METABOLOMICS-ENHANCED GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)

FOR THE QUALITY STANDARDISATION OF *Clinacanthus nutans*

By

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LIST OF ABBREVIATIONS

GC-MS	Gas Chromatography Mass Spectrometry
PCA	Principal Component Analysis
OPLS-DA	Orthogonal Projections To Latent Structures Discriminant Analysis
MVDA	Multivariate Data Analysis
VIP	Variable Importance For Projection
PC	Principle Component
SIMCA	Soft Independent Modeling Of Class Analogy
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
NOS	Nitric oxide
MMP	Matrix metalloproteinase
XO	Xanthine oxidase
HNE	Human neutrophil elastase
SQS	Squalene synthase enzyme
DPPH	Diphenylpicrylhydrazyl
TLR	Toll-like receptor

LIST OF SYMBOLS AND UNITS

%	Percentage
g	Gram
mg	Milligram
w/w	Water per water
mg/mL	Milligram per mililiter
m	Meter
mm	Milimeter
°C	Degree celcius
mL	Mililiter
µL	Microliter
°C/m	Degree celcius per minute
rpm	Rotation per minute
cm	Centimetre

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ABSTRACT

Clinacanthus nutans, a widely used medicinal plant, is extensively grown in tropical Asia and Southest Asian countries. *C. nutans* with its broad spectrum of pharmacological activities has been traditionally used to treat cancer, inflammatory disorders, diabetes, insect bites and skin problems. In order to improve the accuracy and consistency of control phytomedicine preparations worldwide, standardisation is necessary. Gas chromatography coupled with mass spectrometry (GC-MS) in particular is a fast and accurate method widely applied in diagnostics, functional genomics and screening purposes. Following plant extraction and derivatisation, hundreds of metabolites from different chemical groups can be characterized in one analytical run. In this study, GC-MS based metabolomics approach has been applied to standardise the extracts of *C. nutans* leaves from eight different locations. Lupeol, betulin, stigmasterol and squalene were used as biomarkers to discriminate the group among the samples. Multivariate analysis through Principal Component Analysis (PCA) and Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) models were applied to obtain an overview and discriminate the distribution of samples from different locations. Results from biplot showed that squalene, stigmasterol and betulin present in the samples from Kangar, Alor Setar and Manjung while lupeol presents in the samples from Sungai Ara and Kepala Batas. Squalene was detected in sample from Taman Herba IPPT. The findings suggested that lupeol, stigmasterol, betulin and squalene could be added as markers to the GC-MS analysis of *C. nutans* in maintaining its quality control. In conclusion, the

combination of multivariate statistical analysis with GC-MS data–mining proved to be a powerful tool for metabolomics in the discrimination and quality control of *C. nutans*.

ABSTRAK

Clinacanthus nutans, ubatan herba yang digunakan secara meluas, berkembang pesat di Asia tropika dan negara-negara Asia Tenggara. Aktiviti farmakologi *C. nutans* yang berkembang luas telah digunakan secara tradisional untuk merawat kanser, gangguan keradangan, kencing manis, gigitan serangga dan masalah kulit. Untuk meningkatkan kejituan dan ketekalan persediaan fitoubat kawalan di seluruh dunia, pempiawaian diperlukan. Gabungan kromatografi gas dengan spektrometri jisim (GC-MS) khususnya adalah kaedah yang cepat dan tepat digunakan secara meluas dalam diagnostik, genomik berfungsi dan tujuan penyaringan. Selepas proses pengekstrakan tumbuhan dan derivatisasi, beratus-ratus metabolit dari kumpulan kimia yang berlainan boleh dicirikan dalam satu ujian analitikal. Dalam kajian ini, pendekatan metabolomik GC-MS telah digunakan untuk memiawaikan ekstrak *C. nutans* dari lapan lokasi yang berbeza. Lupeol, betulin, stigmasterol dan squalena digunakan sebagai penunjuk untuk mendiskriminasi kumpulan di antara sampel. Analisis multivariat melalui Prinsip Analisis Komponen Utama (PCA) dan Unjuran Ortogonal kepada Analisis Diskriminasi Struktur Pendam (OPLS-DA) telah digunakan untuk mendapatkan gambaran keseluruhan dan mendiskriminasi pembahagian sampel dari lokasi yang berlainan. Hasil biplot menunjukkan bahawa squalena, stigmasterol dan betulin hadir dalam sampel dari Kangar, Alor Setar dan Manjung dan lupeol yang dibentangkan dalam sampel dari Sungai Ara dan Kepala Batas. Squalena juga dikesan dalam sampel dari Taman Herba IPPT. Penemuan ini menunjukkan bahawa lupeol, stigmasterol, betulin dan squalena boleh dijadikan sebagai

