



BIOASSAY GUIDED ISOLATION AND
CHARACTERIZATION OF COMPOUNDS WITH
ANTIOXIDANT ACTIVITIES FROM THE LEAVES OF
Entada spiralis RIDL. (SINTOK)

BY

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ABSTRACT

Entada spiralis Ridl. known as Sintok or Akar Beluru is the woody climber plant belong to Leguminoceae family. *E. spiralis* is an excellent foaming agent due to the presence of saponin. Thus, *E. spiralis* stem bark is established used as natural body soap, shampoo and washing agent. The possession of antioxidant activity has been revealed by *E. spiralis* ability to treat syphilis, insect bites and superficial skin diseases. This research aims to screen the major compounds by phytochemical screening test, verify the antioxidant compounds from crude extracts and fractions via *in-vitro* antioxidant activity assays, isolate the antioxidant compounds through chromatographic methods and characterize the structure of antioxidant compounds by using various spectroscopic techniques. The crude extract of *E. spiralis* leaves was prepared by macerating the leaves with petroleum ether, chloroform, and methanol solvent sequentially. Determination and evaluation of antioxidant activity was done through *in-vitro* study by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, and 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation. Folin-Ciocalteu's reagent method was used to measure total phenolic content and flavonoid content was evaluated by calorimetric method. The crude extract of *E. spiralis* leaves was fractionate by vacuum liquid chromatography (VLC) and liquid-liquid extraction. Isolation of antioxidant compounds was further with column chromatography technique and characterized by spectroscopic analysis. Methanol, chloroform, and petroleum ether crude extracts were analyzed with 5.53 %, 2.05 % and 2.55 % yield. Saponin and flavonoid were the major components. Methanol extract exhibited the highest antioxidant activity with IC₅₀ (DPPH); 40.23 ± 2.66 ug/mL and IC₅₀ (ABTS); 5.09 ± 0.53 ug/mL. The highest possession of phenolic content and flavonoid content; 124.67 ± 6.63 mg GAE/g and 51.67 ± 2.17 mg QE/g were also displayed by the methanol extract. Isoscutellarein, kaempferol-3-C-rutinoside, kaempferol-3-O-glucoside, and kaempferol glycoside were isolated from liquid-liquid extraction fraction while stearic acid and ethyl oleate were isolated from the fraction of vacuum liquid chromatography. IC₅₀ (DPPH) of isoscutellarein; 112.18 ± 2.21 ug/mL, kaempferol-3-C-rutinoside; 136.04 ± 0.52 ug/mL, kaempferol-3-O-glucoside; 301.01 ± 3.02 ug/mL, and stearic acid; 187.49 ± 1.37 ug/mL. Ethyl oleate and kaempferol glycoside were showed negative results towards DPPH. Whereas, IC₅₀ (ABTS) of isoscutellarein; 17.50 ± 0.26 ug/mL, kaempferol-3-C-rutinoside; 34.10 ± 1.13 ug/mL, kaempferol-3-O-glucoside; 14.65 ± 0.11 ug/mL, stearic acid; 5.69 ± 0.06 ug/mL, ethyl oleate; 474.37 ± 2.91 ug/mL, and kaempferol glycoside; 124.82 ± 6.60 ug/mL. In conclusion, the active crude extract and fraction was verified by several *in-vitro* antioxidant assays. Six antioxidant compounds were successfully isolated from this research study.

