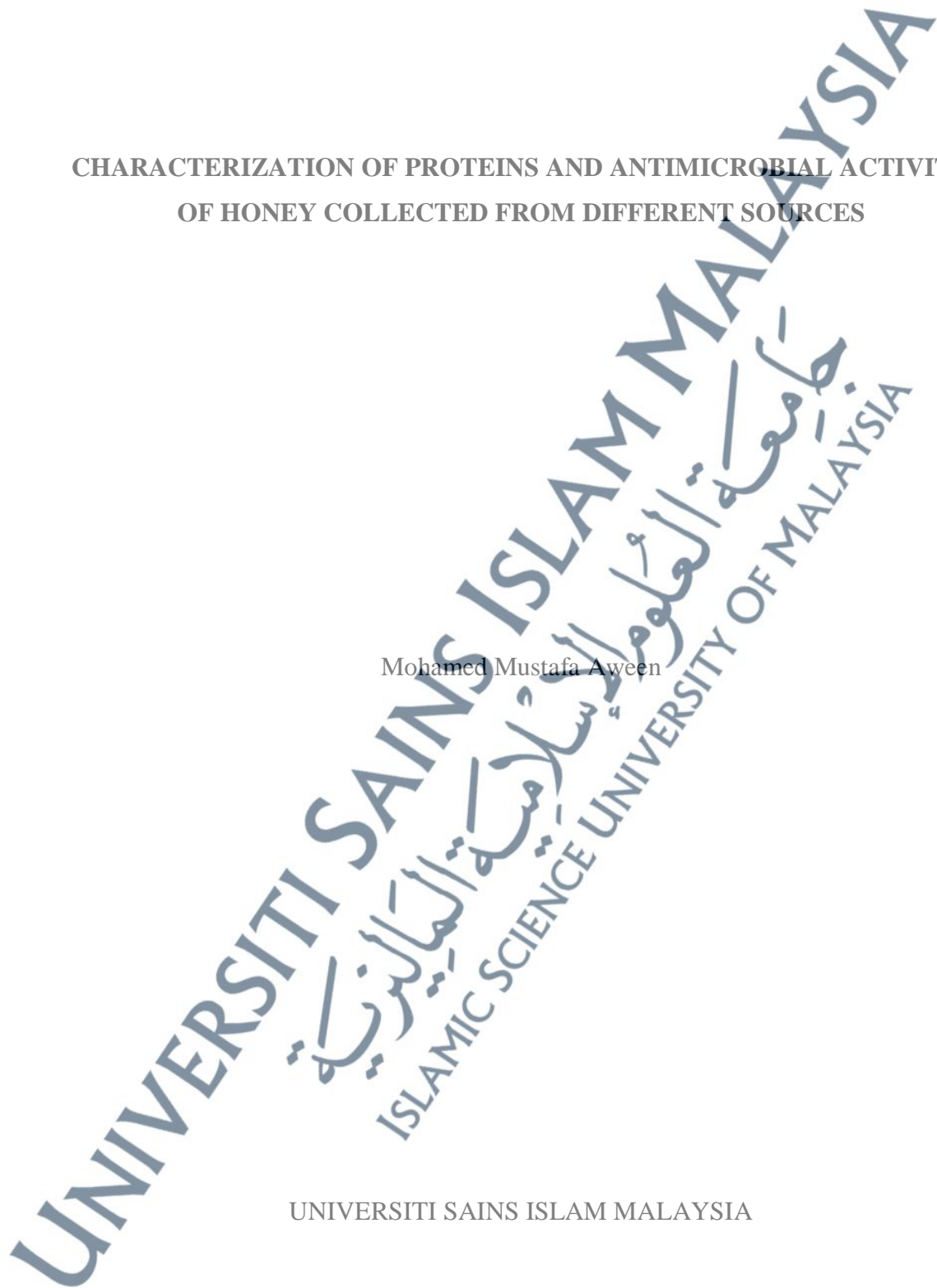


CHARACTERIZATION OF PROTEINS AND ANTIMICROBIAL ACTIVITY  
OF HONEY COLLECTED FROM DIFFERENT SOURCES

Mohamed Mustafa Aween



UNIVERSITI SAINS ISLAM MALAYSIA

**CHARACTERIZATION OF PROTEINS AND ANTIMICROBIAL ACTIVITY  
OF HONEY COLLECTED FROM DIFFERENT SOURCES**

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(Matric. No. 4120060)

