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ISOLATION AND CHARACTERIZATION OF CONDENSED TANNIN FROM Acacia mangium WILLD. AND ITS ANTIBACTERIAL ACTIVITY

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By

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Chairman : Associate Professor Loong Yik Yee, PhD
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Three experiments have been conducted to extract and evaluate the ability of tannins to bind with proteins and also their inhibition towards the growth of several types of pathogenic skin bacteria. The types of tannins and their molecular weight are believed to be the main factors influencing their activity towards proteins and microbes. In the first experiment, condensed tannin was extracted from several parts of *Acacia mangium* including leaves, stems, and pods. Sephadex LH-20 was used to separate condensed tannin (CT) from other phenolic compounds contains in the crude extracts. The amount of purified condensed tannin was measured by modified Vanillin-HCl assay method using cathechin as the standard. The concentration of extracted condensed tannin was found to be different in each part. The amount of tannins was calculated based on dry matter (DM) basis. Leaves were found to contain the highest amount which is 3.28% DM, whereas in stems and pods contains 0.98% DM and 1.54% DM, respectively.

In second experiment the protein-binding affinity (PBA) of extracted condensed tannins was determined using a protein precipitation assay with bovine serum albumin (BSA) used as the standard proteins. The affinity was measured based on b-value (the b-value represent the quantity of condensed tannin needed to bind half of the maximum precipitable BSA). The average b-value of isolated CT was 0.53 with standard deviation of 0.048.

In the third experiment, the antibacterial activity of extracted CT was evaluated. There were three sub-experiment were conducted to evaluate the activity. The first experiment was to measure the zone of inhibition of tested bacteria against 100 mg/mL CT extracts by agar diffusion test. Then, the minimum inhibitory were measured by using microdilution test. Lastly in the third experiment, the log reduction of numbers of bacteria based on time incubation and also by concentration was quantified by time-kill assay.

From the study, it showed that all tested bacterium were susceptible to the CT at the concentration of 100 mg/mL, with diameter zone of inhibition of ranged between 12.0 to 13.0 mm. However, the minimum inhibitory concentrations (MIC) were different between the isolates. Compared to resistant strains, *S. aureus* ATCC 29213 was found to be inhibited by CT extract at low concentration (1 mg/mL) and it also was killed after 6 hours exposure with 10 mg/mL CT extracts. On the other hand, all the MRSA isolates had varied inhibitory effects. The MIC values were in the range of 2 mg/mL to 10 mg/mL and most of the isolates were inhibited at 5 mg/mL. Based on the collected data it shows that condensed tannins had the higher content in *A. mangium* leaves. Eventhough, the amount were lower in stems and pods, their affinity to bind protein were almost similar. These compounds also shows a good antibacterial effects against *S. aureus* (including resistant strains).

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

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Oleh

NASYATUL EKMA BINTI MOHD HUSSIN

Januari 2016

Pengerusi : Profesor Madya Loong Yik Yee, PhD
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Tiga eksperimen telah dijalankan untuk mengekstrak dan menilai keupayaan tanin untuk mengikat protein dan merencat pertumbuhan beberapa jenis bakteria berbahaya yang biasa dijumpai pada kulit manusia. Jenis tanin serta berat molekul dipercayai merupakan faktor utama mempengaruhi aktiviti tanin ini terhadap protein dan mikrob.Dalam eksperimen pertama, tanin tersejat (CT) telah diekstrak daripada beberapa bahagian pokok Acacia mangium, termasuk dedaun, batang, dan buah. Sephadex LH-20 telah digunakan bagi mengasingkan CT daripada beberapa jenis sebatian fenolic lain yang terdapat di dalam ekstrak mentah. Jumlah CT telah dikira menggunakan kaedah Vanillin-HCl assay yang telah diubahsuai, menggunakan cathechin sebagai standard. Kepekatan tanin dilihat berbeza bagi setiap bahagian tersebut. Dedaun mempunyai kandungan tanin tersejat (CT) yang paling tinggi iaitu 3.28% berat kering, manakala dalam batang dan buah mengandungi 0.98% berat kering dan 1.54% berat kering, setiap satunya.

Dalam eksperimen kedua, keupayaan mengikat protein (PBA) oleh ekstrak tanin tersejat (CT) telah dikenalpasti melalui ujian pemendakan protein dengan menggukan serum albumin lembu (BSA) sebagai protein standard. Keupayaan tersebut telah dikira melalui jumlah nilai-b (nilai b ini mewakili jumlah CT yang diperlukan untuk mengikat separuh dari maksimum BSA yang termendak). Jumlah purata nilai-b untuk tanin yang telah diasingkan daripada setiap bahagian ialah 0.53 dengan sisihan piawainya adalah 0.048.

