# UV-VIS SPECTROPHOTOMETRIC-BASED METABOLITE PROFILING OF *CLINACANTHUS NUTANS* LEAVES POSSESSING ANTIOXIDANT ACTIVITY

**By**

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**This thesis is dedicated to my beloved parents, the most valuable and precious people in my life**

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# ABBREVIATIONS

| | |
|---|---|
| UV | Ultra violet |
| VIS | Visible |
| SIMCA | Soft independent modelling of class analogy |
| MVDA | Multivariate data analysis |
| *C. nutans* | *Clinacanthus nutans* |
| m | Meter |
| cm | Centimeter |
| min | Minutes |
| mL | Millilitre |
| g | Gram |
| mg | Milligram |
| ˚C | Degree Celsius |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| nm | Nanometer |
| λ | Lambda (wavelength) |

| | |
|---|---|
| μL | Microliter |
| mM | Millimolar |
| PCA | Principal component analysis |
| PLS | Partial least square |
| OPLS-DA | Orthogonal partial least square discriminant analysis |
| W | Water |
| E | Ethanol |
| C | Chloroform |
| P | Petroleum ether |
| PC | Principal component |

# ABSTRAK

Pendekatan metabolomiks merupakan satu kajian yang tidak berat sebelah secara kualitatif dan kuantitatif yang komprehensif bagi semua metabolit yang sedia ada dalam sampel biologi. Metabolomiks berasaskan tumbuhan mengenalpasti metabolit tumbuhan berdasarkan analisis fitokimia berskala luas. Komposisi bioperubatan dan penggunaannya dalam pemakanan dan perubatan telah menarik perhatian kepada metabolomiks tumbuhan. Kajian ini bertujuan untuk mengesan dan mengenalpasti potensi metabolit aktif yang berkesan untuk aktiviti antioksidan dalam *Clinacanthus nutans* (Burm.f) Lindau (*C. nutans*) menggunakan pendekatan metabolomiks berdasarkan spektrofotometri UV-Vis. Kaedah ultrasonikasi telah digunakan untuk pengekstrakan daun tumbuhan yang menggunakan 4 pelarut yang berbeza kekutuban. Data spektrofotometri dipindahkan ke perisian SIMCA versi 13.0.3 (Umetrics AB, Umeå, Sweden) untuk analisis data multivariat (MVDA) menggunakan analisis komponen utama (PCA), separa-kurangnya dua struktur terpendam (PLS), dan analisis diskriminan PLS orthogonal (OPLS-DA). Analisis kemometrik ini digunakan untuk membezakan pelbagai ekstrak *C. nutans*. Semua model mempunyai kebolehulangan yang tinggi dan keupayaan ramalan berdasarkan pelbagai perkakas diagnostik. OPLS-DA menunjukkan pemisahan yang ketara daripada 4 kluster ekstrak (nilai p kurang dari 0.0001), yakni petroleum eter, kloroform, etanol, dan akues. Pemisahan antara 4 ekstrak ini dicatatkan oleh panjang gelombang 266, 267, 265 nm dalam PLS dan 332, 333, 331 nm dalam OPLS-DA. Ekstrak etanol dan akues mempunyai korelasi positif dengan aktiviti antioksidan (E > W). Tambahan pula, ekstrak etanol daun *C. nutans* mempunyai

aktiviti hapus-sisa tertinggi 2,2-polibrominat-1-pikilhidrazil (DPPH) berbanding dengan ekstrak lain. Sebatian aktif yang berpotensi bertanggungjawab untuk aktiviti antioksidan dalam ekstrak etano ini ialah metabolit dengan julat panjang gelombang 303-365 nm seperti orientin, homoorientin, shaftoside, vitesin, isovitesin dan asid kafeik. Kajian ini menonjolkan potensi UV-Vis spektrofotometri untuk pendekatan metabolomiks bagi menilai variasi metabolit dalam sampel ke arah pengetahuan yang lengkap tentang tumbuhan dengan aktiviti antioksidan mereka.

# ABSTRACT

A metabolomics approach is an unbiased qualitatively and quantitatively comprehensive study of all the existing metabolites in a biological sample. Plant-based metabolomics seek to identify plant metabolites based on a wide-scale phytochemical analysis. Biomedical composition of plant and its usage in nutrition and medicine have drawn universal attention to the plant metabolomics. This study aims to detect and identify potential active metabolites responsible for antioxidant activity in *Clinacanthus nutans* (Burm.f) Lindau (*C. nutans*) leaves extracts using UV-Vis spectrophotometric-based metabolomics approach. Ultrasonication method was applied for the leaf extraction of this prominent medicinal plant using 4 different polarities of solvents. Spectrophotometric data were transformed to SIMCA software version 13.0.3 (Umetrics AB, Umeå, Sweden) for multivariate data analysis (MVDA) using principal component analysis (PCA), partial least squares to latent structures (PLS), and orthogonal PLS discriminant Analysis (OPLS-DA). This chemometric analysis was applied to differentiate between various extracts of *C. nutans*. All models had high reproducibility and predictive ability based on the various diagnostics tools. OPLS-DA showed the clearest discrimination of the 4 clusters of the extracts (*p*-value of less than 0.0001) i.e. petroleum ether, chloroform, ethanol, and aqueous. The discrimination of the 4 extracts were recorded with wavelengths of 266, 267, 265 nm in PLS and 332, 333, 331 nm in OPLS-DA. Ethanol and water extracts have the positive correlation with antioxidant activity (E > W). Moreover, ethanolic extract of *C. nutans* leaves showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity compared to other extracts. Potential

active compounds responsible for antioxidant activity in this medicinal plant ethanolic extract were metabolites with the wavelength ranged from 303 to 365 nm such as orientin, homoorientin, shaftoside, vitexin, isovitexin, and caffeic acid. This study highlighted the potential of using UV-VIS spectrophotometry for metabolomic approach to assess metabolite variation in samples and moving towards a comprehensive knowledge of plants with antioxidant activity.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the study

