GCMS – BASED METABOLOMICS FOR BIOMARKER DISCOVERY OF *CLINACANTHUS NUTANS* AND ITS TISSUE CULTURE

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ABSTRACT

Plant metabolomics is a data-driven approach that plays an important role in systems biology in identifying and describing chemical matrices of plant cells that correlate to biochemical phenotypes. Gas chromatography coupled with mass spectrometry (GC-MS) is a powerful and robust analytical tool widely applied in separating and identifying phytochemical constituents of plant metabolites nowadays. *Clinacanthus nutans* is a medicinal plant currently of public interest due to its use in treating various chronic diseases such as herpes and cancer. The present study aimed to analyse and compare the metabolic profiles of *Clinacanthus nutans* of different ages using GC-MS approach. As a result, seven compounds with medicinal interest were found to accumulate in all of the four different ages of *C. nutans* plants (one-three-year old purchased plant, re-cultivated three-, six- and twelve-month old plant). These seven biomarker compounds for *Clinacanthus nutans* were squalene, Vitamin E, stigmasterol, campesterol, sitosterol, lupeol and betulin. Of these plants, the six-month old re-cultivated plant was found to accumulate the highest amount of the compounds, hence the profile of the plant was chosen for further comparison with the tissue cultures induced from *Clinacanthus nutans.* In total, eleven secondary metabolites were detected in the leaves, stems, and roots of purchased, re-cultivated and, regenerated plants as well as in the adventitious roots and calluses of *Clinacanthus nutans.* The metabolites were phytol, squalene, didecan-2-yl phthalate, Vitamin E, β/γ -tocopherol, stigmasterol, campesterol, β-sitosterol, α/β-amyrin, lupeol, and betulin. The presence of phytol, squalene, didecan-2-yl phthalate, Vitamin E and its derivatives, campesterol, and amyrin were reported for the first time in this species. Highest quantities of squalene, Vitamin E, campesterol, stigmasterol, and β-sitosterol were accumulated in the regenerated plants, 2.76 ± 0.27, 0.85 ± 0.08, 7.36 ± 0.85, 1.86 ± 0.04, and 11.68 ± 0.91 g/kg (on dry matter basis), respectively. Meanwhile, the six-month old re-cultivated plant' root possessed the highest amount of lupeol and betulin, corresponding to 498.19 ± 22.50 and 4.44 ± 0.31 g/kg, respectively. In addition, the induced calluses and adventitious roots also showed the accumulation of the above-mentioned metabolites especially lupeol, with 248.03 ± 71.68 and 81.27 ± 1.84 g/kg, respectively. Through analyses of the metabolic profiles, the chemical compounds detected were postulated to have originated via the biosynthetic pathways of phytosterols and triterpenoids. Interestingly, more phytochemical compounds in *Clinacanthus nutans* were detected in the current work than previously reported and they could potentially be used as marker compounds for the standardisation and development of herbal drugs and nutraceutical products.

ABSTRAK

Metabolomik Berasaskan Kromatografi Gas Spektrometri Jisim (GC-MS) untuk Penemuan Penanda Biologi daripada Clinacanthus nutans dan Kultur Tisunya