Thesis submitted in fulfillment for the degree of  
Doctor of Philosophy in Science and Technology

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UNIVERSITI SAINS ISLAM MALAYSIA

NILAI

January 2016

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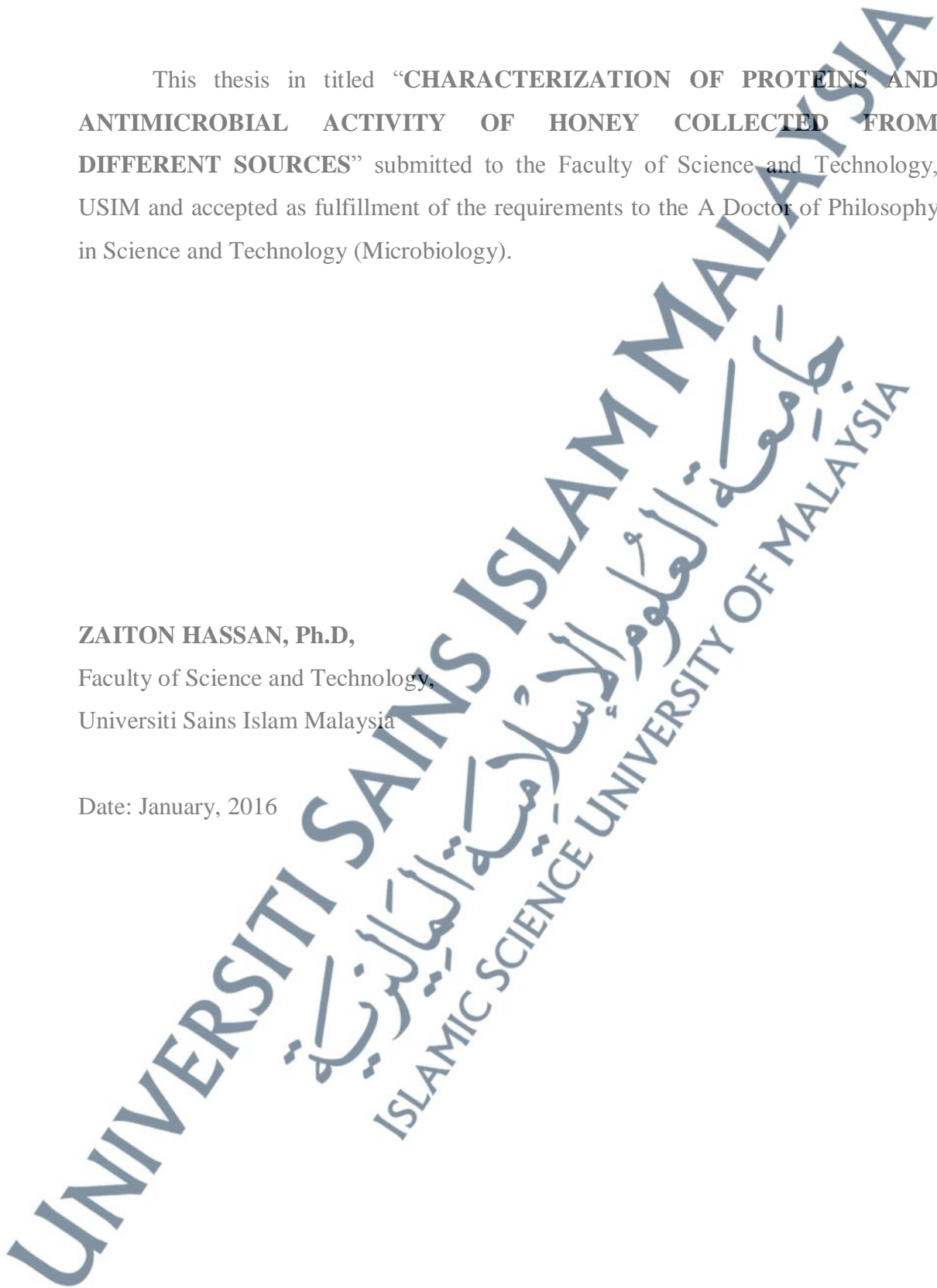
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## APPROVAL

