BIOASSAY GUIDED ISOLATION OF BIOACTIVE COMPONENTS FROM THE STEM BARK OF *ENTADA SPIRALIS* RIDL. (SINTOK)

BY

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ABSTRACT

Entada spiralis Ridl. which is locally known as 'sintok' or 'beluru' is a perennial plant species belonging to the family of Leguminoceae. Traditionally, the stem bark had been used as shampoo when rubbed in water to treat scalp and body care, while the decoction of its root had been used to treat syphilis and bloody defecation. The bioactivity studies against dermatophytes and bacteria and phytochemical studies of the stem bark of this species are the first time reported in this thesis. The froth test of the stem bark of *E. spiralis* indicated the presence of saponin, and the Liebermann-Burchand test had confirmed the existence of triterpenoid saponin. The extract of the stem bark of *E. spiralis* was prepared by extracting with petroleum ether, chloroform and methanol sequentially. Methanol extract (polar extract) gave the highest percent yield. The bioactivity assay results revealed that *Microsporum gypseum* was the most susceptible dermatophyte towards all extracts with minimum inhibitory concentration MIC values of methanol extract at 0.78 mgl/mL. *Staphylococcus epidermidis* was found to be the most susceptible bacteria towards all extracts with MIC values of methanol extract at 0.195 mg/mL, 3.125 mg/mL for chloroform extract and for petroleum ether extract at 0.78 mg/mL. Methanol extract was further fractionated using vacuum liquid chromatography (VLC) since it displayed promising antimicrobial activity against dermatophytes and bacteria. Among all fractions that were assayed for bioactivity, fraction FA1 was found to be the most effective fraction with MIC at 0.097 mg/mL and 0.195 mg/mL against *Trichophyton mentagrophytes* and *M. gypseum* respectively. Other fractions showed higher MIC value at 3.125 mg/mL for fraction FA2, FA3 and FA5. The fraction FA4 gave MIC value at 6.25 mg/mL. Fraction FA1 had also been screened to contain a greater number of active compounds compared to other fractions with respect to the number of clear zone of inhibition through the agar overlay bioautography assay. The active compounds from fraction FA1 were known as terpenoid compounds as detected by spraying the reference chromatogram with vanillin/sulfuric acid reagent. Therefore, fraction FA1 was chosen for isolation using separation methods of circular chromatography on silica gel (chromatotron) and solid phase extraction (SPE). About ten, compounds were successfully isolated and were indentified based on spectroscopic analysis of UV, HPLC, MS, FTIR, 1-D NMR (^{1}H, ^{13}C and DEPT 135) and 2-D NMR (H-H COSY, HMQC, HMBC) at 600 MHz. AC1 compound was identified as penta-2-acetoxy-β-D-digitoxopyranosyl-(1→2)-fructofuranosyl-(6→4)-β-D-glucopyranosyl-(1→4)-glucopyranosyl-(1→2)-β-D fructofuranosyl-(6→1)-β-D-glucopyranosyl-(4→1)-acetylglucosamine. AC2 was elucidated as β,D-glucopyranosyl(1→2)-β,D-glucopyranosyl)-(1→3)-β,D-xylopyranosy-l(1→4)-α,L-rhamnopyranosyl)-(1→3)-β,D-glucopyranos yl(1→3)-β,D-glucopyranoside. AC3 was known as 16-β-D-arabinofuranosyl-15-β-D-xylopyranosyl-5,8,9,10-tetrahydroxyl-17,18,19,20-tetramethyl diterpene ester. AC5 was identified as 28-α,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylamino-β-D-glucopyranosyl) triterpene ester. AC9 was elucidated as 4-(β-D-glucopyranosyl)-1-hydroxybenzene-(20-hydroxyl-18,19-dimethylcyclotetradecanol) benzoate. AC4 and AC7 were classified as triterpenoid ester. AC8 and AC10 were determined as diterpenoid ester.