Metabolomik tumbuhan merupakan satu pendekatan berasaskan data yang memainkan peranan penting dalam penyelidikan biologi sistem untuk mengenal pasti dan menghuraikan matriks kimia yang terkandung di dalam sel-sel tumbuhan yang berhubungkait dengan fenotip biokimia. Gabungan di antara kromatografi gas dengan spektrometri jisim (GC-MS) merupakan alat analisis yang jitu digunakan secara meluas dalam kajian metabolit tumbuhan bagi mengasingkan dan mengenal pasti sebatian kimia tersebut. Clinacanthus nutans merupakan sejenis tumbuhan perubatan yang menjadi tumpuan pada masa kini di kalangan orang umum berikutan laporan bahawa ianya boleh digunakan untuk merawat pelbagai jenis penyakit kronik seperti herpes dan kanser. Sehubungan dengan itu, kajian ini bertujuan untuk menganalisis dan membandingkan profil-profil metabolik Clincanthus nutans pada peringkat umur yang berbeza. Hasil kajian menunjukkan tujuh sebatian terdapat dalam kesemua empat peringkat umur yang berbeza di Clinacanthus nutans (satu-tiga tahun tumbuhan yang dibeli, tumbuhan ditanam semula dengan tiga-, enam- dan dua belas- bulan). Tujuh sebatian petanda biologi untuk Clinacanthus nutans tersebut adalah skualena, Vitamin E, stigmasterol, kampesterol, β-sitosterol, lupeol, dan betulin. Di antara keempat-empat peringkat umur Clinacanthus nutans ini, Clinacanthus nutans yang ditanam semula mengandungi sebatian yang terbanyak pada usia enam bulan, justeru, menjadi penanda aras dalam analisis dan perbandingan profil-profil metabolik dengan kultur tisunya melalui pendekatan GC-MS. Sebelas metabolit sekunder telah dikenalpasti di dalam ekstrak etil asetat daun, batang, dan akar daripada tumbuhan yang dibeli, yang ditanam semula, dan yang dijana semula melalui kultur tisu serta di dalam akar adventitius dan kalus Clinacanthus nutans, iaitu fitol, skualena, didekan-2-il ftalat, Vitamin E, β/γ-tokoferol, stigmasterol, kampesterol, β-sitosterol, α/β-amirin, lupeol, dan betulin. Kehadiran fitol, skualena, didekan-2-il ftalat, Vitamin E, β/γ-tokoferol, kampesterol, dan α/β-amirin adalah pertama kali dilaporkan dalam spesies ini. Kandungan skualena, Vitamin E, kampesterol, stigmasterol, dan β-sitosterol adalah yang tertinggi di dalam tumbuhan yang dijana semula dengan masing-masing mempunyai 2.76 ± 0.27, 0.85 ± 0.08, 7.36 ± 0.85, 1.86 ± 0.04, dan 11.68 ± 0.91 g/kg (perkadaran berasaskan jisim bahan kering). Sementara itu, akar tumbuhan yang ditanam semula (enam bulan) mengandungi lupeol dan betulin tertinggi, iaitu 498.19 ± 22.50 dan 4.44 ± 0.31 g/kg. Tambahan lagi, kalus dan akar adventitius juga menunjukkan pengumpulan sebatian kimia lupeol di mana masing-masing mengandungi 248.03 ± 71.68 dan 81.27 ± 1.84 g/kg. Penanda biologi yang berpotensi untuk Clinacanthus nutans diandaikan melalui laluan biosintesis fitosterol dan triterpenoids dengan menganalisis profil metabolik dan pangkalan data. Unsur-unsur fitokimia yang ditemui dalam Clinacanthus nutans melalui kajian ini adalah lebih banyak berbanding dengan kajian lain yang telah dilaporkan sebelum ini. Maklumat yang terdapat dalam kajian ini akan memainkan peranan penting dalam pempiawaian dan penghasilan ubat-ubatan herba dan produk nutraseutikal.

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LIST OF ABBREVIATIONS

%	-	percentage
µm	-	micrometer
°C	-	degree Celcius
°C/min	-	degree Celcius per minute
A.D.	-	Anno Domine
AMDIS	-	Automated Mass Spectral Deconvolution and Identification System
B.C.	-	System Before Christ
BAP	-	6-Benzylaminopurine
CAS	-	Chemical Abstracts Service
cm	-	centimeter
DW	-	Dry Weight
EI	-	Electron Ionization
eV	-	electron Volt
g	-	gram
GC	-	Gas Chromatography
GC-MS	-	Gas Chromatography-Mass Spectrometry
h	-	hour
HCl	-	Hydrochloric acid
HPTLC	-	High Performance Thin Layer Chromatography
IBA	-	Indole-3-butyric acid
ID	-	Identity
IUPAC	-	International Union of Pure and Applied Chemistry
L	-	Liter
LC	-	Liquid Chromatography
LC-MS	-	Liquid Chromatography-Mass Spectrometry
LDL	-	Low-Density Lipoprotein
M	-	Molarity
m/z	-	mass-to-charge ratio