This thesis in titled “**CHARACTERIZATION OF PROTEINS AND ANTIMICROBIAL ACTIVITY OF HONEY COLLECTED FROM DIFFERENT SOURCES**” submitted to the Faculty of Science and Technology, USIM and accepted as fulfillment of the requirements to the A Doctor of Philosophy in Science and Technology (Microbiology).

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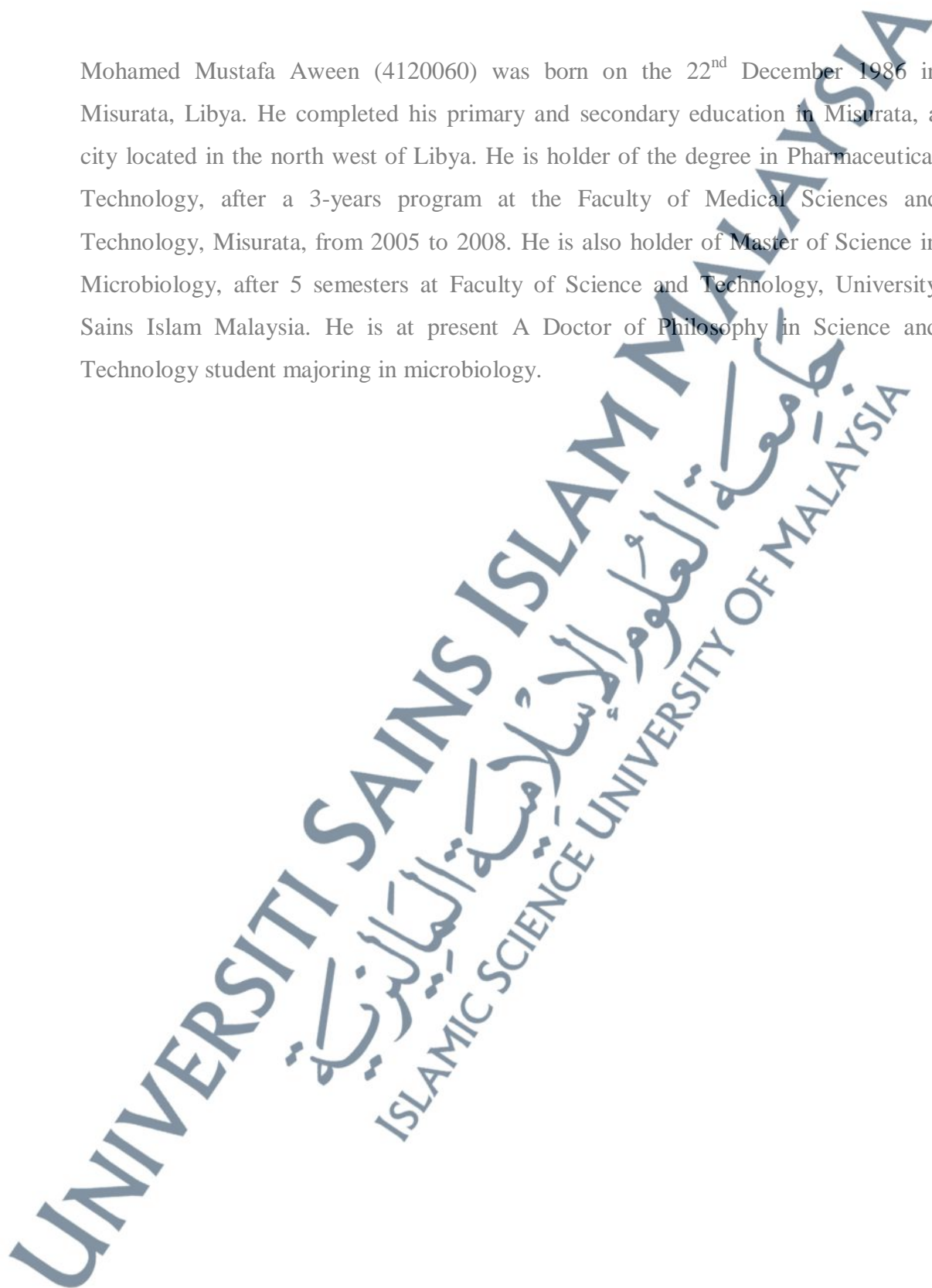
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## ABSTRAK

