DISEASE SCREENING AND ANTIMICROBIAL ACTIVITY OF STINGLESS BEES (*Heterotrigona itama* Cockerell AND *Geniotrigona thoracica* Smith) IN MALAYSIA

By

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Chair : Lau Wei Hong, PhD
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Honeybees are vulnerable to diseases and pests. The presence of pests and diseases in honeybee colonies had caused the honey industry to face many difficulties in the past. Stingless bees are the alternative producer for honey production. The occurrence of honeybee diseases in stingless bees have been reported in oversea and the health status of stingless bees in Malaysia is unknown. Thus, this was the first attempt in Malaysia to screen the health of stingless bees to see if they were free from honeybee diseases. Adults of *Heterotrigona itama* and *Geniotrigona thoracica* were collected from eight different farms and tested negative to seven known honeybee diseases. During the sampling, one *G. thoracica* colony at the Department of Plant Protection, UPM was detected having white mycelia on its propolis, and another *H. itama* colony at the Infoport Halal Hub, UPM was collapsed with white dust covered on the propolis. Isolation of the fungi were done, and pure colonies were obtained. The fungus isolated from the *G. thoracica* colony was confirmed as *Lasiodiplodia theobromae*, while the fungus isolated from *H. itama* colony was confirmed as *Aspergillus caelatus* by morphological and molecular identification. A pathogenicity test was conducted using both the fungus species and last instar larvae the stingless bees. The LC_{50} value of 9.16 x 10^3 conidia/µl was recorded in *H. itama* larvae infected *with L. theobromae.* It was not pathogenic to *G. thoracica* (22% mortality). When *H. itama* and *G. thoracica* were inoculated with *A. caelatus*, 100% mortality was obtained in both stingless bee species. The LC_{50} value were 7.58 x 103 conidia/µl for *H. itama* and 6.24 x 10^3 conidia/µl for *G. thoracica*. *A. caelatus* was confirmed pathogenic to both *H. itama* and *G. thoracica*. Antimicrobial susceptibility test was carried out using the hemolymph of the last instar larvae of *H. itama* to test against *A. caelatus*. No inhibition was observed in the treatment with either induced or non-induced hemolymph at the end of the broth microdilution assay. The positive control, intraconazole, had recorded 70.23% inhibition. Again, *A. caelatus* was confirmed very pathogenic to *H. itama* and the insect host immunity was able to

fight against *A. caelatus*. The antimicrobial susceptibility test was not carried on *G. thoracica* with both fungus species and *H. itama* with *L. theobromae* as the bee colonies were stolen during the Covid-19 pandemic.

Keywords: Stingless bees, Entomopathogenic fungus, Fungal disease, Bee health status

SDGs: No poverty, Zero hunger, Responsible consumption and production

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

PEMERIKSAAN PENYAKIT DAN AKTIVITI ANTIMICROBIAL KELULUT (*Heterotrigona itama* Cockerell DAN *Geniotrigona thoracica* Smith) DI MALAYSIA