mg-	-	milligram
mg/mL	-	milligram per milliliter
min	-	minute
mL	-	milliliter
mL/min	-	milliliter per minute
mm	-	millimeter
MS	-	Murashige and Skoog
NAA	-	1-Naphthaleneacetic acid
NaOH	-	Sodium hydroxide
NIST	-	National Institute of Standards and Technology
NMR	-	Nuclear Magnetic Resonance
PCA	-	Principal Component Analysis
PCDL	-	Personal Compound Database and Library Manager
PLS-DA	-	Partial Least Square-Discriminant Analysis
ppm	-	part per million
rpm	-	revolution per minute
TIC	-	Total Ion Chromatogram
V	-	volt
v/v	-	volume per volume
VIP	-	Variable Importance Projection

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Plants belonging to the family Acanthaceae (commonly known as lipstick plants) are dicotyledonous and flowering group that comprised of about 250 genera and almost 4,000 species. Most of the plants in this family are widely distributed herbs, shrubs, and trees, while some are epiphytes (Prasad, 2014: 8630). The genus *Clinacanthus* is the major group of angiosperm under this family. Two species belonging to *Clinacanthus* have been described, namely, *Clinacanthus nutans* (Burm. f.) Lindau and *Clinacanthus spirei* Benoist. *Clinacanthus nutans* var. *robinsonii* Benoist, *Clinacanthus burmanni* Nees and *Justicia nutans* Burm. f. are junior synonyms of *Clinacanthus nutans* (The Plant List, 2016). Plants in the Acanthaceae family are valued for their medicinal properties attributed to the presence of phytochemical constituents namely alkaloids, phenols, terpenoids, tannins, quinones, cardiac glycosides, saponins, carbohydrates, flavonoids and proteins (Prasad, 2014).

Clinacanthus nutans (*C. nutans*) is widely distributed in Southeast Asian countries such as Malaysia, Thailand and Indonesia where it is used in traditional medicine (Sakdarat, Shuyprom, Pientong, Ekalaksanan and Thongchai, 2009: 1858). In Sabah, this species is commonly known as Sabah Snake Grass, which has recently gained popularity amongst the locals particularly for its purported efficacy in treating cancer-related diseases. The leaves of *C. nutans* can be consumed raw or made into juice by blending them with other fruits and vegetables. In folklore medicine, this plant has been used to treat diabetes mellitus, fever, diarrhoea, dysuria (Uawonggul, Thammasirirak, Chaveerach, Chuachan, Daduang and Daduang, 2011: 1984), herpes simplex skin infection, shingles, insect bites (Thawaranantha, Balachandra, Jongtrakulsiri, Chavalittumrong, Bhumiwasdi and Jayavasu, 1992 :289; Tuntiwachwuttikul, Pootaeng-On, Phansa and Taylor, 2004: 27), preventing and relieving radiation-induced oral mucosities in the head and neck of cancer patients (Putwatana, Sanmanowong, Oonprasertpong, Junda, Pitiporn and Narkwong, 2009: 82), skin rash treatment, snake and insect bites, herpes simplex virus (HSV) infection, and varicella zoster virus (VZV) lesions (Sookmai, Ekalaksananan, Pientong, Sakdarat and Kongyingyoes, 2011: 243). Nonetheless, there have been few studies that outlined the metabolite profile of *C. nutans*, especially the Sabah conspecific that are available in the market.

