GENETIC AND CHEMICAL VARIATION OF *Clinacanthus nutans* FROM NORTHERN REGION OF PENINSULAR MALAYSIA

NOOR ZAFIRAH BINTI ISMAIL

UNIVERSITI SAINS MALAYSIA

2018

GENETIC AND CHEMICAL VARIATION OF *Clinacanthus nutans* FROM NORTHERN REGION OF PENINSULAR MALAYSIA

by

NOOR ZAFIRAH BINTI ISMAIL

Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

July 2018

ACKNOWLEDGEMENT

First and foremost I am very thankful to Allah S.W.T for blessing me with good health and strength from the beginning of my research until submitting my thesis.

I am forever grateful to my best supervisor ever, Dr. Hasni Arsad for his priceless guidance, patience and motivation to complete my thesis. I would like to thank Prof Ahmad Sofiman Othman and Prof Mohammed Razip Samian for giving me invaluable ideas during my studies.

My appreciation also goes to MyBrain15, The Ministry of Higher Education, Malaysia and USM Fellowship, Universiti Sains Malaysia for funding my studies. I am thankful to Fundamental Research Grant Scheme: 203/CIPPT/6711340 for funding this research project.

Besides that, I would like to thank all my laboratory mates, science officers from Advanced Medical and Dental Institute and Drug Discovery and Development Facilities, Universiti Sains Malaysia for their assistance in my research.

My sincere thankfulness goes to my beloved parents, Ismail Hassan and Farizah Mahamood for their continuous support, help and motivation throughout my studies. Finally, I am grateful to everyone who involved directly or indirectly in completing my thesis.

TABLE OF CONTENTS

CHAPTER 3 - METHODOLOGY

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A	Absorbance
AFLP	Amplified fragment length polymorphism
AMDI	Advanced Medical and Dental Institute
AMOVA	Analysis of Molecular Variance
ARC	Animal Research Centre
bp	Base pair
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Data System
CAPS	Cleaved amplified polymorphic sequence
CBOL	Consortium for the Barcode of Life
CCA	Canonical Correspondence Analysis
DAF	DNA amplification fingerprinting
DCA	Detrended Correspondence Analysis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphates
DPPH	1-Diphenyl-2-picryl-hydrazyl
EDTA	Ethylenediaminetetraacetic acid
EMR	Effective multiplex ratio
EtBr	Ethidium bromide
GAE	Gallic acid equivalent
GC-MS	Gas chromatography–mass spectrometry
G_{st}	Gene differentiation

H	Nei’s gene diversity
HSV	Herpes simplex virus
I	Shannon’s Index
ITS	Internal transcribed spacer
ITS2	Internal transcribed spacer 2
ISSR	Inter-simple sequence repeats
K2P	Kimura-2-Parameter
KJN	Jeniang, Kedah
KKK	Kuala Ketil, Kedah
KSP	Sungai Petani, Kedah
*mat*K	Maturase K
MEGA 6.0	Molecular Evolutionary Genetics Analysis 6.0
MI	Marker index
Ne	Effective number of alleles
NCBI	National Center for Biotechnology Information
NIST	National Institute of Standards and Technology
NJ	Neighbor-joining
N_m	Gene flow
PBF	Batu Feringgi, Penang
PBM	Batu Maung, Penang
PCR	Polymerase chain reaction
PIC	Polymorphic information content
PPB	Polymorphism loci
PPS	Pongsu Seribu, Penang

PTG	Tasek Gelugor, Penang
QE	Quercetin equivalent
RAMP	Random amplified microsatellite polymorphism
RAPD	Random amplified polymorphic DNA
*rbc*L	Ribulose-bisphosphate carboxylase
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RP	Resolving power
SBP	Sungai Batu Pahat, Perlis
SCAR	Sequence characterized amplified regions
STS	Sequence-Tagged Sites
TAE	Tris acetate-EDTA
TEAC	Trolox equivalent antioxidant activitiy concentration
*trn*H-*psb*A	Chloroplast intergenic spacer region
UPGMA	Unweighted pair group method arithmetic
USDA	United States Department of Agriculture
USM	Universiti Sains Malaysia
VZV	Varicella-zoster virus

VARISASI GENETIK DAN KIMIA *Clinacanthus nutans* DARI KAWASAN UTARA SEMENANJUNG MALAYSIA