A metabolomics approach is an unbiased qualitatively and quantitatively comprehensive study of all the existing metabolites in a biological sample. Metabolomics aim to represent a comprehensive assessment of the entire components in a specific biological system (e.g., cell, tissue, and organ), and recognise as many metabolites as possible (Shen et al., 2013). Metabolite profiling, a potent tool to discover biological issues, can be defined as simultaneous quantification of the certain groups of metabolites in a given biological matrix which has gained more and more interest in the recent years. Measurements of metabolites would enhance our knowledge about biological responses to every possible stimulation.

Plant-based metabolomics seeks to identify plant metabolites based on a wide-scale phytochemical analysis. It is a relatively fresh research field albeit numerous studies have been conducted in this area. Metabolomics or indeed small molecule-omics (Hall, 2006) analyses in plants can be challenging due to the chemical diversity of a vast number of metabolites. Biomedical composition of plant and its usage in nutrition and medicine have drawn universal attention to the plant metabolomics. Medicinal plant markets have been dramatically increasing around the world (Organization, 2003) dealing 60 billion of US dollars annually (Tilburt & Kaptchuk, 2008). Over 35,000 species of plants universally and more than 1,300 in Malaysia have been used for their medicinal values (Jantan, 2004). By

now, over 50,000 metabolites from the plant kingdom and thousands of metabolites from single plant have been characterised (Wikipedia, Retrieved August 17, 2014). However, the plant metabolome is still poorly defined and the identification process for specific compounds remains challenging (Shen et al., 2013).

Recently, finding naturally occurring antioxidants has come to the fore (Kant et al., 2013). It is estimated that more than % 65 of plant species have therapeutic value such as antioxidant properties (Krishnaiah et al., 2011). Antioxidants are able to scavenge free radicals in the cells and decrease oxidative stress. Therefore, they have beneficial therapeutic effects facing with various diseases such as cancers, inflammations and cardiovascular diseases (Krishnaiah et al., 2011).

Malaysia as a megadiverse tropical country has numerous medicinal plants. One-fourth of conventional medical drugs comes from plants located in tropical rainforest areas. Surprisingly, only less than 5 percent of these tropical rainforest herbs have been scientifically investigated (Jantan, 2004). Therefore, this study focused on a tropical herb named *Clinacanthus nutans* due to the increasing public demand for the use of natural products as well as to provide a basis for further research.

UV-Vis spectrophotometry is one of the techniques used in metabolomics. It is simple, rapid, inexpensive and powerful method (Khoshayand et al., 2010) which is suitable for identification of components in biological materials without a need for preliminary separation stage (Ragupathy and Arcot, 2013). Meanwhile, it has been gaining growing

attention in metabolomics and agriculture fields and has been applied for a large number of plants (Luthria et al., 2008). One of the primary steps for the discovery of new drugs is phytochemical screening to find potential compounds (Jantan, 2004).

Nowadays most of the data as well as metabolomics data are multivariate because of complex research plans and easy usage of advanced instruments. Thus, there is a need for multivariate data analysis to avoid inefficient and inappropriate analysis (Wiklund, 2008). Metabolite profiling provides many data points for each parameter that are suitable for data mining (Kopka et al., 2004). Analysis via different statistical methods such as soft independent modelling of class analogy (SIMCA) can be used to detect main data patterns, correlations and clusters. This analysis drives unbiased knowledge possession by introducing unknown relations (Kopka et al., 2004). Therefore, in this study, SIMCA software was used for multivariate data analysis (MVDA) that provides information for subsequent trial and determining 'diagnostic' metabolites.

MVDA is a proper statistical tool for handling great spectroscopic data sets and is utilised in classifying samples based on their components (Javadi et al., 2014). It also projects all variables at the same time, without any risk of missing information, and finds unknown trends using reliable models (Wiklund, 2008). This concept has lately been introduced to organise huge data sets.

## 1.2 Objectives of study

### 1.2.1 Main objective

To detect and identify potential active metabolites responsible for antioxidant activity in *C. nutans* leaves extracts using UV-Vis based metabolomics approach.

### 1.2.2 Specific objectives

i) To determine the antioxidant activity of *C. nutans* extracts (comprehensive extraction).

ii) To analyse various metabolites in *C. nutans* leaves extracts using UV-Vis spectrophotometric method.

iii) To identify and discriminate potential active compounds responsible for antioxidant activity of *C. nutans* extracts using MVDA.