Several compounds have been identified from *C. nutans* by using different solvent systems, namely, stigmasterol (Dampawan, 1976), lupeol and β-sitosterol (Dampawan, Huntrakul and Reutrakul, 1977: 16), betulin (Lin, Li and Yu, 1983: 337), six known C-glycosyl flavones (Teshima, Kaneko, Othani, Kasai, Lhieochaiphant, Picheansoonthon and Yamasaki, 1997: 557), five sulfur-containing glycosides (Teshima, Kaneko, Othani, Kasai, Lhieochaiphant, Picheansoonthon and Yamasaki, 1998: 832), two glycoglycerolipids (Satakhun, 2001), a mixture of nine cerebroside and a monoacylmonogalatosylglycerol (Tuntiwachwuttikul *et al.*, 2004), eight compounds derived from chlorophyll (Sakdarat *et al.*, 2009), four new sulfur-containing compounds and three compounds known as entadamide A, entadamide C and trans-3-methylsulfinyl-2-propenol (Tu, Liu, Cheng, Hsu, Du, El-Shazly, Wu and Chang, 2014: 20384), gendarucin A and its isomer, 3,3-di-O-methylellagic acid, ascorbic acid, two isomeric oxoprolinates (Khoo, Mediania, Zolkeflee, Leong, Ismail, Khatib, Shaarib and Abas, 2015: 125) and polysaccharide–peptide complex CNP-1-2 (Huang, Li, Cui, Chen and Sun, 2015: 707).

Propagation of *C. nutans* is carried out mainly through the stem cutting technique. However, this method is relatively inefficient for mass-propagating enough plants to meet increasing demands by the phytomedicine industry. Alternatives in the form of *in vitro* tissue culture technologies may potentially help overcome this impediment as the approach could induce rapid mass propagation for large-scale re-vegetation, consequently in obtaining secondary metabolites

(Vanisree, Lee, Lo, Nalawade, Lin and Tsay, 2004: 2). In addition, plants grown through micropropagation may produce novel compounds that are not found the in the stock plant due to its independent growth with various geographical, seasonal and environmental factors compared to parent plants (Rao and Ravishankar, 2002: 103).

Metabolomics is an attractive area of study as it is more closely linked to phenotypes as compared to other branches of 'omics'. By studying genomics, transcriptomics, proteomics and metabolomics through different protocols, a framework that strengthens the relationships amongst all these 'omics' can be attained. In turn, the data generated can be consolidated to acquire a comprehensive snapshot of how cells, organs and organisms perform as well as the changes that might happen in the dynamics and nature of metabolome, thus helping us understand their roles in nature (Roessner and Bowne, 2009: 363). Metabolic profiling has great potential to provide further biological information about plants; for example in elucidating the mode, mechanism and pathways for the synthesis of secondary metabolites. Metabolite profiling is a targeted approach for the identification and quantification of a limited number of metabolites related through similar chemical structures or metabolic pathways (Dunn, Bailey and Johnson, 2005: 612). Mass spectrometry is ideal for this kind of analysis because it can detect and resolve a broad range of metabolites with speed, sensitivity and accuracy (Dettmer, Aronov and Hammock, 2007: 55). Metabolomics data generated by using these instruments were analysed with statistical methods for data interpretation and analysis.

1.2 Problem Statement and Significance of Study

Clinacanthus nutans is a medicinal plant currently of public interest largely due to anecdotal accounts on their medicinal values. In Sabah, locals consume the plants by purchasing the aerial parts (tightened as a bunch of leaves and stems) from suppliers at local markets around Kota Kinabalu. These parts are usually blended with green apple juice, and are believed to be able to treat various ailments including nasal and stomach cancer, kidney stone, and hypertension. To date, the medicinal properties of only a few lipstick plants have been verified such as discovery of the bioactive compound andrographolide derived from *Andrographis paniculata*, which had demonstrated anticancer activity against diverse human cancer cells (Ajaya, Sridevi, Vijaya, Nanduri and Rajagopal, 2004: 295). In addition, although the phytochemical constituents of *C. nutans* have been reported elsewhere, the majority of these involved only the aerial parts (the consumed parts) and did not include the below-ground components.

