EFFECT OF VERMICOMPOST APPLICATION ON BIOACTIVE COMPOSITION AND ANTIOXIDANT PROPERTIES OF *Clinacanthus nutans* Lindau

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2019

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF BIOTECHNOLOGY

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2019

UNIVERSITY OF MALAYA

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Matric No: SGF 160006

Name of Degree: Master of Biotechnology

Title of Dissertation:

"EFFECT OF VERMICOMPOST APPLICATION ON BIOACTIVE COMPOSITION AND ANTIOXIDANT PROPERTIES OF *Clinacanthus nutans* Lindau"

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EFFECT OF VERMICOMPOST APPLICATION ON BIOACTIVE COMPOSITION AND ANTIOXIDANT PROPERTIES OF *Clinacanthus nutans* Lindau

ABSTRACT

Vermicompost is the product derived from composting of organic materials through the use of earthworms. The use of vermicompost as an alternative to chemical fertilizer is gaining attention, parallel to the rise in awareness on organic farming and sustainable agriculture. In this study, the effect of vermicompost application on the total phenolic content (TPC), total flavonoid content (TFC), total chlorophyll content (TCC), total anthocyanin content (TAC), total carotenoid content (TC) and antioxidant properties of *Clinacanthus nutans* Lindau were evaluated. The stability of all compounds after extract storage was also assessed. A field study employing a randomized complete block design (RCBD) with four treatment groups consisting of control plants (CC), plants supplied with chemical fertilizer (CF), plants supplied with vermicompost (VC) and plants supplied with both chemical fertilizer and vermicompost (MF) was conducted. Data analysis showed that CF plants contained the highest TPC (181.53 ± 35.58 mg GAE/g dry weight) and highest TCC (6090.97± 144.90 µg/g dry weight). On the other hand, CC plants showed the highest reading for TFC (276.25 ± 3.09 mg QE / g dry weight) and TC (520.47± 16.47 µg/g dry weight). Moreover, the antioxidant properties of the extracts were also assessed using DPPH, ABTS and FRAP assays. It was found that CC plants had the highest antioxidant potential against both DPPH and ABTS radicals and also the highest FRAP value. On the other hand, MF plants showed the lowest antioxidant potential. After 2 and 4 weeks of extract storage at -20°C and +4°C, TPC, TFC, TAC, TCC and antioxidant potential of the extracts were found to decrease but TC

for all extracts increased significantly. Interestingly, extracts from VC and MF showed the lowest percentage of total phenolic, total flavonoid and total chlorophyll loss after 2 weeks of extract storage at -20^{o}C and +4^{o}C as compared to CC and CF. Besides that, extract from VC plants also exhibited the highest increase in TC after 2 and 4 weeks of extract storage at -20^{o}C. At this juncture, it could be deduced that the application of vermicompost had little effect in the expression of phenolics, flavonoids, anthocyanin, chlorophyll and carotenoid in *C. nutans* Lindau plants. However, the extract from plants treated with vermicompost (both VC and MF) showed better stability as compared to CC and CF after being stored at different storage conditions. Pearson correlation analyses showed a significant correlation between TPC, TFC, TAC and TCC with FRAP, DPPH and ABTS.

Keywords: total phenolic content, antioxidant, extract storage, vermicompost, *Clinacanthus nutans* Lindau

KESAN APLIKASI VERMIKOMPOS KE ATAS KOMPOSISI BIOAKTIF DAN CIRI ANTIOKSIDA OLEH *Clinacanthus nutans* Lindau

ABSTRAK

Vermikompos ialah produk yang diperoleh daripada pengkomposan bahan organik melalui penggunaan cacing tanah. Penggunaan vermikompos sebagai alternatif kepada baja kimia semakin mendapat perhatian, selari dengan peningkatan kesedaran mengenai penanaman organik dan pertanian mampan. Dalam kajian ini, kesan aplikasi vermikompos terhadap jumlah kandungan fenolik (TPC), flavonoid (TFC), klorofil (TCC), antosianin (TAC), karotenoid (TC) dan potensi antioksida dalam *Clinacanthus nutans* Lindau telah dinilai. Kestabilan kandungan kesemua kompaun selepas ekstrak disimpan juga dinilai. Satu kajian lapangan menggunakan reka bentuk blok lengkap rawak (RCBD) dengan empat kumpulan rawatan yang terdiri daripada pokok kawalan (CC), pokok yang dibekalkan dengan baja kimia (CF), pokok yang dibekalkan dengan vermikompos (VC) dan pokok yang dibekalkan dengan kedua-dua baja kimia dan vermikompos MF) telah dijalankan. Analisis data menunjukkan bahawa pokok CF mengandungi TPC tertinggi (181.53 ± 35.58 mg GAE /g berat kering) dan TCC (6090.97± 144.90 μg/g berat kering). Selain itu, pokok CC pula menjukkan nilai TFC (276.25 ± 3.09 mg QE/g berat kering) dan nilai TC tertinggi (520.47± 16.47 μg/g berat kering). Potensi antioksida dalam ekstrak juga dinilai melalui ujian DPPH, ABTS dan FRAP. Pokok CC telah didapati mempunyai potensi antioksida tertinggi terhadap kedua-dua radikal DPPH dan ABTS dan juga mempunyai nilai FRAP tertinggi. Sebaliknya, pokok MF menunjukkan potensi antioksida paling rendah. Selepas 2 dan 4 minggu ekstrak disimpan pada suhu -20 ºC dan +4ºC , kesemua kandungan TPC, TFC, TAC, TCC dan potensi antioksida dalam ekstrak tersebut didapati berkurangan. Namun

kandungan TC bagi semua ekstrak meningkat secara signifikan. Menariknya, ekstrak dari pokok VC dan MF menunjukkan peratusan kehilangan fenolik, flavonoid, klorofil, dan antosianin yang paling rendah berbanding pokok CC dan CF. Di samping itu, pokok VC juga menunjukkan peratusan peningkatan karotenoid yang paling tinggi selepas 2 dan 4 minggu disimpan pada -20ºC. Berdasarkan ini, dapat disimpulkan bahawa aplikasi vermikompos tidak mempunyai pengaruh yang besar dalam ekspresi kandungan fenolik, flavonoid, antosianin, klorofil dan karotenoid dalam pokok *C. nutans* Lindau. Namun demikian, ekstrak dari pokok yang dirawat dengan vermikompos menunjukkan kestabilan yang lebih baik berbanding CC dan CF selepas ekstrak disimpan pada kondisi yang berbeza. Analisis korelasi Pearson menunjukkan korelasi yang signifikan antara nilai TPC, TFC, TAC dan TCC dengan FRAP, DPPH dan ABTS.

Kata kunci: kandungan fenolik, antioksida, penyimpanan ekstrak, vermikompos, *Clinacanthus nutans* Lindau

ACKNOWLEDGEMENTS

In the name of Allah, the most gracious and the most merciful, all praise goes to Allah for His blessings, guidance and for helping me to complete my project work satisfactorily on time.

My utmost thanks to my supervisor, Dr. Jamilah Syafawati Yaacob and my co-supervisor, Assoc. Prof. Dr. Noor Zalina Mahmood for their help and support throughout the process of conducting my research. Without their kind guidance, this research may not have been possible.

I would like to thank all those who have been very helpful and supportive during the completion of this project. This include Kak Norsyuhada Japri for helping me obtain the samples from Pusat Penyelidikan Bioteknologi Glami Lemi (PPBGL) for my research, the students from Dr. Sujatha's laboratory in Institute of Graduate Studies(IGS), and Kak Aisyah from Bioprocess laboratory, Institute of Biological Science for giving me the permission to use the equipments in their laboratory.

In addition, I would like to express my appreciation to all my labmates for their company, support and help. I would also like to thank my roommate/coursemate, Tang Chen Seok for always being there sharing our ups and downs together during the journey to complete our Masters. I am forever grateful to be surrounded by friends who are kind and understanding.

Last but not least, I owe my deepest gratitude to my parents and family for their tremendous help, unconditional support, and continuous encouragement.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	:	Alpha
β	:	Beta
^{o}C	:	Degree celcius
<	:	Less than
≤	:	Less than or equal to
>	:	More than
-	:	Negative
%	:	Percentage
+	:	Positive
µg/g	:	Microgram per gram
µg/mL	:	Microgram per millilitre
µL	:	Microlittre
ABTS	:	2, 2-azino-bis (3-ethylbenzotiazoline-6-sulfonic acid)
$AlCl_3.6H_2O$	:	Aluminium chloride
ANOVA	:	Analysis of variance
BCB	:	β–carotene bleaching
cm	:	Centimetre
CO_2	:	Carbon dioxide
Cu	:	Copper
cv.	:	Cultivar
DCFH-DA	:	Dichloro-dihydro-fluorescein diacetate
ddH_2O	:	Double distilled water
DF	:	Dilution factor
DMRT	:	Duncan's Multiple Range Test
DPPH	:	2,2-diphenyl-1-picrylhydrazyl

EPP	:	Ethyl phenylpropiolate
Fe	:	Ferrum/ Iron
Fe^{2+}	:	Ferrous
Fe^{3+}	:	Ferric
$FeSO_4$	:	Iron(II) sulfate
FRAP	:	Ferric reducing power
FYM	:	Farmyard manure
g	:	Gram
g^{-1}	:	Per gram
GC-MS	:	Gas chromatography–mass spectrometry
HPLC	:	High Performance Liquid Chromatography
IFN	:	Interferon
IMO	:	Indigenous Microorganisms
K	:	Potassium
$K_2S_2O_4$	:	Potassium persulfate
kg	:	Kilogram
kg ha^{-1}	:	Kilogram per hectare
MBC/MFC	:	Minimum bactericidal or fungicidal
MeOH	:	Methanol
Mg	:	Magnesium
mg	:	Milligram
mg g^{-1} DW	:	mg per gram of dry weight
mg GAE/g DW	:	mg of gallic acid equivalents/g dry weight
mg ha^{-1}	:	Milligram per hectare
mg/ml	:	Milligram per millilitre
MIC	:	Minimum inhibitory concentration

min^{-1}	:	Per minute
mm	:	Millimetre
mM	:	Millimolar
mMol TE/g	:	Millimolar per gram Tellurium
Mn	:	Molybdenum
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	:	Molar weight
N	:	Nitrogen
Na_2CO_3	:	Sodium carbonate
$NaC_2H_3O_2.3H_2O$	:	Sodium acetate
NH^{4+}	:	Ammonium
nm	:	Nanometre
NO^{3-}	:	Nitrate
ORAC	:	Oxygen radical absorbance capacity
P	:	Phosphorus
PGRs	:	Plant growth regulators
PMA	:	Phorbol myristate acetate
PPO	:	Polyphenol oxidase
ROS	:	Reactive oxygen species
SPSS	:	Statistical Package for the Social Sciences
t ha^{-1} or t/ha	:	Tonne per hectare
TNF	:	Tumor necrosis factor
TPTZ	:	2,4,6-Tri(2-pyridyl)-s- triazine
UV	:	Ultraviolet
v/v	:	Volume per volume
Zn	:	Zinc

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Vermicomposts are organic materials broken down by the interactions between microoganism and earthworms in a mesophilic process, to produce fully stabilized and organic soil amendments with low C: N ratios (Ramasamy & Suresh, 2010). Besides having large particulate surface area that provides many micro-sites for the microbial activity, vermicompost also contains significant quantities of nutrients and biologically active metabolites. Vermicompost which is rich in NPK (nitrogen 2-3%, phosphorus 1.55-2.25% and potassium 1.85-2.25%), micronutrients and beneficial soil microbes is gaining a lot of interest as a greener replacement for chemical fertilizers to maintain and further improve soil quality. The application of vermicompost as an alternative to chemical fertilizer not only can produce healthier plants, but it can also increase resistance in plants towards pests and diseases, avoid wastes from filling up landfill and it is also a quicker and cost-effective technique of composting. Vermicompost significantly stimulates the growth of a wide range of plant species including several medicinal plants (Chiluvuru *et al.*, 2009) horticultural crops (Kashem *et al.*, 2015; Sundararasu & Neelanarayanan, 2012; Yang *et al.*, 2015; Zucco *et al.*, 2015), fruit crops (Acevedo & Pire, 2004; Cabanas-Echevarría *et al.*, 2005), ornamentals (Chattopadhyay, 2014; Sardoei *et al.*, 2014) and forestry species (Donald & Visser, 1989; Lazcano *et al.*, 2010a; Lazcano *et al.*, 2010b).

Clinacanthus nutans Lindau is an important medicinal plant from the family Acanthaceae. It is a tall, erect herbaceous perennial shrub, growing up to 1 metre in height and is found distributed throughout the tropical regions including Southeast Asia and China. The plant is locally known in Malaysia as Belalai Gajah due to the slightly curved stem supporting the leaves which resembles the curve of an elephant's trunk

(Zakaria *et al.*, 2016). The leaves of *C. nutans* are used as a remedy against venomous snake bites, scorpion and insect stings in Malaysia and Thailand (Uawonggul *et al.*, 2006) resulting in the vernacular name, Sabah snake grass.

Figure 1.0: *Clinacanthus nutans* Lindau plant (adapted from Zulkipli *et al.*, 2017).

Clinacanthus nutans Lindau is classified as one of Malaysian high-value herbal products in the Entry Point Project 1 (EPP1) under the Agriculture sector, which is one of the identified National Key Economic Areas (NKEAs). This plant has been widely used for the treatment of various diseases such as cancer, herpes simplex virus (HSV), varicella-zoster virus (VZV) lesions, skin rashes and kidney problem (Farsi *et al.*, 2016). *C. nutans* contains bioactive phytochemicals comprised of secondary metabolites that are produced naturally to protect the plant against environmental stresses and free radicals. The phytochemicals include flavanoids, alkaloids, phenolic compounds and

other bioactive compounds. These phytochemicals extracted from the plant demonstrate various biological activities such as antioxidant (Yong *et al.*, 2013), antimicrobial (Arullappan *et al.*, 2014), anti-inflammatory (Wanikiat *et al.*, 2008), antivenom (Uawonggul *et al.*, 2006) and anticancer activities (Farooqui *et al.*, 2016).

1.1 Problem Statement

In recent years, chemical fertilizers have been the key components for providing crop nutrient needs. Though they promote the growth of crops, the application of chemical fertilizers in the field may have negative effects on the environment. Besides contributing greatly to the deterioration of the environment, usage of chemical fertilizers also results in loss of soil fertility, less agricultural productivity and soil degradation. The price of chemical fertilizers is also higher compared to organic fertilizers. To overcome the various problems due to application of chemical fertilizers, organic fertilizers such as vermicompost can be used as a better alternative. At present, using organic manures and biofertilizers, such as vermicompost has led to a decrease in the application of chemical fertilizers and provide high quality products that are free of harmful agro-chemicals for human safety (Mahfouz & Sharaf-Eldin, 2007; Malik *et al.*, 2009). In addition to that, application of vermicompost may reduce environmental pollution that is caused during industrial manufacturing of the chemical fertilizers.

1.2 Research Gap

Based on literature review, very limited publication was found on the effect of vermicompost application towards availability of phytoconstituents and bioactivity of *C. nutans*. Previous works conducted on application of vermicompost only focused on the effects of its application on plant growth, which can be measured physically by a number of parameters such as the shoot length, number of leaves, leaf length, leaf

width, plant height and stem diameter. However, the effects of vermicompost application on the availability of bioactive compounds in plants remain in doubt.

1.3 Significance of Study

This study which was carried out on *C. nutans* Lindau can provide valuable information on the capability of vermicompost as an alternative to chemical fertilizer to enhance plant growth as well as its ability to affect the availability of bioactive phytoconstituents in this species.

1.4 Objectives of Study

The objectives of the present study were as follows:

1. To evaluate the effect of vermicompost application on the presence of bioactive compounds in *C. nutans* Lindau.
2. To analyze the effect of vermicompost application on the antioxidant properties of *C. nutans* Lindau.
3. To evaluate the effect of different storage conditions on the stability of bioactive compounds in *C. nutans* Lindau.

CHAPTER 2: LITERATURE REVIEW

2.1 Vermicompost

2.1.1 Process of Vermicomposting

Vermicomposting is a mesophilic process in which microorganisms and certain species of earthworms such as *Lumbricus rubellus* and *Eisenia ferida* are used to help in the process of decomposing. The presence of various microorganisms such as bacteria, fungi and cilliates in the composting bin help to break down organic matter (Edwards *et al.*, 2004).

Figure 2.1 shows a simple and basic process of vermicomposting as reported by Wang *et al.* (2009)**.** In vermicomposting, worm beds and sufficient supply of worms are required. In general, the worm beds are filled with biosolids that were digested and dewatered, in which raw liquid sludge was being placed. Additional pretreatment might be needed if the anaerobic digestion is used before the conversion of earthworm. A bulking agent like wood chips may be used in situation where the moisture is high to keep the bed porous and aerobic. Biosolids were generally placed in the absence of bulking agent and the worm bed resembles a simple tray. During the composting process, windrows may also be used. The worms then need to be separated from the castings once the biosolids had been consumed, by using an earthworm harvester which is a drum screen that rotates horizontally. The end product of the breakdown of organic matter, called as vermicompost (or also known as worm castings, worm humus or worm manure) will exit through the screen openings and the worms will plunge down the length of the drum (Wang *et al.*, 2009)**.**

During vermicomposting, the earthworms act as a mechanical grinder by aerating, grinding, crushing, degrading and stimulating the biosolids, thus changing the organic matter physically and chemically in a short period of time (Sinha *et al.*, 2010). Apart from the earthworms' activities, mesophilic microorganism also aids in the conversion of biosolids by stimulating the degradation of the organic matter. The formation of the castings resulted in a larger surface area available for the colonization of microorganisms and hence indirectly stimulated the population of microbes.