## 1.3 Literature review

### 1.3.1 *Clinacanthus nutans*

Sabah snake grass scientifically named *Clinacanthus nutans* (Burm.f) Lindau (*C. nutans*) from the family Acanthaceae is extensively grown in tropical Asia and is a prominent traditional medicinal herb in Thailand and Malaysia. It is also called as “belalai gajah”, “phaya yo” and “e zui hua” in Malay, Thai and Chinese language, respectively. The therapeutic properties of *C. nutans* have been partially examined (Aslam et al., 2014). Table 1.1 shows the taxonomy of *C. nutans* plant.

Table 1.1: *C. nutans* Taxonomy (Aslam et al., 2014)

| Kingdom | Plantae (Plants) |
|---|---|
| Class | Equisetopsida C. Agardh |
| Subclass | Magnoliidae Novák ex Takht |
| Superorder | Asteranae Takht |
| Order | Lamiales Bromhead |
| Family | Acanthaceae Juss |
| Genus | Clinacanthus Nees |

### 1.3.2 Morphology

*C. nutans* is a shrub approximately 1 to 3 m high with pubescent branches. Leaves are green and narrowly lanceolate, 2.5-13 cm long, and 0.5-1.5 cm wide. Stems are terete, straite and glabrescent (Tinh, 2014). Figure 1.1 shows the picture of *C. nutans* plant.

Figure 1.1: *C. nutans* plant in “My Medicinal Herbs Garden” at Integrative Medicine Cluster, IPPT, USM

### 1.3.3 Phytochemical contents

Some investigations about *C. nutans* constituents based on different properties have been reported in various studies. Currently, the detected phytochemicals include stigmasterol -β-Dglucoside, 3-amino-4,5-dihydroxyfuran-2(3H)-one, stigmasterol, lupeol, β-sitosterol, belutin, six known C-glycosyl flavones, vitexin, isovitexin, shaftoside, isomollupentin-7-O-β- glucopyranoside, orientin, isoorientin (Tinh, 2014), catechin, quercetin, kaempferol, luteolin, caffeic acid, gallic acid (Ghasemzadeh et al., 2014), clinamide A, clinamide B, clinamide C, 2-cis-entadamide A, entadamide A, entadamide C, trans-3-methylsulfinyl-2-propenol (Tu et al., 2014), five sulfur-containing glycosides, two glycoglycerolipids, a mixture of nine cerebrosides, a monoacyl monogalatosyl glycerol [(2S)-1-O-linolenoyl- 3-O-b-Dgalactopyranosylglycerol] (Sakdarat et al., 2009), 132-hydroxy-(132S--)chlorophyll, 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, phaeophorbide a (Sakdarat et al., 2006), n-pentadecanol, eicosane, 1-nonadecene, heptadecane, dibutylphthalate, n-tetracosanol-1, heneicosane, behenic alcohol, 1-heptacosanol, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester, nonadecyl heptafluorobutyrate, eicosayl trifluoroacetate, 1,2-benzenedicarcoxylic acid, dinonyl ester, phthalic acid, dodecyl nonylester (Yong et al., 2013), 19 monoglycosyl diglycerides, for example, 1,2-*O*-dilinolenoyl-3-*O*-β-D-glucopyranosyl-*sn*-glycerol (Janwitayanuchit et al., 2003), myricyl alcohol, trigalactosyl and digalactosyl diglycerides (Aslam et al., 2014).

Despite all these studies, there is still lack of metabolite profiling of this plant extract possessing antioxidant activity. Hence, the present study was designed to reach *C. nutans* metabolite profile by chemometric techniques using UV-Vis spectrophotometer.

### 1.3.4 Traditional values

Malaysian people use *C. nutans* as herbal tea and boil the fresh leaves with water. They tend to consume this plant because of its antioxidants and nutrients. Moreover, cancer patients used this plant as a cheap house regime (Aslam et al., 2014).

*C. nutans* is applied for diabetic myelitis, fever and diuretics. In Thailand, skin rashes, insect and snake bite, varicella zoster virus and herpes simplex virus lesions are treated by fresh leaves alcoholic extract (Aslam et al., 2014).

People consume the leaves with different methods. Some just take the raw leaves and some use as fresh drinks. They blend it with other drinks, for instance sugarcane, apple juice or green tea (Aslam et al., 2014).

### 1.3.5 Plant antioxidant activity

Previous studies indicated that ethanolic extract of *C. nutans* has antioxidant activity and protective impact against free radical-induced hemolysis (Patchareewan et al., 2007). However, its antioxidant activities are less than green tea (Jr-Shiuan et al., 2012).

Recently, phytochemicals from chloroform extract of *C. nutans* showed a strong radical scavenging activity compared to aqueous and methanol extracts (Yong et al., 2013).

The phytochemicals from cold solvent extraction of *C. nutans* are potential antioxidant agents. Among 3 different solvents, petroleum ether extracts exhibited the strongest radical scavenging activity of 82.00 ± 0.02 %, compared with ascorbic acid (88.7 ± 0.0 %) and á-tocopherol (86.6 ± 0.0 %) (Arullappan et al., 2014).

*C. nutans* dried tea leaves with various drying methods and different infusion periods were examined to measure their antioxidant activity, total flavonoids content and phenolics content. Unfermented samples showed higher antioxidant activity as the phenolics compounds drop because of fermentation. This study indicated that herbal tea of *C. nutans* is a strong antioxidant (Lusia Barek, 2015).