ABSTRAK

Clinacanthus nutans merupakan tumbuhan ubatan yang berharga dan telah mendapat perhatian sejak kebelakangan ini kerana nilai farmakologinya. Walau bagaimanapun, terdapat kurang maklumat tentang hubungan genetik dan fitokimia bagi tumbuhan ini di lokasi yang berbeza. Oleh itu, objektif kajian ini adalah untuk mengenal pasti identiti *C. nutans* menggunakan penanda kodbar asid deoksiribonukleik (DNA), menilai kepelbagaian genetik menggunakan 17 pencetus 'random amplified polymorphic deoxyribonucleic acids' (RAPD), lapan pencetus 'inter-simple sequence repeats' (ISSR) dan 136 pencetus 'random amplified microsatellite polymorphisms' (RAMP) serta menentukan kandungan flavonoid, polifenol, aktiviti antioksidan dan kandungan fitokimia *C. nutans* menggunakan kromatografi gas-spektrometri jisim (GC-MS). Sebanyak 80 aksesi *C. nutans* dari lapan lokasi yang berbeza di kawasan utara Semenanjung Malaysia telah diambil. *C. nutans* dapat dikenal pasti identitinya melalui koleksi baucar dan jujukan produk tindak balas rantaian polymerase (PCR) menggunakan penanda kodbar DNA iaitu *mat*K, *rbc*L dan *trn*H-*psb*A. Analisis jujukan produck PCR menunjukkan bahawa *C. nutans* boleh dikenal pasti dan *trn*H-*psb*A dipilih sebagai penanda yang sesuai untuk tumbuhan ini. DNA genomik telah berjaya diamplifikasi menggunakan sepuluh pencetus RAPD, lima pencetus ISSR dan 37 pencetus RAMP dalam analisis variasi genetik. *C. nutans* menunjukkan peratusan polimorfisme yang tinggi di peringkat spesies berbanding peringkat populasi. RAMP adalah penanda kepelbagaian genetik yang terbaik berbanding RAPD dan ISSR dengan menunjukkan

nilai purata yang tertinggi dalam kandungan maklumat polimorfisme (PIC), indeks penanda (MI) dan kuasa penyelesaian (RP). Kajian fitokimia menunjukkan ekstrak *C. nutans* metanol 80.0% mempunyai ukuran aktiviti antioksidan yang tinggi berbanding kandungan fenolik dan flavonoid. Ekstrak *C. nutans* dari sampel lokasi KKK (Kuala Ketil, Kedah, Malaysia) mempunyai aktiviti antioksidan (54.34 mg TEAC/100g), kandungan flavonoid (30.80 mg QE/100g) dan kandungan fenolik (44.13 mg GAE/100g) yang paling tinggi berbanding sampel dari lokasi lain. Analisis GC-MS menunjukkan kandungan kimia dalam ekstrak *C. nutans* dari lokasi berbeza mempunyai kepelbagaian dalam peratusan kelimpahan relatif (RA). Analisis Kesepadanan Kanonikal (CCA) menunjukkan bahawa variasi genetik dan kandungan kimia dalam populasi *C. nutans* berkait rapat dengan beberapa faktor seperti tanah dan faktor persekitaran. Kesimpulannya, kajian ini memberikan data asas bagi genetik dan kandungan kimia *C. nutans* di lokasi yang berbeza untuk penilaian kualiti ubatan dari tumbuhan.

GENETIC AND CHEMICAL VARIATION OF *Clinacanthus nutans* FROM NORTHERN REGION OF PENINSULAR MALAYSIA

ABSTRACT

Clinacanthus nutans is a valuable medicinal plant which has gained more attention in the last few years mainly because of its pharmacological properties. Despite this, there is little information available about the genetic and phytochemicals of the plant in different locations. Therefore, the objectives of this study were to identify *C. nutans* using deoxyribonucleic acid (DNA) barcode loci, evaluate genetic diversity by using 17 primers of 'random amplified polymorphic deoxyribonucleic acids' (RAPD), eight primers of 'inter-simple sequence repeats' (ISSR) and 136 primers of 'random amplified microsatellite polymorphisms' (RAMP) and determine the total flavonoid, phenolic contents, antioxidant activity and phytochemical contents of *C. nutans* using gas chromatography-mass spectrometry (GC-MS). A total of 80 *C. nutans* accessions from eight different locations in the northern region of Peninsular Malaysia were harvested. The plant was identified using voucher collection and the sequence of polymerase chain reaction (PCR) products using DNA barcode markers namely *mat*K, *rbc*L, and *trn*H-*psb*A. The PCR product sequence analysis showed that *C. nutans* was identified and *trn*H-*psb*A was chosen as the suitable marker for *C. nutans* identification. Genomic DNA had successfully amplified ten primers of RAPD, five primers of ISSR and 37 primers of RAMP by using PCR in genetic variation analysis. *C. nutans* showed low percentage polymorphism at the population level compare to species level. The RAMP markers were the most useful marker compared to RAPD and ISSR markers

by showing the highest mean value of polymorphic information content (PIC), marker index (MI) and resolving power (RP). The phytochemical study revealed that 80.0% methanol *C. nutans* extracts had higher measurement of antioxidant activity compared to the total flavonoid and phenolic contents. *C. nutans* extracts from KKK (Kuala Ketil, Kedah, Malaysia) sample exhibited high antioxidant activities (54.34 mg TEAC/100g), total flavonoid (30.80 mg QE/100g) and total phenolic (44.13 mg GAE/100g) compared to samples from other locations. The GC-MS analysis showed that chemical compounds found in *C. nutans* extract from different locations had different variation in relative abundance (RA) percentage. The Canonical correspondence analysis (CCA) showed that genetic and phytochemical content variations in *C. nutans* population correlate with several factors such as soil characteristics and environmental factors. In conclusion, this study provides baseline data for genetics and chemical compounds of *C. nutans* in different locations for quality evaluation of phytomedicine.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Clinacanthus nutans (Burm.f.) Lindau (*C. nutans*) locally known as Sabah Snake Grass is a member of the Acanthaceae family widely found in South-East Asia. It has been used in many local remedies and its extracts have been used to treat skin rashes, snake bites, insect stings and inflammation as well as cancer (Alam et al., 2016). It also works as an antiviral against the varicella-zoster virus (VZV) and herpes simplex virus (HSV) (Sakdarat et al., 2006; Wanikiat et al., 2008; Arullappan et al., 2014; Alam et al., 2016).