penanda kepada analisis GC-MS *C. nutans* untuk mengekalkan kawalan kualitinya. Kesimpulannya, gabungan analisis statistik multivariate dengan penambangan data GC-MS terbukti berguna untuk metabolomik dalam diskriminasi dan kawalan mutu *C. nutans.*

CHAPTER 1

INTRODUCTION

1.1 Research Background

Recently, medicinal plants have become prominent in our community due to their mystical healing properties and therapeutic values (Noor *et al.*, 2017). The knowledge on the usage of medicinal plants is important to maintain human health apart from sustaining the quality, efficiency and safety of the plant (Kumari, 2016). Thus, standardisation is necessary for the utilisation of plants because it provides the establishment of constant chemical profiles, biological activities and quality assurance for herbal medicines (Patra *et al*., 2010). Standardisation is a method of prescribing a standard, constant parameter and definitive quantitative and qualitative values which leads to quality assurance, efficacy and safety (Folashade, Omoregie and Ochogu, 2012).

Metabolomics, a comprehensive classification on the small metabolites in a biological system is the suitable way to achieve the quality standardisation in medicinal plants (Commisso *et al.*, 2013). Metabolomics approach through the metabolite composition analysis have been recognised as a great tool for characterisation of plant phenotypic (Dai *et al.*, 2010). In addition, an organism's physiology and changes in environment are accurately reflected by the metabolic phenotypes (Fiehn, 2002). This approach is extensively used in the metabolic phenotypes characterisation of herbal plants with different species and ecotypes. Currently, for a great potential enhancement of the crops quality, metabolomics

approach is used for the analysis of the metabolite content among individual groups with many natural variance (Schauer and Fernie, 2006). GC-MS is widely used as a tool for quality standardisation in metabolomics. GC-MS provides high sensitivity, good separation, low cost and also user-friendly (Kanani, Chrysanthopoulos and Klapa, 2008). GC-MS technique is fast, accurate method which and has been applied in diagnostics, screening and functional genomics purposes due to its ability to characterise hundreds of metabolites from different chemical groups in one analytical run (Rohloff, 2015). GC-MS is an integral tool due to its ability to produce reproducible molecular fragmentation patterns which is very suitable for metabolite identification. Thus, for metabolomics studies, GC-MS is the most effective, sensitive and steadfast tool for analysis (Qiu and Reed, 2014).

Clinacanthus nutans (C. nutans), generally identified as Belalai Gajah or Sabah Snake Grass is one of the famous herbal plants in Malaysia (Lau, Lee and Chin, 2014). This plant is consumed as salad or tea due to its ability to treat diabetes mellitus, skin rashes, diuretic, lesions and snake or insects bites (Kunsorn *et al.*, 2013). *C. nutans* extract was reported to possess anti-inflammatory, antiviral and analgesic effects (Mai *et al.*, 2016). These effects are influenced by the phytochemical contents of triterpenoids, flavonoids, glycerides, steroids, glycoglycero-lipids, cerebrosides present in this plant (Sakdarat *et al.*, 2009). *C. nutans* extract with high phenolic content has high efficacy against hypercholesterolemia-induced oxidative stress which contributes to oxidative stress related disease management (Sarega *et al.*, 2016).

