UNIVERSITI PUTRA MALAYSIA

PHYTOCHEMICAL ANALYSES OF BELALAI GAJAH (Clinacanthus nutans Burm. f. Lindau) LEAVES BUTANOL FRACTION

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

May 2018

DEDICATION

This thesis is dedicated to my Ibu

for her love, endless support and encouragement

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Clinacanthus nutans is a medicinal shrub that has been increasingly used as an anti-viral regimen treatment in the form of water decoction or infusion. However, there are very few reports on phytochemical constituent studies focus on the polar fraction of this plant. Therefore, the aim of this study was to isolate and identify the phytochemical constituents present in the butanol fraction of the methanolic extract from the leaves of *C. nutans*. The butanol fraction was analyzed by LC-DAD-ESIMS/MS and twenty-eight compounds were tentatively identified including five types of apigenin derived compounds, two types of luteolin derived compounds, seven sulphur-containing compounds, three caffeoyl derived compounds and two other metabolites, named methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate (**137**) and 3,3-di-*O*-methylellagic acid (**138**). The tentatively identified compounds from apigenin derivatives were vicenin 2 (**164**) with its two isomers, isoschaftoside (**168**), schaftoside (**11**), neoschaftoside (**169**), gendarucin A (**136**) with its isomer, isovitexin (**10**), vitexin (**9**) and apigenin (**145**). The compounds identified from luteolin derivatives were isoorientin (**14**), orientin (**13**) and isoorientin 2”-*O*-apiofuranoside (**146**). The sulphur-containing compounds named clinacoside A (**15**), clinacoside B (**16**), clinamide E (**44**), clinacoside C (**17**), cycloclinacoside A1 (**18**), entadamide A (**36**) and clinamide B (**33**) were also tentatively identified in the butanol fraction. Caffeoyl derivatives, forsythoside A/H/I (**165**/**166**/**167**) were identified from the same fraction, together with methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5 oxoprolinate (**137**) with its isomer and 3,3-di-*O*-methylellagic acid (**138**) with its isomer. Identification of compounds from LC-DAD-ESIMS/MS data was supported by the literatures and MS databases. Subsequently, the fractionation and isolation of compounds from the butanol fraction were performed by various chromatography methods, including centrifugal partitioning chromatography (CPC), column chromatography and recycling high performance liquid chromatography (R-HPLC).

Isolation of the butanol fraction of the methanolic *C. nutans* extract yielded seven compounds including isoorientin (**14**), orientin (**13**), schaftoside (**11**), isovitexin (**10**), vitexin (**9**), clinacoside B (**16**) and clinamide E (**44**). The structures of the isolated compounds were characterized based on collections of physical, spectroscopic (NMR and MS) data as well as comparison with literatures. In conclusion, present study successfully revealed the applications of LC-DAD-ESIMS/MS, various chromatography techniques and NMR analysis to identify and characterize the polar phytochemical constituents in the butanol fraction of the methanolic *C. nutans* extract.

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

ANALISIS FITOKIMIA PECAHAN BUTANOL DAUN BELALAI GAJAH (*Clinacanthus nutans* Burm. f. Lindau)