خلاصة البحث

يرجع أصل نبتة إنتادا سبيراليس، أو المعروفة باسم سينتوك أو أكار برلورو، إلى فصيلة البقوليات. تعتبر هذه النبتة عامل إرغاء ممتاز نظرا لاحتوائها على الصابونين، ولذلك يستعمل لحاء سيقانها كصابون، وشامبو، وغسول طبيعي للجسم. تم التأكد من حيابة هذه النبتة على نشاط مضاد للأكسدة من قدرتها على علاج مرض الزهري، ولدغات الحشرات، وأمراض الجلد السطحية. تهدف الدراسة لفحص المركبات الرئيسية بواسطة الفحص الكيميائي النباتي، والتحقق من المركبات المضادة للأكسدة في المستخلصات الخامة وأجزاء المستخلصات عن طريق اختبارات النشاط المضاد للأكسدة في الوسط المخبري، وعزل المركبات المضادة للأكسدة من خلال الطرق الكروماتوغرافية، وتحديد أشكال المركبات باستخدام تقنيات طيفية مختلفة. تم استخراج المستخلصات الخامة عن طريق نقع الأوراق في أثير البترول، والكلوروفورم، والميثانول بالتتابع. تحديد وتقييم النشاط المضاد للأكسدة بفحوصات الوسط المخبري باستخدام 2،2-ثنائيالفيثيل-1-بيكريليهيدرازيل (DPPH) الجذرية، 2،2-أزينو بيس (3-إيثيلبنزينثولين-6-حمض السلفونيك) (ABTS) الموجبة الجذرية. استخدام طريقة كاشف فولين-سيو كالتيو لقياس المحتوى الفينولي الكلي وتم تقييم محتوى الفلافونويد بالطريقة السعيرية. تمت تجزئة المستخلص الخام عن طريق الكروماتوغرافيا السائلة المفرغة، وطريقة استخلاص السائل-سائل. تم عزل المركبات المضادة للأكسدة كذلك بتقنية الكروماتوغرافيا العمودية وتمييزها بالتحليل الطيفي. تحليل المستخلصات الخامة للميثانول، واكلوروفورم، وأثير البترول بنسب إنتاج 5.53% و 2.05% و 2.55%. والصابونين، والفلافونويد كانتا الأكثر كمية. أظهر مستخلص الميثانول أعلى نشاط مضاد للأكسدة بنسبة IC_{50} (DPPH) قدرته بـ 2.66 ± 40.23 ميكروغرام/مل و IC_{50} (ABTS)، و 0.53 ± 5.09 ميكروغرام/مل. أعلى محتوى للفينول والفلافونويد أيضا في مستخلصات الميثانول وبنسبة 6.63 ± 124.67 ملغ/GAE و 2.17 ± 51.67 ملغ/QE. تم عزل الإيسوسكوتالارين، كامبفيرول-3-C-روتينوسيد، كامبفيرول-3-O-غلوكوسيد، وكامبفيرول جليكوسيد من السائل-سائل للأجزاء، بينما الحامض الدهني، وأوليت الإيثيل من جزء الكروماتوغرافيا السائلة المفرغة. IC_{50} (DPPH) للإيسوسكوتالارين 2.21 ± 112.18 ميكروغرام/مل، والكامبفيرول-3-C-روتينوسيد 136.04 ± 0.52 ميكروغرام/مل، والكامبفيرول-3-O-غلوكوسيد 3.02 ± 301.01 ميكروغرام/مل، والحامض الدهني 1.37 ± 187.49 ميكروغرام/مل. أظهرت أوليت الإيثيل والكامبفيرول جليكوسيد نتائج سلبية نحو DPPH IC_{50} (ABTS) للإيسوسكوتالارين 0.26 ± 17.50 ميكروغرام/مل، والكامبفيرول-3-C-روتينوسيد 1.13 ± 34.10 ميكروغرام/مل، والكامبفيرول-3-O-غلوكوسيد 0.11 ± 14.65 ميكروغرام/مل، والحامض الدهني 0.06 ± 5.69 ميكروغرام/مل، وإيثيل الأوليت 2.91 ± 474.3 ميكروغرام/مل، والكامبفيرول جليكوسيد 6.6 ± 124.8 ميكروغرام/مل. في الختام، التحقق من نشاطات المستخلصات الخامة وأجزائها من قبل عدد من اختبارات مضادات الأكسدة في المختبر. تم عزل ستة مركبات مضادة للأكسدة بنجاح في هذه الدراسة البحثية.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Sciences (Pharmaceutical Chemistry).

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DECLARATION

I hereby declare that this dissertation is the result 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.

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LIST OF ABBREVIATIONS

LLE	Liquid-liquid extraction
VLC	Vacuum liquid chromatography
TLC	Thin layer chromatography
Prep-TLC	Preparative thin layer chromatography
BDE	Bond dissociation energy
SET	Single electron transfer
HAT	Hydrogen atom transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
ABTS	2, 2' - azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
FRAP	Ferric reducing antioxidant power
ORAC	Oxygen radical absorbance capacity
TRAP	Total radical-trapping antioxidant parameter
CUPRAC	Cupric reducing antioxidant capacity
TPC	Total phenolic content
TFC	Total flavonoid content
NMR	Nuclear magnetic resonance spectrometer
FTIR	Fourier transform infrared spectrometer
LC-MS	Liquid chromatography-mass spectroscopy
GC-MS	Gas chromatography-mass spectroscopy
HPLC	High performance liquid chromatography
UV-Vis	Ultraviolet visible
HMBC	Heteronuclear multiple bond correlation
COSY	H-H correlation spectroscopy