Currently, most frequent reproduction method of *C. nutans* is based on stem-cutting rather than sexual reproduction as the latter process given low reproduction rate and time-consuming (Fong et al., 2014). *In vitro* tissue culture of *C. nutans* also had been used for rapid propagation (Chen et al., 2015). However, Fong et al. (2015) reported that, vegetative propagation and *in vitro* tissue culture have negative long term impacts on the ecology of *C. nutans,* including incapability to adapt to environmental changes and disease resistance which can lead to increase risk of species extinction because of low genetic diversity.

At present there is less information on the genetic diversity of *C. nutans.* A literature review revealed only one published work by Fong et al. (2014)

which documented the genetics of this species with only using fewer samples of *C. nutans* leaves and markers. Therefore, random amplified polymorphic DNA (RAPD), inter-simple sequence repeats (ISSR) and random amplified microsatellite polymorphism (RAMP) were selected to evaluate the genetic diversity of *C. nutans* populations at different locations using polymerase chain reaction (PCR). This study was useful for phylogenetic and evolutionary studies of *C. nutans* and the genetic improvement of the species using marker-based breeding techniques. These markers contribute to long-term objectives in identifying diverse parental lines by targeting important traits while providing information on genetic resistance to wilting, insect pests and other diseases (Arif et al., 2009).

C. nutans is often misidentified with *Clinacanthus siamensis* due to the similar morphologies especially in leaves and flowers (Kunsorn et al., 2013; Shim et al., 2013; Fong et al., 2014; Alam et al., 2016) (Figure 1.1). A study by Kunsorn et al. (2013) showed that microscopic and macroscopic analysis of both plants show similar morphology and cell component but the identification from measurement index such as palisade ratio, stomatal index and stomatal number were different. Hence, identification of *C. nutans* using DNA barcoding markers namely *trn*H-*psb*A, *rbc*L and *mat*K were used to assure significance quality for standardisation and authentication of *C. nutans* from adulteration and substitution from *C. siamensis*. Besides that, these plants have different pharmacological characteristics in anti-HSV type 1 and type 2 activities

(Kunsorn et al., 2013; Alam et al., 2016). Thus, DNA barcoding technique can help to uncover the fraud in herbal product industries since herbal products are unidentifiable by morphology (Ghorbani et al., 2017).

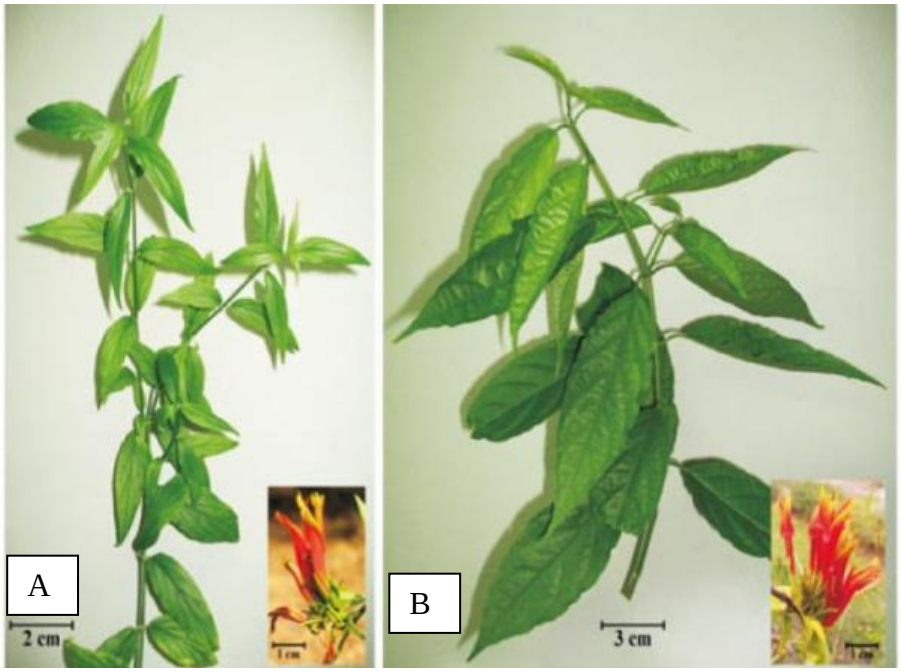

Figure 1.1: Comparison of *C. nutans* and *C. siamensis* leaves and flowers. The photo of the leaves was credited to Kunsorn et al. (2013). (A) *C. nutans* and (B) *C. siamensis*.