Terdapat beberapa sebatian yang terlibat dalam aktiviti antimikrob pada madu. Kajian ini adalah untuk menentukan sama ada peptida menyumbang kepada aktiviti antimikrob terhadap beberapa patogen tahan rintangan kepada pelbagai antibiotik. Sampel madu yang diperolehi dari pelbagai sumber (Malaysia, Libya dan New Zealand) telah dinilai bagi ciri-ciri antimikrob terhadap *Staphylococcus aureus*, *Salmonella Typhimurium*, *Escherichia coli*, *Bacillus subtilis* dan *Pseudomonas aeruginosa* menggunakan lima kaedah. Aktiviti antimikrob madu telah dikesan pada kepekatan yang lebih rendah (12%) dan kaedah plat microtiter adalah kaedah terbaik. Pengubahsuaian pH kepada pH 3, 5 dan 7 menghasilkan sedikit perubahan pada aktiviti antimikrob bergantung kepada sumber madu. Aktiviti antimikrob madu tidak dipengaruhi oleh pemanasan pada suhu 80°C dan 100°C selama 10 minit, tetapi sedikit peningkatan aktiviti antimikrob dikesan setelah pemanasan pada 121°C selama 10 minit. Perawatan sampel madu dengan enzim pepsin dan chymotrypsin menurunkan aktiviti antibakteria semua sample madu kecuali madu Tualang (H026) dan madu Acacia (H031) yang menunjukkan peningkatan antimikrob yang signifikan ( $p < 0.05$ ) terhadap semua patogen, menunjukkan protein terlibat dalam aktiviti antimikrob. Kandungan protein pada madu adalah berbeza (0.315 kepada 1.426 mg/ml): H026 (madu Tualang, 1.426 mg / ml), H020 (madu Hannon, 1.289 mg / ml), H032 (madu Acacia, 1.203 mg / ml), H031 (madu Acacia, 0.567 mg / ml), H035 (madu Manuka, 0.570 mg / ml), dan H030 (madu Acacia, 0.315 mg / ml). Jumlah kandungan asid amino di dalam madu adalah dalam lingkungan  $1.290 \pm 0.027$  hingga  $2.670 \pm 0.580$  g/100 g dan jumlah asid amino ini berbeza di antara semua sampel madu. Madu Acacia mengandungi lapan daripada asid amino perlu terutamanya threonine ( $0.044 \pm 0.013$  g/100g) dan tertinggi dalam asid amino tidak perlu (asid aspartik dan tyrosina). Kandungan serina, glysina, methionina dan lysina dikesan paling tinggi pada madu Alseder. Kandungan peptida yang diperolehi melalui kaedah OPA adalah paling tinggi pada sampel H026 (madu Tualang, 1.542 mg / ml) diikuti oleh H032 (madu Acacia, 1.140 mg / ml) dan jumlah yang paling rendah adalah dari sampel H035 (madu Manuka, 0.076 mg / ml). Peptida tidak dapat dikesan pada sampel H020 (madu Hannon) dan H027 (madu Manuka). Sebahagian kecil terkumpul 7 hingga 13 yang diperolehi dari Sephadex 50G kromatografi turus halus menunjukkan protein dengan berat molekul yang berbeza-beza (6-250 kDa) dapat dikesan menggunakan SDS-PAGE. Pecahan protein sampel madu kemudiannya dipecahkan menggunakan RP-HPLC. Aktiviti antimikrob adalah berkaitan dengan kandungan peptide dimana sebahagian kecil terkumpul 7 daripada sampel H032 (madu Acacia) memberikan nilai peptida tertinggi (3.27 mM) dan aktiviti antimikrob yang tinggi (78, 53%), manakala sebahagian kecil terkumpul 3 dan 4 dari H026 (madu Tualang) menunjukkan kandungan peptida yang lebih rendah (3.11 dan 2.76 mM) tetapi aktiviti antimikrob yang lebih tinggi (96.02 dan 78.67%). Pecahan 7 ke 13 yang diperolehi daripada kromatografi Sephadex G-50 Fine menunjukkan protein dengan berat molekul yang berbeza (6 ke 250 kDa) dikesan menggunakan SDS-PAGE. Pecahan 9 daripada sampel H026 (madu Tualang) dan pecahan 11 daripada sample H032 (madu akasia) menunjukkan kandungan peptida yang tinggi (0.937 dan 0.45 mg/ml) dan aktiviti antibakteria sederhana (41.02 dan 37.26%), masing-masing. Lima belas peptida *de novo* telah dikesan daripada H026 (madu Tualang) dan lima peptide *de*