خلاصة البحث

يعتبر نبات الـ Entada spiralis من النباتات المعمرة وهو ينتمي إلى عائلة الـ Leguminoceae. استخدم **لحاء** الساق شعبياً في الشامبو لتدليك فروة الرأس لعلاج فروة الرأس والعناية ببشرة الجسم, بينما استخدمت الخلاصة المغلية للجذور لعلاج الزهري والتغوط الدموي. يعرض هذا البحث لأول مرة دراسة الفعالية الحيوية وكيمياء العقاقير للحاء قشرة الساق ضد الفطور الجلدية والجراثيم. أظهر اختبار زبد الساق لهذا النبات وجود سابونينات, وكما أثبت اختبار Liebermann-Burchand وجود تري تربينويدسابونين. تم استخلاص لحاء الساق باستخدام بترواليوم ايتر, كلوروفوم و ميتانول. وقد أعطى الاستخلاص بالميتانول (خلاصة قطبية) أعلى مردود. أظهرت الدراسة الحيوية أن فطور الـ Microsporum gypseum هي أكثر الفطور الجلدية تأثراً بالخلاصات الثلاث السابقة وقد كان التركيز الأدنى الفعال للخلاصة الميتانولية 0.78 مغ/مل. أكثر الجراثيم تأثراً بالخلاصات السابقة كانت الـ Staphylococcus epidermidis وذلك بتركيز أدنى فعال 0.195, 3.125, 0.78 مغ/مل لكل من خلاصة الميتانول, الكلوروفوم وال الايتر على التوالي. تم فصل مكونات الخلاصة الميتانولية باستخدام تقنية التفريغ اللوني السائل VLC باعتبارها أظهرت أفضل النتائج كمضاد فطري. من بين كل الأجزاء, أظهر الجزء FA1 أعلى فعالية وذلك بتركيز ادنى فعال 0.097 و 0.195مغ/مل ضد الـ Trichophyton mentagrophytes و M. gypseum على التوالي. أظهرت الأجزاء FA2, FA3 و FA5 فعالية عند التراكيز 3.125 مغ/مل. أظهر FA4 فعالية عند تركيز 6.25 مغ/مل. وقد تم دراسة الجزء FA1 باعتباره يحتوى على عدد أكبر من المركبات الفعالة مقارنة مع باقي الأجزاء كما أظهرت مناطق التثبيط على طبقات الآغار في اختبار البصمة الحيوية. وقد كانت المركبات الفعالة في FA1 عبارة عن تربينويد كماأظهر رذ الكروماتوغرام بكاشف فانيلين/حمض الكبريت. لذا تم اختبار الجزء FA1 للاستخلاص باستخدام الاستشراب الدائري على السيليكاجل (كروماتوترون) في الطور الصلب. وقد تم عزل عشر مركبات بنجاح وتم تحديد البنية باستخدام تقنيات المطيافية: الأشعة فوق البنفسجية, الاستشراب السائل عالي الضغط, الكتلة الجزيئية, الاشعة تحت الحمراء, وطيف الرنين المغناطيسي أحادي وثنائي البعد (H-H COSY, HMQC, HMBC) باستخدام حقل 600 ميغاهرتز. تم تعريف مركب AC1 على أنه: penta-2-acetoxy-β-D-digitoxopyranosyl-(1→2)-fructofuranosyl-(6→4)-β-D-glucopyranosyl-(1→4)-glucopyranosyl-(1→2)-β-D fructofuranosyl-(6→1)-β-D-glucopyranosyl-(1→4)-acetylglucosamine. وتم تحديد مركب AC2 على انه: β,D-glucopyranosyl(1→2)-β,D-glucopyranosyl)-(1→3)-β,D-xylopyranosy-l(1→4)-α,L-rhamnopyranosyl)-(1→3)-β,D-glucopyranos yl(1→3)-β,D-glucopyranoside. بينما مركب AC3 كان:16-β-D-arabinofuranosyl-15-β-D-xylopyranosyl-5,8,9,10-tetrahydroxyl-17,18,19,20-tetramethyl diterpene ester. مركب AC5 تم تعريفة: 28--α,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylamino-β-D-glucopyranosyl) triterpene ester. بينما AC9: 4-(20-hydroxyl-18,19-)-β-D-glucopyranosyl)-1-hydroxybenzene-(dimethylcyclotetradecanol) benzoate. كلاً من AC4 و AC7 تم تصنيفه على أنه تربينويد استر بينما AC8 و AC10 تم تعريفهما على أنهما ديتربينويد استر.