The prominent concerns relating to the quality of medicinal plants are the differences of environmental conditions in the cultivation site in which can contribute to the differences of phytochemical compounds (Hu et al., 2007). Fong et al. (2015) reported that the chemicals of *C. nutans* remains uncertain whether different locations with different environmental conditions have an effect on the concentration of secondary metabolites, mainly flavonoids and phenolics. Therefore, the qualities of phytochemical contents of *C. nutans* in different locations need to be conducted. In this study, the total phenolic, flavonoid contents, antioxidant activity and Gas chromatography-mass spectrometry (GC-MS) analysis were used to determine phytochemical contents of *C. nutans* from different locations.

1.2 Hypothesis

1.2.1 Null hypothesis

C. nutans contains no genetic and phytochemical variations in different locations.

1.2.2 Alternative hypothesis

C. nutans contains different genetic and phytochemical variations in different locations.

1.3 Objectives of the Study

The objectives are outlined as follows:

a) To identify *C. nutans* using *trn*H-*psb*A, *rbc*L and *mat*K DNA barcode markers

b) To determine the genetic diversity of *C. nutans* populations from different locations using RAPD, ISSR and RAMP markers and

c) To determine the total flavonoid, phenolic contents, antioxidant activity and phytochemical contents using GC-MS analysis of *C. nutans* populations from different locations.

CHAPTER 2

LITERATURE REVIEWS

2.1 Botanical description of *C. nutans*

All recorded population of *C. nutans* were found in Malaysia, Thailand, Indonesia, Vietnam and China (Chelyn et al., 2014). This plant comes from the family Acanthaceae and can be found in most habitats; dense or open forests, bushes, valleys, damp fields, sea shores and marine regions, swamps as well as mangrove areas (Alam et al., 2016). *C. nutans* has its own common name which is Sabah Snake Grass and Belalai Gajah in Malaysia, Dandang Gendis and Ki Tajan in Indonesia, Phaya Yo and Phaya Plongtong in Thailand and E Zuihua in China (Farsi et al., 2016). This species can be classified in the kingdom Plantae, phylum Magnoliophyta, class Magnoliopsida, subclass Asteridae, order Lamiales and family Acanthaceae (Alam et al., 2016).

Figure 2.1 shows *C. nutans* at cultivated lands which can grows up to 1 metre in height and has cylindrical stems which are yellow when dry, densely striated and subglabrous. The leaves are blade lanceolate-ovale, lanceolate or linear-lanceolate, which can grow up to 0.3 cm to 2.0 cm and paired in opposite arrangements of the curved stem (Shim et al., 2013). Both of the leaf surfaces are pubescent (covered with short and soft hairs) when young which later become glabrescent (without hairs). It contains secondary veins with four to six leaves on each side of the midvein and abaxially elevated and convex on both surfaces when dry (South China Botanical Garden, 2008). The petiole is sulcate and bifariously pubescent (Alam et al., 2016) and sometimes can grow up to 5.0 cm to 7.0 cm or more (GlobinMed, 2015).

The flowers are a dull red with a green-based corolla (3.0 cm to 4.2 cm) with a calyx about 1.0 cm long in the presence of grandular-pubescent. The stamen is exerted from the throat of corolla whereas the ovary is compacted into two cells, which has two ovules in each cell. The styles are filiform and shortly bidentate. The capsule is oblong basally wrapped into 4-seeded short stalks. The flowers are basely yellow or greenish yellow and dense cymes at the top of the branches and branchlets which are covered with 5-alpha cymules (Alam et al., 2016).

A

B

C

Figure 2.1: *C. nutans* (A) *C. nutans* in cultivated land of Tasek Gelugor, Penang, (B) Apical shoot and (C) Flower. The photo of the flower was credited to GlobinMed (2015).

2.2 Ethnomedicinal uses of *C. nutans*

This plant is often employed to cure many illnesses in various traditional treatments. In Malaysia, it has been reported that *C. nutans* has gained popularity among Malaysians in treating cancer (P'ng et al., 2013). However, the effectiveness has not yet been scientifically proven as an alternative treatment for cancer patients. Malaysians consumed *C. nutans* by blending the leaves and drinking it as juice (Yahaya et al., 2015) or boiled with water and consumed as an herbal tea (Alam et al., 2016). The traditional Malaysian medicine also utilise the leaf for antioxidant properties in complementary and alternative medicine (Shim et al., 2013).

In Thailand, the Thai Ministry of Public Health declared *C. nutans* as one of the leading medicinal plants for health care and is used in the treatment of snake and insect bites, HSV, VZV and skin rashes, (Sakdarat et al., 2009; Yahaya et al., 2015). In Thailand, the leaves are consumed as raw vegetables or mixed with fruit juices of apple, green tea or sugar cane. Besides that, they also serve *C. nutans* leaves as a fresh drink or refreshing beverage (Shim et al., 2013). In addition, Sookmai et al. (2011) reported that alcohol extracts of fresh leaves were used externally for the treatment of HSV and VZV lesions, skin rashes, snake and insect bites. For hepatitis infection, extracts from the infusion or decoction of dried leaves and stems are recommended for the treatment. Fever

and dysuria (painful or difficult urination) also have been treated by using the dried leaves (Shim et al., 2013; Yahaya et al., 2015). Basically, Thailand has been using *C. nutans* in many traditional healthcare treatments including anti-inflammatory, anti-venom, anti-diabetic, analgesic, anti-rheumatism, antioxidant and antiviral agent (Kunsorn et al., 2013).