*novo* daripada H032 (madu Acacia). Oleh itu, kajian ini menunjukkan bahawa madu mengandungi peptida antibakteria dari sumber yang telah dikenalpasti dan *de novo* yang menyumbang kepada aktiviti antimikrob madu.

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## ABSTRACT

A number of compounds have been implicated for the antimicrobial activity of honey. This study was carried out to determine if peptides contributed to antimicrobial activity against selected multiantibiotic resistant pathogens. Honey samples obtained from different sources (Malaysian, Libyan and New Zealand) were evaluated for the antimicrobial properties against *Staphylococcus aureus*, *Salmonella* Typhimurium, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* using five methods. The antimicrobial activity of honey was detected at lower concentration (12%) and microtiter plate method was the best. Adjusting the pH of honey to 3, 5 and 7 resulted in slight changes in antibacterial activity either increase or decrease depending on source of honey. The antibacterial activity of honey samples was not affected by heating at 80 and 100°C for 10 min, but a slight increase in activity was detected after heating at 121°C for 10 min. Treating honey samples with the enzymes chymotrypsin and pepsin decreased antibacterial activity of honey samples except for Tualang honey (H026) and Acacia honey (H031) which showed significant ( $p < 0.05$ ) increase against all pathogens, indicating protein were involved in the antimicrobial activity. The protein content of honey samples varied from 0.315 to 1.426 mg/ml, H026 (Tualang honey, 1.426 mg/ml), H020 (Hannon honey, 1.289 mg/ml), H032 (Acacia honey, 1.203 mg/ml), H031 (Acacia honey, 0.567 mg/ml), H035 (Manuka honey, 0.570 mg/ml), and H030 (Acacia honey, 0.315 mg/ml). Total amino acids content of honey was in the range of  $1.290 \pm 0.027$  to  $2.670 \pm 0.580$  g/100g and consists of variable amounts of essential amino acids among the honey samples. Acacia honey contains eight of the essential amino acids especially threonine ( $0.044 \pm 0.013$  g/100g) and, highest concentrations of the non-essential amino acids (aspartic acid and tyrosine). High concentration of serine, glycine, methionine and lysine were detected in Alseder honey compared to other honey samples. The peptide content as evaluated by OPA method showed that H026 (Tualang honey) was 1.542 mg/ml followed by H032 (Acacia honey, 1.140 mg/ml) and the lowest amount was shown by H035 (Manuka honey, 0.076 mg/ml). Peptide was not detected in H020 (Hannon honey) and H027 (Manuka honey). Protein fractions of honey samples were further separated by RP-HPLC. The antimicrobial activity seems to be related to the peptide content; fraction 7 from H032 (Acacia honey) showed the highest peptide content (3.27 mM) and high antibacterial activity (78.53%), while fractions 3 and 4 from H026 (Tualang honey) showed slightly lower peptide content (3.11 and 2.76 mM, respectively), but higher antibacterial activity (96.02 and 78.67%). Fractions 7 to 13 obtained from Sephadex G-50 Fine column chromatography showed proteins with different molecular weights (6 to 250 kDa) as detected using SDS-PAGE. Fractions 9 from sample H026 (Tualang honey) and fractions 11 from sample H032 (Acacia honey) showed high peptide content (0.937 and 0.450 mg/ml) and moderate antibacterial activity (41.02 and 37.26 %, respectively). Fourteen *de novo* peptides were detected from of Tualang honey and five *de novo* peptides from Acacia honey. Therefore, this study showed that honey contains antibacterial peptides both from known sources and *de novo* that contribute to the antimicrobial activity of honey.