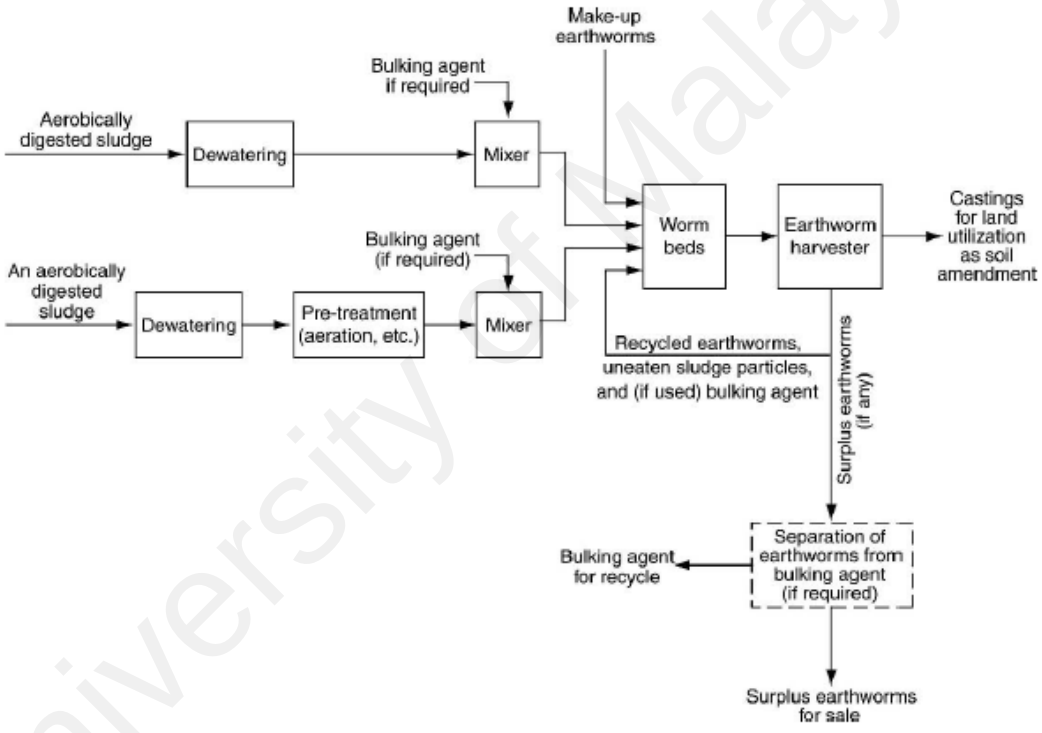


Figure 2.1: Basic process of vermicomposting (adapted from Wang *et al.*, 2009).

2.1.2 Nutrient Composition of Vermicompost

The nutrients level in vermicompost is dependent on the source of raw materials and earthworm species. Vermicompost contains various plant macro- and micronutrients. Most of the nutrients which are present in inorganic forms are readily available and released gradually through mineralization of the organic matter, thus constituting a slow-release fertilizer that constantly supplies the plant with the required nutrients

(Chaoui *et al.*, 2003). Vermicompost has been known as a nutritive organic fertilizer that is not only rich in NPK (nitrogen 2-3%, potassium 1.85-2.25% and phosphorus 1.55-2.25%) but also micronutrients, beneficial soil microbes like 'nitrogen-fixing bacteria' and 'mycorrhizal fungi' and are scientifically proven as 'miracle' growth promoters & protectors (Sinha *et al.*, 2010).

2.1.2.1 C/N Ratio

Organic carbon and inorganic nitrogen both have important roles for living organisms. Carbon is mainly required as a source of energy to maintain the growth and reproduction while nitrogen is utilized for the synthesis of protein and other cell components. During the composting process, carbon and nitrogen must be available in the substrate at the right ratio to ensure the plant's proper nutrition and growth. The C to N (C/N) ratio is often used as a maturity indicator for organic waste (Pattnaik & Reddy, 2010) and it is an important parameter for fertilizer because plants are unable to assimilate mineral nitrogen unless the ratio is about 20:1 (Senesi, 1989). It was also reported that C/N ratio of less than 20 demonstrates a gratifying level of maturity (Senesi, 1989) and an advanced stabilization of organic waste while C/N ratio that is higher than 20 indicates degradation, which resulted in a decrease of the biological activity (Haug, 1993).

Earthworms act as mechanical blenders and help to lower the carbon content by consuming the fresh organic matter, breaking it down, modifying its biological, physical and chemical status and using the carbon as a source of energy during respiration. This action by the earthworms will gradually reduce the C/N ratio of the matter, increasing the surface area exposed to microorganisms and making it more favorable for microbial activity and further decomposition (Dominguez & Edwards, 2004). A study was

conducted by Sailila *et al.* (2010) on the nutrient elements of different agricultural wastes from vermicomposting activity. The vermicompost produced in the study were made from 5 different feed materials such as goat manure, paddy straw, spent mushroom paddy straw compost, sawdust and spent mushroom sawdust compost. As evident in Table 2.1, the C/N ratio of vermicompost made from goat manure was the lowest followed by spent mushroom paddy straw compost, paddy straw, spent mushroom sawdust and sawdust compost. Abu Bakar *et al.* (2014) on the other hand, studied the vermicomposting of vegetable waste amended with different sources of agro-industrial by-products by using the earthworm *Lumbricus rubellus.* Results from the study showed that vermicompost made up of cow dung and vegetable waste at the ratio of 1.75:1.75 kg produced the lowest C/N ratio (19.62±0.11) and it also showed the greatest loss in C/N ratio (-31.37%). On the other hand, the combination treatment of cow dung and vegetable waste at 1.17:2.33 kg yielded the highest C/N ratio (27.17±0.95) and exhibited the highest gain in C/N ratio (+73.28%).

Table 2.1: C/N ratio of vermicompost from each treatment (adapted from Sailila *et al.*, 2010).

Vermicompost	C/N ratio
T_1	9.86
T_2	31.52
T_3	11.07
T_4	107.41
T_5	83.30

T1: goat manure; T2: paddy straw; T3: spent mushroom substrate (paddy straw); T4: saw dust; T5: spent mushroom substrate (saw dust).

2.1.2.2 Major Nutrients (NPK)

Nitrogen (N), Phosporus(P), and Potassium (K) are the important macronutrient elements that are involved in delivering a quality compost or vermicompost (Abu Bakar *et al.*, 2014). N is an essential component of all protein and its deficiency often leads to the plants became stunted or exhibited slow growth and chlorosis (Basuchaudhuri, 2016). Plants take up nitrogen from the soil in the form of nitrate (NO^{3-}), although ammonium (NH^{4+}) is more likely to be the main source of nitrogen. NH^{4+} must be reduced to NO^{3-} for the building of amino acids and proteins (Basuchaudhuri, 2016). Nitrogens are transported in the soluble forms as amines and amides (Hopkins & Hüner, 2008). Besides that, P is also involved in various plant functions such as in energy transfer, photosynthesis, transformation of sugar and starches and in nutrient movement within plant. Insoluble P is converted into soluble forms with the help of P-solubilizing microorganisms through the enzyme phosphatases present in the gut of the worms (Suthar & Singh, 2008).

On the other hand, K plays many essential roles in the plant development. Besides increasing crop yield and improves quality, K is also required for many plant growth processes such as enzyme activation, stomatal activity, photosynthesis, protein synthesis and in the transport of water and nutrients (Prajapati & Modi, 2012). The microorganisms present in the gut of the worms help in making potassium more available to the plants by converting insoluble K into the soluble form, through the aid of microbial enzymes (Sharma, 2003) such as amylase, lipase, cellulase and chitinase (Chaoui *et al.*, 2003). It has been reported that vermicompost contains an average of 1.5% to 2.2% N, 1.8% to 2.2% P and 1.0% to 1.5% K (Adhikary, 2012). Atiyeh *et al.* (2000) found that vermicompost contains higher amount of nitrates which is the more available form of nitrogen as compared to conventional compost which contains higher

level of ammonium. In the study, it was also found that there were higher availability of N in vermicompost than the conventional compost on a weight basis and the supply of nutrients such as P and K were significantly increased with the addition of vermicompost (Atiyeh *et al.*, 2000).

It has been reported that the mineralization of organic matter containing proteins (Bansal & Kapoor, 2000; Kaushik & Garg, 2003) and the conversion of ammonium-nitrogen into nitrate (Atiyeh *et al.*, 2000; Suthar & Singh, 2008) are the two important factors that resulted in the enhancement of N in vermicompost. N levels in the substrate can be increased through the action of earthrworms such as by the addition of their nitrogenous excretory products, mucus, body fluid, enzymes, and even through the decaying dead tissues of worms in vermicomposting subsystem (Suthar, 2007). Pramanik *et al.* (2007) on the other hand have reported that the acid produced through the decomposition of organic matter by the earthworms is what resulted in the increase of P and K contents of vermicompost. The increase in total nitrogen, phosphorus, and potassium were also observed in a study conducted by Nath *et al.* (2009) through the vermicompost produced from agriculture, animal and kitchen wastes by earthworm *Eisenia fetida* (Garg & Gupta, 2011). Results from the study showed that total nitrogen, phosphorus and potassium were higher in vermicompost as compared to other treatments. Likewise, the vermicompost produced from the mixture of rice residues with cow dung by using the earthworm species of *Eudrilus eugeniae* also demonstrated an increase in the nutrients such as calcium, magnesium, phosphorus and potassium (Shak *et al.*, 2014).

2.1.2.3 Micronutrients

All plants require micronutrients to help stimulate their growth. Iron (Fe) which is essential for maintaining growth and productivity, also serves as a cofactor and the catalytic site for many crucial enzymes involved in chlorophyll synthesis. Both Magnesium (Mg) and Manganese (Mn) are important component in the production of chlorophyll and photosynthesis while Zinc (Zn) is the major component for enzyme systems required for plant growth regulation and protein photosynthesis (Kabata-Pendias, 2010). Copper (Cu) also plays a role in several physiological processes in plants such as photosynthesis, respiration, carbohydrate distribution, N and cell wall metabolism, seed production, as well as in disease resistance (Kabata-Pendias, 2010). Only a small quantity of these micronutrients or also known as trace elements is required to stimulate plant growth. However, in high concentration, they are likely to yield unfavourable effects on plant growth.

Earthworms in vermicompost are able to counter the effect by accumulating particular metals through their intestine as well as through the skin, hence reducing specific total potentially hazardous element (Kumar *et al.*, 2008). Analysis of the important micronutrients such as Iron (Fe), Magnesium (Mg), Manganese (Mn) and Zinc (Zn) were carried out in many kinds of experimental vermicomposts. A previous study by Khan & Ishaq (2011) on the chemical nutrient analysis of vermicompost and pitcompost showed that vermicompost contains higher amount of nutrients like Sodium, Calcium, Magnesium, and Chloride as compared to the pit compost and garden soil (control). Nagavallemma *et al.* (2004) also reported that the worm castings from vermicompost made from recycled wastes contain higher percentage (nearly two fold) of both macro and micronutrients than the garden compost (control) as shown in Table 2.2. Similarly, the rice plant (*Oryza sativa*) supplied with the combination of

vermicompost showed the highest uptake of Mg as well as N,P and K in the study conducted by Jadhav *et al.* (1997).

Table 2.2: Nutrient composition of vermicompost and garden compost (adapted from Nagavallemma *et al.*, 2004).

Nutrient Element	Vermicompost (%)	Garden Compost (%)
Organic carbon	9.8 – 13.4	12.2
Nitrogen	0.51 – 1.61	0.8
Phosphorus	0.19 – 1.02	0.35
Potassium	0.15 – 0.73	0.48
Calcium	1.18 – 7.61	2.27
Magnesium	0.093 – 0.568	0.57
Sodium	0.058 – 0.158	<0.01
Zinc	0.0042 – 0.110	0.0012
Copper	0.0026 – 0.0048	0.0017
Iron	0.2050 – 1.3313	1.1690
Manganese	0.0105 – 0.2038	0.0414

2.1.3 The Application of Vermicompost on Other Plants

Vermicompost has been found to significantly stimulate the growth of a wide range of plant species resulting in excellent crop production and enhanced plant growth. Studies have been carried out to investigate the effect of vermicompost application on various crops, such as cereal, fruit, vegetable and medicinal crops.

2.1.3.1 Cereal Crops

Tejada and González (2009) reported that the application of vermicompost on rice crops (*Oryza sativa*) resulted in an increase of the rice yield parameters and the biological properties of soil. A different study was conducted on the effect of organic and inorganic sources of nutrient on the nutrient uptake, yield and yield attributes of rice VC. PRH-10 in India (Kumar *et al.*, 2014). It was found that, when organic and inorganic sources of nutrients were combined, it resulted in an increased of nutrient uptake, yield, and yield attributes of rice as compared to control. The mixture of 125% of recommended dose of fertilizer and 5 t/ha vermicompost resulted in 20.50% higher number of panicles, 23.12% increased panicle length, 13.02% higher panicle weight, 12.90% higher grain weight, 31.15% higher grain yield and higher straw yield (37.12%), protein content (18.77%), N uptake in grain (36.81%) and straw (42.81%), P uptake in grain (32.62%) and straw (31.56%) and K uptake in grain (35.46%) and straw (25.39%) as compared to other treatments.

Other studies were carried out on the different effects of vermicompost application on maize. The application of different amount of vermicompost which include 500-1000 g of vermicompost used for the application time of 10-40 days, and the use of 750 g of vermicompost for 25 days yielded positive effects on the growth of Zea mays. From the study, it was found that the plant height increased by more than 30 cm, the cob weight increased by 30 g, the leaves quantity and production were also increased when vermicompost was applied to the plant as compared to the controls. Increasing application time has also resulted in more than 120 g of cob weight (Manyuchi *et al.*, 2013). A different study by Nasab *et al.* (2015) was conducted to study the effect of different levels of vermicompost on the yield and quality of maize varieties. The treatments included different levels of vermicompost at 0 (control), 4, 8 and 12 t/ha as

main plot and two different maize varities which are 700 and 704 as the subplot. Results from the study showed that biological yield, seed yield, harvest index and percentage of protein were higher when the highest level of vermicompost (12 t/ha) was applied while control showed the lowest results for all parameters measured. 704 type of maize on the other hand, showed better results as compared to the 700 type variety with higher biological yield, seed yield, harvest index and protein percentage. The application of vermicompost has also shown to have significant effect on the height and stalk thickness of maize plant in different growth stages as studied by Kmetova & Kovacik (2014). It was observed that, the statiscally significant highest plant was shown for plant that was supplied with 170 kg ha^{-1} N of vermicompost along with 60 kg ha^{-1} N of mineral nitrogen. The addition of vermicompost alone or together with mineral nitrogen has proven to have positive effects on the increase in the plant stalk thickness. A different study was conducted by Sharma & Banik (2014) on the application of vermicompost and fertilizer on the productivity and profitability of baby corn (Zea Mays L.) and soil health. The findings from the study showed that vermicompost applied plots resulted in considerably higher green fodder (17.58 mg ha^{-1}) and higher cob yield with 0.717 mg ha^{-1}. In addition, the application of vermicompost also resulted in built-up soil nutrients which include 145 kg ha^{-1} nitrogen, 16 kg ha−1 phosporus, 190 kg ha^{-1} potassium, 0.78% of organic carbon, enhanced cation exchange capacity, microbial (basal soil respiration, microbial biomass carbon, microbial quotient, and metabolic quotient) and enzyme activities (urease and acid phosphatase). The effect of vermicompost and chemical fertilizers application on growth parameteres of three corn cultivars was also studied by (Amanolahi-Baharvand *et al.*, 2014). The three different corn cultivars used in the study were Single cross 704, Single cross 677 and Single cross 580 while the treatments included three different levels of fertilizers which were 100% urea for chemical fertilizer, 100% vermicompost for organic fertilizer and

integrated fertilizer with half urea and half vermicompost. It was found that, the combination of both vermicompost and urea produced the best results with increased leaf number, leaf area index, stem diameter, plant height, chlorophyll content and remobilization. Guo *et al.* (2015) reported that the use of vermicompost on maize benefit agriculture industry by 304%.

2.1.3.2 Fruit Crops

Studies conducted by (Kashem *et al.*, 2015; Sundararasu & Neelanarayanan, 2012; Yang *et al.*, 2015; Zucco *et al.*, 2015) have reported that vermicompost can effectively enhance the growth and yield of tomato (*Lycopersicum esculentum*) by improving the physicochemical properties of the soil. Goel and Sidhu (2012) also agreed that the application of vermicompost has positive impacts on tomato as it significantly influenced plant height, leaves number, fruits, flowers and the stem diameter as compared to controls. A different study was conducted by Dhanalakshmi *et al.* (2014) on the impact of vermicompost addition on vegetable plant growth. Vermicompost promoted excellent growth in tomato which resulted in higher percentage of seed germination, increased shoot and root length, and higher branch and leaf number. Based on the results obtained, tomato showed early seed germination (4.77 days) as compared to control (varied between 6.03 to 8.87 days). The addition of the highest level (75%) of vermicompost to tomato produced highest root length which is 8.77cm, 12 cm, and 15.30 cm for 30,60 and 90 days respectively. The shoot length also showed the highest result when 75% of vermicompost was added with 51.67, 74.53, 95.73 cm after 30, 60 and 90 day of planting. The branch number of tomato was around 14.00 to 19.33 cm and the leaf number was 17.00cm. All of the results were higher in vermicompost-supplied plant as compared to control.

There were several studies conducted on the effect of vermicompost application on strawberry. The combination of 8 tonnes per hectar of vermicompost with 6 tonnes per hectar of poultry manure recorded the greatest plant growth for strawberry. It can be observed that vermicompost resulted in the highest plant height (24.27cm), plant spread (33.80 cm), number of plant leaves, petiole length (8.53 cm), number of flowers or plant (3.20) and number of plant fruits (92.83 g). The same treatment also showed the highest yield as compared to control. A study by Arancon *et al.* (2003) reported that there was a significant improvement of the growth and yield of strawberry. Two types of vermicompost which are made of food waste and recycled paper waste were applied to the plots planted with strawberries at the rates of 5 t ha^{-1} or 10 t ha^{-1} in 2000. Strawberries in plots that were treated with vermicompost showed increased leaf areas, number of strawberry suckers, number of flowers, shoot weights, and total yield of strawberries. The results were greater as compared to the strawberries in plots that were supplied with inorganic fertilizer.

The use of vermicompost was also tested on the growth of Citrus reticulatus which is a type of Indian orange. The study revealed that vermicompost application could significantly enhanced the quantity and quality of oranges as compared to the plants that were not supplied with vermicompost. It was later found that the application of 10 kg of vermicompost resulted in increased fruit number, fruit weight and fruit yield (Makode, 2015).

2.1.3.3 Vegetable Crops

Several studies were conducted on the effect of vermicompost on different types of beans. The application of 20% vermicompost has resulted in the highest pod weight, pod number, pod dry weight and pod length in three different beans studied which

include bush bean, winged bean and yard long bean. Furthermore, photosynthetic rate was shown the highest in winged bean that was grown with 20% vermicompost. Not only that, the highest yield and highest protein content for all the three types of beans were also found in the treatment of 20% vermicompost (Islam *et al.*, 2016). The application of vermicompost also showed positive effects on plant height, number of leaves per plant, increase in growth and yield pots of cluster bean (*Cyamopsis tetragonolobus*) as studied by Chavan *et al.* (2015) and significantly enhanced pore space and water holding capacity of *Phaseolus vulgaris* beans (Manivannan *et al.*, 2009). A different study by Singh *et al.* (2011) on the effects of vermicompost, fertilizer and mulch on plant growth, nodulation and pod yield of french bean (*Phaseolus Vulgaris* L.) reported that the application of 3.75 t ha^{-1} vermicompost and N P2O5 K2O at the ratio of 8:13:10 resulted in a 28 to 63% increase of shoot length, primary branches number, the fresh and dry weight of shoots.