Oleh

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Clinacanthus nutans ialah sejenis tumbuhan renek ubatan yang semakin banyak digunakan sebagai rawatan regimen anti-virus dalam bentuk rebusan air atau seduhan. Walau bagaimanapun, hanya sedikit laporan mengenai kajian sebatian fitokimia yang memberi tumpuan kepada pecahan polar dari tumbuhan ini. Oleh itu, matlamat kajian ini adalah untuk mengasingkan dan mengenal pasti sebatian fitokimia yang terdapat dalam pecahan butanol dari ekstrak metanol daun *C. nutans*. Pecahan butanol telah dianalisis dengan menggunakan LC-DAD-ESIMS/MS dan dua puluh lapan sebatian yang merangkumi lima jenis sebatian terbitan apigenin, dua jenis sebatian terbitan luteolin, tujuh sebatian yang mengandungi sulfur, tiga sebatian terbitan kaffeol dan dua metabolit lain dinamakan metil 2-[sikloheks-2-en-1-il(hidroksi)metil]-3-hidroksi-4-(2-hidroksietil)-3-metil-5-oksoprolinat (**137**) dan asid 3,3-di-*O*-metilellagik (**138**). Sebatian yang dikenal pasti secara tentatif dari terbitan apigenin adalah vicenin 2 (**164**) dengan dua isomernya, isosaftosida (**168**), saftosida (**11**), neosaftosida (**169**), gendarusin A (**136**) dengan isomernya, isoviteksin (**10**), viteksin (**9**) dan apigenin (**145**). Sebatian yang dikenal pasti dari terbitan luteolin adalah isoorientin (**14**), orientin (**13**) dan isoorientin 2”-*O*-apiofuranosida (**146**). Sebatian yang mengandungi sulfur yang dinamakan klinakosida A (**15**), klinakosida B (**16**), klinamida E (**44**), klinakosida C (**17**), sikloklinakosida A1 (**18**), entadamida A (**36**) dan klinamida B (**33**) turut dikenal pasti dalam pecahan butanol. Terbitan kaffeol, forsitosida A/H/I (**165/166/167**) telah dikenal pasti dari pecahan yang sama, bersama dengan metil 2-[sikloheks-2-en-1-il(hidroksi)metil]-3-hidroksi-4-(2-hidroksietil)-3-metil-5-oksoprolinat (**137**) dengan isomernya dan asid 3,3-di-*O*-metilellagik (**138**) dengan satu isomernya. Pengenalpastian sebatian daripada data LC-DAD-ESIMS/MS disokong oleh maklumat terdahulu dan pangkalan data MS. Selepas itu, pemecahan dan pengasingan sebatian dari pecahan butanol telah dilakukan dengan pelbagai kaedah kromatografi, termasuk kromatografi pembahagian terpencar (CPC), kromatografi turus dan kitar semula kromatografi cecair berprestasi tinggi (R-HPLC).

Pengasingan pecahan butanol daripada ekstrak metanol *C. nutans* telah menghasilkan tujuh sebatian yang merangkumi isoorientin (**14**), orientin (**13**), saftosida (**11**), isoviteksin (**10**), viteksin (**9**), klinakosida B (**16**) dan klinamida E (**44**). Struktur sebatian yang dipencilkan telah dicirikan berdasarkan koleksi fizikal dan spektroskopi (NMR dan MS) data bersama perbandingan maklumat terdahulu. Kesimpulannya, kajian ini berjaya mendedahkan applikasi LC-DAD-ESIMS/MS, pelbagai teknik kromatografi dan analisis NMR untuk mengenal pasti dan mencirikan sebatian polar fitokimia dari pecahan butanol dari ekstrak metanol *C. nutans*.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree Master of Science. The members of the Supervisory Committee were as follows:

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4.35a HMBC Spectrum of Compound 44 in CD_3OD 114

4.35b HMBC (Continuation) Spectrum of Compound 44 in CD_3OD 115

LIST OF ABBREVIATIONS

1D	One-Dimensional
2D	Two-Dimensional
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
ACN	Acetonitrile
C_5D_5N	Deuterated Pyridine
CC	Column Chromatography
CD_3OD	Deuterated Methanol
$CHCl_3$	Chloroform
$CDCl_3$	Deuterated Chloroform
CID	Collision-Induced Dissociation
COSY	Correlation Spectroscopy
CPC	Centrifugal Partitioning Chromatography
d	Doublet
DAD	Diode Array Detector
dd	Doublet of Doublet
DGDG	Digalactosyl Diglyceride
dH_2O	Deionized Water
DIP-MS	Direct Injection Probe Mass Spectrometer
DMSO-d_6	Deuterated Dimethylsulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
dt	Doublet of Triplet
EA	Ethyl Acetate
ESI	Electrospray Ionization