The prominent concerns relating the phytomedicines qualities are the origin of plants originated from different geographical places can contribute to the differences of phytochemical compounds (Wang *et al.*, 2004). Thus, it leads to the variations of metabolites composition and effects the standardisation of herbal drug (Chawla *et al.*, 2013). Therefore, quality standardisation on *C. nutans* needs to be conducted using metabolomics approach to provide a comprehensive and equitable analysis of all metabolites including metabolites with low molecular number that exist in a biological sample (Fiehn, 2001). GC-MS technique is the most reliable method to provide a high-throughput way for metabolites quantification to standardise the *C. nutans* as it combines the electron ionization (EI) that has very high separation power and repeatable retention times with a sensitive, versatile and selective mass detection (Koek *et al.*, 2006).

In this study, the analysis of the normalised GC-MS data were done using soft independent modelling of class analogy (SIMCA) to identify the patterns, correlations and clusters of the data (Kamboj, 2012). For multivariate analysis, the processed data were inserted into the SIMCA-P software Version 13 (Umetrics AB, Umeå, Sweden) by using pareto scaling parameter (Madala *et al.*, 2014). Principal Component Analysis (PCA) model was recognised as the initial step of the data analysis and the scores plot shown the sample separation in the population (Kowalczuk *et al.*, 2015). Moreover, Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) preferably employed in the statistical analysis because of the clearly samples discrimination within the population based on their group (Javadi *et al.*, 2014). The effectiveness of the model was described

using the R^2 and Q^2 value, which represented the goodness of fit and accuracy for prediction, respectively. Furthermore, the compounds which contributed significantly to the discrimination of the samples, namely as squalene, betulin, lupeol and stigmasterol were analysed by the loading plot of PCA and OPLS-DA models.

1.2 Objectives

This study embarks the following objectives:

1. To standardise the extracts of *C. nutans* from different locations using biomakers.
2. To apply metabolomics approach in quality standardisation using GC-MS.
3. To classify and discriminate the *C. nutans* extracts using multivariate data analysis (MVDA).

CHAPTER 2

LITERATURE REVIEW

2.1 *Clinacanthus nutans (C. nutans)*

2.1.1 Plant

C. nutans is locally known as Belalai Gajah or Sabah snake grass in Malaysia (Raya *et al.*, 2015). In Indonesia, this plant is called as Ki Tajam (Sunda), Dandang Gendis (Java) and Gendis (Central Java) (Zulkipli *et al.*, 2017). In Thailand, this plant is called as Saled Pangpon Tua Mea which represents the saliva of the female mongoose (Arullappan *et al.*, 2014). The synonym names for *C. nutans* (Burm. F) Lindau are *Clinacanthus burmanni* Nees and *Clinacanthus burmanni* var. *robinsonii* Benoist (Aslam, Ahmad and Mamat, 2015).

2.1.2 Taxonomy hierarchy of *C. nutans*

The taxonomy of *C. nutans* is summarised in Table 2.1.

Table 2.1: Taxonomy classification of *C. nutans* (Alam *et al.*, 2016)

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Subclass	Asteridae
Order	Lamiales
Family	Acanthaceae
Genus	*Clinacanthus* Lindau
Species	*C. nutans* (Burm. f.) Lindau

2.1.3 Plant morphology

The leaves of *C. nutans* are pale green in colour with narrowly elliptic-oblong and in paired opposite arrangement. It is called as Belalai Gajah due to the curve shape of the stem that look like the curve of an elephant's trunk (Shim, Aziana and Khoo, 2013). This perennial herb has pubescent branches with striate, glabrescent and cylindrical stems which grows up to one meter tall. The structure of the leaves is simple with lanceolate shape that has measurement of 2.5 to 13.0 cm long and 0.5 to 1.5 cm wide (Alam *et al.*, 2016). The shape of the leaves is apex acute and exculpate with dentate margins. At young, both surfaces of the leaves are pubescent but later they change to glabrescent. The petiole normally has the length of 0.3 to 2.0 cm with bifarious pubescent and sulcate morphology. The flowers are greenish yellow or sordidly yellow in colour and always covered with 5-alpha cymules at the top of branches. The calyx of the flower is made up of grandular pubescent with about one cm long. The corolla length is about 3.0 to 4.2 cm and is in dull red color. The capsule is oblong basally and wrapped into 4 seeded short stalk. Figure 2.1 shows the *C. nutan* plant.