### 1.3.6 Other studies

*C. nutans* has shown anti-viral, antioxidant, anticancer, and anti-inflammatory activities (Yong et al., 2013) and also protective effect against oxidative induced hemolysis (Aslam et al., 2014). To be more specific, some of the studies conducted on this plant are noted in this section.

*C. nutans* extracts was unable to antagonise cobra venom effect (Cherdchu et al., 1977) and did not show significant potential toward scorpion venom (Uawonggul et al., 2006).

Topical formulation of *C. nutans* extract reduces the varcella zoster virus pain in infected patients earlier than placebo group (Sangkitporn et al., 1995) and its cream has been successfully examined for herpes zoster treatment (Charuwichitratana et al., 1996). In line with all antiviral studies of *C. nutans*, Yoosook et al. (1999) also investigated about its anti-HSV-2 activities but the results revealed that it is not potential to treat this virus. (Yoosook et al., 1999). In another study, Janwitayanuchit et al. (2003) investigated about 19 isolated monoglycosyl diglycerides inhibitory activity on 2 types of herpes simplex virus (HSV-1, HSV-2) which 1,2-O-dilinolenoyl-3-O-beta-D-glucopyranosyl-sn-glycerol demonstrated a great inhibitory effect toward both types of HSV (Janwitayanuchit et al., 2003). Also, three isolated chlorophyll related compounds from the leaves of *C. nutans* showed anti-herpes simplex activity in pre-viral entry step (Sakdarat et al., 2009). Furthermore, simplex virus type-2 prior to infection is significantly inhibited or inactivated by *C. nutans* extracts (Vachirayonstien et al., 2010). Bibliographic resources for 151 patients with herpes infection reported the effectiveness of *C. nutans* extracts (Kongkaew & Chaiyakunapruk, 2011). Kunsorn et al. (2013) described the recognition methods to differentiate *Clinacanthus nutans* and *Clinacanthus siamensis* as well as confirming their anti-HSV activity (Kunsorn et al., 2013).

In addition, an experiment surveyed the effects of Thai herbs, including *C. nutans* in black tiger shrimp pathogenic bacteria (Supamattaya et al., 2005). *C. nutans* extract indicated significant anti-inflammatory activities due to *in vivo* inhibition of neutrophil activity (Wanikiat et al., 2008). Methanolic extract of *C. nutans* leaves was once orally applied in male mice and did not lead to death or any undesirable result (P'ng et al., 2012). In addition, 14 days oral administration of this plant activated AChE function resulted in regulating cholinergic neurotransmission in heart, liver and kidney of mice (Lau et al., 2014). Plus, *C. nutans* leaves extracts have higher protective activity on *E. coli* super-coiled plasmid DNA integrity compared to green tea extracts (Jr-Shiuan et al., 2012).

In the other study, chloroform extract of *C. nutans* revealed a high antiproliferative activity against cancer cell lines compared to aqueous and methanol extracts (Yong et al., 2013). The phytochemicals from cold solvent extraction of *C. nutans* are potential antimicrobial and cytotoxic agents. Petroleum ether extract exhibited the highest cytotoxic activity against K-562 and HeLa cells among 3 solvents (Arullappan et al., 2014).

A cross sectional, descriptive study of 240 cases with adult-onset diabetes in Malaysia documented that 62.5% of them had used complementary and alternative medicine which half of them had used biological therapy including 4 herbs. 7.9% of patients had used *C. nutans* in their early therapy (Ching et al., 2013).

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Materials

Lists of used equipment and chemicals are presented in Table 2.1 and Table 2.2.

### 2.1.1 Equipment

Table 2.1: List of instruments used in this study.

| Instrument | Company & Model |
|---|---|
| Herb grinder | Retsch, ZM 200, Haan, Germany |
| Analytical balance | Sartorius, M-Pact, Goettingen, Germany |
| Fume hood | Azteclab, HOOD 1.2, Selangor, Malaysia |
| Ultrasonic cleaner (sonicator) | WiseClean, WUC-A10H, Wertheim, Germany |
| Centrifuge | Hettich, EBA 21, Buckinghamshire, England |
| Vacuum pump | Vacuubrand, MZ 2C NT, Wertheim, Germany |
| Refrigerator 4-8˚C | LG Electronics, GR-V242RL, Seoul, Korea |
| Rotary evaporator | Eyela, N-1100, Tokyo, Japan |
| Freeze dryer (a) | Genevac, EZ-2 .3 Elite, New York, USA |
| Freeze dryer (b) | Eyela, FDU-1200, New York, USA |
| Micro plate reader | BMG Labtech, FLU0star Omega, Germany |
| UV/VIS Spectrophotometer | Perkin Elmer, Lambda 25, USA |