Besides that, the application of vermicompost can also enhanced the productivity of potato, spinach and turnip by improving the soil quality (Ansari & Sukhraj, 2010). From the experiment conducted, there has been a significant improvement of the soil quality after the 6 tonnes per ha of vermicompost was added. The vermicompost requirement for leafy crops like spinach was lower which is at 4 tonnes per ha whereas for tuber crops which include turnip and potato required higher level of vermicompost at 6 tonnes per ha. Peyvast *et al.* (2008) studied the effect of vermicompost application as soil supplement on the growth and yield of parsley. Results showed that plant height and the total leaves number increased after vermicompost was added and the plant grown in combination of 10:100 of vermicomposted cattle manure with soil yielded greater total yields and amounts of potassium, phosphorus, and total soluble solids. Other studies of vermicompost application on vegetable crops include chilli (*Capsicum annuum*) and

bhendi or lady's finger (*Abelmoschus esculentus*) as studied by Kumar & Lekeshmanaswamy (2016). The study focused on the germination, growth and seed production of the two vegetables. Application of vermicompost resulted in maximum percentage of seed germination and seedling length of chilli and bhendi. It was also noted that the fruits number and weight were increased as a result of vermicompost addition. The growth of a vegetative crop *Pisum sativum* is also possitively influenced by the application of vermicompost. In a study by Khan & Ishaq (2011) for a period of one month, it was found that the pots containing vermicompost showed the most optimum plant growth. This is due to the fact that vermicompost was rich in nutrients like Potassium, Nitrate, Sodium, Calcium, Magnesium and Chloride.

Several studies were also carried out on the effect of vermicompost on the growth of bell pepper fruits (*Capsicum annum*). In a study conducted by Narkhede *et al.* (2011), chemical fertilizer of urea and vermicompost were used for the plot treatment. The fertilizer was given at the rate of 0 (control), 5, 10, 15 and 20% concentration. Better results were obtained after vermicompost application as compared to chemical fertilizer. This is evident by the significant increased in plant height, leaf length and fruit yield of pepper plants in plots treated with vermicompost with 20% of vermicomposting plot has resulted in maximum leaf chlorophyll content. Llaven *et al.* (2008) studied the effect of vermicompost made of earthworm-processed sheep manure on the growth, productivity and characteristics of bell pepper fruits (*Capsicum annum*) (cv. 'Ancho supremo'). The experiment involved six treatment which include the mixture of of vermicompost and soil in the ratios of 0:1, 1:1, 1:2, 1:3, 1:4, and 1:5 (v/v). The parameters for the plant are measured 21 and 90 days after transplanting. There was a significant increase of 8 cm in plant size for the plant treated with 1:3 vermicompost: soil treatment as compared to soil which was not given any fertilizer. Besides that, the plants with 1:2 and 1:3

vermicompost:soil treatment also showed more number of flowers and higher number of marketable fruits per plant after 90 days of transplanting. Plants treated with 1:4 vermicompost:soil treatment on the other hand showed higher content of nitrogen and higher titratable acidity values as compared to others.

2.1.3.4 Medicinal Crops

Yousefi *et al.* (2013) studied the effect of different vermicompost levels and photoperiod on greenhouse production of stevia (*Stevia rebaudiana* Bertoni). The treatments involved in this experiment include 3 vermicompost levels which are 0 (control), 10 and 20% v/v, while the 3 different photoperiods include short winter days, interrupting night for 1.5 hours at midnight and long days. The result from the study showed that the treatment of 20% vermicompost with interrupting nights produced the highest leaf yield. The same treatment also showed increased amount of chlorophyll a, total chlorophyll and carotenoid content as compared to other treatments. Vermicompost has also shown to be effective in enhancing the growth and development of a rare, endangered and threatened medicinal plant known as *Piper Longum*. The leaf number, branch number, shoot height, shoot fresh weight, root length , fresh and dry weight of root were greater for the plant that was treated with vermicompost mixed soil. In addition, vermicompost application had also resulted in higher Leaf Area Ratio (LAR), Relative Growth Rate (RGR) and Net Assimilation Rate (NAR) as compared to control (Sahoo & Gupta, 2017).

Chiluvuru *et al.* (2009) reported that vermicompost application resulted in significant increase of plant growth, yield, and chlorophyll content in two important medicinal plants which are *Vigna radiata* and *Centella asiatica*. In the study, the two plants were applied with different concentrations of vermicompost involving 0 (control), 10%, 20%

and 30% of vermicompost. It was found that the maximum response for both plants were shown after the application of 20% vermicompost. Amooaghaie & Golmohammadi (2017) investigated the effect of different levels of vermicompost on the growth, essential oil and health of *Thymus Vulgaris*. 0 (control), 25, 50 and 75% of vermicompost were applied. Overall results showed that the best seedling emergence indices was shown by the application of 25% vermicompost while 50% vermicompost resulted in maximum length, fresh and dry weight of aerial parts and root, chlorophyll and carotenoid contents, photosynthetic efficiency, and the highest content of essential oil. The highest level of vermicompost which is at 75% has proved to enhance the suppresion of pathogens.

Other studies involved the use of vermicompost on the growth, yield and profitibility of two different turmeric plants which are kasthuri turmeric (*Curcuma aromatica* Salisb.) and (*Curcuma longa* L.) variety- Megha Turmeric-1 as studied by Shamrao *et al.* (2013) and Sarma *et al.* (2015) respectively. The results from the study showed that vermicompost application significantly influenced plant growth and yield as evident by the higher plant height, and maximum fresh and dry rhizome yield of 456.99 and 82.56 g^{-1} plant respectively. However, the vermicompost-treated plants gave the lowest B: C ratio (2.37) resulting in vermicompost to not be the most cost effective treatment. This may be due to the higher cultivation cost incurred for the purchase of vermicomposts that are readily available in the market. In the study on turmeric (*Curcuma longa* L.) variety- Megha Turmeric-1, the combination of vermicompost with FYM, neemcake and *Trichoderma harzianum* resulted in increased plant height, number of plant fingers, girth of rhizome, weight of rhizome and the highest yield of rhizome with maximum benefit-cost ratio. The results obtained were greater as compared to control. The influence of three types of vermicompost which are cowdung vermicompost, leaf ash

vermicompost and poultry feather vermicompost were assesed on the yield and the alkaloid content of *Withania somnifera* which is a type of medicinal crop. The plant that was treated with poultry feather vermicompost showed the best results with higher shoot length, root length, shoot dry weight, root dry weight, shoot wet weight, root wet weight, shoot: root ratio. Furthermore, the alkaloid content was also higher in vermicompost-treated plant (Raja & Veerakumari, 2013).

The positive effects for the combination application of organic fertilizer were also reported in several studies.The combination of plant growth promoting bacteria and vermicompost in an experiment carried out by Befrozfar *et al.* (2013) resulted in the highest wet and dry yield, essence yield, chlorophyll a, chlorophyll b and the total chlorophyll of basil (*Ocimum basilicum* L.) The effect of vermicompost and amino acids application on the flower yield and essential oil production from *Matricaria chamomile* L. was studied by (Hadi *et al.*, 2011). There were 5 treament groups involved and they consist of 0 (control), 5, 10, 15 and 20 tons per hectare while amino acids were sprayed during budding stage, flowering stage and the combination of budding and flowering stage. The results from the study depicted that 20 ton of vermicompost per hectare gave out the best results as evident by the highest plant height, flower head diamer, fresh and dry flower yield and significant essential oil content.

2.1.4 Other Beneficial Roles of Vermicompost

Vermicompost has important roles in improving water holding capacity and water penetration of soil. The high level of humus produced from *Eisenia fetida* earthworm's castings help to clump the soil particles together to form channels for the passage of air. (Bhat & Khambata, 1959; Capowiez *et al.*, 2009; Ghabbour, 1966) reported that water

penetration in soils was improved by more than 50% through the action of earthworms that helps regenerate compacted soils. The presence of humic acid in humus also provide sites for the binding of plant nutrients which include calcium, iron, potassium, sulfur and phosphorus. Besides that, humic acid also helps making organic matter ready for plants to use by dissolving unresolved minerals, help stimulating the growth of roots and helps overcome stress for plants (Li *et al.*, 2010).

The worm casting which is a biologically active material contain more than thousands of bacterias, enzymes and leftover plant materials that were not digested by the earthworms. The level of bacterial population in the vermicast is greater than the population of bacterias present in ingested soil or the earthworm's gut. Tomlin *et al.* (1995) has reported that there was 10 to 20 times higher of microbial activity in the worm castings as compared to other organic matter. Among the important soil microbes present include the actinomycetes, mycorrhizal fungi, nitrogen-fixing bacterias and phosphate solubilizing bacterias. This finding is supported by a study conducted by Sinha *et al.* (2010) who found that there was more than 10/gm of total bacterial count in vermicompost as compared to other types of composts and the microorganisms present comprised of actinomycetes, azotobacter, rhizobium, nitobacter and phosphate solubilizing bacterias that ranges from 102-106 per gram of vermicompost. The slow released nutrients that are contained within the vermicast are readily available to the plants. Castings which are produced by the earthworm and are covered with mucus membrane dissolve slowly rather than resulting in leaching of immediate nutrients. The product has great effect on the structure of soil, porosity, aeration and has higher capabilities to hold water as it can hold two to three times more water than their weight in soil. Sinha *et al.* (2010) also reported that worm castings can protect plant roots from the high temperature, lessen the risk of erosion and control weeds.

Nielson (1965) reported that the humus produced from worm's castings helps protect plants from the attack of harmful soil-borne/foliar plant pathogens, fungi, nematodes, bacteria and pests. There is extensive research on the suppressive effect of vermicompost on plant diseases. Vermicompost has proved to have significant effects in disease suppressions caused by pathogens such as *Pythium, Phytophthora, Fusarium, Rhizoctonia and Verticillium.* Studies on the ability of vermicompost to supress plant diseases were conducted using different organic materials which include from dairy solids (Kannangara *et al.*, 2000) sewage sludge (Szczech & Smolińska, 2001) and cattle manure (Simsek-Ersahin, 2011; Szczech, 1999).

Vermicompost also helps in protecting plants from the attack of pests. Arancon *et al.* (2002) conducted a study to investigate the effect of vermicompost application on the suppression of insect pests on pepper (*Capiscum annuum*), cabbage (*Brassica oleracea*) and tomato (*Lycopersicum esculentum*). It was found that vermicompost application resulted in 20-40% suppresion of insect pests which involve aphids (*Myzus persicae*), mearly bugs (*Pseudococcus spp*.) and cabbage white caterpillars (*Peiris brassicae).* The use of vermicompost could also inhibit diseases caused by soil born pathogens as studied by (Arancon *et al.*, 2003). Significant suppresion of plant-parasitic nematodes were observed in the experiment involving pepper, strawberries and grapes. The suppressive effect of vermicompost on pests may be due to the high amount of microorganisms present in the vermicompost which function to protect the plant by outcompeting plant pathogens for food resources. This action by the microbes causes the plant pathogens to starve themselves and the microbes also occupied available sites by blocking the plant pathogens access to plant roots. Furthermore, the sporulation of plant pathogen such as *Botrytis uriecinerea*, *Sclerotinia sclerotiorum*, *Corticium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum* were also reduced through the application

of vermicompost extract. The result from a study conducted by Szczech (1999) on the effect of vermicompost addition to tomato showed that, the infection caused by a fungal plant pathogen, *Fusarium lycopersici* was significantly reduced. However, in a different study by Szczech & Smolińska (2001) it was found that there was no significant suppressive effect of vermicompost produced from sewage sludge on *Phytophthora nicotianae.*

2.1.5 Impact of Vermicompost on Plant Secondary Metabolites and Antioxidant Properties

The effect of vermicompost on plant secondary metabolites and antioxidant properties of various plant extracts were previously studied. It has been found that *Manihot esculenta* plants (Medan and Sri Pontian variety) that were treated with vermicompost had the highest total phenolic content as compared to plants treated with empty fruit bunch compost and inorganic fertilizer. Not only that, the total flavonoid content in vermicompost treated plants also showed 24 to 30% higher flavonoids as compared to plants treated with inorganics fertilizer. In addition, both varieties of plants also showed high antioxidant activity in response to vermicompost application (Nur *et al.*, 2013). In a different study by Wang *et al.* (2010), the influence of vermicompost made of cow manure were studied on the antioxidant activities of Chinese cabbage (*Brassica campestris* ssp. *chinensis*). The treatments involve the mixture of vermicompost and soil at ratio of 0:7, 1:7, 2:7, 4:7 and 7:0 (w/w). Total phenols and total flavonoids of the plant extracts increased significantly by 62%, 18%, 200%, 25%, and 17% when treated with the five different ratios of vermicompost respectively as compared to full soil treatment. The antioxidant activity of the extract assessed by DPPH assay was also higher in plant treated with 4:7 vermicompost. The author suggested that the high content of humic compounds present in vermicompost may

influence the strong antioxidant activity shown by vermicompost treated plant. The potential of vermicompost produced from plant waste was also reported by Theunissen *et al.* (2010). Plants that were grown under vermicompost fertilization has shown higher total phenolics as compared to those grown under Osmocote fertilization. This results is due to the slow and gradual release of plant-available nutrients from vermicompost or organic fertilizer relative to Osmocote.

2.2 *Clinacanthus nutans* Lindau

2.2.1 Description of *C. nutans* Lindau

Clinacanthus nutans Lindau or *C. nutans* is known by different names across the world. “Sabah snake grass” or “belalai gajah” are the vernacular names given for *C. nutans* in Malaysia; “dandang gendis” in Indonesia; “phaya yo” or “phaya plongtong” in Thailand; “Bim Bip,” “Xuong Khi,” or “Manh Cong” in some regions of Vietnam; and “e zui hua” or “you dun cao” among the Chinese community (Panyakom, 2006; Roosita *et al.*, 2008; Smitinand, 1980; Watson & Preedy, 2008). The plant is native to countries in Southeast Asia which include South China, Indonesia, Malaysia, Mynamar, Singapore, Thailand and Vietnam (Farsi *et al.*, 2016). *C. nutans* is taxonomically classified by kingdom: Plantae; phylum: Magnoliophyta; class: Magnoliopsida; subclass: Asteridae; order: Lamiales; family: Acanthaceae; genus: *Clinacanthus*; and species *nutans* Lindau (Fazil *et al.*, 2016).

Figure 2.2: Botanical picture of *Clinacanthus nutans* Lindau.

C. *nutans* is a herb shrub that can grow up to 1 metre height. Fong (2015) reported that the plant leaves are simple, opposite with leaf blades having the shapes of lanceolate-ovate, lanceolate or linear-lanceolate and a dimension of 2.5-13.0 cm long and 0.5-1.5 cm wide as can been observed in Figure 2.2. The stems of the plant are cylindrical, terete and striate while the plant petiole has the length of 2-15 mm (Wu *et al.*, 2011). During the young stage, both surfaces of *C. nutans* leaves are pubescent then becoming glabrescent when mature. The base of the leaves are cuncate, obtuse rounderd or truncate while the leaves apex is acuminate (Alam *et al.*, 2016).

2.2.2 Medicinal Properties of *C. nutans* Lindau

Many studies have reported the medicinal properties possessed by *Clinacanthus nutans* Lindau. It was known that the medicinal plants have anti-inflammatory, anti-cancer, antibacterial, antivenom and antioxidant activities.

2.2.2.1 Anti-inflammatory Activity of *C. nutans*

Wanikiat *et al.* (2008) has conducted a study on the anti-inflammatory effects of *C.nutans* extract in models of ethyl phenylpropiolate (EPP)- induced ear odema and carrageenan-induced paw oedema in rats. In the study, topical application of dose-dependent ethyl phenylpropiolate(EPP) was applied to the inner and outer surfaces of the male rats' ears to induce ear oedema while intra-dermal injection of carrageenan was given on the subplantar of the right hind paw to induce paw oedema. The result showed that *C. nutans* extract inhibit the formation of ear oedema significantly at all time points which are after 15 minutes, 30 minutes, 1 hour and 2 hours. Besides that, oral administration of the *C. nutans* extracts at doses of 50,100 and 200 mg/kg also significantly reduced the carrageenan-induced oedema in the hind paw. The author suggested that the reduced activity of myeloperoxidase in the rat odema is due to the possibility of *C. nutans* extract to control the release and synthesis of inflammatory mediators such as kinin, serotonin and prostaglandins during the early phase of inflammations (Mahdy & Webster, 2014).These findings are in agreement with previous reports by Satayavivad *et al.* (1996) which reported the anti-inflammatory activity exhibited by *C. nutans* extract. There was no signs of acute or subacute toxicity in rats after given oral administration of 540 to 1300 mg/kg *C. nutans* leaf extract daily for six weeks (Satayavivad *et al.*, 1996). Pongphasuk *et al.* (2003) also studies the anti-inflammatory and analgesic activities of several plant extracts which include *C. nutans* extract in mice using carrageenan induced paw edema. The 95% ethanolic extract from *C. nutans* leaves showed less than 35% reduced oedema and low analgesic activity as compared to other extracts which are *C. rotundus*, *N. arbor tristis* and *G. mangostana*. The anti-inflammatory activity of *C. nutans* is contributed by its immunomodulatory property. The immunomodulatory activity of *C. nutans* was highlighted in a study which aims to investigate the modulatory effects of plant extracts from Thai medicinal

plants on proinflammatory cytokines-induced apoptosis in human keratinocyte HaCaT cells (Thongrakard & Tencomnao, 2010). Plant extracts (1–100 μg/mL) containing a mixture of Thai traditional herbs which included *C. nutans* showed significant and mild inhibition of IFN-gamma/TNF-alphainduced apoptosis in HaCaT keratinocyte.