C. nutans have also received much attention in China where they used the entire plant of *C. nutans* to treat inflammatory conditions (hematoma, contusion or bruise, rheumatism, sprains and strains of injuries) (South China Botanical Garden, 2008; Watson and Preedy, 2008; Alam et al., 2016). *C. nutans* is also useful in the regulation of relieving pain, menstrual cycles, setting of fractured bones, anaemia and jaundice (Ailiah, 2011; Alam et al., 2016). In Indonesia, medicines from *C. nutans* are prepared by using a handful of the fresh leaves boiled with five glasses of water and left to simmer until the water levels recede to three glasses and given to the patient as a dose of one glass each time for diabetes, dysuria and fever (Ailiah, 2011; Alam et al., 2016).

2.3 Pharmacological and bioactivity studies of *C. nutans*

C. nutans has been used as a medicinal plant in different regions of Asia due to their diverse pharmacological effects. Due to its pharmacological effects, different kinds of topical preparations such as tablet, cream, capsule, lotions, herbal tea, concentrated extract and secondary metabolites products are available in the market (Alam et al., 2016). The published literature in Table 2.1 shows variation of pharmacological and bioactivity studies of *C. nutans*.

Table 2.1: Pharmacological and bioactivity studies of *C. nutans*

Pharmacological studies	**Part uses**	**Extraction/Fraction**	**Dose tested/ route of administration**	**Animals/Cell line culture**	**Experimental model**	**Results**	**References**
Cytotoxic study	Roots	Methanol extract	0.01 to 0.05 mg/mL	MCF-7 cells	*In vitro*	IC_{50}: 0.04 mg/mL	Teoh et al. (2017)
		Ethyl acetate extract	0.01 to 0.05 mg/mL	MCF-7 cells	*In vitro*	IC_{50}: 0.03 mg/mL	
	Leaves	Petroleum ether extract	0.02 mg/mL	HeLa cells	*In vitro*	IC_{50}: 0.02 mg/mL	Arullappan et al. (2014); Alam et al. (2016)
Dengue virus	Aerial part	80.0% ethanol extract	31.04 mg/mL	Naive Huh-7 cells	*In vitro*	Moderate anti-dengue virus activity	Tu et al. (2014); Alam et al. (2016); Aslam et al. (2016)
Cholinergic modulation	Leaves	Methanol extract	0.25 mg/mL to 1.00 mg/mL	Male mice	*In vivo*	Acetylcholinesterase activity was found highest in mice liver, brain, kidney and heart	Aslam et al. (2014); Lau et al. (2014); Alam et al. (2016)
Anti-inflammatory activity	Aerial part	80.0% ethanol extract	10.00 mg/mL	Human neutrophils	*In vitro*	Strongest elastase release inhibitory effect at 68.3%	Tu et al. (2014); Alam et al. (2016)
	Leaves	Methanol extract	0.00 to 1.00 mg/mL	Rats	*In vivo*	Extracts induced powerful dose	Wanikiat et al. (2008)

Table 2.1: Continued

Anti-HSV type 1 activity	Leaves	n-Hexane fraction extract	0.10 mg/mL	Plaque reduction assay using Vero cell line	*In vitro*	0.03 mg/mL inhibited HSV-1	Kunsorn et al. (2013)
Anti-HSV type 2 activity	Leaves	Methanol extract	0.10 mg/mL	Plaque reduction assay using Vero cell line	*In vitro*	0.07 mg/mL inhibited HSV-2	Kunsorn et al. (2013)
Acute toxicity study	Leaves	Methanol extract	0.25, 0.30, 0.50, 0.60 and 0.90 mg/mL	Rats	*In vivo*	No toxicological effects in liver and kidney	P'ng et al. (2013); Alam et al. (2016)
	Leaves	Methanol extract	5.00 mg/mL	Rats	*In vivo*	No clinical signs of toxicity, mortality and body weight changes in both acute and subchronic toxicity studies.	Zakaria et al. (2016)
Antioxidant activity	Leaves	Petroleum ether extract and methanol extract	4.00 mg/mL	DPPH assay	*In vitro*	Inhibition at 82.0%	Arullappan et al. (2014); Alam et al. (2016)
	Stem	Petroleum ether extract and methanol extract	10.00 mg/mL	DPPH assay	*In vitro*	Inhibition at 70.0%	Arullappan et al. (2014); Alam et al. (2016)
Anti-viral activity on VZV	Aerial part	Topical formulation	7 to 14 days (5 times)	Human	Clinical trial	VZV lesion healed and reduced pain	Alam et al. (2016)

2.4 Identification of plant

There are traditional approaches to identify plants, which are organoleptic methods (identification by smell, sight, touch and taste) and morphological characteristics (identification by texture, colour and shape). At the family level, plants can be easily recognized morphologically through characteristics of their leaves (simple, opposite and decussate where the leaves are arranged in opposite pairs), flowers (zygomorph) and ovary (superior) (Alam et al., 2016). However, these methods require an expert to identify the plant species (Techen et al., 2014). Besides that, most of the medicinal plant materials are in the form of dried or powdered materials (Vassou et al., 2015). Thus, it is much easier to use DNA to authenticate the plant materials as it is more accurate and can be done using a very small amount of material.