EtOH	Ethanol
FA	Formic Acid
FDA	Food and Drug Administration
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric Acid
HeLa	Human Cervical Cancer Cell Line
HepG2	Human Liver Hepatocellular Carcinoma Cell Line
HESI	Heated Electrospray Ionization
HMBC	Heteronuclear Multiple Bond Coherence
HPLC	High Performance Liquid Chromatography
HSCCC	High-Speed Counter-Current Chromatography
HSQC	Heteronuclear Single Quantum Correlation
HSV	Herpes Simplex Virus
Hz	Hertz
IMR-32	Human Neuroblastoma Cell Lines
J	Coupling Constant
K-562	Human Erythroleukemia Cell Line
K_d	Distribution Coefficient
LC	Liquid Chromatography
LC-DAD-ESIMS/MS	Liquid-Chromatography-Diode Array Detection-Electrospray Ionization-Tandem Mass Spectrometry
LC-MS	Liquid Chromatography Mass Spectrometry
LS-174T	Human Colon Adenocarcinoma Cell Line
MeOH	Methanol
MGDG	Monogalactosyl Diglyceride

MPLC	Medium Pressure Liquid Chromatography
MS	Mass Spectrometry
m/z	Mass to Charge Ratio
NCI-H23	Human Lung Cancer Cell Line
NO	Nitric Oxide
NP	Normal Phase
NP TLC	Normal Phase Thin Layer Chromatography
ORAC	Oxygen Radical Absorbance Activity
ppm	Parts Per Million
PTLC	Preparative Thin Layer Chromatography
Raji cells	Human Burkitt's Lymphoma Cell Line
R-HPLC	Recycling High Performance Liquid Chromatography
RP	Reverse Phase
RP TLC	Reverse Phase Thin Layer Chromatography
s	Singlet
SGC-7901	Human Gastric Cancer Cell Line
SNU-1	Human Gastric Cancer Cell Line
TIC	Total Ion Chromatogram
TLC	Thin Layer Chromatography
TMS	Tetramethyl Silane
TPC	Total Phenolic Compound
UPLC-MS/MS	Ultra-Performance Liquid Chromatography-Mass Spectrometer
WHO	World Health Organization
YRV	Yellow-Head Rhabdovirus

CHAPTER 1

INTRODUCTION

1.1 Background

Plants have been used as natural medicines to treat human diseases and injuries. Despite the exponential growth of modern medical practices, medicinal plants still play a vital role in drug discovery due to their availability, cost efficiency and high tolerability with few side effects in the human body. According to the World Health Organization (WHO), over 80% of the world's population lives in the rural areas depends on the different forms of traditional medicine as fundamental healthcare (Cragg & Newman, 2013; Kayne, 2009). For example, 80% of the African population still uses medicinal plants as their primary source of medicine. Following this trend, 30-50% of all the medicinal preparations used by the Chinese population comes from traditional herbal preparations (Kayne, 2009). To date, a total of 122 pure compounds obtained from 94 plants are used as drugs in countries hosting WHO-Traditional Medicine Centers and 97 of these compounds are used for related or associated ethnomedical purposes (Cragg & Newman, 2013).

In Malaysia, over 20% of the known vascular plants species reportedly contain medicinal properties and have been used as traditional medicines by various ethnic groups (Conservation and Environmental Management Division, 2006; Alsarhan *et al.*, 2014). Table 1.1 describes some of the selected medicinal plants used by different races in Malaysia.

Table 1.1 : Selected List of Medicinal Plants Used by Different Races in Malaysia

Scientific name	Local name	Traditional used	Parts used	Way of consuming	Racial groups	References
Eurycoma longifolia Jack	Tongkat Ali	Cure fever, afterbirth medication and dysentery	Roots	Water decoction	Malays and aborigines	Khanam *et al.*, 2015
Labisia pumila (Blume) Fern. Vill	Kacip Fatimah	Regain body strength, facilitation of birth and gonorrhea	Leaves, stem and roots	Water decoction	Malays and aborigines	Chua *et al.*, 2012
Areca catechu L.	Pinang	Treat sore throat, anti-inflammatory properties and regain body strength	Ground nuts	Water decoction	Chinese and aborigines	Peng *et al*., 2015
Piper nigrum L.	Lada Hitam	Treat indigestion, antiseptic remedy for tooth-decay and gum swellings	Dry fruits	Poultice	Chinese	Alsarhan *et al.*, 2014
Centella asiatica (L.) Urb.	Pegaga	Treat dysentery, hemorrhoids and dizziness	Leaves and stems	Salad and juice	Indians	Maulidiani *et al*., 2016
Carica papaya L.	Betik	Aid in digestion, treat warts and jaundice	Leaves and flesh	Salad and poultice	Aborigines	Aravind *et al.*, 2013;
Amaranthus spinosus L.	Bayam Duri	Treat internal bleeding, eczema and snake bites	Seeds, stems, leaves and roots	Poultice and water decoction	Kadazan Dusun (Sabah)	Kumar *et al.*, 2010