ESI	Electrospray ionization
oops	Out of plane stretching
SEM	Standard error of mean
CHCl ₃	Chloroform
MeOH	Methanol
EA	Ethyl acetate
DCM	Dichloromethane
Hex	Hexane
PE	Petroleum ether
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
AlCl ₃	Aluminium trichloride
CDCl ₃	Deuterated chloroform
CD ₃ OD	Deuterated methanol
¹ H	Proton
¹³ C	Carbon
Ar	Benzene
O-H	Hydroxyl
C=O	Carbonyl
C-H	Alkyl
C=C	Alkene
C-O	Ether
glu	Glucose
rha	Rhamnose
ppm	Parts per million

ug/mL	Microgram per liter
mg	Milligram
mL	Milliliter
uL	Microliter
nm	Nanometer
Hz	Hertz

LIST OF SYMBOLS

J	Coupling constant
δ	Chemical shift
k	Force constant value
s	Singlet
d	Doublet
dd	Doublet of doublet
m	Multiplet
m/z	Mass to charge ratio
n	Nonbonding orbitals
π	Pi bonding
σ	Sigma bonding
π^* and σ^*	Antibonding orbitals
p	Significant value
R^2	Coefficient of determination
IC ₅₀	Inhibition concentration at 50%
R _f	Retention factor value

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Nature's healing power has been explored across centuries and regarded as an ancient concept (Teke et al., 2011). Plants are recognized as being a main source of promising drugs to variety of biological activities such as antioxidants, anticancer, antimicrobials, antidiuretics etc. (Das et al., 2014). Across decades, humans have used different extracts of plant and created their own remedies and formulations in alleviating and treating number of diseases. Generally, the medicinal properties possessed by plants have been contributed from various parts of plants including stem bark, leaves, fruit, and seeds. Most of plants have been declared in possessing their own ethnopharmacological properties which have extensively used by the tribal people across the world since centuries. Therefore, it is believed that nature has provided us the best medicine with their own benefits for treating every human disease (Tiwari, Kumar, Kaur, Kaur, & Kaur, 2011).

Plants with medicinal properties are known to be rich in source of raw drugs, and therapeutic agents in prevention number of diseases and symptoms. The medicinal, nutraceutical, and cosmeticeutical properties exerted by plant are due to the different phytochemicals, bioactive constituents from various parts of the plant. Recently, substances and products originated from plants showing a great interest market caused by their versatile applications (Tiwari et al., 2011). Medicinal plants are managed to synthesize a large number of organic compounds which are categorized into two classes, namely primary and secondary metabolites (Seifu,

Assefa, & Abay, 2012). Mostly, the biological activities that are exhibited by medicinal plants are caused by secondary metabolites which are expressed during plants metabolic pathway (El-Toumy, Mohamed, Hassan, & Mossa, 2011). Plant secondary metabolites can be classified into three main groups according to their biosynthetic origin. They are phenolic and polyphenolic compounds, terpene and alkaloids chemically nitrogen or sulphur bonded (Crozier, Clifford, & Ashihara, 2006).

Primary metabolites are chemical components essential for the growth, development, respiration, and photosynthesis of plant. Examples of primary metabolites are carbohydrate, amino acids, polysaccharides, lipids, and organic acids. Many of them are well distributed among the number of species within plant kingdom. Even though primary metabolites are seen not to be prominent in research field, their role nowadays have captivated researchers' view and mind as being a significant element for plants against herbivores and infections of microbial, act as ultraviolet (UV) protectants as well as provide signal molecules for root nodules to form nitrogen-fixing in legumes. Besides that, primary metabolites also act as seed-dispersing animal's attractants and pollinators and agents for allopathic (Seifu et al., 2012).

Besides primary metabolites, plants also accumulate high concentration of secondary metabolites. Secondary metabolites have been well known due to their potential as sources of new natural drugs, insecticides, antibiotics, and herbicides and their use as flavoring agents, dyes, perfumes, fibers, oils, waxes, and glues (Seifu et al., 2012). Across years, most of researchers have given their full attention on exploring these metabolites and trying so much to isolate as many as possible the