### 2.1.2 Chemicals and reagents

Table 2.2: List of chemicals used in this study

| Chemicals | Manufacturers |
| --- | --- |
| Ethanol 99.7% | QReC, New Zealand |
| Chloroform | QReC, New Zealand |
| Petroleum ether 60-80 ˚C | QReC, New Zealand |
| 2,2-Diphenyl-1-picrylhydrazyl (DPPH) | Sigma-Aldrich, Steinheim, Germany |
| Butylated hydroxyanisole | Sigma-Aldrich, Missouri, USA |
| Caffeic acid (≥95%, HPLC) | Sigma-Aldrich, China |
| (+)-Catechin | Sigma-Aldrich, Fluka, France |
| Gallic acid (99%) | Merck Schuchardt OHG, Hohenbrunn, Germany |
| Kaempferol (≥90%, HPLC) | Sigma-Aldrich, Germany |
| Orientin | Chroma Dex, 1J13, United States |
| Homoorientin (Isoorientin) | Chroma Dex, 1O11, United States |
| Pheophorbide a | Sigma-Aldrich, CDSO13345, United States |
| Purpurin | ACROS, New Jersey, USA |
| Quercetin (≥95%, HPLC) | Sigma-Aldrich, India |
| Vitexin (≥95%, HPLC) | Sigma-Aldrich, Fluka, Bulgaria |
| Isovitexin | Chroma Dex, A1087B, United States |

## 2.2 Methods

### 2.2.1 Plant identification

The botanical identity of *C. nutans* was characterised by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia (Voucher no. SK 1980/11).

### 2.2.2 Leaf extraction

Dry leaves of *C. nutans* were bought from Manjung, Perak, Malaysia. The leaves were pulverised into fine powder using herb grinder and kept in room temperature. A total of 15 replicates for each of the 4 different solvent polarities including petroleum ether, chloroform, ethanol (99.7%) and distilled water were prepared, resulting in 60 samples for analysis. For each sample, based on the ratio 1:25 in a comprehensive extraction, 5 g of obtained fine powder was weighed and sonicated for 30 min by ultrasonicator after immersing in 125 mL of each respective solvent. Then, the extract was centrifuged at 6000 rpm for 15 min to separate supernatant (liquid part) from pellet. The supernatant was filtered using a vacuum pump and Whatman filter paper number one. The labeled extract was covered and kept in the 4 ˚C fridge until the next usage. At last, different methods were used for drying the extracts due to the type of the solvents. For drying the 15 water extracts, we used a freeze-drier (Eyela) which gave us solid crystal extracts within 3 days. They were then converted into the fine powder using a mortar and pestle. For drying 15 chloroform extracts, another freeze-drier (Genevac) was used which generated pasty extracts within 1.40 hours adjusting as low boiling point (low BP) solvent. However, for drying petroleum ether and ethanol extracts, a rotary evaporator was used which was adjusted with the temperature 60 ˚C and the spin of 2 rotation. It was managed to get pasty extracts which were neither liquid nor too

dry. Finally, all of our 4 solvent extracts (60 samples) labeled and kept in desiccator for further analysis.

Percentage yield of extraction for the 4 different types of extracts is calculated according to the following equation:

$$\text{Percentage yield } = \frac{\text{Weight of obtained extract}}{\text{Weight of extracted leaves}} \times 100$$

### 2.2.3 Antioxidant properties

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was conducted by following an antioxidant protocol (Rockenbach et al., 2011) with some modifications to examine antioxidant activity of four different *C. nutans* extracts (i.e., petroleum ether, chloroform, ethanol and water).

One flat bottom 96 well cell culture plate was used for each solvent due to the 5 repeats for each sample and 3 repeats for each concentration of standard. Butylated hydroxyanisole, the standard, was dissolved in ethanol (99.7%) to gain 1.0 mg/ml concentration followed by a serial dilution based on the following equation to get 7 different concentrations (1.0, 0.8, 0.6, 0.4, 0.2, 0.1 and 0.0 mg/ml):

$$M_1V_1 = M_2V_2 *$$

* M= Molarity, V= Volume

Among these 7 concentrations, the concentration of 0.0 which is only DPPH and ethanol was considered as control.

In addition, all 60 samples were prepared by dissolving 2 mg of each sample in 1 ml of their respective solvent which was whether petroleum ether, chloroform, ethanol or distilled water. For preparing DPPH (0.1 mM) solution, 3.94 mg DPPH was added to 0.1 liter ethanol:

$$\text{DPPH weight } = 0.1 \text{ L ethanol } \times 394.33 \times 0.0001 \text{ M}$$

33.3 μL of each sample extract and standard was thoroughly mixed with 1 mL of freshly made ethanolic DPPH (0.1 mM) in a dark room. Then, it was kept in dark for 30 min at room temperature. After incubation, the absorbance was detected at a wavelength of λ=517 nm by micro plate reader equipped with Omega software to send the data to excel file, calculate the average of each sample and standard, and draw the calibration curve for standard. Lastly, the scavenging percentage of DPPH was calculated according to the following equation and arranged in one excel sheet:

$$\%\ DPPHsc = \left[\frac{absorbance\ control - absorbance\ sample}{absorbance\ control}\right] \times 100$$

### 2.2.4 UV/VIS Spectrophotometric analysis

An ultraviolet-visible spectrophotometer equipped with Lambda 25 software provided digital information obtained from absorbance spectra in the wavelength range of 250-600 nm included ultra violet and visible light with one data point in each nanometer. Meanwhile, a reference blank based on the extract solvent was used for all measurements to calibrate spectrometer (Ragupathy & Arcot, 2013). Depends on the nature of the solvents, different cuvettes were utilized. For ethanol and water extracts, plastic cuvette and for chloroform and petroleum ether extracts, quartz cuvette were used. After preparing 1 mg/ml of all samples as the stock, each sample was further diluted with its own solvent to get an acceptable peak in the spectrum. Hence, ethanol extract was diluted 5 times by adding 5 ml ethanol, water 6.6 times and chloroform 2.5 times while petroleum ether did not need to be diluted. The experiment was conducted for all 4 types of extracts with 15 repeats for each and 5 replicates for each repeats resulting in 300 times readings. Digital data was auto saved in ASCII file. Then, we compressed all 300 files in one excel sheet where we could label and arrange whole data. Besides, an average was calculated for 5 replicates of each repeats and data were edited by adding antioxidant activity to them as Y variable.