Oleh

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Lebah madu terdedah kepada penyakit dan perosak. Kehadiran perosak dan penyakit dalam koloni lebah madu menyebabkan industri madu menghadapi pelbagai kesukaran pada masa lalu. Kelulut adalah pengeluar alternatif untuk pengeluaran madu. Kejadian penyakit lebah madu dalam kalangan kelulut telah dilaporkan di luar negara dan status kesihatan kelulut di Malaysia tidak diketahui. Justeru, ini merupakan percubaan pertama di Malaysia untuk menyaring kesihatan kelulut untuk melihat sama ada mereka bebas daripada penyakit lebah madu. Dewasa *Heterotrigona itama* dan *Geniotrigona thoracica* dikumpulkan dari lapan ladang berbeza dan diuji negatif kepada tujuh penyakit lebah madu yang diketahui. Semasa persampelan, satu koloni *G. thoracica* di Jabatan Perlindungan Tumbuhan, UPM telah dikesan mempunyai miselia putih pada propolisnya, dan satu lagi koloni *H. itama* di Hab Halal Infoport, UPM telah runtuh dengan debu putih yang dilitupi pada propolis. Pengasingan kulat telah dilakukan, dan koloni tulen diperolehi. Kulat yang diasingkan daripada koloni *G. thoracica* telah disahkan sebagai *Lasiodiplodia theobromae*, manakala kulat yang diasingkan daripada koloni *H. itama* telah disahkan sebagai *Aspergillus caelatus* melalui pengecaman morfologi dan molekul. Ujian patogenik telah dijalankan menggunakan kedua-dua spesies kulat dan larva instar terakhir iaitu lebah tanpa sengat. Nilai LC_{50} sebanyak 9.16 x 10^3 konidia/µl direkodkan dalam larva *H. itama* yang dijangkiti *L. theobromae*. Ia tidak patogenik kepada *G. thoracica* (22% kematian). Apabila *H. itama* dan *G. thoracica* disuntik dengan *A. caelatus*, 100% kematian diperolehi dalam kedua-dua spesies kelulut. Nilai LC_{50} ialah 7.58 x 10^3 konidia/µl untuk *H. itama* dan 6.24 x 10^3 konidia/µl untuk *G. thoracica*. *A. caelatus* telah disahkan patogenik kepada kedua-dua *H. itama* dan *G. thoracica*. Ujian kerentanan antimikrob telah dijalankan menggunakan hemolymph larva instar terakhir *H. itama* untuk menguji terhadap *A. caelatus*. Tiada perencatan diperhatikan dalam rawatan sama ada hemolimfa teraruh atau tidak teraruh pada akhir ujian mikrodilusi sup. Kawalan positif, intraconazole, telah merekodkan perencatan 70.23%. Sekali

lagi, *A. caelatus* disahkan sangat patogenik kepada *H. itama* dan imuniti perumah serangga dapat melawan *A. caelatus*. Ujian kerentanan antimikrob tidak dijalankan pada *G. thoracica* dengan kedua-dua spesies kulat dan *H. itama* dengan *L. theobromae* kerana koloni lebah telah dicuri semasa pandemik Covid-19.

Kata kunci: Keulut, Kulat entomopatogenik, Penyakit kulat, Status kesihatan lebah.

SDG: Tiada kemiskinan, Sifar kelaparan, Penggunaan dan pengeluaran yang bertanggungjawab
.

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LIST OF ABBREVIATIONS

MBRDT	Malaysian Beekeeping Research and Development Team
MARDI	Malaysian Agricultural Research and Development Institute
IOBC	Isolation organization for Biological Control
CCD	Colony collapse disorder
MIC	Minimum inhibitory concentration
EEP	Ethanolic extract of propolis
DWV	Deforwed wing virus
BQCV	Black queen cell virus
ABPV	Acute bee paralysis virus
IAPV	Israeli acute paralysis virus
CBPV	Chronic bee paralysis virus
SB	Sacbrood virus
AFB	American foulbrood bacteria
EFB	European foulbrood bacteria
mg	Milligram
g	Gram
µg	Microgram
ng	Nanogram
µM	Micromolar
µl	Microlitre
ml	Millilitre
s	Seconds
min	Minutes
h	Hour

°C	Degree celsius
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
PCR	Polymerase chain reaction
bp	Base pair
dH2O	Sterile distilled water
p.i	Post-inoculation

CHAPTER 1

INTRODUCTION

1.1 Background of Study

In Malaysia, honey is produced mainly by honeybees and stingless bees (kelulut). Honeybees are referred as *Apis mellifera* (European bee) and *Apis cerana* (Asian bee) while stingless bees are referred *Geniotrigona thoracica*, *Heterotrigona itama*, *Lepidotrigona terminata*, and *Tetragonula leaviceps* in Malaysia. The honey yield from *A. cerana* is about 5-9 kg per colony (Ismail, 2014), while *A. mellifera* are expected to produce up to 50 kg per colony. Despite the high honey production, honeybees are prone to pests and diseases (Ismail and Ismail, 2018). Stingless bees in Malaysia produce an average of 4 kg honey per colony each year (Mustafa et al., 2018). The stingless bee honey industry can collect RM 3.03 billion in annual sales if the industry is further developed (Bernama, 2020). The total market value of this stingless bee honey is RM33.6 million and the potential market value is RM 67.2 million.