ABSTRAK

Entada spiralis Ridl. adalah sejenis spesis pokok saka dari famili Leguminoceae. Ia juga dikenali dengan nama 'sintok' atau 'beluru'. Batang pokok ini lazimnya jika digosokkan bersama-sama dengan air boleh digunakan secara tradisional sebagai syampu, sabun mandi dan juga merawat kegatalan kulit kepala. Rebusan akar pokok boleh merawat penyakit sifilis dan juga berak berdarah. Kajian keaktifan biologi terhadap beberapa kulat dan bakteria penyakit kulit dan kajian fitokimia batang pokok merupakan kajian yang pertama dilaporkan. Ujian buih menunjukkan komponen saponin sebagai komponen utama sementara Ujian Liebermann-Burchand mengesahkan saponin dari jenis triterpenoid saponin. Ekstrak batang pokok disediakan dengan menggunakan pelarut-pelarut seperti petroleum ether, klorofom dan metanol. Ekstrak batang pokok dari pelarut metanol memberikan peratus hasil yang paling banyak. Keputusan ujian aktiviti antimikrob menunjukkan kulat dari jenis *Microsporum gypseum* paling sensitif terhadap semua ekstrak di mana nilai kepekatan minimum bagi perencatan menggunakan ekstrak metanol adalah 0.78 mg/ml. Bakteria dari jenis *Staphylococcus epidermis* pula adalah yang paling sensitif terhadap semua ekstrak di mana nilai kepekatan minimum bagi perencatan menggunakan ekstrak methanol adalah 0.195 mg/ml, bagi ekstrak klorofom sebanyak 3.125 mg/ml dan 0.78 mg/ml bagi ekstrak petroleum eter. Ekstrak metanol telah dipilih untuk proses pengasingan melalui teknik kromatografi cecair vakum kepada fraksi-fraksi kecil kerana ekstrak ini menunjukkan aktiviti antimikrob yang memberangsangkan, di samping ia mempunyai kandungan triterpenoid saponin. Komponen tulen AC1 dan AC2 berjaya dipisahkan melalui teknik ini. Keputusan ujian aktiviti antimikrob menggunakan fraksi-fraksi dari ekstrak metanol pula menunjukkan fraksi FA1 adalah yang paling aktif terhadap kulat seperti *Trichophyton mentagrophytes* dengan nilai kepekatan minimumnya sebanyak 0.097 mg/ml dan kulat *M. gypseum* dengan nilai kepekatan minimum terhadap perencatan sebanyak 0.195 mg/ml. Manakala fraksi-fraksi lain seperti fraksi FA2, FA3 dan FA5 menunjukkan nilai kepekatan minimum yang agak tinggi terhadap perencatan iaitu sebanyak 3.125 mg/ml dan fraksi FA4 mempunyai nilai kepekatan minimum terhadap perencatan yang paling tinggi iaitu sebanyak 6.25 mg/ml. Keputusan ujian kromatografi lapisan nipis yang dilapisi agar yang mengandung mikrob menunjukkan fraksi FA1 paling banyak mengandungi komponen-komponen yang aktif terhadap kulat dan bakteria berbanding dengan fraksi-fraksi lain. Kehadiran komponen-komponen yang aktif menyebabkan terbentuknya zon perencatan di atas kromatografi lapisan agar. Komponen-komponen aktif ini adalah dari jenis komponen kimia yang dinamakan terpenoid yang telah dikenalpasti hasil dari ujian penyemburan menggunakan campuran asid sufurik dan vanillin. Oleh itu fraksi FA1 dipilih untuk menjalani proses pemisahan komponen-komponen yang aktif. Proses pemisahan menggunakan teknik pemisahan kromatografi telah berjaya memisahkan sebanyak sepuluh komponen secara keseluruhan dari batang pokok iaitu AC1, AC2, AC3, AC4, AC5, AC6, AC7, AC8, AC9 dan AC10. Teknik pengenalpastian struktur telah dilakukan dengan menggunakan analisa spectroskopi 1 dimensi (1D) dan dua dimensi (2D). Sebanyak lima komponen telah lengkap dikenal pasti strukturnya dan penamaan kimia. Komponen tulen AC1 dikenal pasti sebagai penta-2-acetoxy-β-D-digitoxopyranosyl-