Dalam ekperimen ketiga, aktiviti anti-bakteria oleh ekstrak CT telah dinilai. Terdapat tiga sub-eksperimen telah dijalankan untuk menilai aktiviti tersebut. Experimen pertama adalah untuk mengira zon perencatan bagi bakteria yang diuji ke atas 100 mg/mL ekstrak CT menggunakan ujian difusi/penyebaran agar. Kemudian, efek perencatan yang minimum telah dikira menggunakan kaedah ujian pencairan mikro. Dalam eksperimen terakhir, pengurangan jumlah log bakteria telah dikira menerusi masa inkubasi dan juga menerusi kepekatan, menggunakan kaedah *time-kill*.

Daripada kajian ini, ia telah menunjukkan bahawa semua bakteria yang diuji adalah terdedah kepada CT pada kepekatan 100 mg/mL, dengan zon diameter perencatan dalam lingkungan 12 ke 13 mm. Walau bagaimanapun, kepekatan minima untuk perencatan adalah berbeza diantara jenis-jenis strain. *S. aureus* ATCC 29213 telah dikenalpasti mampu direncatkan oleh ekstrak CT pada kepekatan yang rendah (1 mg/mL) berbanding strain yang mampu tahan/ resistan. Ia juga telah mati selepas terdedah selama 6 jam dengan 10 mg/mL ekstrak CT. Pada masa yang lain, kesemua jenis MRSA mempunyai aktiviti perencatan yang berbeza. Nilai MIC adalah dalam lingkungan 2 mg/mL hingga 10 mg/mL dan kebanyakan jenis tersebut telah direncatkan pada 5 mg/mL. Daripada data yang telah disimpan, didapati bahawa kandungan CT dalam daun *A. mangium* adalah tinggi. Walaupun kandungannya adalah rendah dalam batang dan buah, kemampuan ia untuk mengikat protin adalah lebih kurang sama. Ekstrak CT juga menunjukkan ia mempunyai efek anti-bakteria yang baik ke atas *S. aureus* (termasuk strain yang mampu tahan/resistan).

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I certify that a Thesis Examination Committee has met on 18 January 2016 to conduct the final examination of Nasyatul Ekma binti Mohd Hussin on her thesis entitled "Isolation and Characterization of Condensed Tannin from *Acacia mangium* Willd. and its Antibacterial Activity" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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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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Declaration by Members of Supervisory Committee

This is to confirm that:

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
CT	Condensed tannin
CTs	Condensed tannins
Da	Dalton
DM	Dry matter
g	gram
HT	Hydrolysable tannin
M	Molar
mg	milligram
µg	microgram
µL	microliter
µm	micrometer
nm	nanometer
pH	Puissance Hydrogen
S	Susceptible
I	Intermediate
R	Resistance
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
HCl	Hydrochloric acid
m	meter
mm	millimeter
mL	millilitre
%	percent
v/v	volume over volume
w/v	weight over volume
min	miniute
°C	Degree Cecius
std dev	standard deviation
C-C	Carbon-carbon link
PBA	Protein binding affinity
ATCC	American Type Culture Collection
MH	Muller-Hinton
TPC	Total plate count
ND	Not defined
sp.	species
PEG	Polyethylene Glycol
NaCl	Sodium chloride
NaOH	Sodium hydroxide
N	Normality

CHAPTER 1

INTRODUCTION

The wide diversity of structurally unique compounds, produced by tropical plant was documented and well addressed by most researchers in the world as the sources of new mechanisms, materials, therapeutic and prophylactic agents to cure and prevent human diseases (Mazni, 2016). To date, there are more than 500,000 species of higher plant are present in the Earth, but only small percentage were investigated for phytochemically and pharmacological screening (Sharma and Patel, 2013). It is essential to utilize this resource by exploiting its biomolecules that posse's biological activity to bring beneficial use in healthcare and the hope of new cures to important existing diseases.

Tannins are high molecular weight phenolic compounds present in many plants. They are water soluble polyphenolic polymers and have a high capacity to form complexes mainly with proteins. Tannins are molecules that have a potency to bind with proteins, cellulose, starches and minerals. This results in the formation of insoluble compounds which is resistant to the decomposition processes. Tannins can be found in various parts of plants, including barks, wood, leaves, buds, stems, fruit, seed, roots, and plant galls. In all of these structures, tannin help to protect the plant form being infected by bacteria or fungi. One of the mechanisms is by precipitating the enzyme and other proteins produced by the bacteria or fungi (Hassanpour, 2011). There are two groups of tannins; hydrolysable tannins and condensed tannins.