### 2.2.5 Phytochemicals wavelengths

A number of detected phytochemicals for *C. nutans* in previous studies were chosen and their UV wavelengths for the maximum absorbance were found using spectrophotometer or in literature review. These compounds are caffeic acid, catechin, quercetin, gallic acid, pheophorbide a, purpurin, orientin, homoorientin, kaempferol, shaftoside, vitexin, and isovitexin. These information were added to the SIMCA to see whether these phytochemicals are existed in our extracts or not.

### 2.2.6 Statistical analysis

All obtained data from UV-Vis spectra and antioxidant activity were transformed to SIMCA software version 13.0.3 (Umetrics AB, Umeå, Sweden) for multivariate data analysis (MVDA) using PCA-X, PLS and OPLS-DA after the pre-processing step to detect outliers and clean the data. By excluding outliers, the data equaled to 14 repeats for each type of extracts.

PCA (Principal Component Analysis), which is normally the first step for any multivariate analysis, was used for getting an overview and a summary of our data to appraise the main differences among the samples. This unsupervised model classified the data, identified the pattern and trends as well as finding outliers (Wiklund, 2008).

PLS (Partial Least Square) is a common prediction and regression tool to see how things are various from each other and to show the correlations and relationships. It is a supervised model given the DPPH activity as Y variable. In PLS, one or more than one X-variables relate with one or more than one Y-variables by regression (Khatib, 2015).

OPLS-DA (Orthogonal Partial Least Square Discriminant Analysis or Orthogonal Projection of Latent Structure Discriminant Analysis) is an extension of PLS-DA which is used in classification studies, model interpretation and biomarker identification. It is a supervised model guided by known information of classes that finds responsible variables for class discrimination (Wiklund, 2008). Basically, it divides variations into 2 categories

which are the variations correlated to response and the variations uncorrelated to response. Therefore, irrelevant variations are filtered out (Nordin et al., 2015).

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1 Plant extraction

Ultrasonication, a new extraction method, was used to extract biochemical from *C. nutans* leaves. This technique is simple, cheap, efficient, and fast which uses less organic solvents compared with conventional methods (Wang & Weller, 2006).

Different polarity solvents such as petroleum ether, chloroform, ethanol and distilled water were utilised in this study to increase the extraction of bioactive compounds with varying polarities. The polarity index of solvents are shown in Table 3.1.

Table 3.1: Polarity index of solvents used in this study

| Solvent | Polarity index |
|---|---|
| Petroleum ether | 0.1 |
| Chloroform | 4.1 |
| Ethanol | 5.2 |
| Water | 9.0 |

42 g extract was obtained from 300 g fine powder of *C. nutans* dried leaves which included 18 g distilled water extract, 15 g ethanol extract, 6 g chloroform extract and 3 g petroleum ether extract. Among these extracts, water was in powder form and the rest had

pasty texture. Also, percentage yield of extraction for each solvent was calculated and simplified in Table 3.2.

Table 3.2: Percentage yield of extracts

| Solvent | Percentage yield (%) |
|---|---|
| Petroleum ether | 4 |
| Chloroform | 8 |
| Ethanol | 20 |
| Water | 24 |

### 3.2 Antioxidant properties

Antioxidant assay was conducted using DPPH radical scavenging method according to (Rockenbach et al., 2011) with some modifications. DPPH is a stable free radical in room temperature with a maximum absorbance at 517 nm in ethanol. Antioxidants, proton donating substances, scavenge DPPH from purple to yellow causing a lower absorbance. All 4 types of *C. nutans* extracts (petroleum ether, chloroform, ethanol, and water) were tested for their DPPH radical scavenging ability. Butylated hydroxyanisole, a synthetic antioxidant (Krishnaiah et al., 2011), was used as standard in this study. The results for DPPH free radical scavenging activity percentage for each *C. nutans* extract are individually demonstrated in Figure 3.1. Also, in Figure 3.2, all 4 extracts are compared between all their samples and between the averages of them. Standard error of the mean (SEM) is shown in Figure 3.2 (b).

At 2 mg/ml, ethanol extract and water extract showed the highest DPPH radical scavenging activity of % 15.90 and % 9.88, respectively. The high content of metabolites found in *C. nutans* ethanol extract such as orientin, homoorientin, shaftoside, vitexin, isovitexin, and caffeic acid, which are flavonoids and phenolics, might be the reason for having a higher antioxidant potentiality in this extract. Flavonoids and phenolics are the reason of antioxidant activity in the wide variety of plants (Abdel-Farid et al., 2014).

Although previous studies have introduced chloroform (Yong et al., 2013) and Petroleum ether (Arullappan et al., 2014) as the most potent *C. nutans* extracts for free radical scavenging activity, they have used different extraction methods with this study. Method of extraction is one of the parameters that influence the amount of phenolic compounds (Upadhya et al., 2015). However, ethanolic extract of *C. nutans* has also shown antioxidant activity (Patchareewan et al., 2007).