2.2.2.2 Anticancer Activity of *C. nutans*

Studies have been carried out to evaluate the anticancer activities of *C. nutans*. A recent study by Zakaria *et al.* (2017) evaluates the anti cancer effects of *C. nutans* extract on human cervival cancer cell line or also known as HeLa. The author suggested that aqueous extract of *C. nutans* could be a potential and promising therapeutic substance for the prevention and treatment of cancer especially for cervical cancer. This is due to the significant cytotoxic effect exerted by the extract on HeLa cells (IC_{50}=12±0.82 μg/ml). Besides that, aqueous extract of *C. nutans* also induced cell death on HeLa cells via apoptosis based on the Hoechst 33258 nuclear staining performed. Chemopreventive study of *C. nutans* ethanolic extract on cervical cancer lines was carried out in a different study by Othman *et al.* (2016)**.** The results showed inhibition of the growth of cancer cells at IC_{50} value of 1.08 mg/ml by the exthanol extract of *C. nutans*. GCMS analysis that was carried out on the extract showed presence of Lupeol, lup-20(29)-en-3-one, β-Amyrin, Linoleic Acid ethyl ester and Squalene. Lupeol is suggested as the primary compound that may contribute to the anticancer activity of *C. nutans*. Anticancer activity of *C. nutans* extracts using five solvents which are hexane, chloroform, ethyl acetate, methanol and water were also tested on different human cancer cell lines involving small cell lung cancer (A549), nasopharygeal cancer (CNE1) and liver cancer (HepG2) cells. In the study, antiproliferative action of the five solvents on human cancer cell lines were assessed using MTT assay. Then, flow cytometry was performed on the most potent anticancer

extract to study the changes in the cell cycle, and DCFH-DA assay was conducted to quantify the intracellular levels of reactive oxygen species (ROS). Caspase assay kits was also used to analyze the involvement of the caspase pathway in apoptosis induction and GC-MS analysis was carried out to identify phytoconstituents in the extracts. It was found that hexane extract showed the most potent antiproliferative effect against the three human cancer cell lines while ethyl acetate extract was only antiproliferative against nasopharygeal cancer cells. In addition, hexane extract of *C.nutans* induced apoptosis in all three cell lines as evident by the increased percentage of cells in sub-G1 phase. Hexane extract also showed caspases upregulation at concentration higher than 100 µg/mL across all human cancer cells and 31 compounds were identified in the GC-MS analysis of the hexane extract (Ng *et al.*, 2017). In vitro anticancer activity in *C. nutans* extracts using different solvent which are 100% ethanol, 100% methanol, 50% ethanol, 50% methanol and water were tested on K562 chronic myeloid leukemia cells, HCT 116 colorectal carcinoma cells, and CCD-18Co normal colon fibroblasts. The results showed that no significant cytoxicity was observed at 200 and 100 µg/ml on all tested cell lines. After the methanol extract was subjected to fractionation, significant cytotoxicity was obtained in in fractions number 3, 4, 14 and 16 at 200 and 100 µg/ml. The percentage of inhibition at 200 µg/ml was 80±1.0%, 34±4.2% , 93±1.0 and 46±10% for fraction 3,4,14 and 16 respcetively while at 100 µg/ml, only fraction 14 showed more than 50% of growth inhibition. The author suggested that fraction 14 of *C. nutans* methanol extract could be a candidate anti-colon cancer therapy (Kameh *et al.*, 2013)**.**

2.2.2.3 Antibacterial Activity of *C. nutans*

C. nutans extract has been reported to possess strong bactericidal activity against several pathogens from aquatic animals. The extract also showed mild antibacterial effect against the gram negative bacteria, *Aeromonas hydrophila* in fish and shrimp (Direkbusarakom *et al.*, 1998). The growth of several types of microorganisms such as

Bacillus cereus, *Escherichia coli*, *Salmonella enteric Tryphimurium* and *Candida albicans* were also significantly inhibited by the crude *C. nutans* extract produced by solvents petroleum ether, ethyl acetate and methanol.

The antibacterial assay used were minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal (MBC/MFC) assays. The author suggested that *C. nutans* extract could be a potential antioxidant agent as it showed inhibition against all tested bacterias (Arullappan *et al.*, 2014). Antibacterial activity of *C. nutans* was also tested against different bacteria strains which include *Propionibacterium acnes, Staphylococcus aureus, Staphylococcus epidermis, Bacillus cereus* and *Escherichia coli* by using microbroth dilution method and minimum inhibitory concentrations (Yang *et al.*, 2013). There was significant inhibition on all microorganisms tested especially on *S. aureus* and *E.coli*. *C. nutans* extract showed antibacterial activity against *Propionibacterium acnes* (MIC > 12.5 mg/ml), *Staphylococcus aureus* (MIC 12.5 mg/ml), *Staphylococcus epidermidis* (MIC > 12.5 mg/ml), *Bacillus cereus* (MIC > 12.5 mg/ml) and *Escherichia coli* (MIC 12.5 mg/ml). The extract was also formulated into ointment in which its antibacterial efficacy was tested against four bacterias involving *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas* aeruginosa through disc diffusion method. The oinment containing methanol extract from *C. nutans* leaves depicted significant antibacterial effect against all microorganisms tested with zone of inhibition ranges from 8.00±2.00 to 18.67±2.09 mm (Sekar & Rashid, 2016). In a different study by Raina & Hassan (2016), aqueous and methanol extracts of *C. nutans* were used to determine its antibacterial effect on the two most common freshwater microorganisms which are *Streptococcus agalactiae* and *Aeromonas hydrophila*. The minimum inhibitory concentration value of C. nutans methanol extract against *A. hydrophila* and *S. agalactiae* were 25 mg/mL and 50 mg/mL respectively. Aqueous extract however did not show any microbial activity.

2.2.2.4 Antivenom Activity of *C. nutans*

C. nutans plant has been used traditionally for a long time in southern Thailand and Malaysia as a treatment for snakes bites (Naja naja siamensis or cobra) and other venomous insects such as scorpions and bees (Daduang *et al.*, 2005). The initial study conducted on the antivenom activity of *C. nutans* aqeous extract against the Naja naja simensis venom showed that the extract failed to inhibit the neuromuscular transmission in isolated rat phrenic-nerve diaphragm preparations (Cherdchu *et al.*, 1977). In addition, the mice which received lethal dose of the venom from the cobra snake also did not survive for a long time. However, Uawonggul *et al.* (2006) conducted a study to investigate the antivenom activity of *C. nutans* plant extract against the venom of other poisonous insect which is Heterometrus laoticus scorpion in chick embryonic fibroblast cells. Scorpion venoms can attack the systemic and cardiovascular systems due to the toxins contained that can affect ion channels (mainly sodium and potassium) and other components which include serotonin, neorotransmitters, lipids and enzymes (Gueron & Liron, 1992; Ismail & Abd-Elsalam, 1988). This can cause patients to suffer from severe local pain associated with inflammation, edema, swelling, and redness. In the study, the treatment of *C. nutans* aqueous extract at 0.706 mg/ml showed moderate antiscorpion venom activity that resulted in 50% inhibition of the chick embryonic fibroblast cell lysis. Results from the study showed that higher dose of the extract which is at 0.706 mg/ml demonstrated better cell antilysis activity but lower cell viability as compared to the usage of lower dose (0.406 mg/ml). *C. nutans* extract was also tested against honey bee venom and it was found that 0.14-1.08 mg/ml of extract exhibited low antibee venom activity with low cell viability in the primary culture of chick embryonic fibroblast cells (Uawonggul *et al.*, 2006). The reported studies supported the potential use of *C. nutans* extract as an antivenom agents for scorpions and the author suggested that the antivenom activity of *C. nutans* extract is due to its anticell lysis as

evident by the study with the scorpion's venom and not because of the antineuromuscular blockage as reported by Cherdchu *et al.* (1977).

2.2.2.5 Antioxidant Activity of *C. nutans*

The antioxidant activity of *C. nutans* has been evaluated in a number of *in vitro, ex vivo*, and *in vivo* studies. In a study by Pannangpetch *et al.* (2007), the ethanolic extract of *C. nutans* leaves has been subjected to antioxidant screening by using DPPH radical scavenging assay, the ferric reducing antioxidant power (FRAP) assay, and phorbol myristate acetate (PMA)-induced reactive oxygen species production in rat macrophages. Results from the study showed that *C. nutans* extract possess antioxidant activities and protective effects against free radicals. The IC_{50} value for DPPH assay of the extract obtained was 110.40±6.59. This result showed that *C. nutans* extract has moderate scavenging activity with 67.65±6.59% maximum effect. Ferric reducing activity of the ethanolic extract was 59 times less pottent than ascorbic acid. Furthermore, PMA-induced free radicals was also significantly reduced by *C. nutans* extract at concentrations of 30, 100 and 300 μg/ml with the fluorescent intensity of 58.72±5.52, 51.92±8.49 and 53.50±6.17 respectively. In another study by Ghasemzadeh *et al.* (2014), plant age was shown to be as a determining factor for bioactivity. The 12-month-old buds and leaves showed high radical scavenging activities with DPPH IC_{50} of 64.6 μg/mL. The activity of the extracts was compared to those of hydroxytoluene (68.0%), caffeic acid (70.4%), and α- tocopherol (71.2%) at a concentration of 100 μg/ml.

Arullappan *et al.* (2014) also studies the antioxidant activity of *C. nutans* extract by using DPPH radical scavenging assay. Petroleum ether, ethyl acetate and methanol crude extracts were produced from powdered leaves and stem barks of *C. nutans*. The results from the study showed that petroleum ether extract exhibited higher DPPH

radical scavenging activity with 82% more than ethyl acetate and methanol crude preparation. On the other hand, Shiuan *et al.* (2012) has conducted a study on the protective activity of a *C. nutans* extract against oxidative stress on plasmid DNA integrity in *Escherichia coli*. In the study, the extract was tested and compared to the superoxide dismutase activity and total phenolic contents of green tea. It was found that *C. nutans* extract has higher reference activities than the green tea extract. However, it was also reported that the addition of riboflavin increase the protective effects of *C. nutans*.

In vitro antioxidant activity of *C. nutans* was carried out using different solvents which are n-hexane, dichloromethane, ethyl acetate and ethanol. The antioxidant capacity of all extracts were assessed using DPPH radical scavenging activity assay, oxygen radical absorbance capacity (ORAC) and ß-carotene bleaching activity assays. It was found that among all the four extracts, ethanol and ethyl acetate depicted stronger antioxidant activity ($p<0.05$). This is evident by the lower DPPH activity shown by ethanol and n-hexane extracts and lower DPPH means higher antioxidant capacity. This result is in agreement with a study conducted by Yong *et al.* (2013) which also reported that higher antioxidant activity can be seen in chloroform extract of *C. nutans* leaves as compared to methanol and aqueous extract. In his study, the *C. nutans* chloroform extract had the strongest antioxidant activity against DPPH free radicals and galvinoxyl radicals, but it was less effective against nitric oxide and hydrogen peroxide radicals (Yong *et al.*, 2013). For β–carotene bleaching (BCB) assay, the extract that showed the highest antioxidant activity was n-hexane (720 mg Teq/100 g extract) and the lowest antioxidant activity shown by ethanol extract (120 mg Teq/100 g extract). On the other hand, the highest antioxidant activity of *C. nutans* for ORAC assay can be seen in ethanol extract, followed by ethyl acetate, dichloromethane and n-haxane with readings

229.5 mMol TE/100 g extract, 181.6 mMol TE/100 g extract, 115.5 mMol TE/100 g extract, and 114.3 mMol TE/100 g extract respectively (Sulaiman *et al.*, 2015).

2.2.3 Phytochemical Constituents

Phytochemicals are biologically active substances or also known as secondary metabolites that are contained within most plants whose function is for protection. There are immense number of biotic and abiotic environmental stresses in all natural habitats (Mazid *et al.*, 2011). A wide variety of bacteria, viruses, fungi, nematodes, mites, insects, mammals and other herbivorous animals are responsible for reducing the productivity of crops (Mazid *et al.*, 2011). As stationary autotrophs, plants need to ensure their survival towards various challenges by developing survival strategies such as engineering their own pollination and seed dispersal, overcoming deprivation of nutrients for the synthesis of food, surviving solar radiation and the coexistence of herbivores and pathogens in their immediate environment (Kennedy & Wightman, 2011). Plants therefore produced secondary metabolites through biochemical pathways in vegetative (e.g. leaf, stem and root) and reproductive (e.g. flower, fruit and seed) regions in response to environmental stresses (Hermsmeier *et al.*, 2001; Reymond *et al.*, 2000).

Thre are three main groups of plant secondary metabolites based on their biosynthetic origin and they include terpenes, alkaloid and phenolic compounds (Croteau *et al.*, 2000) (Figure 2.2).

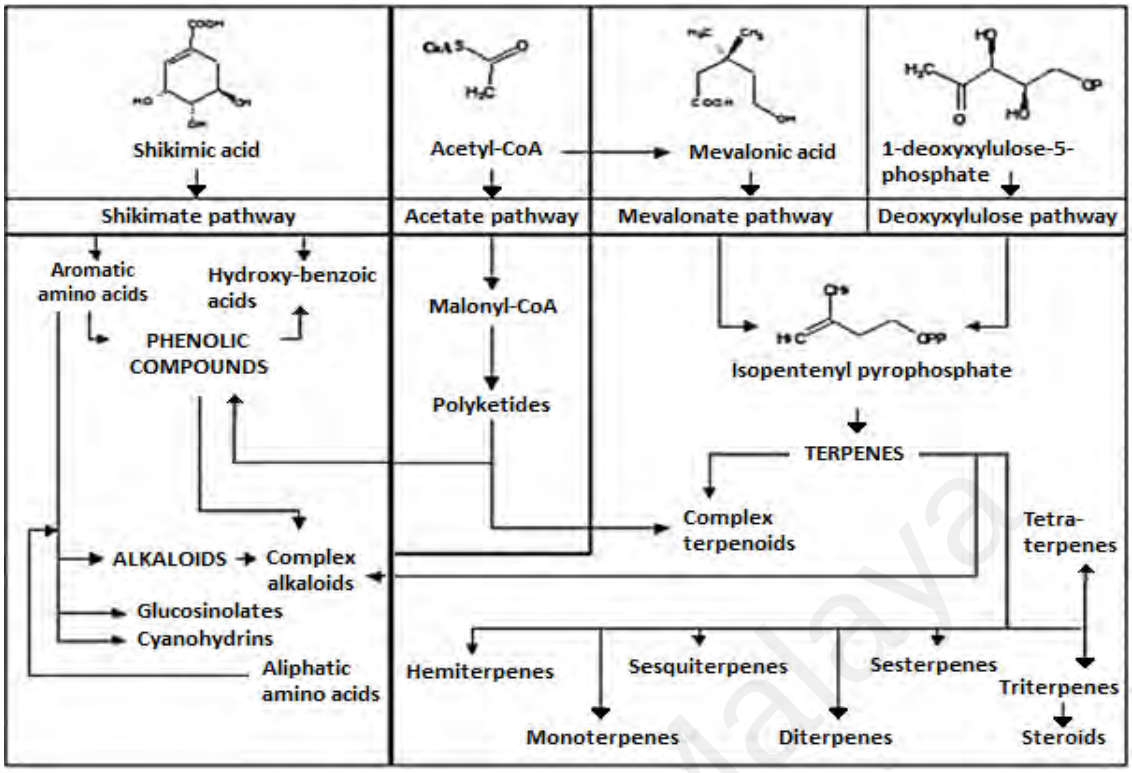


Figure 2.3: Biosynthetic pathways of secondary metabolites in plants (adapted from Ribera & Zuñiga, 2012).

The largest class of secondary metabolite comprised of terpenes which consists of more than 30 000 lipid-soluble compounds derived biosynthetically from acetyl-coA or glycolytic intermediates as can be seen in Figure 2.3 (Mazid *et al.*, 2011). Five major subclasses of terpenes include monoterpenes, sesquiterpenes, diterpenes, triterpenes and Polyterpenes (Mazid *et al.*, 2011). Terpenes are categorized based on the total isoprene units attached and they basically contained at least one or more 5-carbon units of isoprene. The classification is : 1 = hemiterpenes, 2 = monoterpenes, 3= sesquiterpenes, 4 = diterpenes, 5 = sesterpenes, 6 = triterpenes and 8 = tetraterpenes (Kennedy & Wightman, 2011).

Alkaloids is the second largest metabolite group of over 12 000 cyclic nitrogen-containing compounds that are present within 20% of vascular plant species (Kennedy & Wightman, 2011). The medicinally important group is commonly classified based on the similarity of the structures (for example, indole alkaloids) or common precursor

which include benzylisoquinoline, tropane, pyrrolizidine, or purine alkaloids (Kennedy & Wightman, 2011). Amino acids such as aspartic acid, lysine, tyrosine and tryptophan are among the common amino acids that are responsible for the production of alkaloid (Pearce *et al.*, 1991).

Phenolic compounds is the third largest group of secondary metabolite which is produced by the precursors from shikimate or phenylpropanoid pathway. These important compounds can be grouped into eight different classes which are phenolic acids, flavonoids, stilbenes, coumarins, quinones, lignans, curcuminoids and tannins based on the number of aromatic ring it possessed (Figure 2.4). Phenolics would have at least one aromatic ring with one or more hydroxyl groups, which can undergo reactions like esterification, methylation, etherification or glycosylation (Fresco *et al*., 2006). Besides that, phenolic compounds are also grouped according to the nature and complexity of the basic carbonaceous skeleton, the degree of skeletal modification and the link between the base unit and other molecules, including primary and secondary metabolites (Ewané *et al.*, 2012).

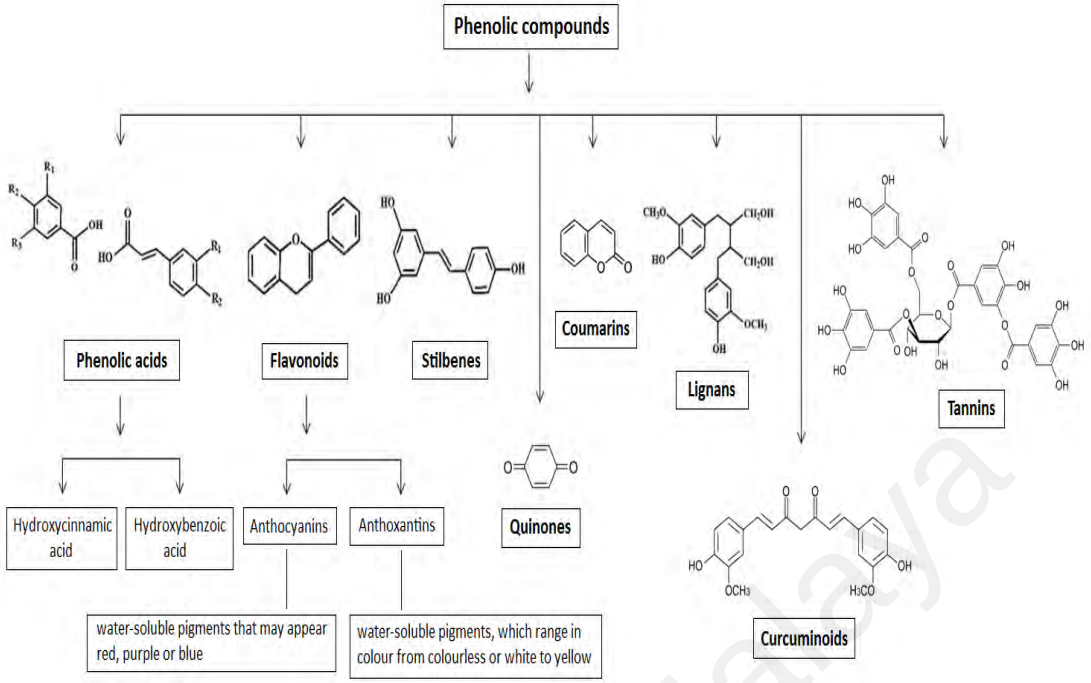


Figure 2.4: Classification of phenolic compounds (adapted from Fong, 2015).