(a) (b)

(c) (d)

Figure 2.1:
(a) *C. nutans* plant at Taman Herba, IPPT USM
(b) Flowers of *C. nutans*, (c) leaves of *C. nutans*, (d) stems of *C. nutans*
(images adapted from Zulkipli et al., 2017)

2.1.4 Chemical compounds

The main chemical compounds isolated from *C. nutans* are β-sitosterol , lupeol, stigmasterol, C-glycosyl flavones likes isoorientin, vitexin, shaftoside, isovitexin, isomollupentin-7-0-β-glucopyranoside and orientin, sulphur containing glucosides, glycoglycerolipids and a mixture of nine cerebrosides and monoacrylmonogalactosylglycerol (Tuntiwachwuttikul *et al.*, 2004). The aerial parts of *C. nutans* using ethanol extract has revealed that this plant comprises of four new sulfur-containing compounds name as clinamides A - C and 2-*cis*-entadamine A (Tu *et al.*, 2014). It was reported that the leaves extracts of this plant using hexane and chloroform contain 132-hydroxy-(132-S)-chlorophyll-b, 132-hydroxy-(132-R)-chlorophyll-b, 132-hydroxy-(132-S)-phaeophytin-b, 132-hydroxy-(132-R)-phaeophytin-b, 132-hydroxy-(132-S)-phaeophytin-a, 132-hydroxy-(132-R)-phaeophytin-a, purpurin-18-phytyl ester and phaeophorbide-a (Sakdarat *et al.*, 2006). The chloroform extract of *C. nutans* leaves has produced three chlorophyll derivatives (phaeophytins) name as 132-hydroxy-(132-R)-phaeophytin b, 132-hydroxy-(132-S)-phaeophytin-a and 132-hydroxy-(132-R)–phaeophytin (Sakdarat *et al.*, 2009). Yong et al. (2013) has reported that 14 chemical compounds have been identified using GC-MS technique. The compounds were eicosane, behenic alcohol, n-pentadecanol, 1,2-benzenedicarboxylic acid, dibutylphathalate, 1-heptacosanol, phthalic acic dodenyl nonylester, eicosayl trifluoacetate, dinonyl ester, henecoisane, n-pentadecanol, nanodecyl heptafluobutyrate, mono(2-ethylhexyl) ester and 1-nanodecene. The main constituent found was 1,2-benzenedicarboxylic acid with relatively 28.6 % of

peak area whereas others were less than 2%. In an ethanol extract of *C. nutans* leaves, C-glycosidic flavones likes vitexin, isovitexin, orientin, isoorientin and shaftoside have been identified and the results were validated by high performance liquid chromatography (HPLC) method for quantification and quality control purposes (Chelyn *et al.*, 2014) . The methanol extract of *C. nutans* leaves contains flavonoids, saponins, diterpenes, phenolics and phytosterols with 15% w/w yield with approximately 1.77 mg gallic acid equivalents per g of total phenolic content (Yang *et al.*, 2013).

2.1.5 Ethnomedicinal uses

C. nutans has been used to treat diarrhoea, fever, dysuria and diabetes mellitus. In Thailand, the fresh leaves are used to treat skin infection of herpes simplex, lesions of varicella-zoster virus, insect bites and shingles (Tuntiwachwuttikul *et al.*, 2004). The leaves and branches parts also have the ability to relieve oral inflammation symptoms, burns, nettle rash and scalds (Cheeptham and Towers, 2002). Some people consumed it as raw materials or as fresh drink by mixing it with sugarcane, green tea or apple juice. The dried leaves are served as tea by soaking it in hot water. In China, *C. nutans* is used as inflammation treatment for hematoma, eye's bruises, rheumatism and anxieties. This plant also helps in controlling menstrual function, releasing pain, repairing bone fractured and preventing jaundice. In Asian countries, it helps in treating gout, uric acid, kidney problem, uterine fibroid and nasal cavity cancer (Arullappan *et al.*, 2014).