Before meliponiculture bloom in Malaysia, Apiculture industry was acting as an additional source of income. Besides, Beekeeping is a source of food, raw materials for industries (beeswax candles and lubricants), medicine (honey, propolis, beeswax, and bee venom) (Ismail and Ismail, 2018). *Apis mellifera* produces more honey than *A. cerana* but they are prone to disease and pests. Colony Collapse Disorder (CCD), which occurred in 1996 and 1997 had caused many hurdles to the beekeeping development in Malaysia, where almost all the bees were destroyed. *Varroa destructor*, a mite that was carried by the *A. mellifera* did spread to other colonies and eventually killed them (Ismail, 2016), *Aethina tumida* is also a pest to honeybees in Malaysia and other countries such as Italy (Palmeri et al., 2014), United States and Africa (Evans et al., 2000). Other than pests, diseases associated with honeybees, namely American Foulbrood Disease, and European Foulbrood Disease (OIE, 2021) were reported in Malaysia. The disease and pests of bees are monitored by the Department of Agriculture (DOA), Malaysia. The *Varroa* sp. outbreak that occurred in 1997-2008 has caused honey production to decrease and this outbreak is suspected due to the imported queens from other countries. With the communication made with some local bee-keepers, it is known that the diseased colonies were normally discarded by burning. Since the honeybee was prone to disease and pest, new alternative species that was free from disease was needed. The introduction of stingless beekeeping in 2004 by MARDI was expected to fulfill the requirement (Ismail and Ismail, 2018).

Stingless bees species such as *G. thoracica*, *H. itama*, *L. terminata*, and *T. leaviceps* are the most commonly domesticated species from about 38 species of stingless bees found in Malaysia (Mustafa et al., 2018; Jaafar, 2012). As of

2021, there are 67,300 colonies of stingless bee recorded in Malaysia in which 37,000 colonies are found in Peninsular Malaysia and 30,300 colonies are found in West Malaysia (Sarawak, Sabah, and Labuan) (OIE, 2021). Many guidelines were made to protect beekeeping industry. Such guidelines include MS 2679:2017-Good Agriculture Practice: Farming of bee (tribe Apini) and the stingless bee (tribe Meliponini), and also MS2683:2017 Kelulut (stingless bee) honey specification (OIE, 2021). In 2019, the National Kelulut Honey Industry Development Plan 2020-2030 was launched with the aim to make the kelulut honey industry a new source of stable and sustainable income (Nation, 2020). The government also introduced myGAP (Malaysian Good Agricultural Practice) certification to ensure the safety of honey products to meet the world standards (Bernama, 2019).

Similar to honeybees, stingless bees had encountered pest problem in their colonies. Black Soldier fly (*Hermetia illucens*) infestation was reported in 2016 (Hashim et al., 2017) and this eventually caused a loss of over RM 1 million (Mustafa et al., 2018). Sap beetle (*Haptoncus luteolus*) and *A. tumida* were also identified as a pest to stingless bees (OIE, 2021; Hashim et al., 2017). The presence of pests and diseases in honeybee colonies had caused the honey industry to face many difficulties in the past. Similarly, if stingless bees have similar disease problem, the local beekeeping farming and honey production will face a big challenge like what happened in the late '90s. Honeybee diseases have been reported in the stingless bee colonies in Brazil (Teixeira et al., 2020; Uiera-Vieira et al., 2015) Australia (Purkiss and Lach, 2019), Argentina (Alvarez et al., 2018) and North America (Guzman-Novoa et al., 2015). Diseases of honeybee has been detected in stingless bees in overseas, this has increased the curiosity to determine if stingless bees in Malaysia are also affected by honeybee disease. Thus, this was the first attempt to screen the health status of stingless bees in Malaysia.

1.2 Objectives of the study

The objective of this study are as follows;

1. To assess the health of stingless bee (*H. itama* and *G. thoracica*) colonies in Selangor, Negeri Sembilan and Melaka.
2. To identify and characterize the fungi found on the colonies of stingless bees.
3. To reconfirm the antimicrobial susceptibility of *H. itama* and *G. thoracica* against the pathogen, *A. caelatus* at lower conidial concentration.

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