The largest and most diverse group among the eight subclasses of phenolic compounds is flavonoid with approximately 6000 identified compounds (Kennedy & Wightman, 2011). A phenylbenzopyrine structure (C6-C3-C6) which consists of two aromatic benzene ringis linked by a heterocylic pyrane ring formed the basic structure of all flavonoids (Figure 2.5). Flavonoids are further divided into subgroups which consist of flavones, flavonols,flavonones, flavanonol, isoflavones, and flavan-3-ols. The condensation of six-member ring with benzene ring forms a α-pyrone (flavonols and flavanones) or its dihydroderivative (flavonols and flavanones). Different position of the benzenoid substituent further divides the flavonoid into flavonoids (2-position) and isoflavonoids (3-position). The hydroxyl group at position number 3 and the C2-C3 double bond on flavonols differentiate it from flavanones (Narayana *et al.*, 2001). The difference of all the subgroups of flavonoids are based on the oxidation leven and the substitution pattern of carbon ring (Middleton, 1998).

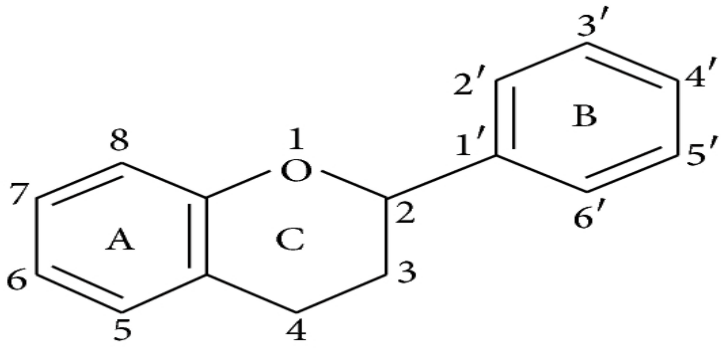


Figure 2.5: Basic structure of flavonoid (adapted from Kumar & Pandey, 2013).

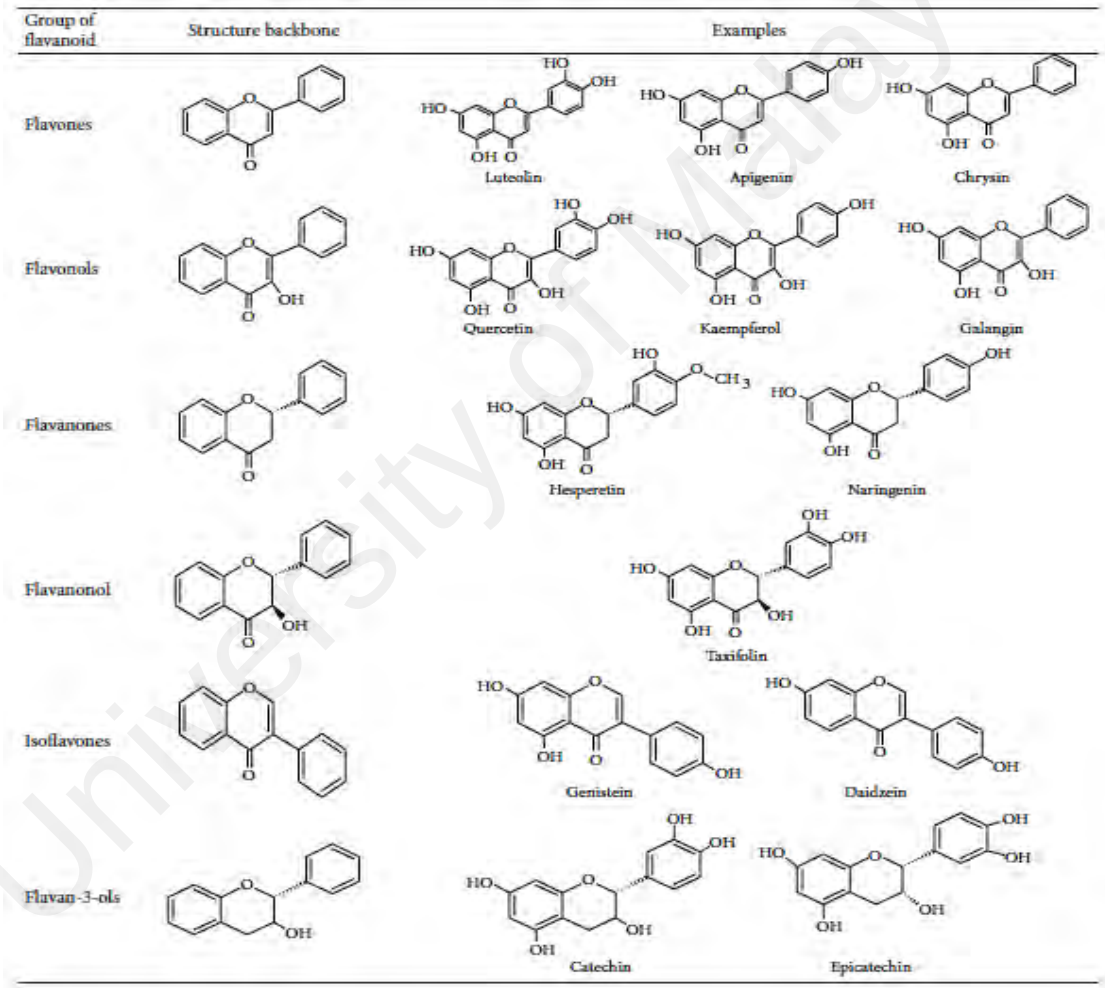


Figure 2.6: Structure of different groups of flavonoids (adapted from Kumar & Pandey, 2013).

The phytochemical screening carried out on *C. nutans* revealed the presence of saponins, phenolics, flavonoids, diterpenes and phytosterols (Yang *et al.*, 2013). *C. nutans* also contains terpenoids and other potentially bioactive compounds, including

sulphur-containing glucosides, lipids, chlorophyll derivatives and benzonoids as summarized in Table 2.3. However, to date, no alkaloids have been detected in *C. nutans* extract.

Table 2.3: Phytochemical compounds present in different parts of *C.nutans* (adapted from Fong, 2015).

Compound Class	**Compound**	**Plant Part(s)**	**Extract(s)**	**Reference(s)**
Steroids	Stigmasterol β-sitosterol	Stem Leaf	Petroleum	Dampawan (1976) Dampawan *et al.* (1977)
Triterpenoids	Lupeol	Stem	Petroleum	Dampawan *et al.* (1977)
Phenolic acids	Caffeic acid Gallic acid	Leaf Leaf buds	Methanol	Ghasemzadeh *et al.* (2014)
Flavonoids	Catechin Kaempferol Luteolin Quercetin	Leaf Leaf buds	Methanol	Ghasemzadeh *et al.* (2014)
C-glycosyl flavones	Vitexin Isovitexin Shaftoside Isomollupentin 7-O-ϐ-glucopyranoside Orientin Isoorientin	Stem Leaf	Butanol and water soluble portions of methanol Ethanol	Teshima *et al.* (1997) Chelyn *et al.* (2014) Mustapa *et al.* (2015)
Sulphur-containing glucosides	Clinacoside A Clinacoside B Clinacoside C Cycloclinacose A1 Cycloclinacosede A2 Clinamide A Clinamide B Clinamide C 2-cis-entadamide A	Stem Leaf	Butanol and water soluble portions of methanol Ethanol	Teshima *et al.* (1998) Tu *et al.* (2014)
Glycerides	Digalactosyl diglycerides Trigalactosyl diglycerides	Leaf	Uknown	Janwitayanuchit *et al.* (2003)

Table 2.3, continued.

Cerebrosides Monoacylmonogalactosyl-glycerol	1-*O*-*б*-D glucosides of phytosphingosines 2*S*)-1-*O*-linolenoyl-3-*O*-*б*-D-galactopyranosylglycerol	Leaf	Ethyl acetate soluble fraction of ethanol	Tuntiwachwuttikul *et al.* (2004)
Chlorophyll derivatives (phaeophytins)-related to chlorophyll a and b	132-hydroxy-(132-*R*)-phaeophytin a 132-hydroxy-(132-*R*)-phaeophytin b 132-hydroxy-(132-*S*)-phaeophytin a 132-hydroxy-(132-*S*)-phaeophytin b 132-hydroxy-(132-*R*)-chlorophyll b 132-hydroxy-(132-*S*)-chlorophyll b purpurin 18 phytyl ester phaeophorbide a	Leaf	Chloroform Hexane	Shuyprom (2004) Sakdarat *et al.* (2006) Sakdarat *et al.* (2009) Sittiso *et al.* (2010)
Benzenoids	1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester	Leaf	Chloroform	Yong *et al.* (2013)

2.2.4 Impact of Storage Duration and Temperature on Plant Secondary Metabolites and Antioxidant Properties

Duration and storage temperature has significantly influenced the content of plant secondary metabolites and antioxidant levels. Galani *et al.* (2017) studied the effect of storage temperatures which are at 4°C and 15°C on total phenolics and antioxidant capacity of eleven potato *Solanum tuberosum* varieties. It was found that the antioxidant activity of the potatoes assesed by DPPH and ABTS assays increased during the early days of storage but decreased to a level comparable or lower than the original value, irrespective of storage temperatures. At room temperature, total phenolic content of the potatoes fluctuated but increased after storage. Meanwhile in incubator and cold storage, the content of total phenolics fluctuated during the storage period but the content was higher on the last day as compared to the starting day of the experiment.

Factors such as environmental stresses which include low-temperature storage, strong light, wounding and attacks from pathogen have been reported to induce the generation of phenolic compounds via the activation of phenylalanine ammonia-lyase (PAL) through the phenylpropanoid pathway which affected the content of the (Galani *et al.*, 2017). The effects of storage on phenols and antioxidant activity of leaf, stem and rhizome extracts of *Anemopsis californica* were studied. Results showed that the extracts were stable, with 97 and 98% stability during the first 60 days when stored at 4°C and −20°C respectively (Del-Toro-Sánchez *et al.*, 2015). Total phenolic contents decreased significantly after this time period and the best storage temperature was recorded at −20°C after 180 days as the rhizome extract was the most stable conserving approximately 79% of total phenols and 73% of total flavonoid. The antioxidant activity was also assessed by DPPH and ABTS assays. For DPPH assay, all methanolic extracts of *Anemopsis californica* showed up to 95% stability when stored at −20°C for 60 days. After 180 days, the stem extract decreased to 70% of DPPH inhibition. The extracts also showed high inhibition of the ABTS radical with 95% and 98% stability when stored at 4°C and −20°C respectively. In this study it was observed that stem extract showed higher antioxidant stability as compared to rhizome. The author suggested that phenolic concentration, polarity and chemical structure of phenolic compound are factors affecting the antioxidant activity of the plant extracts. In addition, higher temperature and light are factors causing the change and degradation of polyphenols (Del-Toro-Sánchez *et al.*, 2015). In a different study by Moldovan *et al.* (2016), the effects of storage temperature on the phenolic content of Cornelian cherry (*Cornus mas* L.) fruits extracts were investigated. Results from the study showed that the highest loss of phenolic compounds were observed in extracts stored at temperature higher than 50°C after 60 days of storage. On the other hand, extracts stored at 2°C showed 22.6% of losses while no significant change was observed in extracts stored at 22°C for 60 days.

2.2.5 Past Research on the Use of Fertilizer to Improve the Growth of *C. nutans*

Malek *et al.* (2014) studied the effects of natural zeolite (clinoptilolite) and urea as fertilizers on *C. nutans.* In the study, there were seven types of fertilizers applied on *C. nutans* which include control (without fertilizer), only urea, only zeolite, 3g of urea with zeolite, 6g of urea with zeolite, 3g of IMO-plus and 6g of IMO-plus. The effects of the seven types of fertilizers on the growth of *C. nutans* were compared. Zeolites possess high ammonium adsorption capacity, good ability to retain water and nutrients that are essential to plants and it can slowly release N and P into soil, thus making it widely used in agricultural area as a controlled release fertilizer (Ayan *et al.*, 2005). Zeolite type clinoptilolite was used in this study due to its porous structure with high cation exchange capacity (CEC), high adsorption, catalysis, dehydration capacities and it has high affinity for NH_4^+. It is used as a promoter for better plant growth by improving the value of fertilizers, maintaining valuable nitrogen and improving the quality of resulting manures and sludges (Park *et al.*, 2005).

IMO-plus on the other hand, is a mixed fertilizer containing organic compound, zeolites, effective microorganisms and N, P and K nutrients. The results from this study showed that the addition of zeolite in urea and other fertilizers can improve the productivity and growth of *C.nutans*. This is due to the property of zeolite that enables it to hold nutrients, especially N element and slowly releasing them from the fertilizer to the plant. Because of this, all plants could obtain enough N for their growth, especially for their leaves (Jalali, 2005). On the other hand, the plants did not grow well after being applied with only zeolite because it does not contain sufficient nutrients such as ammonium, nitrate or phosphate that are needed for plant growth. Besides that, the plants that were fertilized by only urea showed slower plant growth and this is possibly due to the leaching of ammonium from urea.

2.3 Summary of Literature Review

As a conclusion, vermicomposting is a simple process of decomposing and it has the potential to improve the nutrient value and the growth of a wide range of plant species due to the presence of various plant macro- and micro-nutrients. On the other hand, *Clinacanthus nutans* Lindau is a medicinal plant that is known not only for its anti-inflammatory, anti-cancer, antibacterial and antivenom activity, but also its antioxidant activity. The effects of natural zeolite (clinoptilolite) and urea as fertilizers has been conducted on *C. nutans* in the past res earch to improve its growth.

CHAPTER 3: METHODOLOGY

3.1 Sample Collection

In this project, a field study employing a randomized complete block design (RCBD) was conducted at Glami Lemi Biotechnology Research Centre (PPBGL), Jelebu, Malaysia by Syuhada Japri. There were three blocks and four treatments involved with three replications. The treatment groups consist of control plants (CC) which were not supplied with any fertilizer, plants supplied with 10 t ha^{-1} of NPK green fertilizer (CF) (N: 15%; P: 15%; K: 15%), plants supplied with vermicompost at 15 t ha^{-1} (VC) (N: 2.06%; P: 0.53%; K: 2.47%) and plants supplied with mixed fertilizer (containing both 15 t ha^{-1} of vermicompost and 5 t ha^{-1} of NPK green fertilizer; MF). The purpose of using different rates of fertilizers in this study was to identify the optimum dosage of fertilizer needed for the growth of *C. nutans* and the different rates of fertilizers were chosen based on previous study carried out by Suthar (2012). All treatments were applied on the plots as a soil conditioner two weeks before the planting process. The leaves from three replicates of *C. nutans* plants of each of the three different blocks per treatment were harvested in February 2017, resulting in a total of nine samples for each treatment. *C. nutans* was identified by comparing the plant with a herbarium specimen and a voucher specimen (KLU 49509) was deposited at the Herbarium of Rimba Ilmu, Institute of Biological Sciences, University of Malaya, Kuala Lumpur, Malaysia.

3.2 Extraction of Plant Materials

Based on Figure 3.1, after the leaves were harvested, the methanolic extract of *C. nutans* was prepared in which the fresh leaves were freeze-dried using a Labconco freeze dryer (Labconco Corporation, MO 64132 United States) at -50°C and subjected to solvent extraction by using methanol. Briefly, 3 g of freeze-dried leaves were soaked

in 90 ml 70% methanol and ground using mortar and pestle. Then, the sample mixture was incubated in solvent at 4°C for 24 hours followed by filtration using filter paper. The extraction process was repeated using the residue obtained from the filtration. The filtrates were pooled and evaporated to dryness by using a rotary evaporator at 45°C to yield the methanolic extracts. The concentrated extract was adjusted to a concentration of 20 mg/ml using 70% methanol before it was stored at 4°C and -20 °C until further analysis. Storage temperature 4°C and -20 °C were selected for this experiment as the temperatures can be easily controlled in the laboratory and previous studies have proven that these storage temperatures can significantly affect the antioxidant activity and total phenolic content of plant extracts (Del-Toro-Sánchez *et al.*, 2015; Genova *et al.*, 2012; Samad *et al.*, 2016)

For the extraction of chlorophyll, the fresh leaves were freeze-dried using a Labconco freeze dryer (Labconco Corporation, MO 64132 United States) at -50°C. Then, 3g of the freeze dried leaves were soaked in 90 ml 70% methanol and ground using chilled pestle and mortar. A small quantity of magnesium carbonate was added to prevent conversion of chlorophyll to phaeophytin under acidic condition. The experiment was conducted in low light as pigments are light-sensitive. The sample mixture in solvent was incubated at 4°C for 24 hours and then centrifuged using a Universal 32 R centrifuge (Hettich Zentrifugen, D-78532 Germany) at 9050xf for 5 minutes at 4°C.

For extraction of the carotenoid for HPLC, 1.0 g of the freeze-dried samples of *C. nutans* was rehydrated with 1.0 ml distilled water. The mixture was then soaked overnight in darkness at room temperature, in 5 ml of acetone:methanol (7:3). Then, the mixture was vortexed and centrifuged at 13500 *g* for 2 minutes, where the supernatant

was then transferred into a 50 ml graduated polypropylene centrifuge tubes covered with foil. To remove fine particulates, the supernatant was centrifuged again at 13500 *g* for 5 minutes and stored at 4°C in the dark, prior to analysis. The extraction of carotenoids was carried out by adding an equal volume of hexane and distilled water to the sample mixture, vortexed and centrifuged at 13500 *g* for 1 minute. The upper layer containing the carotenoids was collected and dried under a gentle stream of Oxygen-free nitrogen gas. Then, the vials were immediately capped, sealed with parafilm and stored at -80 °C until subsequent analysis.

3.3 Phytochemical Screening of Bioactive Compounds in *C. nutans*

The presence of various phytochemicals such alkaloid, tannin, phenol, flavonoid and saponin in the samples were analyzed based on the methods described by Solihah *et al.* (2012).

3.3.1 Test for Alkaloids

1 mL of *C. nutans* extract was stirred with 5 mL (1%) HCl on a steam bath (60°C) for 15 minutes and filtered. There were three methods used to test for alkaloids;

Alkaloids (I): 1 mL of Dragendorff reagent was added to 1 mL filtrate. The formation of cloudy orange solution indicated the presence of alkaloids.

Alkaloids (II): 1 mL of Mayer reagent was added to 1 mL filtrate. Appearance of slight yellow colour of the solution was the indicator for alkaloids.

Alkaloids (III): 1 mL of Wagner reagent was added to 1 mL filtrate. The observation of turbid brown colour indicated the presence of alkaloids.

3.3.2 Test for Tannins

1 mL extract was added to 1 mL of 3% $FeCl_3$. A greenish black precipitate signified the presence of tannins

3.3.3 Test for Phenols

2 mL extract was placed in water bath and warmed at 45-50°C. Then 2 mL of 3% $FeCl_3$ was added. The formation of green or blue colour indicated the presence of phenols.