DNA barcoding technique has a great influence and is widely accepted within the scientific community (Coissac et al., 2016). It has been widely used since the mitochondrial cytochrome c oxidase I (COI) gene, was suggested as the DNA barcode for identification of the species in animals (Hebert et al., 2003). Subsequently, much progress has been made for determining the DNA barcode for plants with many candidates being proposed such as *mat*K, *rbc*L, ITS and ITS2 barcode markers, which are short DNA sequences between 400 bp to 800 bp. Vassou et al. (2015) reported that there is no single universal DNA barcode marker for plants and each marker has its own benefits and difficulties. Hence, many plastid DNA sequences have been studied as possible barcode loci.

Studies by Kunsorn et al. (2013) and Suesatpanit et al. (2017) showed that ITS and ITS2, respectively could not discriminate *C. nutans* from *C. siamensis*. Therefore, we attempted to use another universal DNA marker such as *trn*H-*psb*A, *rbc*L and *mat*K from (Consortium for the Barcode of Life) CBOL Plant Working Group for identification of *C. nutans*. This information will serves as a guide to provide a suitable plant identification marker for *C. nutans* and other Acanthaceae species.

2.5 Genetic variation in plant

The study of genetic variation of *C. nutans* has received little research attention to date. Among the publications about *C. nutans*, only one article on genetics of *C. nutans* had been published by Fong et al. (2014). They only used two molecular markers namely RAPD and ISSR to detect the homogeneity of *C. nutans* from *C. siamensis* in Malaysia, Thailand and Vietnam. According to Fong et al. (2015), *C. nutans* have been propagated by vegetatative propagation in which effect the quality of *C. nutans* genetics. The vegetative propagation can cause genetic erosion (loss of genetic variation) as it can lead to a clonal growth where one clone (genet) may consist of several individuals (ramets) in a population (Meloni et al., 2013).

Thus, genetic variation in plant is significant for survivability and adaptability as it provides the necessary adaptation and enables changes in the genetic composition for the plants to cope with the changes in the environment

(Booy et al., 2000). Plant with high genetic variation inherits good traits and reduces the unfavourable inherited traits which make the plant resistant to disease or environment (Brown et al., 2009). However, the plants with uniform genetics are more likely to become extinct due to the plants cannot survived towards unfavourable environment and outbreak of diseases (Govindaraj et al. 2015).

2.5.1 Molecular markers for assessment of genetic variation

Molecular markers have been used widely in plant genetic research to observe the pattern of genetic diversity among species. The assessments of genetic diversity within and among populations are usually done at molecular levels such as DNA analyses (Mondini et al., 2009; da Costa et al., 2017). DNA-based molecular markers have more advantages as it can produce different genetic qualities (dominant or co-dominant, amplify anonymous or characterised loci, contain expressed or non-expressed sequences and do not involve environmental conditions) (Mondini et al., 2009). Table 2.2 shows the advantages and disadvantages of different types of genetic variation DNA molecular markers that have been used recently. The advantages and disadvantages of DNA markers provide some explanations but there is no single DNA-marker approach with a clear and appropriate application that can enhance the research area in genetic diversity efficiently (Kumar et al., 2009).

Table 2.2: Advantages and disadvantages of different types of molecular markers (Semagn et al., 2006)

Types of molecular markers	Advantages	Disadvantages
PCR-based markers		
Arbitary or semi arbitrary primed PCR techniques -RAPD -ISSR -Amplified fragment length polymorphism (AFLP) -DNA amplification fingerprinting (DAF) -RAMP Site targeted PCR techniques developed from known DNA sequenced -Cleaved amplified polymorphic sequence (CAPS) -Sequence Characterized Amplified Regions (SCAR) -Sequence-Tagged Sites (STS)	-Small amount of genomic DNA is required -DNA sequences can be amplified from preserved tissues -Radioisotopes has been eliminated in most techniques -Required small laboratory in terms of equipments, facilities and cost -Generate high polymorphisms that can produce many genetic markers within a short time -Able to screen many genes at once	-High molecular weight DNA -Subjectively determined criteria for acceptance of bands in the analysis -Loss of small bands -Highly standardised experimental procedures are needed because of they are sensitivity to the reaction conditions
Hybridisation-based molecular marker -Restriction fragment length polymorphism (RFLP)	-Able to screen many genes at once -Codominant inheritance -Provide locus specific markers -No sequence information required -Easy to score due to large size differences between fragments	-Requires high quantity and quality of DNA -Depends on development of specific probe libraries for the species - Requires radioactively labelled probes -Level of polymorphism is low and few loci detected per assay -Time consuming, laborious and expensive

The ideal genetic diversity markers have been described by many researchers since DNA markers revealed high polymorphisms and codominant inheritance, while also being frequently distributed throughout the genome (Kumar et al., 2009).