2.1.6 Pharmacological activities

C. nutans has been reported to possess significant antibacterial effect against *S. aureus*, *E. coli, P. acnes, B. cereus* and *S. epidermidis* as the minimum inhibitory concentration (MIC) more than 12.5 mg/mL (Yang *et al.*, 2013). Recently, it was reported that the aqueous extract of *C. nutans* inhibited anaphylaxis, a life-threatening allergic response through the IgG-mediated pathway (Kow *et al.*, 2016). A clinical trial has been done on the treatment of *C. nutans* cream towards the herpes zoster infection. Based on this study, it shows that *C. nutans* cream is more effective compared to the placebo in patients (Charuwichitratana *et al.*, 1996). A study has been done by Narayanaswamy et al., 2016 in evaluating the docking behaviour of 11 chemical constituents of *C. nutans* on nitric oxide synthase (NOS), matrix metalloproteinase (MMP 2 and 9), xanthine oxidase (XO), human neutrophil elastase (HNE) and squalene synthase (SQS) enzyme. Results showed that isoorientin and isovitexin have potentials to dock and bind with all the six targeted enzymes while orientin and vitexin failed to dock and bind with four enzymes except NOS and HNE. For β-sitosterol, it could dock and fix with HNE and MMP 9 but interestingly all the selected ligands from *C. nutans* have the tedency to dock and bind with HNE (Narayanaswamy *et al.*, 2016). These findings are important to understand the mechanism of action of *C. nutans* as inhibitor towards the associated disorders of wound healing, hyperlipidemia and hyperuricemia. The leaf crude extract of of *C. nutans* was reported to possess anti-inflammatory properties in inhibiting the activation of Toll-Like Receptor-4 (TLR-4). By using the polar and non-polar extracts, a significant concentration-

dependent reduction in LPS-stimulated nitric oxide, LPS-induced TLR-4 activation in HEK-Blue^{TM}–hTLR4 cells and LPS-stimulated cytokines production in RWA264.7 macrophages were observed (Mai *et al.*, 2016). Furthermore, the inhibition of TLR-4 activation produced potent anti-inflammatory effects since TLR-4 is the upstream receptor that activated both NF κB and IRF3 signaling, the hallmarks of inflammation (Hatziieremia *et al.*, 2006). It has been recorded that the ethanolic extract of *C. nutans* possesses antioxidant property and showed positive influence towards the free-radical induced hemolysis. It could scavenge DPPH with maximum scavenging activity of 67.65±6.59% and has an IC_{50} of 110.4±6.59 µg/mL (Pannangpetch *et al.*, 2007). In addition, the extract demonstrated a significant inhibition of peroxide production in rat macrophages stimulated by phorbol myristate acetate (PMA) and protected red blood cell against AAPH-induced hemolysis with an IC_{50} of 359.38±14.02 mg/ml. Thus, these results showed the possibility to improve the oxidative damage by employing the *C. nutans* extract as antioxidant substances. Study has been done to demonstrate the antioxidant properties of the extract against the hypercholesterolemia-induces oxidative stress in rats (Sarega *et al.*, 2016). The findings showed that *C. nutans* extract is rich in multiple natural antioxidants, thus it is a good source of functional ingredients in managing the oxidative stress-related diseases.