3.3.4 Test for Flavonoids

There are two methods used to test for flavonoids;

Flavonoids (I): 1 mL extract was added to 1 mL of 10% lead acetate. Then, it was gently shaken and a muddy brownish precipitate indicated the presence of flavonoids.

Flavonoids (II):1 mL extract was added to 1 mL 10% $FeCl_3$. The mixture was shaken. A wooly brownish precipitate indicated the presence of flavonoids.

3.3.5 Test for Saponins

Approximately 0.2 mL extract was mixed with 5 mL distilled water. It was shaken vigorously for 5 minutes. Persistence appearance of foams was the indicator for saponins.

3.4 Quantification of Bioactive Compounds

The amount of bioactive compounds such as flavonoids, anthocyanins and phenolic compounds as well as chlorophyll and carotenoid were quantified, based on the procedures described below:

3.4.1 Total Chlorophyll Content

Total chlorophyll content was measured by using the method described by Lichtenthaler & Buschmann (2001). The concentrations for Chl *a* (c_a), Chl b (c_b), and the sum of leaf carotenoids (c_{x+c}) were calculated with the following equations,

$$\text{Chlorophyll } \alpha \text{ (mg/L)} = 16.72\ A665.2 - 9.16\ A652.4$$

$$\text{Chlorophyll b (mg/L)} = 34.09\ A652.4 - 15.28\ A665.2$$

$$\text{Total carotenoid (mg/L)} = (1000\ A470 - (1.63\ \text{Chl a}) - (104.96\ \text{Chl b}))/221$$

3.4.2 Total Anthocyanin Content (TAC)

The total anthocyanin content was determined by using the pH differential method (Wrolstad *et al.*, 1995). The methanolic extracts of *C. nutans* were diluted separately with two types of buffer; potassium chloride (0.025M) at pH 1.0 and sodium acetate (0.4M) at pH 4.5 using the ratio 1:4 (1 part test portion and 4 parts buffer). The absorbance was measured at wavelengths of 530 nm and 700 nm using UV-200-RS spectrophotometer (MRC Ltd.) The concentration of anthocyanin pigment was measured by using the following formula:

$$\text{Anthocyanin pigment content (mg/L)} = \frac{(\text{A} \times \text{MW} \times \text{DF} \times 1000)}{(\varepsilon \times 1)}$$

$$\text{Where, A} = (\text{Abs}_{520} - \text{Abs}_{700})\text{pH1.0} - (\text{Abs}_{520} - \text{Abs}_{700})\text{pH4.5}$$

$$\text{MW(cyanidin} - 3 - \text{glucoside)} = 449.2\ \text{g/mol}$$

$$\text{DF} = \text{dilution factor}$$

$$\varepsilon = 26{,}900$$

3.4.3 Total Flavonoid Content (TFC)

Aluminium chloride colorimetric method as reported by Lin & Tang (2007) was used to quantify the total flavonoid content in *C. nutans* extract. Briefly, 0.5 mL of each extract was mixed with 1.5 mL of 70% methanol, 0.10 mL of 10% aluminium chloride, $AlCl_3$ ($AlCl_3.6H_2O$), 0.10 mL of sodium acetate ($NaC_2H_3O_2.3H_2O$) (1 M) and 2.80 mL of distilled water. Then the absorbance was measured at 415 nm using a UV-200-RS spectrophotometer (MRC Ltd.) after 40 minutes of incubation. The concentration of flavonoids was calculated by preparing a calibration curve using quercetin as the standard (0.15–0.4 mg/mL). The flavonoid concentration was expressed as quercetin equivalents in mg per gram of dry weight (mg g^{-1} DW) of extract. All assays were carried out in triplicate.

3.4.4 Total Phenolic Content (TPC)

Folin-Ciocalteu's method was used to quantify the total phenolic content of *C. nutans* methanolic extracts, according to the method described by Sun *et al.* (2007) with minor changes. Briefly, Folin-Ciocalteu's reagent was diluted 10-fold with deionised water. 0.1 mL of *C. nutans* methanolic extract was mixed with 0.75 mL of the diluted Folin–Ciocalteu reagent and incubated for 10 minutes at room temperature. Then, 0.75 mL of 2% sodium carbonate (Na_2CO_3) solution was added. The mixture was allowed to stand in the dark for 45 minutes before measuring the absorbance at 765 nm using UV-200-RS spectrophotometer (MRC Ltd., Holon, Israel) against a blank, containing the solvent (70% methanol). The TPC values were determined from a calibration curve prepared with a series of gallic acid standards (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 mg/ml). Results were expressed as mg of gallic acid equivalents/g dry weight (mg GAE/g DW).

3.4.5 Total Carotenoid Content by HPLC

In this study, the samples were also screened for 8 types of carotenoid which include neoxanthin, violaxanthin, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene and lutein using method described by Rashidi Othman (2017).

Quantification of carotenoid content was conducted by using High Performance Liquid Chromatography (HPLC), on an Agilent 1200 series model that comprised of a binary pump with autosampler injector, micro vacuum degassers, thermostatted column compartment and a diode array detector. The separation was carried out using HPLC column ZORBAX SB-C_{18} end capped (5 μm, 4.6 × 250 mm) reverse phase column (Agilent Technologies, USA). There were two types of eluents used in this analysis; (A) acetonitrile:water (9:1 v/v) and (B) ethyl acetate. The solvent gradients used were; 0-40% solvent B (0-20 min), 40-60% solvent B (20-25 min), 60-100% solvent B (25-25.1 min), 100% solvent B (25.1-35 min) and 100-0% solvent B (35-35.1 min) at a flow rate of 1.0 ml min^{-1}. Then, the re-equilibration of the column was done in 100% solvent A for 10 minutes prior to the next injection and 10 μL of injection volume was used. The column was kept at 20°C. Carotenoid peaks were detected between wavelengths range of 350 to 550 nm. The identity and amount of the carotenoids was calculated based on standard curves prepared using neoxanthin, violaxanthin, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene and lutein standards.

3.5 Determination of the Antioxidant Activity

The methanolic extracts were assessed for their antioxidant potential against free radicals, based on the procedures described below:

3.5.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity Assay

DPPH free radical scavenging activity of the *C. nutans* methanolic extracts were analyzed following standard procedure described by Sulaiman & Ooi (2012). First, 50 µL of *C. nutans* extract at six different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/mL) were added to 150 µL of DPPH solution (0.2 mM) in each well of a 96-well plate. The mixture was then incubated at room temperature for 30 minutes. Then, 50 µL of methanol was added to DPPH solution as blank. At the end of the incubation period, the Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA) was used to measure the absorbance value at 515 nm. All the extracts were assayed in triplicate. The obtained data were then used to determine the concentration of the sample required to scavenge 50% of the DPPH free radicals (IC_{50}). The percentage of inhibition was plotted against the concentration and the IC_{50} was obtained from the fitted linear curve. A lower IC_{50} denotes a more potent antioxidant.

3.5.2 ABTS (2, 2-azino-bis (3-ethylbenzotiazoline-6-sulfonic acid)) Radical Scavenging Activity Assay

The colorimetric method described by Shao *et al.* (2014) was used to perform the ABTS scavenging activity assay. First, the preparation of ABTS radical cation was performed by mixing 10 ml of 2.6 mM $K_2S_2O_4$ solution with 10 ml of 7.4 mM ABTS solution. Then, the mixture was stored for 12 hours at room temperature in a dark room before further use. After that, double distilled water (ddH_2O) was used to dilute the mixture and it was adjusted to produce an absorbance reading of 0.70 ± 0.2 at 734 nm. A 20 µL of sample at six different concentrations (0.5, 1, 1.5, 2.0, 2.5, and 3.0 mg/mL) was then added to 200 µL ABTS solution and the mixture was incubated at room temperature for 30 minutes. The absorbance reading was measured at 734 nm using Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA) and the assay was

performed in triplicate. The percentage of inhibition was calculated according to the following formula:

$$\% \text{ inhibition} = [(A_{blank} - A_{sample})/ (A_{blank})] \times 100$$

The antioxidant capacity of the test extracts is expressed as $IC_{50,}$ which is the concentration necessary for 50% reduction of ABTS radicals.

3.5.3 FRAP (ferric reducing antioxidant power) Assay

The methods described by Benzie & Strain (1996) and Kong *et al.* (2013) with modifications were used to perform the FRAP assay. Briefly, three reagents were prepared which include 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6,tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid (HCl) and 20 mM iron chloride ($FeCl_3$). The preparation of FRAP reagent was done by mixing the TPTZ solution with acetate buffer in 40 mM HCl and 20 mM $FeCl_3$ at a ratio 80:8:8:9.6 (v/ v/ v), respectively. Then, 300 µL of FRAP reagent was added to 10 µL of *C. nutans* extract at six different concentrations (0.5, 1, 1.5, 2.0, 2.5, and 3.0 mg/mL) after 30 minutes of incubation at 37°C. Subsequently, the absorbance was measured at 593 nm by using the Multiskan Go plate reader (Thermo Scientific, Waltham, MA, USA). The calibration curve was constructed using aqueous solutions of known ferrous ion concentration ($FeSO_4.7H_2O$) and the FRAP values obtained from the curve was expressed as mmol Fe^{2+}/100g sample. The intense blue colour indicated the antioxidant ability of the *C. nutans* extract to reduce Fe (II)-2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) complex to Fe (II)-TPTZ and it was linearly related to the amount of the antioxidant present.

3.6 Statistical Analysis

All data obtained in this study were subjected to statistical analysis using ANOVA and mean values were compared using Duncan's Multiple Range Test (DMRT) in SPSS software version 24. Other than that, Pearson correlation analysis was carried out to determine the relationship between antioxidant potential with the amount of bioactive phytoconstituents present in the extracts.

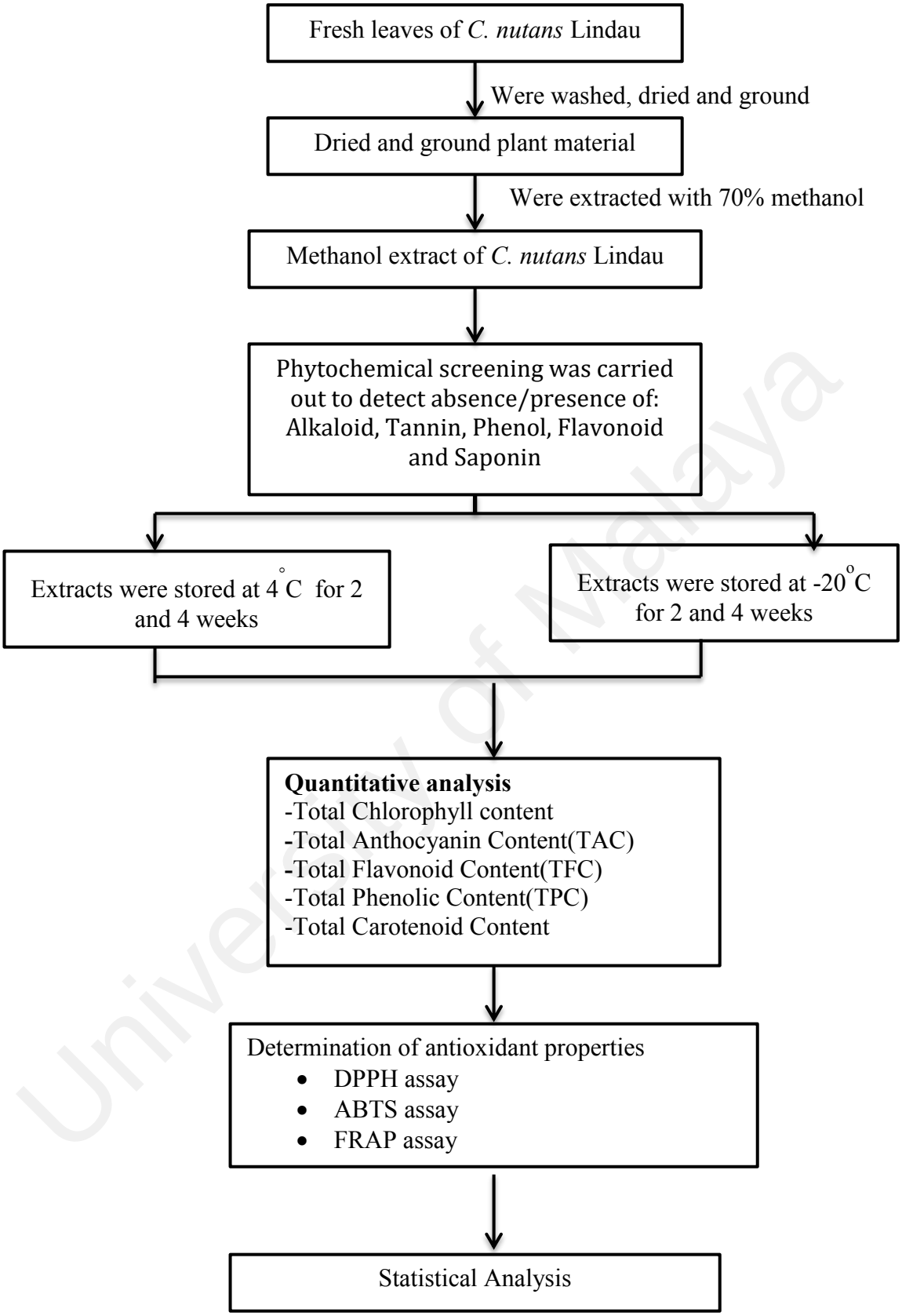


Figure 3.1: Schematic representation of methanol extraction from *Clinacanthus nutans* Lindau leaves for the quantitative and antioxidant analysis.

CHAPTER 4: RESULTS

4.1 Phytochemical Screening

In the present study, qualitative screening of *C. nutans* methanolic extract revealed the presence of phytochemical compounds. Based on Table 4.1, all *C. nutans* methanolic extract from each treatment showed the presence of phenols, flavonoid and saponin while negative results for alkaloid and tannin.

Table 4.1: Qualitative analysis of the phytochemical properties of *C. nutans* methanolic extract.

Phytochemical	*C. nutans* Methanolic Extract			
	No fertilizer (CC)	Recommended dose of NPK fertilizer (CF)	Vermicompost at 15 t ha^{-1} (VC)	Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer (MF)
Alkaloid	-	-	-	-
Tannin	-	-	-	-
Phenol	+	+	+	+
Flavonoid	+	+	+	+
Saponin	+	+	+	+

\+ = Present
\- = Absent

4.2 Measurement of Pigment and Bioactive Compound

4.2.1 Analysis of Chlorophyll and Carotenoid Content through Spectrophotometry

In this study, the chlorophyll and carotenoid content in methanolic extracts of *C. nutans* Lindau were measured and they were expressed as µg per g dry weight of each extract. Based on Table 4.2, CF exhibited the highest amount of chlorophyll *a* (1890.92 ± 94.10 µg per g dry weight), followed by MF, CC and VC. Statiscally, there was no significant differences for initial chlorophyll a content between all treatments ($p \leq 0.05$).

Results from the present study revealed that the *C. nutans* extracts contained higher amounts of chlorophyll *b* than chlorophyll *a*. Based on Table 4.2, VC showed the highest chlorophyll *b* content (4213.76 ± 54.72 µg per g dry weight), followed by CF, CC, and MF. The highest total chlorophyll content was shown by CF plant extract (6090.96 ± 144.90 µg per g dry weight) followed by VC, CC and MF. Moreover, CF extract also showed the highest total carotenoid content (520.47± 16.47 µg per g dry weight), followed by CC, VC and MF. Statistically, there were significant differences observed between VC and MF extracts with CC and CF at $p \leq 0.05$.

Table 4.2: Effect of plant growth supplements on total carotenoid and total chlorophyll contents in methanolic extracts of *C. nutans* leaves.

Treatment	Sample ID	Total carotenoid content (µg/g DW)	Total chlorophyll content (µg/g DW)	Chlorophyll a content (µg/g DW)	Chlorophyll b content (µg/g DW)
No fertilizer	CC	470.19 ± 27.56^{b}	5845.19 ± 35.49^{ab}	1786.44 ± 60.85^{a}	4058.74 ± 29.75^{ab}
Recommended dose of NPK fertilizer	CF	520.47± 16.47^{c}	6090.96 ± 144.90^{b}	1890.92 ± 94.10^{a}	4200.04 ± 92.65^{b}
Vermicompost at 15 t ha^{-1}	VC	359.40 ± 11.63^{a}	5975.00 ± 38.23^{ab}	1761.24 ± 72.93^{a}	4213.76 ± 54.72^{b}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	MF	335.70 ± 6.82^{a}	5801.34 ± 70.29^{a}	1824.34 ± 71.10^{a}	3977.00 ± 6.36^{a}

Each value is the mean ± standard error consisting of three replicates. Means with different letters within the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

Table 4.3 showed the ratio of pigment contents in methanolic extracts of *C. nutans*. Before extract storage, the weight ratio of Chl *a* and Chl *b* (Chl *a/b* ratio) for CC, CF, VC and MF were 0.44, 0.45, 0.42 and 0.46 respectively.Besides that, the weight ratio of chlorophyll *a* and *b* to total carotenoid (C(a+b)/C(x+c)) were also evaluated. The ratios were 12.82, 11.84, 16.77 and 17.34 for CC, CF, VC and MF respectively.

Table 4.3: Effect of plant growth supplements on chlorophyll a to chlorophyll b (C*a*/C*b*) ratios and total chlorophyll to total carotenoid (C*a* + C*b*)/ (C (x + c)) ratios in methanolic extracts of *C. nutans* leaves.

Treatment	Sample ID	C*a*/C*b* ratio (µg/g DW)	(C*a* + C*b*)/ (C (x + c)) ratio (µg/g DW)
No fertilizer	CC	0.44 ± 0.02^{a}	12.82 ± 0.84^{a}
Recommended dose of NPK fertilizer	CF	0.45 ± 0.02^{a}	11.84 ± 0.57^{a}
Vermicompost at 15 t ha^{-1}	VC	0.42 ± 0.02^{a}	16.77 ± 0.57^{b}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	MF	0.46 ± 0.02^{a}	17.34 ± 0.43^{b}

Each value is the mean ± standard error consisting of three replicates. Means with different letters within the same column are significantly different at p≤0.05 according to Duncan's multiple range test (DMRT).
*C*a* chlorophyll *a*, C*b* chlorophyll *b*, C*a* + C*b* total chlorophyll *a* and *b*, C(x+c) total carotenoid (xanthophyll and carotene)

4.2.2 Analysis of Carotenoid Content and Composition through HPLC

In Table 4.4, the analyzed carotenoid content and composition from *C. nutans* methanolic extract can be observed. The *C. nutans* extract from CF showed the highest total carotenoid content (144.67 ± 70.86 µg/g dry weight) followed by CC, VC and MF. Four types of carotenoids were detected and they include violaxanthin, lutein, α-Carotene and β-Carotene. According to the results, CF plant extract exhibited the highest amount of violaxanthin (5.73 ± 0.22 µg/g dry weight) and lutein (551.70 ± 4.13 µg/g dry weight). Interestingly, the application of vermicompost resulted in higher amount of α-Carotene and β-Carotene as compared to control. A contrasting situation occurred with MF which showed the highest α- Carotene (24.40 ± 4.26 µg/g dry weight) and β-Carotene (20.67 ± 3.96µg/g dry weight) level, despite the fact that it possesses the lowest content of violaxanthin (3.75 ± 0.03µg/g dry weight) and lutein (189.52 ± 22.03µg/g dry weight). CC on the other hand, showed the absence of α- Carotene and β-Carotene.