Several medicinal plants such as *E. longifolia* and *L. pumila,* based on ancestor knowledge have been scientifically proven to possess certain medicinal properties. According to folklore, *E. longifolia* is used to cure fever, dysentery and as a health tonic for post-partum recovery. It has been reported that *E. longifolia* possesses antimalarial, antitumor, anticancer, antidiabetic, aphrodisiac, anxiolytic and antiparasitic activities (Khanam *et al*., 2015). *L. pumila,* is known to relieve menstrual symptoms and ease childbirth and is consumed as a health tonic to regain body strength after birth. It has been scientifically proven that *L. pumila* contains antioxidant activity, estrogenic activity and antibacterial and antifungal effects (Chua *et al.*, 2012). Due to this scientific breakthrough, the increased in economic value of this particular medicinal plant has to lead to intensive logging and land clearance to commercially grow and manufacture medicine from natural sources. However, this practice is inefficient as the active compounds with accurate mechanisms and potency of the medicinal plants are unknown. The aforementioned bioactivities primarily arise from the presence of secondary metabolites in the medicinal plants.

Secondary metabolites are compounds that do not participate directly in the development or reproduction of an organism, nevertheless, they are crucial for protection, competition and synergistic communication between species and potentially for organism survival (Croteau *et al.*, 2000; Pichersky & Gang, 2000; Vasconsuelo & Boland, 2007; Llorent-Martinez *et al.*, 2015). Secondary metabolites can be grouped into three different categories based on their biosynthetic origin; alkaloids, terpenoids and flavonoids (Croteau *et al.*, 2000). These secondary metabolites can be obtained from the plant extracts through a series of isolation methods to determine the specific bioactive compounds that are responsible for the bioactivity (Jones & Kinghorn, 2012).

From 1983 to 1994, over 520 new drugs derived from plant sources were approved by the US Food and Drug Administration (FDA) or comparable governing bodies in other countries (De Smet, 1997). Twenty-six new drugs were approved by the FDA from 2000 to 2006 (Saklani & Kutty, 2008). Extensive research on secondary metabolites from the medicinal plants with unidentified pharmacological activities has been conducted for years to attain either new plant-derived drugs, semi-synthetic plant-derived drugs or synthetic drugs derived from natural product sources. For example, formulated capsaicin (**1**) (Figure 1.1) cream, which is extracted from *Capsicum,* has been extensively used to relieve post herpetic neuralgia caused by diabetic neuropathy or osteoarthritis since the early 1990s (De Smet, 1997). Furthermore, galantamine (**2**) (Figure 1.1) obtained from the bulbs of *Galanthus* spp. shows positive results towards anti-acetylcholinesterase activity and can be applied as a potential candidate to treat Alzheimer's patients. In addition, alkaloid quinine (**3**) (Figure 1.1) found in the bark of *Cinchona* trees was identified as a competitive regimen due to its antimalarial activity. Vinorelbine (**4**) (Figure 1.1) derived from the alkaloids vinblastine and vincristine obtained from *Catharanthus roseus* shows a promising anti-malignancy effect in lung cells and is an example of a semi-synthetic plant-derived drug approved by the US FDA in 1994 (De Smet, 1997). In addition, vinorelbine also shows a progressive effect on antitumor activity in advanced breast cancer cells and lymphoma cancer cells (De Smet, 1997).