Many researchers have begun to take the first step in DNA fingerprinting of genetic diversity with simple markers such as RAPD and ISSR as they only need low qualities of template DNA while no sequence data for designing primer is needed (Idrees and Irshad, 2014). It is also low in cost, very high genomic abundance and random distribution throughout the genome which can generate multiple bands per reaction (Tomar et al., 2014). These techniques are suitable in providing alternate tools for genetic diversity yet result in precise data that need to have restricted fragments of the species. RAPD technique is one of the many successful tools used to analyse genetic diversity. This technique is quite simple as it is least time consuming and non-laborious, incurring low cost and does not require cloning, sequencing nor characterisation of the genome of the species (Bardakci, 2001). Furthermore, RAPD does not need precise data about DNA sequence of the target organisms (Mbwana et al., 2006).

ISSR has also been used after RAPD markers. Both markers exhibit dominant alleles. The RAMP marker is based on set PCR markers which have combined characteristics of RAPD and microsatellite markers (Grover and Sharma, 2016; Avila-Treviño et al., 2017). Based on previous studies, RAMP had been used successfully in genetic studies of various plant crops such as *Prunus* sp. (Cheng et al., 2001), *Phoenix dactylifera* (Soumaya et al., 2013) and *Moringa oleifera* (Avila-Treviño et al., 2017). In this study, three different genetic markers were chosen namely RAPD, ISSR and RAMP for genetic diversity determination of *C. nutans* from different locations.

2.6 Phytochemicals variation

Phytochemical compounds varied in different locations due to the plant adaptation towards the environment (Khadivi-Khub et al., 2014). According to Kumar et al. (2017), the changing in environmental conditions such as temperature, different soil moisture, soil fertility and wind patterns associated with climate change will affect the flowering, fruiting and phytochemical contents of the plant. A study from China showed that *Potentilla fruticosa* had a variation of phytochemical contents due to the variation in temperature, latitude, climate and season and fertility of soil (Liu et al., 2016). Therefore, in order to assure the efficiency and the quality of the medicinal plants, it is essential to monitor availability of the chemical compounds in different location variations of a particular species.

C. nutans received much attention as a medicinal plant but the phytochemicals of *C. nutans* remains unclear whether the locations with different environmental characteristics have an effect on the phytochemical contents. As reported by Fong et al., (2015), there were variations of phenolic and flavonoid contents of *C. nutans* methanol extracts in different locations. The variations of phenolic and flavonoid of *C. nutans* samples were higher from Thailand compared to Malaysia. *C. nutans* that have grown at higher elevations with cooler air temperatures showed higher total phenolic content than *C. nutans* that grown at lower elevations with warmer air temperatures. This study also in line with *Thalictrum foliolosum* that showed phenolic and flavonoid content increased at higher altitudes (Pandey et al., 2017).

From the literature above, many studies that have been conducted by the researchers in order to find antioxidant activities, phenolic and flavonoid content of *C. nutans* (Ghasemzadeh et al., 2014; Lusia Barek et al., 2015; Raya et al., 2015; Sulaiman et al., 2015). However, the results may be attributed to the different varieties, which contain different antioxidant, phenolic and flavonoid compounds. Therefore, it is important to know the chemical properties of the plant in different locations as they might contain useful compounds that can benefit to human health and other living things.

2.6.1 Chemicals of *C. nutans*

Phytochemicals are beneficial especially in medicinal plants, which are valuable gifts of nature and serving as the foundation for human and animal diets (Krishnamoorthy et al., 2014). There are various phytochemical compounds that have been detected in *C. nutans*. *C. nutans* is known to has phenols, flavonoids (Sarega et al., 2016), glycosides (Chelyn et al., 2014), alkaloid (Teshima et al., 1998; Alam et al., 2016), saponins (Ho et al., 2013; Abdullah and Kasim et al., 2017; Zulkipli et al., 2017), tannins and amino acids (Sekar and Rashid, 2016).

Phenolic compounds are the largest group of phytochemical and accounts for the most of the antioxidant activity in plants (Saxena et al., 2013). It is usually assumed that plants which are having more phenolic content show high antioxidant activity but complementary investigations are suggested in order to determine the bioactive element (Sadeghi et al., 2015). The extracts of methanol and ethanol from *C. nutans* leaves and stems were subjected to a polyphenol determination including total polyphenols and antioxidant activity evaluation. The analysis of the extracts showed the results of vitexin, isovitexin, schaftoside, isomollupentin 7-O-β-glucopyranoside, isoorientin orientin as well as sulphurous glycosides namely clinacosides A-C, cycloclinacosides A1 and A2 (Chelyn et al., 2014; Quah et al., 2017).

A mixture of cerebrosides and monoacylmonogalactosyl glycerol (2S)-1-*O*-linolenoyl-3-*O*-b-dgalactopyranosylglycerol (Sakdarat et al., 2009; Alam et al., 2016) were extracted from the ethyl acetate soluble fraction of the ethanol extract of the fresh *C. nutans* leaves. *C. nutans* extracts of hexane and chloroform were used for the isolation of 13-hydroxy-(13-*S*)-phaeophytin b, pupurin-18-phytyl ester and phaeophorbide (Ayudhya et al., 2001; Alam et al., 2016). Moreover, digalactosyl diglycerides and trigalactosyl which were isolated from the leaves extracts were effective in anti-HSV treatments (Janwitayanuchit et al., 2003).

