malaysian stingless bee honey's physicochemical qualities do not meet the International Codex Standard of honey and only partially comply with the Malaysian Standard for Kelulut Honey (MS2683:2017). Notably, the moisture content of SBH often exceeds the International Codex Standard (ICS), which sets the limit at less than 20%.

However, the majority moisture content of SBH is within the range set in MS2683:2017, which is less than 35%. The discovery of trehalulose sugar as one of the leading sugar compositions in stingless bee honey has necessitated a thorough examination of SBH's physicochemical profile. The latest amendment of MS2683:2017 only acknowledged the presence of trehalulose without specifying the limit range for its concentration. This finding, coupled with the reported beneficial effects of trehalulose sugar, such as anti-diabetic, acariogenic, and low glycemic index properties, has underscored the need for a new international standard exclusively for SBH. Therefore, determining the physicochemical properties, especially the quantification of trehalulose in SBH, will not only enhance Malaysia's existing stingless bee honey standard, MS 2683:2017 but also pave the way for a new international standard for SBH.

Based on the literature, SBH also has high antioxidant activities (Cheng et al., 2019; Majid et al., 2020). They can scavenge free radicals, transfer hydrogen atoms or single electrons, and chelate metal ions (Santos-Sánchez et al., 2019). SBH's chemical composition varies in physicochemical characteristics and antioxidant capacities based on geographical location (Silva et al., 2013; Biluca et al., 2016; Shamsudin et al., 2019; Braghini et al., 2021; Gomes et al., 2022). Studies on SBH antioxidant capacities from various geographical areas are still lacking, though a few have evaluated the antioxidant activities and identification of bioactive compounds in honey (Tuksitha et al., 2018; Shamsudin et al., 2019; Biluca et al., 2020; Majid et al., 2020; Maringgal et al., 2021).

Prior studies used principal component analysis (PCA) and hierarchical clustering analysis (HCA) to differentiate honey based on its physicochemical and antioxidant properties. However, the sample size was small n= 14 (Kek et al., 2018) and n=6. (Shamsudin et al., 2019). There is an association of physicochemical and bioactive compounds with antioxidant capabilities in honey. However, much research has yet to be done on the correlation between the many samples being examined. Previous research by Zawawi et al. (2022) investigated the correlation of physicochemical characteristics with total phenolic content in smaller sample sizes from Malaysia. Therefore, this study aimed to determine the antioxidant activities of SBH collected from five distinct geographical locations, classify the SBH according to geographical location using PCA, and examine the correlation between physicochemical properties and antioxidant capacities using Pearson's correlation.

1.3 Scope of study

This study focuses on the physicochemical properties, bioactive compounds' quantification, and stingless bee honey's antioxidant activities in different geographical areas in Peninsular Malaysia. The SBH samples were divided into five geographical areas across Peninsular Malaysia based on their distance from the East and West coasts. The choice of different geographical locations is used to study the effect of the geographical location on the composition of the SBH in terms of physicochemical properties, bioactive compounds, and antioxidant activities. The division of samples into five distinct geographical locations is attributed to the rich diversity and abundance of floral species in each area and environmental factors such as weather, seasons, and soil composition. By categorizing samples according to specific distances from each coast (less than 20 km, less than 40 km, and inland), we create a distinct area to understand variation in those specific areas better.

1.4 Objectives

The general objective of this study is to study the physicochemical properties, bioactive compounds, and antioxidant activities of SBH from different geographical areas consisting of inland, east and west coasts and the effects of geographical areas on SBH quality in comparison to the quality standard (ICS and MS2683:2017). The comparison of SBH quality with the standards provides a better understanding of the quality and authenticity of the SBH and improves the current standard of MS2683:2017.

Therefore, the specific objectives are:

1. To optimise sugar profile methodology for trehalulose quantification using HPLC-ELSD.
2. To determine the physicochemical properties and bioactive compounds of SBH from five different geographical areas in Peninsular Malaysia.
3. To determine the antioxidant activities of SBH from five different geographical areas across Peninsular Malaysia and the correlation of bioactive compounds with antioxidant activities in SBH.

1.5 Flow chart of the thesis outline

Chapter 1 – Background of study

Introduction about the stingless bee honey, problem statement and the objectives of the studies.

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Chapter 2 – Literature review

Previous research on the physicochemical properties of stingless bee honey, polyphenol components, and antioxidant activities regarding both the International Codex standard and MS2683:2017, as well as the effects of geographic regions on SBH quality.

↓

Chapter 3 – Optimization of sugar profile methodology using high-performance liquid chromatography with evaporative light scattering detector (HPLC-ELSD)

Isolate trehalulose from SBH for use as a standard and confirm purity with liquid chromatography-mass spectrometry (LCMS).

Further detects and quantifies trehalulose and other sugars using the improved HPLC-ELSD method.

↓

Execute antioxidant activities in SBH (total phenolic content, total flavonoid content, ferric reducing antioxidant power, DPPH and ABTS radical scavenging activities, and oxygen radical absorbance capacity).

Investigate the relationship between bioactive compounds and SBH antioxidant activities.

↓

Chapter 6 – Conclusion and recommendation for further research

To conclude the findings and recommendations for further study.

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