Table 4.4: Analysis of carotenoid content and composition of *C. nutans* extract.

Treatment	Sample ID	Analysis of Carotenoid Content and Composition (µg/g DW)				
		Violaxanthin	Lutein	α-Carotene	β-Carotene	Total Carotenoid
No fertilizer	CC	4.33 ± 0.56^{ab}	209.42 ± 20.95^{a}	ND	ND	56.70 ± 28.94^{a}
Recommended dose of NPK fertilizer	CF	5.73 ± 0.22^{b}	551.70 ± 4.13^{b}	11.83 ±1.11^{b}	9.43 ± 0.91^{b}	144.67 ± 70.86^{a}
Vermicompost at 15 t ha^{-1}	VC	4.37 ± 0.62^{ab}	206.78 ± 40.31^{a}	25.70 ± 4.31^{c}	20.67 ± 3.96^{c}	55.17 ± 20.52^{a}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	MF	3.75 ± 0.03^{a}	189.52 ± 22.03^{a}	24.40 ± 4.26^{c}	14.77 ± 1.98^{bc}	53.13 ± 20.59^{a}

Each value is the mean ± standard error consisting of three replicates.
Means with different letters within the same column are significantly different at p≤0.05 according to Duncan's multiple range test (DMRT).
*ND not detected

4.2.3 Determination of Total Anthocyanin, Total Flavonoid and Total Phenolic Contents

The total anthocyanin (TAC), total phenolic (TPC) and total flavonoid (TFC) contents in the methanolic extracts of *C. nutans* were measured. The TPC was expressed as mg Gallic Acid (GAE) per g dry weight of the extract, while the TFC was expressed as mg Quercetin(QE) per g dry weight of the extract and TAC of *C. nutans* extract was expressed as µg per g dry weight of the extract. Based on Table 4.5, it was evident that the highest TAC was obtained in CC plants (2180.14 ± 338.43 µg/g dry weight), followed by CF, VC and MF (Table 4.5). However, data analysis revealed that the differences observed among the TAC of all samples were not statistically significant. On the other hand, significantly higher TFC was found in the methanolic extracts of CC (276.25 ± 3.09 mg QE/g dry weight) and CF (256.66 ± 45.43 mg QE/g dry weight) plants, compared to VC and MF. CF plant extract was also observed to contain the highest TPC (181.53 ± 35.58 mg GAE/g dry weight), followed by CC, VC

and MF (Table 4.5). The TPC of CF was significantly different from the TPC of CC, VC and MF at $p \leq 0.05$.

Table 4.5: Effect of plant growth supplements on total phenolic, anthocyanin and flavonoid contents in methanolic extracts of *C. nutans* leaves.

Treatment	Sample ID	Total anthocyanin content (µg/g DW)	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QE/g DW)
No fertilizer	CC	2180.14± 338.43[a]	120.48 ± 6.70[a]	276.25 ± 3.09[b]
Recommended dose of NPK fertilizer	CF	1933.52± 66.06[a]	181.53 ± 35.58[b]	256.66 ± 45.43[b]
Vermicompost at 15 t ha^{-1}	VC	1742.86 ± 62.30[a]	98.06 ± 2.27[a]	170.42± 7.55[a]
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	MF	1669.91± 122.12[a]	97.47 ± 18.73[a]	157.30± 26.42[a]

Each value is the mean ± standard error consisting of three replicates.
Means with different letters within the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.3 Determination of Antioxidant Potential of *C.nutans* Methanolic Extracts by Using DPPH, ABTS and FRAP Assays

The antioxidant activities of all extracts against both DPPH (2, 2-diphenyl-1-picrylhydrazyl) and ABTS (2, 2-azino-bis (3-ethylbenzotiazoline-6-sulfonic acid)) radicals and the reducing ability of *C.nutans* extracts by FRAP (ferric reducing antioxidant power) assay were recorded in Table 4.6. The free radical scavenging activity of *C. nutans* methanolic extract were expressed as IC_{50} mg per ml of each extract. The antiradical activity was defined as the concentration of antioxidant necessary to decrease the initial DPPH or ABTS concentration by 50%. On the other hand, the reducing potential was expressed as mg per ml $FeSO_4$ equivalent of each extract. Data analysis revealed that the methanolic extract from control plants (CC) exhibited the highest free radical scavenging activity against DPPH and ABTS radicals, with IC_{50} of 1.18 ± 0.05 mg/ml and 0.98 ± 0.05 mg/ml, respectively (Table 4.6). DPPH and ABTS IC_{50} values for VC were significantly different from CC at $p \leq 0.05$. The

antioxidant activity (denoted by IC_{50}) of the methanolic extracts against both DPPH and ABTS radicals, in decreasing order is CC > CF > VC > MF. In addition, CC plant extract also showed the highest reducing potential with FRAP values of 12.25 ± 0.66 mg/ml followed by CF, VC and MF. The FRAP values of CF, VC and MF were significantly different from CC at $p \leq 0.05$.

Table 4.6: Effect of plant growth supplements on antioxidant potential of *C. nutans* leaves.

Treatment	Sample ID	DPPH IC_{50} (mg/ml)	ABTS IC_{50} (mg/ml)	FRAP values (mg/ml)
No fertilizer	CC	1.18 ± 0.05^{a}	0.98 ± 0.05^{a}	12.16 ± 0.18^{b}
Recommended dose of NPK fertilizer	CF	1.41 ± 0.02^{b}	1.15 ± 0.16^{ab}	10.35 ± 0.65^{a}
Vermicompost at 15 t ha^{-1}	VC	1.67 ± 0.04^{c}	1.41 ± 0.01^{bc}	10.17 ± 0.01^{a}
Vermicompost at 15 t ha^{-1} + 50% NPK fertilizer	MF	2.23 ± 0.02^{d}	1.67 ± 0.17^{c}	10.10 ± 0.07^{a}

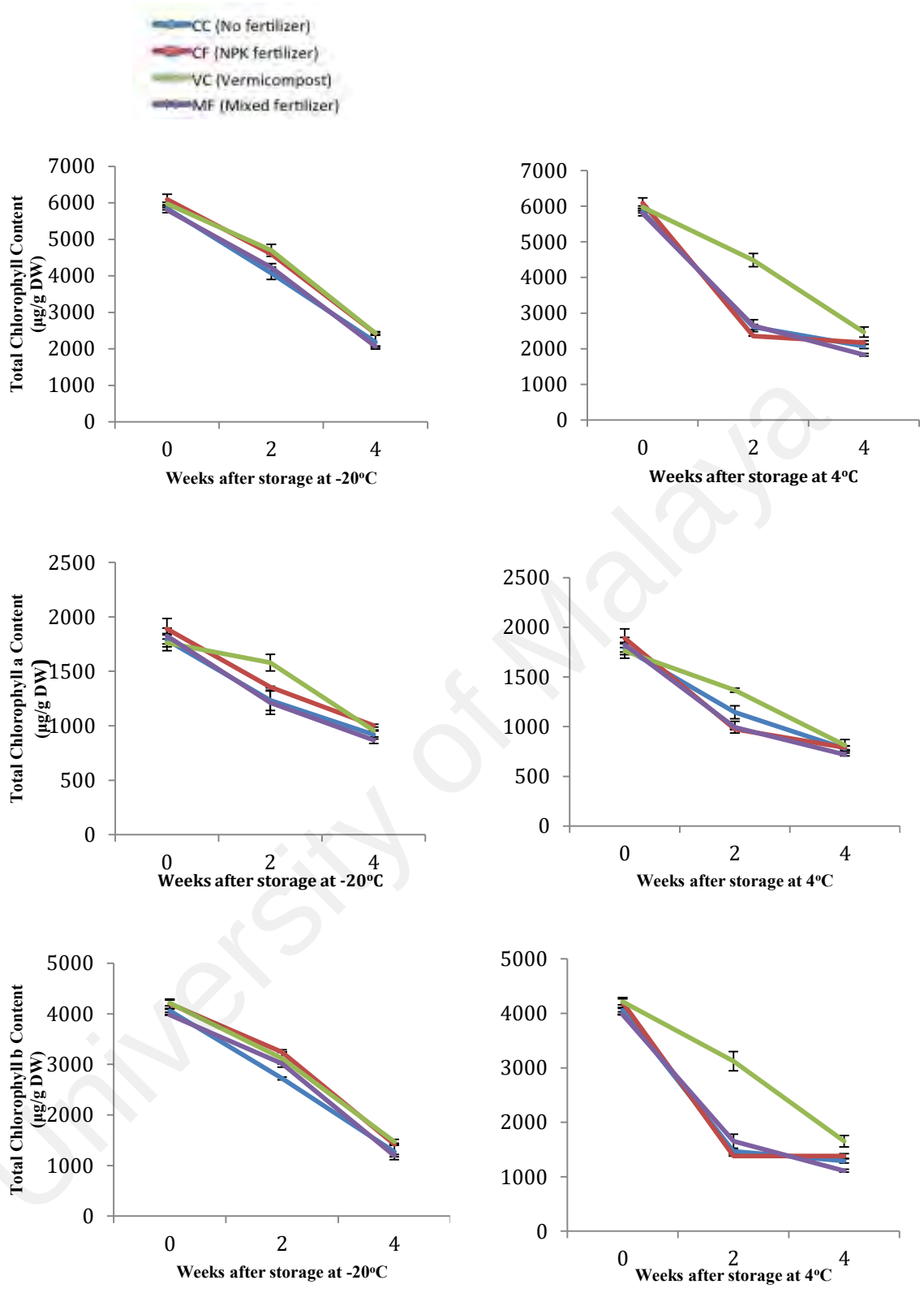
Each value is the mean ± standard error consisting of three replicates.
Means with different letters within the same column are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT).

4.4 Effect of Extract Storage (duration and temperature) on Stability of Pigments and Bioactive Compounds in *C. nutans* Methanolic Extract

4.4.1 Chlorophylls and Carotenoids

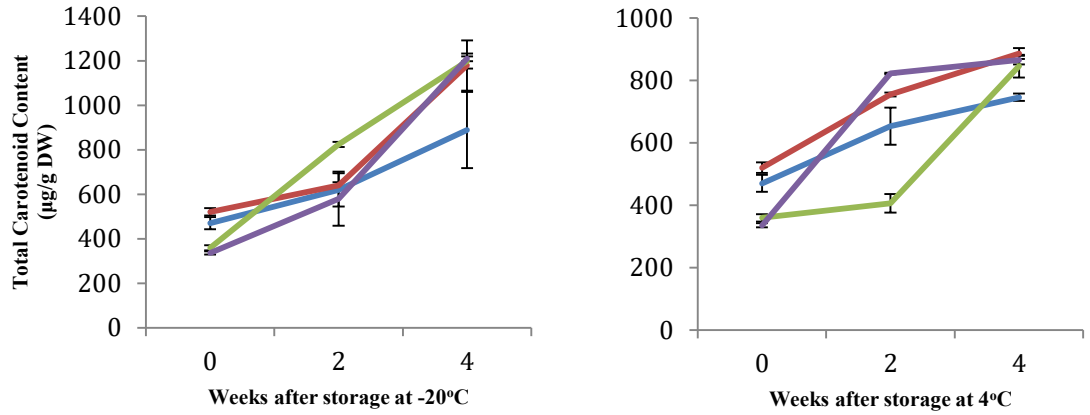
In this study, the TC and TCC of the methanolic extracts after 2 and 4 weeks of storage at -20°C and 4°C were evaluated. As observed in Figure 4.1, the results showed that chlorophyll *a* content in VC exhibited the least reduction, with a decrease in chlorophyll *a* of 10.29% and 22.41% after the extract was stored for 2 weeks at -20°C and 4°C, respectively. After 4 weeks of storage, the content of chlorophyll *a* for VC extract decreased by 45.70% and 53.84% when stored at both -20°C and 4°C, respectively. VC plant extract showed the least reduction in the level of chlorophyll *b* with 25.94% and 60.76% losses when stored at 4°C respectively for 2 and 4 weeks, in

comparison with CC (63.89% and 68.23% respectively) stored in the same conditions. After 4 weeks of storage, VC showed reduction in chlorophyll b content by more than 60% when stored at both -20°C and 4°C. The application of vermicompost was found to have no significant effect on the chlorophyll content of *C. nutans*. However, VC plant extract showed to be more stable when kept at different storage conditions as evident by the lower reduction of total chlorophyll level as compared to extracts from other treatments. The content of total chlorophyll for VC declined by only 21.37% and 24.90% when stored at -20°C and 4°C respectively for 2 weeks and decreased significantly by 59.36% and 58.72% after storage for 4 weeks -20°C and 4°C respectively. In turn, CF exhibited the highest decline in the chlorophyll level by over 60% decrease in the total chlorophyll content after the extract was stored at 4 °C for 2 and 4 weeks. On the other hand, the total carotenoid content showed an increasing trend over 4 weeks of extract storage. VC exhibited higher increase in total carotenoid content with 129.05%% and 233.48% increment after 2 and 4 weeks of storage respectively at -20°C. The increase in total carotenoid content for VC was relatively lower with 135.36% increment when the extract was stored for 4 weeks at 4°C. CC on the other hand showed the least increment in total carotenoid content with only 89.01% and 58.68% increment over 4 weeks of storage at -20°C and 4°C respectively despite it possesses higher total carotenoid content than VC at the beginning of the experiment. CF on the other hand exhibited 126.51% and 70% increment after 4 weeks of extract storage at -20°C and 4°C respectively.



Each value is the mean ± standard error consisting of three replicates.

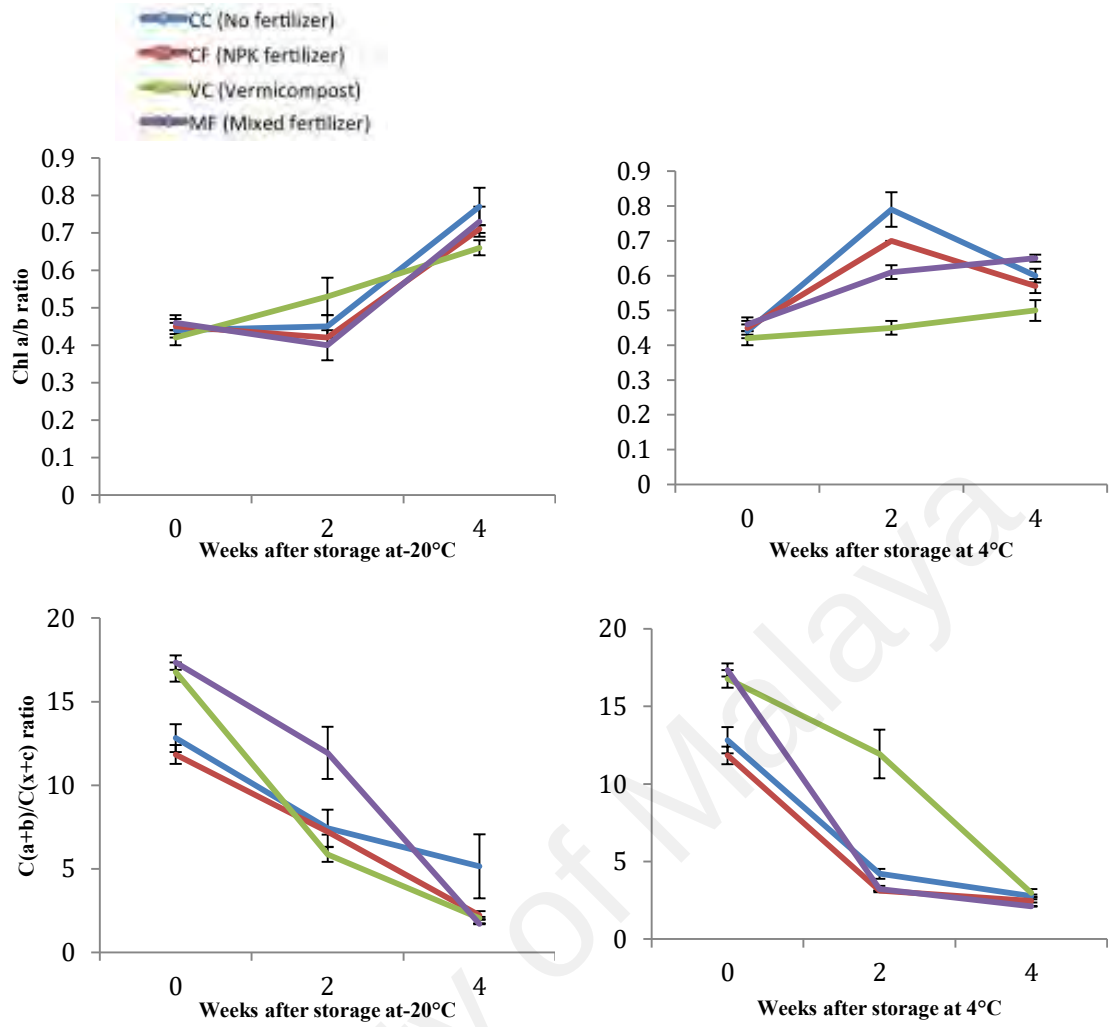
Figure 4.1: Effect of extract storage at -20°C and 4°C on total chlorophyll (TCC), total chlorophyll a, total chlorophyll b and total carotenoid (TC) contents in the methanolic extracts of plants supplemented with different fertilizers.



Each value is the mean ± standard error consisting of three replicates.

Figure 4.1, continued.

Based on Figure 4.2, Chl *a* and Chl *b* (Chl *a/b* ratio) for all *C. nutans* methanolic extracts increased while the weight ratio of chlorophyll *a* and *b* to total carotenoid (C(a+b)/C(x+c)) decreased with the advancement of storage duration. After 4 weeks of storage, the percentage increase of Chl *a/b* ratio when the extracts were stored at -20°C were 64.55%, 56.62%, 55.47% and 58.25% for CC, CF, VC and MF respectively while the Chl *a/b* ratio inreased by 37.11%, 26.70%, 17.64% and 41.05% for CC, CF, VC and MF respectively after 4 weeks of storage at 4°C. On the other hand, the C(a+b)/C(x+c) ratio decreased by 80.0%, 82.45%, 87.81% and 90.17% for CC, CF, VC and MF respectively when the extracts were stored at -20°C for 4 weeks. In turn, the extracts exhibited 77.46%, 79.10%, 82.46% and 87.82% of decrease for CC, CF, VC and MF respectively after 4 weeks of storage at 4°C.