Eight compounds that associated to chlorophyll a and chlorophyll b namely 13^2-hydroxy-(13^2-*S*)-chlorophyll b, 13^2-hydroxy-(13^2-*R*)-chlorophyll b, 13^2-hydroxy-(13^2-*S*)-phaeophytin b, 13^2-hydroxy-(13^2-*R*)-phaeophytin b (13), 13^2-hydroxy-(13^2-*S*)-phaeophytin a (14), 13^2-hydroxy-(13^2-*R*)-phaeophytin a (15), purpurin 18 phytyl ester and phaeophorbide-a were isolated from the chloroform extract of leaves (Sakdarat et al., 2009). According to Tu et al. (2014), four new sulfur-containing compounds namely clinamides A-C (16–18) and 2-cis-entadamide A and three known compounds which are entadamide A, entadamide C and trans-3-methylsulfinyl-2-propenol were isolated from the ethanolic extract of the aerial parts of *C. nutans*.

CHAPTER 3

METHODOLOGY

3.1 Introduction

The research was conducted in the Animal Research Centre (ARC), Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia (USM) involving integrative medicine, oncology and regenerative laboratories. The equipments used in this research are shown in Table 3.1

Table 3.1: Types of equipment used in the research

Equipment	**Type of equipment**
Laboratory balance	Sortorius M-Pact (AX224), Goettingen, Germany
Power supplies	PowerPac™ HC High-Current Power Supply (1645052) Biorad, USA
Agarose gel electrophoresis systems	Mini-Sub® Cell GT Cell (10016027) Biorad, USA
Gel documentation	Syngene Chemi Genius 2 Bio Imaging System, USA
Microwave	Panasonic, Malaysia
Spectrophotometer	NanoDrop 2000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, USA
Micropipettes	Eppendorf Research Plus Pipette, Eppendorf, Hamburg, Germany
Centrifuged machine	Heraeus™ Pico Centrifuge, Thermo Fisher Scientific, USA
Block heater	Block Heater, 3 Block, Digital (SBH130D/3), Stuart, Staffordshire, USA.
Incubator	Incubator shaker KS 4000i control, Ika, Selangor, Malaysia
Freezer (-20^{o}C)	Ardo, Italy
Freezer (-80^{o}C)	Sonyo Electric, Japan

Table 3.1: Continued

Rotary evaporator	Eyela, Buchi N100, USA
Vortex	Vortex 3, Ika, Selangor, Malaysia (Asia)
Blender grinder	Panasonic, Malaysia
Water bath for rotary evaporator	Eyela, Buchi OSB2100, USA
pH meter	CyberScan pH 1500, Eutech Instruments, Singapore
Thermometer	Center 301, Thermometer Type K, Taiwan
Microplate reader	Fluostar Omega, BMG Labtech, Germany
PCR machine	My Cycle ^{TM} Thermal Cycle, Bio-Rad, USA
Homogenizer grinder	IKA RW20 digital Selangor, Malaysia
Gas chromatography-mass spectrometry	Agilent, USA
Freeze drier	Alpha 1-4 LSCplus,Germany
Soil and light tester meter	OEM, China

3.2 Consumables and apparatus

The consumables and apparatus are shown in Table 3.2.

Table 3.2: Type of consumables and apparatus used in the research

Consumables and apparatus	**Manufacturer**
Falcon™ 50 mL Conical Centrifuge Tubes Falcon™ 15 mL Conical Centrifuge Tubes Flat 0.2 mL PCR tube	Thermo Fisher Scientific, USA
Whatman No. 1 filter paper Round bottom flask Whatman® Filter 0.2 µM Greiner CELLSTAR® 96 well plates	Sigma-Aldrich, USA
Laboratory glass bottles with blue screw cap Amber glass bottle Beaker	Duran, Germany
Micropipettes tips 1.5mL Microcentrifuge tube	Axygen, USA
Parafilm M Laboratory Film	Pechiney, Chicago, USA

3.3 Chemicals and reagents

The chemical and reagents used in the research are shown in Table 3.3

Table 3.3: Types of chemical and reagents used in the research

Chemicals and Reagents	**Manufacturer**
All primers	1st BASE Laboratory Sdn Bhd, Malaysia
Polymerase Chain Reaction • 10X iTaq buffer • 50 mM $MgCl_2$ • 10 mM dNTP mix • iTaq DNA polymerase	Bio-Rad, USA
NucleoSpin® Plant II Kit Purification of PCR product	Macherey-Nagel, Germany Wizard® SV Gel and PCR Clean-Up System Kit Promega, USA
Acetic acid (glacial) 100% Methanol 100%	Merck, USA
Ethylenediaminetetraacetic acid (EDTA) Folin-Ciocalteu reagent Tris base Gallic acid Sodium carbonate Aluminium chloride ($AlCl_3$) Quercetin 1-Diphenyl-2-picryl-hydrazyl (DPPH) Ethidium bromide (EtBr)	Sigma-Aldrich, USA
λHindIII DNA Gel Loading Dye (6X)	Thermo Fisher Scientific, USA
1kb DNA ladder 100 bp DNA ladder	Promega, USA
Agarose Powder	Invitrogen Inc, USA
Soil test kit	Luster Leaf, USA