(1→2)-fructofuranosyl-(6→4)-β-D-glucopyranosyl-(1→4)-glucopyranosyl-(1→2)-β-D fructofuranosyl-(6→1)-β-D-glucopyranosyl-(4→1)-acetylglucosamine dengan formula molekul $C_{78}H_{123}NO_{52}$. AC2 dikenal pasti sebagai β,D-glucopyranosyl(1→2)-β,D-glucopyranosyl)-(1→3)-β,D-xylopyranosy-l(1→4)-α,L-rhamnopyranosyl)-(1→3)-β,D-glucopyranosyl-(1→3)-β,D-glucopyranoside dengan formula molekul $C_{35}H_{59}O_{52}$. AC3 dinamakan sebagai 16-β-D-arabinofuranosyl-15-β-D-xylopyranosyl-5,8,9,10-tetrahydroxyl-17,18,19,20-tetramethyl diterpene ester dengan formula molekul $C_{30}H_{46}O_{16}$. AC5 dikenal pasti sebagai 28-α,L-rhamnopyranosyl-18,21,22-trihydroxy-12-en-29-(2-acetylamino-β-D-glucopyranosyl) triterpene ester dengan formula molekul $C_{47}H_{75}NO_{16}$. AC9 dinamakan sebagai 4-(β-D-glucopyranosyl)-1-hydroxybenzene-(20-hydroxyl-18,19-dimethylcyclotetradecanol) benzoate dengan formula molekul $C_{47}H_{62}O_{11}$. AC4 dan AC7 dikelaskan sebagai triterpenoid ester. Manakala komponen AC8 and AC10 dikenali sebagai diterpenoid ester.

APPROVAL PAGE

The thesis of Aiza bt Harun has been approved by the following:

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DECLARATION

I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Aiza Binti Harun

Signature……. Date……

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Affirmed by Aiza Binti Harun

…….. …….

Signature Date

To Asst. Prof. Dr. Siti Zaiton bt Mat So'ad,

for her patience, guidance and belief....

To Sr. Fatimah, Sr, Zihan, Bro. Najib and Bro. Razif,

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LIST OF ABBREVIATIONS

APCIMS	Atmospheric Pressure Chemical Ionization Mass Spectrometry
ATCC	American Type Culture Collection
COSY	Two-dimensional ^{1}H correlation spectroscopy
DEPT	Distortionless Enhancement by Polarization Transfer
EA	Ethyl Acetate
ESIMS	Electron Spray Impact Mass Spectrometry
FTIR	Fourier Transform Infra Red
HCl	Hydrochloric acid
HMBC	^{1}H – detected heteronuclear multiple-bond spectroscopy
HMQC	^{1}H – detected heteronuclear one-bond spectroscopy
HSQC	Heteronuclear Single Quantum Coherence spectroscopy
HPLC	High Pressure Liquid Chromatography
KBr	Potassium bromide
MHA	Muller Hinton Agar
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PE	Petroleum Ether
ppm	part per million
R_f	Retention factor
SDA	Sabouraud Dextrose Agar
SDB	Sabouraud Dextrose Broth
SPE	Solid Phase Extraction
TLC	Thin Layer Chromatography
VLC	Vacuum Liquid Chromatography

CHAPTER ONE
INTRODUCTION

For many years, medicinal plants are the important resources to treat many diseases in the world. In the past two centuries, the chemical investigation and purification of plant extract, which have medicinal properties have yielded numerous purified compounds, which have proven to be indispensable in practice of modern medicine. Phytochemical research is greatly enhanced by the search for bioactive compounds. Therefore the analysis of plant components should thus begin with bioactivity-guided screening and bioactivity-directed fractionation leading to the isolation and characterization of pure biologically active compounds.

Nowadays, the discovery of new antimicrobial agents for human uses has become an urgent need since current drugs increase in severity and extent. Hence, the identification of new and structurally novel natural product with antimicrobial activity will be one of the ways to overcome certain disease-caused microbes, which affected billions of people worldwide. For example, by focusing and targeting saponin-containing medical plants, structural novelty with the required bioactivity is hoped to be achieved more efficiently. This is because saponin is believed to have diverse structures and many show range of pharmacological activities including antimicrobial activities.

Saponins are a class of chemical compounds, glycosylated secondary metabolites found in various plant species and animal domains. They are widely distributed in nature, being present in more the 90 plant families and display a broad spectrum of biological activities which enhanced the interest of phytochemist to further their investigations although saponins have been known for their complex