## المخلص

عدد من المركبات هي المسؤولة على الفعالية المضادة للميكروبات من العسل. هذه الدراسة أجريت لتري ادا البيبتيدات من العسل هي المسؤولة عن فعالية العسل المضادة للميكروبات التي لا تتأثر بالمضادات الحيوية. عينات العسل جابت من عدة مصادر (ماليزيا، ليبيا و نيوزيلاندا) وأختبرت فعاليتها المضادة للبكتيريا ضد ستافيلوكوكوس ايربوس، سالمونيلا تايفيميريوم، ايشيرشيا كولاي، باسيلس سبتيلس و سيدوموناس ابروجيناس باستخدام خمس طرق. الفعالية المضادة للبكتيريا اختبرت حتى بتركيز قليل (12%) وطريقة الميكروتيتير بليتس كانت الأفضل. تثبتت درجة حموضة العسل على 3، 5 و 7 نتج عنه تغيير بسيط في الفعالية المضادة للبكتيريا بلارتفاع أو الانخفاض معتمد على مصدر العسل. فعالية العسل المضادة للبكتيريا لم تتأثر بالتسخين الى درجة حرارة 80 و 100 مئوية لمدة عشر دقائق، لكن زيادة في الفعالية لوحظت عند التسخين الى 121 درجة مئوية لمدة عشر دقائق. تحليل العسل بأنزيمات الكيموتربسين والببسين نتج عنه انخفاض في الفعالية المضادة للبكتيريا ماعدا عسل التوالانق الماليزي (H026) و الأكاشيا الماليزي (H031) والتي اوضحت زيادة ملحوظة ( $p > 0.05$ ) في الفعالية المضادة للبكتيريا ضد كل البكتيريا المختبرة، الذي يبين وجود البروتين في عينات العسل. محتوى البروتين في عينات العسل يتراوح من 0.315 الى 1.426 ملج/مل، H026 (عسل التوالانق، 1.203 ملج/مل)، H020 (عسل الحنون، 1.289 ملج/مل)، H032 (عسل الأكاشيا، 1.203 ملج/مل)، H031 (عسل الأكاشيا، 0.567 ملج/مل)، H035 (عسل المنوكا، 0.570 ملج/مل) و H030 (عسل الأكاشيا، 0.315 ملج/مل). اجمالي الأحماض الأمينية في العسل كانت تتراوح من  $0.027 \pm 1.290$  الى  $0.580 \pm 2.670$  جم/10جم و ايضا يحتوي على الأحماض الأمينية الأساسية باختلاف عينات العسل. عسل الأكاشيا يحتوي على ثمان أحماض أمينية أساسية وخاصة ثيرونين ( $0.013 \pm 0.044$  جم/100جم) وأعلى تركيز للأحماض الأمينية الغير أساسية (حمض الأسبارتيكو الثيروسين). تراكيز عالية من السيرين، الجليسين، ميثيونين واللايسين اكتشفت في عسل السدر مقارنة بعينات العسل الأخرى. محتوى البيبتيدات حلل باستخدام طريقة الأوبي أي (OPA) و بين أن H026 (عسل التوالانق) احتوى على 1.542 ملج/مل ويتبعه H032 (عسل الأكاشيا، 1.140 ملج/مل) والأقل تركيز من البيبتيدات كان من H035 (عسل المنوكا، 0.076 ملج/مل). البيبتيدات لم تكتشف في H020 (عسل الحنون) و H027 (عسل المنوكا). عزلات البروتينات من العسل فصلت باستخدام جهاز الأوبي أنش بي أل سي (RP-HPLC). الفعالية المضادة للبكتيريا للعزلات من الممكن ان تكون مرتبطة بتركيز البيبتيدات. العزلة 7 من H032 (عسل الأكاشيا) أظهرت أعلى تركيز للبيبتيدات (3.27 مل مولر) وفعالية مضادة للبكتيريا عالية (78.53%)، وبينما العزلة 3 و 4 من H026 (عسل التوالانق) أظهرت تركيز بيبتيدات بنسبة بسيطة أقل (3.11 و 2.76 مل مولر، تتابعي) ولكن أعلى في الفعالية المضادة للبكتيريا (96.02 و 78.67%). العزلة 9 من H026 (عسل التوالانق) و العزلة 11 من H032 (عسل الأكاشيا) أظهرت تركيز عالي من البيبتيدات (0.937 و 0.450 ملج/مل، تتابعي) وفعالية مضادة للبكتيريا متوسطة (41.02 و 37.26%، تتابعي). خمسة عشر بيبتيدات غير معروفة صنف من عزلات H026، بينما خمسة بيبتيدات غير معروفة من عزلات H032. يستنتج أن، العسل يحتوي على بيبتيدات غير معروفة ولها فعالية مضادة للبكتيريا وهي مسؤولة عن فعالية العسل المضادة للبكتيريا.