A preliminary metabolite profiling of *C. nutans* purchased from local suppliers was conducted beforehand. It was found that even though the chemical constituents isolated from the plant samples were consistent throughout, the amount of each compound varied greatly across different batches of samples. These variations were linked to the age at which the *C. nutans* were harvested. Ascertaining the exact age of *C. nutans* sold in the market is difficult with estimates ranging between one to three years according to the suppliers. This is because in the stem cutting propagation technique, different parts of a *C. nutans* would usually differ markedly with the shoots being relatively younger compared to other parts of the plant. Once the aerial parts have grown to marketable sizes, they will be cut and sold. A new shoot will eventually grow from the older stems and the propagation process is repeated afterwards.

As *C. nutans* is a highly sought-after plant, it is thus imperative to investigate the metabolite profile of the plants before standardisation and formulation of herbal products can be developed. Since plants of different ages possessed different metabolite profiles, they were re-cultivated using the stem cutting propagation method from a batch of purchased plants in the laboratory (controlled environment). The aerial parts of these *C. nutans* are harvested at the three-, six- and twelve-month to determine the trend in variations in the amount of metabolites produced by the plant at these particular intervals.

In addition to stem cutting, various tissue cultures from *C. nutans* were also induced in this research and their profiles were analysed in order to determine whether the cultures are able to synthesise the chemical compounds as detected in the purchased (intact) *C. nutans*. The present study took into considerations the current conceptual status of biomarkers as the chemical compounds detected in the wild-type plants and re-cultivated plants as well as the dedifferentiated form of the plant such as the callus. Hence, the metabolite profiles of the micropropagated and wild-type *C. nutans* were compared and contrasted qualitatively and quantitatively.

The findings of this study would provide critical insights into how the metabolite profiles of *C. nutans* vary in the course of their ontogeny and when they are subjected to different growth conditions. In the long run, this information would contribute to the end goal of standardising, formulating, and developing commercial products sourced from Sabah *C. nutans*. Besides that, determining the tissue culture protocol that yield *C. nutans* that are able to accumulate chemical compounds in comparable amounts to wild-type plants may offer a viable alternative for the synthesis and isolation of these valuable derivatives.

1.3 Objectives:

This study, which relies on the usage of reliable gas chromatography mass spectrometry to discover the biomarkers in *C. nutans*, has the following objectives:

1. To analyse and identify the metabolite profiles of the *C. nutans* and its tissue cultures of different ages.
2. To generate a comprehensive metabolite database for *C. nutans* and its tissue cultures.
3. To search for potential biomarker compounds of *C. nutans* through the analysis of the metabolite profile and database.

CHAPTER 2

LITERATURE REVIEW

2.1 Acanthaceae Family

The Acanthaceae is one of the families in the mint order (Lamiales) which comprises of approximately 220 genera and nearly 4000 species in the Acanthaceae family in the world (Scotland and Vollesen, 2000: 572). It is a large plant family which consists mostly of shrubs and herbs, but vines and trees occur as well (Acanthaceae, 2015). This family are predominantly distributed in tropical and subtropical regions. The range of habitats extends from marshes and estuaries to extremely dry areas, but most of these plants are found in damp tropical forests (Deng, Hu, Daniel, Wood and Wood, 2011: 369).

The family of Acanthaceous plants has been a specimen of modern research because of the valuable biologically active compounds contained in them (Khurshid, Harun, Aziz, Baki and Golam, 2002: 1264). Phytochemical reports on the Acanthaceae family show that they contain glycosides, flavonoids, benzonoids, phenolic compounds, naphthoquinone and triterpenoids. A study found that Acanthaceae plants possess antifungal, cytotoxic, anti-inflammatory, anti-pyretic, antioxidant, insecticidal, hepatoprotective, immunomodulatory, anti-platelet aggregation and anti-viral potential (Awan, Ahmed, Uzair, Aslam, Farooq and Ishfaq, 2014: 44). The family is known for its horticultural uses and includes ornamental species such as *Acanthus*, *Aphelandra* R. Brown, *Barleria*, *Crossandra* Salisbury, *Eranthemum*, *Fittonia* Coemans, *Justicia*, *Odontonema* Nees, *Pachystachys* Nees, *Ruellia*, *Sanchezia* Ruiz, *Pavon*, *Thunbergia*, and other numerous genera (Deng *et al.*, 2011).

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