2.2 Gas Chromatography - Mass Spectrometry (GC-MS)

2.2.1 Basic principle of GC-MS

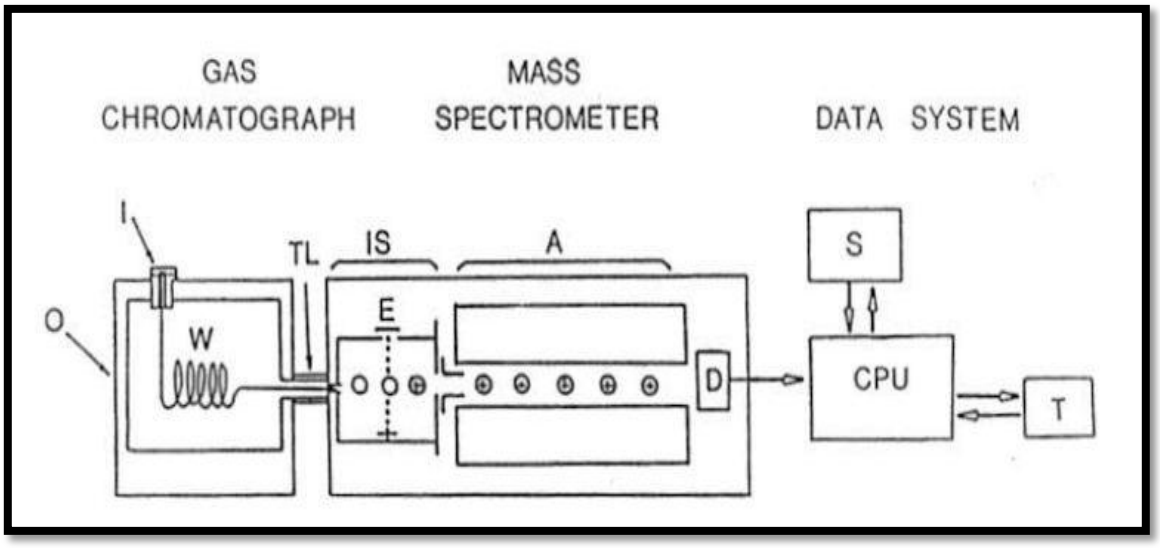

Figure 2.2: Schematic diagram of GC-MS system

(O: Oven, I: Injector, W: WCOT Column, TL: Transfer Line, IS: Ion Source, E: Electron Beam, A: Analyzer, D: Detector, CPU: Central Processing Unit, T: Terminal, S: Data Storage Device)

Chromatography is an analytical technique that involves the process of distribution, separation and partitioning of components between the mobile and stationary phase (Hajšlová and Čajka, 2007). In GC system, gas is used in the mobile phase and the liquid with high molecular weight that deposited with the inner parts of a long capillary tube is used as stationary phase. The GC column is the capillary tubing part where the sample moves through it. Normally the size of the column is 30 to 60 m in length and 0.2 mm of internal diameter. A few microliters of the concentrated solvent extract are injected into the GC column via the injecting port. To sweep the extracted organic compounds through the GC column, inert gases such as N_2, H_2 or He are used. In the GC column, the time

taken for a compound to travel depends on the solubility of the compound in the liquid phase. The retention time will indicate the time taken by a compound to move along the GC column. Generally, lower molecular weight compounds will have lower retention times compared to the higher molecular weight compounds. Any compound with similar polarity to the liquid phase will have greater retention times as it is more soluble in the phase. Hence, by using this concept, the separation of organic compounds in a mixture could be done using GC and the identification of the compound could be assisted by the retention times.

When the compound is eluted from the GC column, it will pass the chamber source of the mass spectrometer which is under high vacuum, where they will be bombarded by the electrons (Hocart, 2010). Due to the energy transferred, this process will lead to the ionisation and dissociation of molecules into various ion fragments. The ions can be singled or multiple charged. The ion then passes through the analyser section and separate based on their mass to charge ratio (m/z). By plotting the abundance ions detected versus the m/z value, a mass spectrum will be obtained. The mass spectrum of a compound acts as a fingerprint which can be used to identify the original organic structure. A positive identification of the sample component can be obtained by matching off the GC retention time of a sample component and its mass spectrum with those of a standard reference compound analysed under the same conditions (Clement and Taguchi, 1991).