Condensed tannins (CT) were known to be able to interact with biological systems through the induction of some physiological effects, such as antioxidant, anti-allergy, anti-hypertensive, and antimicrobial activities (Romani, 2006). Early as in 1980's, condensed tannins were showed to have antiviral activities on Herpes Simplex Virus (HSV-1 and HSV-2).The effect was due to the inhibition of virus adsorption (Fukuchi, 1986). CT compound also was reported to have antiviral activities against respiratory syncytial virus (RSV), influenza A virus (FLU-A), parainfluenza virus (PIV) comparable to ribavirin, and inhibition towards hepatitis-A was observed (Ubillas, 1994). Those inhibitory mechanisms were believed to be either by the binding of the tannin molecules to the protein coat of the virus or to the host cell membrane (Toss, 1999).

Condensed tannins from the other plants such as *Leucaena leucocephala, Phaseolus vulgaris, Fagoypyrum esculentum, Corylus avellana,* and *Juglans nigra,* were shown to have antibacterial activities against some pathogenic bacteria such as *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella enterica* (Mazni, 2016; Ruszard, 2008).
Acacia is a widespread genus of tropical-subtropical trees and shrubs found in Central/South America through Africa to Southeast Asia and Australia. There

were about 1352 species of Acacia species were found worldwide (Maslin, 2003). The predominant Acacia species in Malaysia is *Acacia mangium.* It possesses many advantageous growth characteristics such as a high leaf yield, drought tolerance, adaptation to acidic infertile soils and large temperature variations (Marini, 2011). This plant also was shown to contain tannins. However, the reactivity of these tannins was dependent on the structure and their chemical nature to associate with the substrate (Hoong, 2010).

The emergence of antibiotic-resistant bacteria has become a problem to successful treatment for bacterial infections (Silvia, 2013). Recently, many studies have been done to use biologically active compounds extracted from plant to be used as medicine, especially for bacterial infections (Jigna, 2005). The findings on the antimicrobial activity of plant extracts have also been used as the basic knowledge for many applications. This include as preservation in raw or processed food, in pharmaceuticals, also as alternative medicine (Leela and Satirapipathkul, 2011). There are several types of secondary compounds such as tannins, phenols, steroids and alkaloids. These compounds are deposited in various parts of plants (Balandrin, 1985). They play a major role in plant colour, flavour, texture, also as antioxidant and antibacterial (Hernandez, 1999; Negi, 2005).

A variety of methods have been used in a study of antimicrobial activity of plant bioactive compounds. However, the disc diffusion method of antimicrobial susceptibility test was the most practical method and is a method of choice for the average laboratory (Cleidson, 2007). A growth medium, usually Mueller-Hinton agar, was commonly used in agar diffusion test. This medium was considered the best for the susceptibility testing since it has batch-to-batch reproducibility, low concentration of inhibitors and produces satisfactory results for most of the non-fastidious microorganisms.

In the test, antimicrobial agent was dropped on the paper disc. Once it has on the media, the compounds will start to diffuse outwards from the disc and creates a gradient of antimicrobial concentration in the MH agar such that the highest concentration was found close to the disc with decreasing concentrations further away from the disc. The zone around an antibiotic disc that has no growth was referred to as the zone of inhibition since this approximated the minimum antibiotic concentration sufficient to prevent growth of the test isolate (Lalitha, 2004).

This thesis consisted of three experiments. The first was to extract condensed tannin from several parts of *Acacia mangium* including the leaves, stems, and pods, and then Sephadex LH-20 was used to separate condensed tannin (CT) from other phenolic compounds contained in the crude extracts.

In the second experiment, the affinity of extracted condensed tannin to bind with proteins was examined using protein precipitation assay. This step was important to determine the activity of CT is each sample, and lastly, in the third experiment, the antimicrobial property of condensed tannin extract was tested against several pathogenic bacteria including the resistant strains.

Thus, this study was conducted to accomplish the objectives below:

1. To quantify the amount of condensed tannins isolated from different parts of *Acacia mangium*
2. To examine the activity of extractable condensed tannins to precipitate proteins based on the b-value
3. To determine the antimicrobial activity (inhibition zone, minimum inhibitory concentration, and time-killing assay) of condensed tannins towards the growth of pathogenic bacteria including the drug-resistant strains.

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