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## LIST OF ABBREVIATION AND SYMBOLS

H020	Hannon honey
H025	Alseder honey
H026	Tualang honey
H027	Manuka honey (5+)
H028	Kharoob honey
H030	Acacia honey
H031	Acacia honey 2
H032	Acacia honey 3
H035	Manuka honey 2 (10+)
$\mu\text{L}$	microliter
$^{\circ}\text{C}$	Degree Celsius
a*	redness
BSA	Bovine serum albumin
$\text{CaCO}_3$	Calcium carbonate
CFU/g	Colony forming unit/gramme
cm	Centimetres
$\text{CO}_2$	Carbondioxide
ddH <sub>2</sub> O	Distilled water
dH <sub>2</sub> O	Deionised water
g	Grammes
GC-MS	Gas chromatography-mass spectrometry
AMPs	Antimicrobial peptides
h	Hours
H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HCL	Hydrochloric acid

Hz	Hertz
KDa	Kilodalton
Da	Dalton
Kg	Kilogramme
Kg/g	Kilogramme/gramme
Mg	Milligramme
mg/g	Milligramme/gramme
mg/kg	Milligramme/kilogramme
mg/ml	Milligramme/milliliter
ml	millilitres
mL/kg	millilitres/kilogramme
mM	millimollar
Mmol/g	millimole/gramme
rpm	revolution per minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
OPA	O-phthalaldehyde
v/v	volume/volume
w/v	weight/volume
HPLC	High Performance Liquid Chromatography
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
<i>S. Typhimurium</i>	<i>Salmonella</i> Typhimurium
<i>E. coli</i>	<i>Escherichia coli</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
MAR	Multiple Antibiotic Resistance