2.3 Metabolomics

Metabolomics is defined as the quantitative measurement of the multiparametric metabolic response of living systems towards the pathophysiological stimuli or genetic modification (Nicholson, Lindon and Holmes, 1999). It has been used to recognise disease biomarker, to aid process of drug discovery, to study on nutrition aspect and to analyse the plants, bacteria and environment (Worley and Powers, 2013). Metabolomics is the latest technology used to identify, detect and quantify the metabolites with low molecular weight in the organism's metabolism. Reproducibility characteristics with high peak resolution and sensitivity make GC-MS as a robust metabolomics tool among the various instruments conventionally used for metabolic profiling (Jonsson *et al.*, 2005). Metabolomics can speed up the understanding towards the characteristics of global metabolic, interpretation of mechanisms metabolic and identification of biomarkers metabolic (Peng, Li and Peng, 2015).

Metabolomics study has been used to study the effect of the planting environment towards the tobacco leaf metabolism during the development and senescence period using pseudotargeted GC-SIM-MS and CE-MS methods (Zhao *et al.*, 2015). From the study, it showed that the planting regions and growing periods influenced the metabolism of leaf tobacco. Metabolomics approach also can be used to unravel the metabolic fluctuations of crucial plant molecules and the metabolic flux associated with various genotypes and growth environments (Kusano *et al.*, 2011).

2.3.1 Metabolomics study using GC-MS

GC-MS technique is used widely for plant metabolomics research to facilitate the quantification and identification of the metabolites that involve in the central pathways of primary metabolism like amino acids, sugar, polyamines, organic acids and sugar alcohols. Previous studies have shown that metabolomics study was done using NMR spectroscopy approach in distinguishing the metabolomics variation of *C. nutans* leaves extract due to different drying methods (Hashim *et al.,* 2016). The findings were important especially to select the best drying methods in preserving the *C. nutans* metabolic features. Recent metabolomics studies have shown significant potential for clinical approaches to develop diagnostic and prognostic biomarkers for various diseases (Banoei, Donnelly and Winston, 2014). By using GC-MS for metabolic profiling, we can obtain information on the different metabolic features between the group of healthy and patients. GC-MS offers high separating power and high sensitivity for volatile metabolites and compounds that can be converted to volatile derivatives with 300-500 metabolites in any sample with relative ease in 45 minutes (Solomon and Fischer, 2010).

GC–MS also has been used in plant metabolomics studies (metabolite profiling) for chemotaxonomic study of three *Curcuma spp.* (Xiang *et al.,* 2011), where the authors used essential oils to discriminate among the cultivars and quality control has been done on a common medicinal herb (Tianniam *et al.,* 2010). The principal component partial least-square discriminant analysis is used to visually discriminate among samples; phenological differences in leaves of *Vitis viniera*

spp. (Weingart *et al.,* 2012), in which the authors used retention indices, high-match factors and multivariate statistics to differentiate the two samples and metabolic differences between disease-infected leaves of citrus by volatile headspace analysis (Cevallos-Cevallos *et al.,* 2011). The above examples highlight well the use of GC–MS as an analytical platform for plant metabolites, but more importantly its applicability to the emerging field of plant volatile metabolomics.

2.4 Multivariate data analysis

The PCA and PLS models act as a platform in providing rapid interpretation of information-rich spectral datasets for inferring biological conclusions (Worley and Powers, 2013). PCA and OPLS-DA are powerful tools of statistical modelling which provide insights into the separations between experimental groups based on high-dimensional spectral measurements from GC-MS, NMR, MS or other analytical instruments (Worley and Powers, 2016).

CHAPTER 3

MATERIALS AND METHODS

3.1 Equipments

Table 3.1 shows the instruments used in this study.

Table 3.1: List of instrument used in this study.