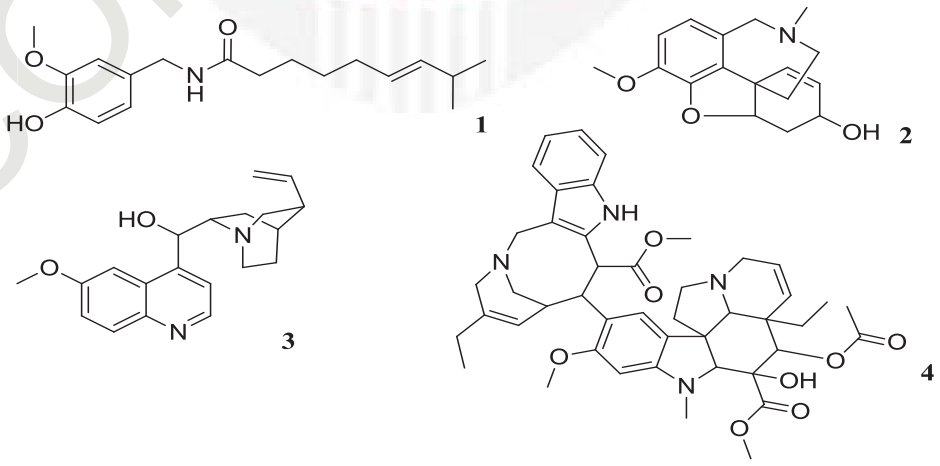

Figure 1.1 : Secondary Metabolites that Contributes to the Bioactivity

1.2 Problem Statement

In recent years, *Clinacanthus nutans* (Burm.f.) Lindau has gained popularity in Malaysia with numerous testimonies due to its ability to combat cancer cells. *C. nutans* is a small shrub that belongs to the family of Acanthaceae, which can be found in South East Asia, especially in Malaysia, Indonesia and Thailand. *C. nutans* is used as a traditional medicine for herpes infections, skin rashes, genital lesions, fever, dysentery, urination difficulty and diabetes (Tuntiwachwuttikul *et al.*, 2003; Tuntiwachwuttikul *et al.*, 2004). It is normally consumed raw or consumed as a fresh fruit juice with addition of other fruits such as green apple or sugar cane.

With the knowledge acquired from the collective folklore stories, it is known that most of the medicinal herbal preparations of *C. nutans*, involved the use of water, either in the form of an infusion or water decoction (Arullappan *et al*., 2014). Therefore, a thorough study on the bioactive compounds present in the water concoction is crucial in order to determine the secondary metabolites responsible for the bioactivity that occurs in the human body. However, the use of water as an extraction solvent does not effectively dissolve most secondary metabolites, which generally belong to various chemical groups, such as alkaloids, terpenoids and flavonoids (Jones & Kinghorn, 2012). The high polarity of water leads to less extraction of secondary metabolites and greater extraction of primary metabolites, such as sugar, hydrophilic protein and salt. Based on the aforementioned reasons, the use of organic solvents particularly methanol, which is closely related to water in terms of hydrogen bonding, is important (Jones & Kinghorn, 2012).

1.3 Objectives

Considering that *C. nutans* has been widely used to treat various diseases, studies on its phytochemical and biological properties, particularly of the polar fraction, have not been exhaustively conducted. Therefore, the current study focuses on the isolation and identification of the phytochemicals present in the butanol fraction of the methanolic extract from the leaves of *C. nutans*. This study emphasizes the newly available technologies such as centrifugal partitioning chromatography (CPC) and recycling high performance liquid chromatography (R-HPLC) for the isolation of compounds. This study was performed based on the following objectives:

1. To identify the chemical constituents in the butanol fraction of the *C. nutans* methanolic extract using liquid-chromatography-diode array detection-electrospray ionization-tandem mass spectrometry (LC-DAD-ESIMS/MS).
2. To isolate the chemical constituents in the butanol fraction of the *C. nutans* methanolic extract using chromatographic techniques.
3. To elucidate and characterize the structure of the isolated compound(s) using various spectroscopic data analyses.

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