Chloroform and petroleum ether extracts showed negative DPPH scavenging percentage. It might be due to an error in handling or inappropriate dilution of plant extracts which can give a negative result of DPPH scavenging capacity. Meanwhile, flavonoids structural conformation (correlated with the presence of hydroxyl groups) effects the interaction of an antioxidant with the free radical. Appropriate dilutions of plant extracts showed a positive reaction with DPPH (Choi et al., 2002).

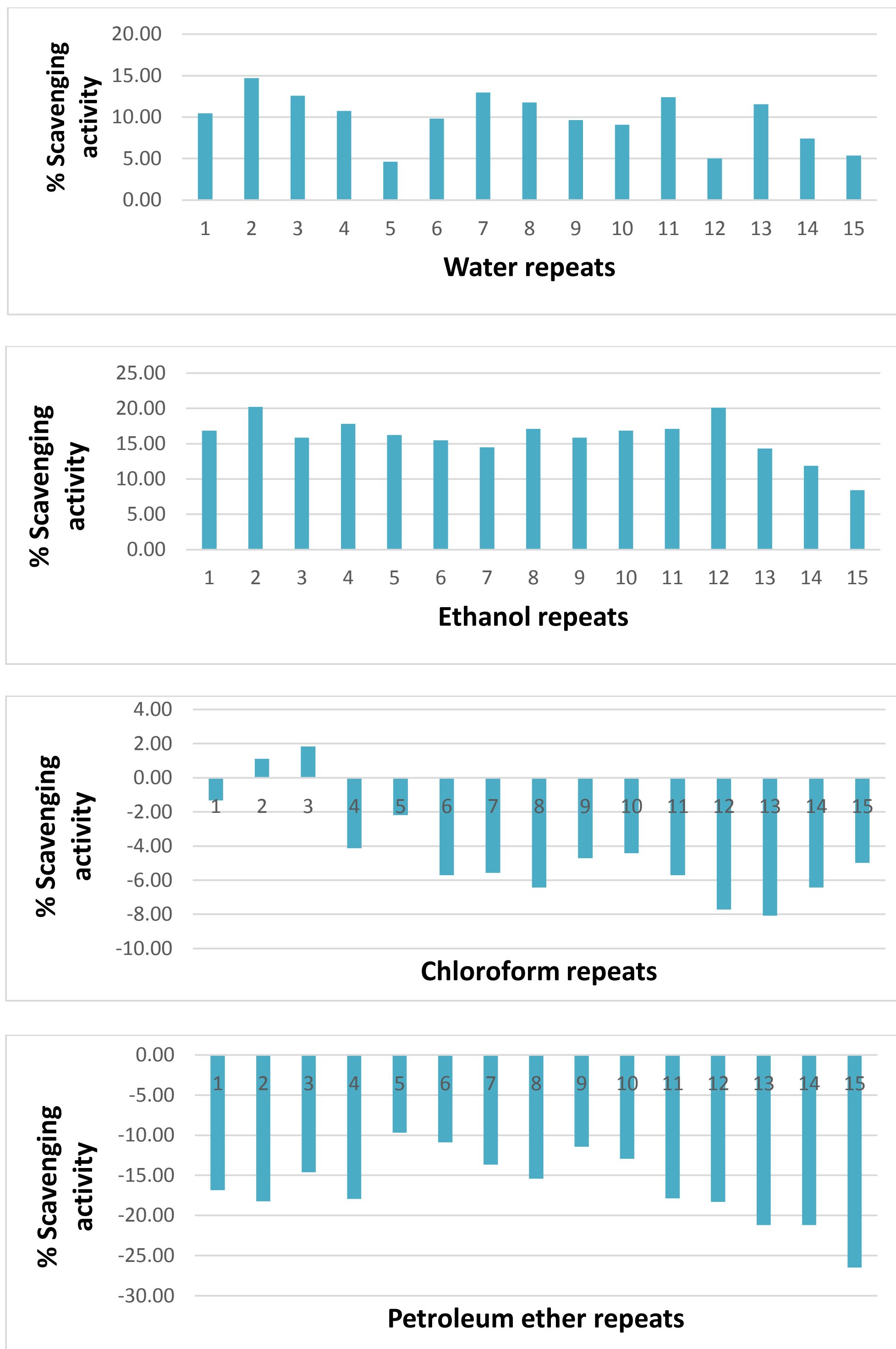
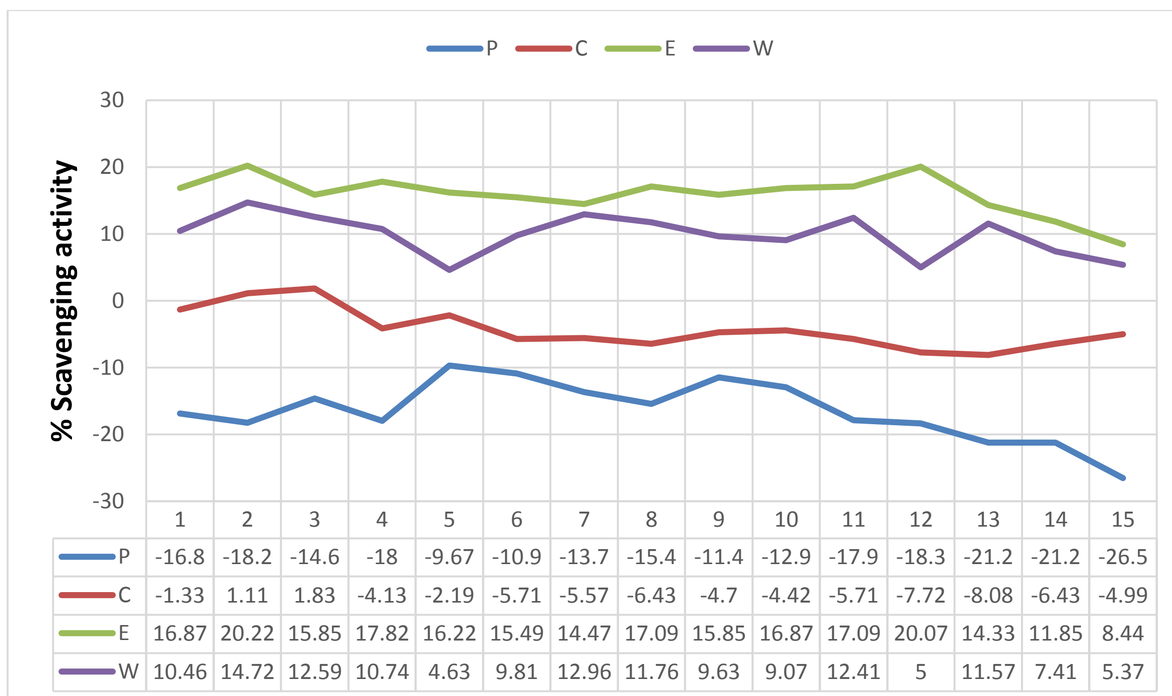
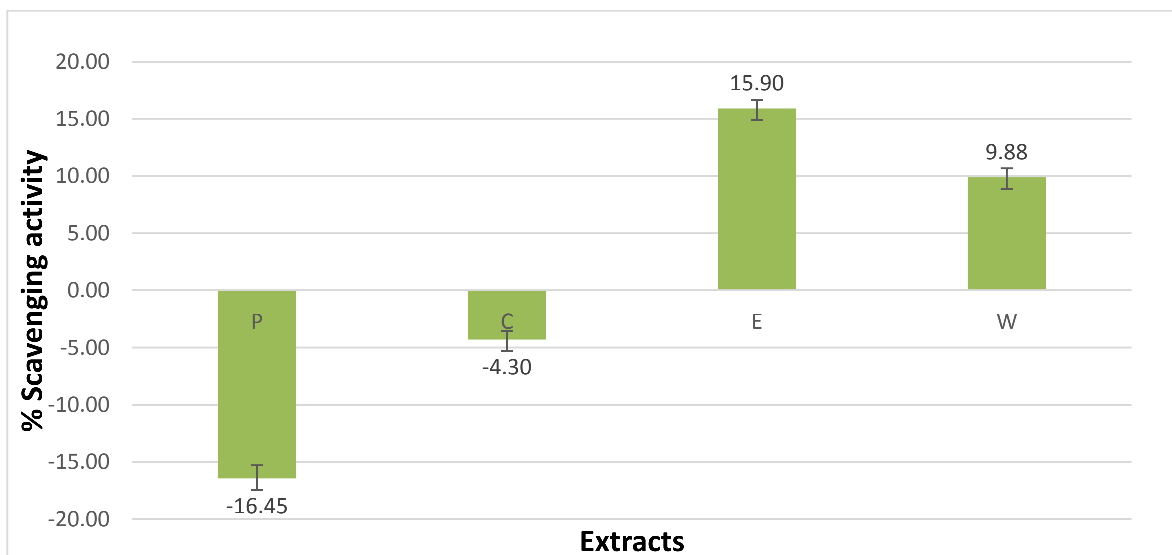



Figure 3.1: Results of DPPH scavenging activity percentage for each *C. nutans* extract (n=15)




| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P | -16.8 | -18.2 | -14.6 | -18 | -9.67 | -10.9 | -13.7 | -15.4 | -11.4 | -12.9 | -17.9 | -18.3 | -21.2 | -21.2 | -26.5 |
| C | -1.33 | 1.11 | 1.83 | -4.13 | -2.19 | -5.71 | -5.57 | -6.43 | -4.7 | -4.42 | -5.71 | -7.72 | -8.08 | -6.43 | -4.99 |
| E | 16.87 | 20.22 | 15.85 | 17.82 | 16.22 | 15.49 | 14.47 | 17.09 | 15.85 | 16.87 | 17.09 | 20.07 | 14.33 | 11.85 | 8.44 |
| W | 10.46 | 14.72 | 12.59 | 10.74 | 4.63 | 9.81 | 12.96 | 11.76 | 9.63 | 9.07 | 12.41 | 5 | 11.57 | 7.41 | 5.37 |

(a)




(b)

Figure 3.2: Results of DPPH scavenging activity percentage for all 4 extracts. (a) between all the samples , (b) between averages of samples. (W= water, E= ethanol, C= chloroform, P= petroleum ether)