Instrument	Model
Ultra centrifugal mill (Herb grinder)	Type ZM 200 Retsch, Haan, Germany
Universal oven	UNE 200-800/UFE 400-800, Schwabach, Germany
Rotary evaporator	R-100 BUCHI, Tokyo, Japan
Ultrasonic Cleaner Set	WiseClean, WUC-A10H, Wertheim, Germany
Analytical balance	Sartorius AX224, Goettingen, Germany
Fume hood	Azteclab, HOOD 1.2, Selangor, Malaysia
Vacuum pump	Vacuubrand, MZ 2C NT, Wertheim, Germany
Centrifuge	Hettich, D-78532, , Buckinghamshire, England
GC-MS	Agilent Technologies 7890 A, New York, US

3.2 Materials

Table 3.1 lists the chemicals and materials used in this study.

Table 3.2: List of materials used in this study.

Chemical	Manufacturer
Ethanol 95%	QReC, New Zealand
Pyridine	Sigma-Aldrich, Steinheim Germany
BSTFA+TMCS (99:1)	Sigma-Aldrich Chemie GmbH, Steinheim Germany

3.3 Methodology

3.3.1 Plant identification

C. nutans was authenticated at the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia (Voucher no. SK 1980/11).

3.3.2 Sample preparation

C. nutans were collected from eight different locations throughout the northern state of Malaysia (Table 3.3). The leaves were freshly harvested and stored at 40 °C oven for drying. Then, the dried leaves were pulverised into powder for extraction process.

Table 3.3: The samples of *C. nutans* from different locations

Sample	Group	State	Location
Sample 1	G1	Perlis	Kangar
Sample 2	G2	Perlis	Beseri
Sample 3	G3	Pulau Pinang	Kepala Batas
Sample 4	G4	Kedah	Alor Setar
Sample 5	G5	Pulau Pinang	Sungai Ara
Sample 6	G6	Pulau Pinang	Taman Herba IPPT
Sample 7	G7	Pulau Pinang	Tasek Gelugor
Sample 8	G8	Perak	Manjung

3.3.3 *C. nutans* extraction

25 grams of *C. nutans* powder was mixed with 250 mL of 95% ethanol in a conical flask. Then, the mixture was sonicated for 30 minutes at room temperature before transferring into 50 mL of centrifuge tube. The centrifugation of the mixture was done at 6000 rpm in 10 minutes. The supernatant was then collected in a schott bottle while the pallet was re-extracted again according to the same procedure for two more cycles. After three cycles, the collected supernatants were filtered through filter paper using vacuum pump. The filtrates were evaporated under the pressure of 175 mbar at 60 °C using rotary evaporator. The dried extracts were left in the desiccators until further use. Percentage yield of the extraction was calculated as follows:

$$\text{Percentage of yield (\%)} = \frac{\text{Weight of extract}}{\text{Weight of leaves}} \times 100$$

3.3.4 Derivatisation of *C. nutans* extracts

Derivatisation process was carried out by mixing 5 mg of *C. nutans* extract with 50 µL of pyridine and 75 µL of bis(trimethysilyl)trifluoracetamine (BSTFA). The mixture was then heated for 20 minutes at 80 °C in the sonicator. The derivatised samples were filtered using a 0.45 µm nylon membrane filter to remove any suspended particulate particle residue. The sample was then transferred to micro volume vial insert for GC-MS analysis (Graikou *et al.*, 2011).

3.4 GC-MS analysis of *C. nutans*

The GC-MS analysis was carried out using the Agilent Technologies 7890 A, New York, US. An aliquot (2 µL) of the derivatised sample was injected into the gas chromatography at a split ratio 1:20 (Graikou *et al.*, 2011). The carrier gas used was helium with flow rate of 1 mL/min. The injector was operated at 200 °C with oven temperature programmed from 100 °C to 300 °C at the rate of 1.2 °C/min (Graikou *et al.*, 2011). Identification of the compounds was conducted according to National Institute of Standards and Technology/Gaithersburg MD USA (NIST MS) search 2.0 (Revathi and Rajeswari, 2015). Each compound was identified quantitatively by estimating the percentage of peak area.