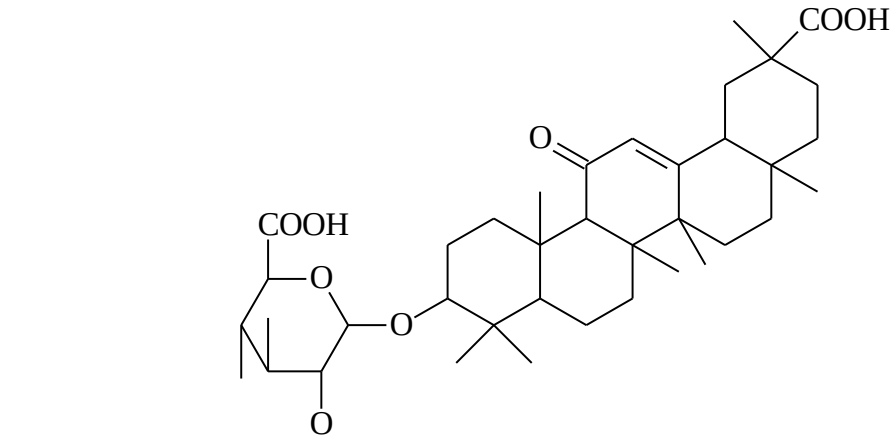
structures. Usually saponins are characterized from their ability to form soapy lathers when agitated with water and they are also known to form colloidal solutions in water and able to precipitate cholesterol. These properties are due to the amphiphilic character of the molecule. From a chemical view, saponin molecules can be divided into two major groups known as glycone (carbohydrate) and aglycone (non-carbohydrate) and sometimes consist of acid. The aglycone for saponin is called sapogenin whereby the nature of the aglycone indicates the saponins to be either steroidal or triterpenoidal. Some common sugars that present in saponin molecules such as D-glucose, D-galactose, L-rhamnose, D-fucose, D-xylose, L-arabinose, D-glucoronic acid and D-galacturonic acid.

Saponins are generally good antifungal and antibacterial agents. However, the antifungal activity is found to be more effective with saponin than the sapogenin and the acetylated saponins, the activity being highly influenced by the number of carbohydrate components. Moreover, when the saponins are in the proximity of cell membranes, their interaction with cholesterol may create pore like structure and leading eventually to the bursting of the membrane (Dubois and Wagner, 2000)

The difference of biological activities of the saponin was due to its chemical structures (Oleszek et al., 1990). Therefore, saponin with different types of chemical structures will responsible for difference biological activities such as antifungal, anticancer, toxicity, reduce digestibility of proteins in ruminants and may express antinutritional properties. Figure 1.1 showed some of different chemical structures of saponin.

A dermatophyte is a parasitic fungus that causes the infections of the skin in animal and human. The term includes the imperfect fungi of the genera *Epidermophyton*, *Microsporum* and *Trichophyton.* The infections are due to their

ability to obtain nutrients from keratinized material. Dermatophytes colonized the keratin tissues and inflammation is caused by host response to metabolic by-product. They are restricted to the nonliving cornified layer of the epidermis because of their inability to penetrate viable tissue of an immunocompetent host. The invasion does elicit a host response ranging from mild to severe. This means that dermatophytes usually do not invade living tissues but colonize the outer layer of the skin. However the presence of dermatophytes and their metabolic products usually induces an allergic and inflammatory eczematous response in the host. According to their habitat, dermatophytes are classified as anthropophilic, zoophilic or geophilic (Ellis, 2006)



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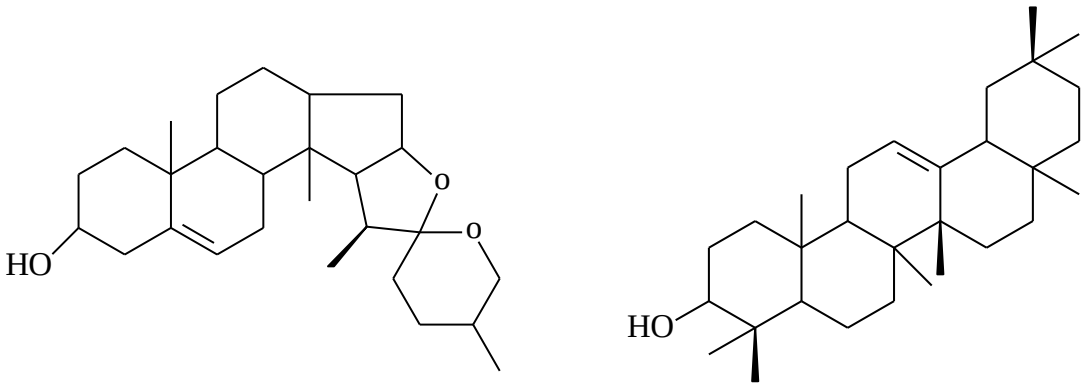

B C

Figure 1.1 Different chemical structure of saponin (Hostettmann and Marston, 1995). A: Glycyrrhizin (triterpenoid saponin); B: Diosgenin (steroidal sapogenin); C: β-Amyrin (triterpenoid sapogenin)