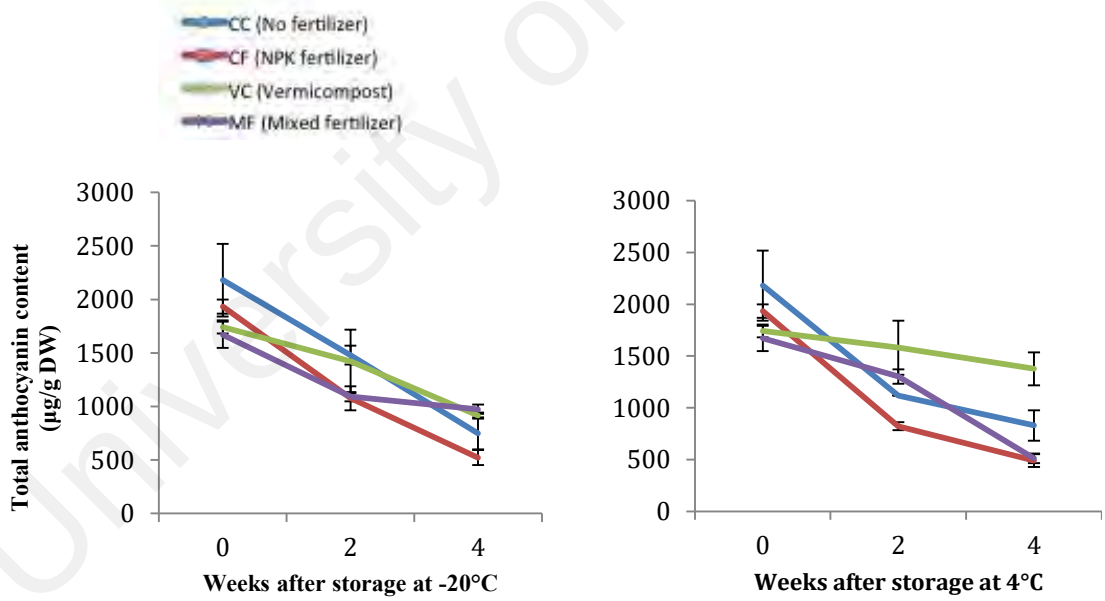
Each value is the mean ± standard error consisting of three replicates.

Figure 4.2: Effect of extract storage at -20°C and 4°C on Chl *a/b* ratio and C(a+b)/C(x+c) ratio in the methanolic extracts of plants supplemented with different fertilizers.

4.4.2 Anthocyanins, Phenols and Flavonoids.

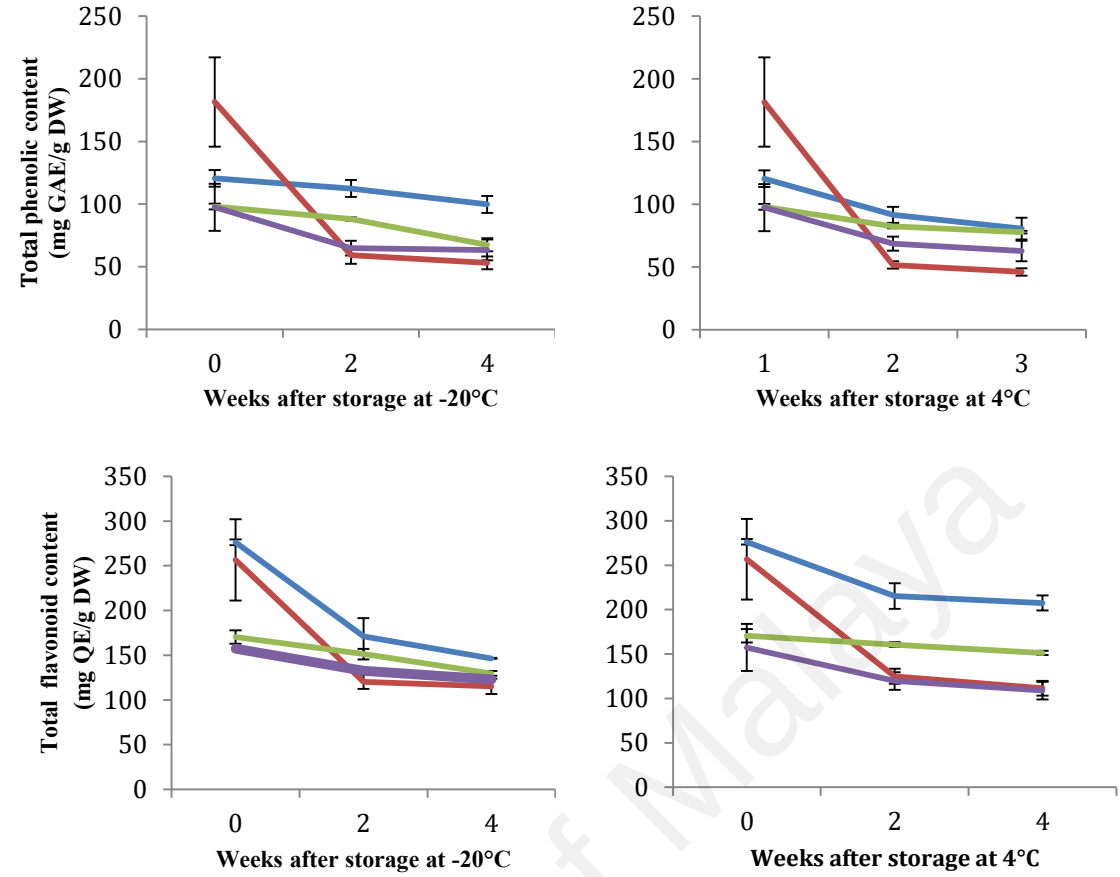
In this study, the TPC, TAC and TFC of the methanolic extracts after 2 and 4 weeks of storage at -20°C and 4°C were also evaluated. As observed in Figure 4.3, the TAC of all plant extracts reduced after 4 weeks of storage at -20°C and 4°C. There was more than 50% of total anthocyanin loss for CC and CF plant extracts after 4 weeks of storage at -20°C and 4°C compared to VC and MF. VC plant extract also showed the lowest percentage of total anthocyanin loss (21.0%) after 4 weeks of storage at 4°C, compared to other extracts which exhibited TAC loss of more than 50%. Data analysis showed that TPC of the extracts decreased gradually with extract storage (Figure 4.3). After 4

weeks of storage at -20°C, the TPC was observed to decrease by 26.62%, 70.75%, 37.04% and 41.19% for CC, CF, VC, and MF extracts, respectively. Plants supplemented with vermicompost (VC) showed the least percentage of TPC loss after extract storage at 4°C, compared to other plant extracts (Figure 4.3). It was also observed that plants supplemented with only NPK fertilizer (CF) showed the highest TPC loss after extract storage at both -20°C and 4°C (Figure 1). Similar results were observed for TFC, where CF plant extracts were found to exhibit the highest TFC loss after extract storage (Figure 4.3). Interestingly, data analysis again revealed that plants supplemented with vermicompost (VC) exhibited the lowest percentage of TFC loss, with a decrease of 23.96% and 11.30% after 4 weeks of storage at -20°C and 4°C, respectively.



Each value is the mean ± standard error consisting of three replicates.

Figure 4.3: Effect of extract storage at -20°C and 4°C on TAC, TPC and TFC of the methanolic extracts of plants supplemented with different fertilizers.



Each value is the mean ± standard error consisting of three replicates.

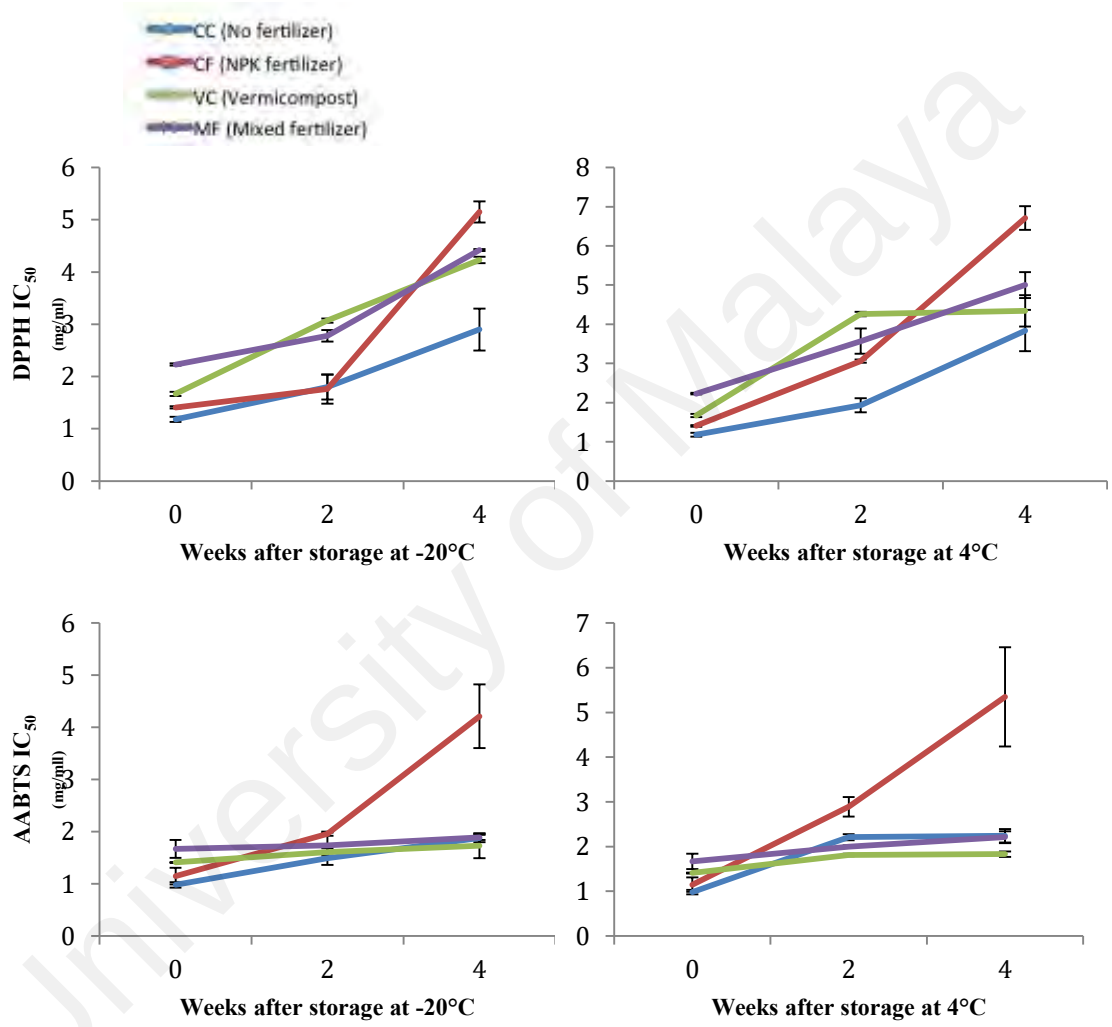
Figure 4.3, continued.

4.5 Effect of Extract Storage (duration and temperature) on Antioxidant Potential of *C. nutans* Methanolic Extract

The antioxidant potential of the plant extracts following extract storage at -20°C and 4°C were monitored (Figure 4.4). After 2 weeks of extract storage at -20°C, the free radical scavenging activities of all extracts against DPPH radicals were found to decrease with increased IC_{50} value of 52.54%, 24.82%, 83.83% and 24.66% for CC_{w2}, CF, VC and MF respectively. Longer storage duration significantly affects the antioxidant potential of extracts. After 4 weeks of storage, the DPPH IC_{50} values significantly increased by 145.76%, 265.25%, 153.30% and 98.21% for CC, CF, VC and MF respectively. The same pattern can be observed when extract is stored at 4°C VC and MF plant extracts showed lower increment of IC_{50} with 159.88% and 124.22% respectively as compared to CC and CF (225.42% and 375.89% respectively) after 4

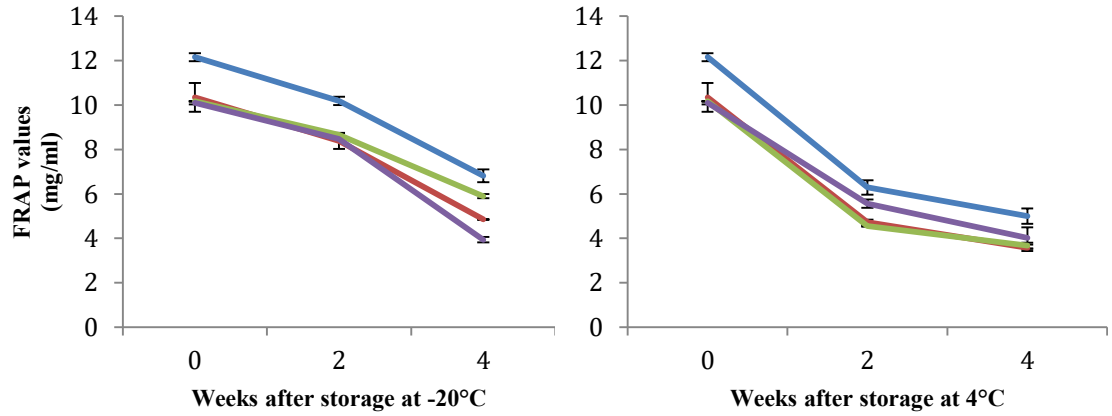
weeks of extract storage. Pearson correlation analyses in Table 4.7, showed strong significant correlation between DPPH IC_{50} values with storage temperature (r = 0.549**) and strong correlation between DPPH IC_{50} values with storage duration (r = 0.710**). Besides that, the radical scavenging activities of the extracts against ABTS radicals also decreased due to storage. After 2 weeks of storage at -20°C, the IC_{50} of VC plant extract increased by only 14.18% as compared to CC (52.04%) and CF (70.43%). IC_{50} of extracts increased by 91.84% and 266.09% respectively upon 4 weeks of storage at -20°C. On the other hand, the IC_{50} value of VC only increased by 22.70%. Higher temperature also has significant effect on *C. nutans* extract. The IC_{50} of all extracts increased when extracts were stored at 4°C. Both CC and CF showed increased IC_{50} values of 128.57% and 365.22% after 4 weeks of storage at 4°C. VC extract proved to be more stable with only slight increment of IC_{50} value (29.79%). Pearson correlation analyses showed moderate significant correlation between ABTS IC_{50} values of all extract with storage temperature (r = 0.350**) and moderate correlation with storage duration (r = 0.391**). The positive correlation proved that the antioxidant activity of *C. nutans* decreased with increased temperature and prolonged storage (higher IC_{50} values denote low antioxidant potential). Furthermore, the reducing potential of *C. nutans* methanolic extract was also influenced by storage temperature and duration. After 2 weeks of storage at -20°C, the reducing potential decreased by 16.20%, 19.03%, 14.85% and 16.14% respectively for CC, CF, VC and MF. VC plant extract showed 41.99% of decreased reducing potential after 4 weeks of storage at -20°C as compared to other extracts with 44.00%, 53.14% and 61.00% of decreased reducing potential for CC, CF and MF respectively. The result showed that VC plant extract is more stable when stored for a longer duration. Reducing potential of all extracts decreased significantly when stored at 4°C. After 2 and 4 weeks of storage, the reducing potential of all extracts decreased by more than 50% with VC showing 54.08% losses and CC,

CF and MF exhibited 58.88%, 65.51% and 60.20% of losses respectively. Pearson correlation analyses showed significant strong negative correlation between FRAP values with storage temperature (r = -0.807**) and strong negative correlation between FRAP values with storage duration (r = -0.794**). The negative correlation showed that FRAP values decreases with increasing temperature and longer storage duration.



Each value is the mean ± standard error consisting of three replicates.

Figure 4.4: Effect of extract storage at -20°C and 4°C on antioxidant activities of the methanolic extracts of plants supplemented with different fertilizers.



Each value is the mean ± standard error consisting of three replicates.

Figure 4.4, continued.

Table 4.7: Correlation between storage temperature and storage duration with antioxidant properties of *C. nutans* extract.

	ST	SD	DPPH	ABTS	FRAP
ST	1				
SD	0.643**	1			
DPPH	0.549**	0.710**	1		
ABTS	0.350**	0.391**	0.545**	1	
FRAP	-0.807**	-0.794**	-0.772**	-0.463**	1

** Correlation is significant at p<0.01
* Correlation is significant at p<0.05
ST= storage temperature; SD= storage duration; DPPH= 2, 2-diphenyl-1-picrylhydrazyl; ABTS= 2, 2-azino-bis (3-ethylbenzotiazoline-6-sulfonic acid; FRAP= ferric reducing antioxidant power.

4.6 Correlation between Bioactive Compounds Composition with Antioxidant Properties of *C. nutans* Extract

Pearson correlation analyses was performed to study the relationship between the bioactive compound composition which comprised of total flavonoid content (TFC), total phenolic content (TPC), total chlorophyll content (TCC), total carotenoid content (TC) and total anthocyanin content (TAC) of *C. nutans* methanolic extracts from different treatment groups and their antioxidant activities based on DPPH, ABTS and FRAP assays. Table 4.8 revealed that there was a significant and moderate correlation showed between TFC and TPC with DPPH and ABTS assays. In addition, TAC showed

strong correlation with DPPH and FRAP while moderate correlation with ABTS assay. Both TFC and TPC also exhibited strong significant correlation with FRAP assay. Besides that, TCC also showed strong and significant correlation with DPPH and FRAP assays while moderate and significant correlation with ABTS assay. The negative correlation indicates that, the increase in TPC, TFC TAC and TCC significantly increased the antioxidant activity against both DPPH and ABTS radicals (denoted by the decrease in the IC_{50} values) while the positive correlation shown with FRAP assay indicates that the reducing potential of the extract increases with increasing level of the bioactive compounds. Results from this analysis proved that phenolics, flavonoids, anthocyanin and chlorophyll may contribute to the antioxidant capacity of *C. nutans* methanolic extract. Pearson's correlation analysis also showed that there was a significant positive correlation found between the DPPH and ABTS antioxidant assays with TC while significant negative correlation between FRAP assay and TC. This correlation is obtained because the carotenoid level in *C. nutans* methanolic extract continued to increase after storage even after the antioxidant activity of the extract decreased.

Table 4.8: Correlation between bioactive compounds composition with antioxidant properties of *C. nutans* extract.

	TFC	TPC	TAC	TCC	TC	DPPH	ABTS	FRAP
TFC	1							
TPC	0.834**	1						
TAC	0.399**	0.429**	1					
TCC	0.429**	0.499**	0.663**	1				
TC	-0.369**	-0.347**	-0.554**	-0.685**	1			
DPPH	-0.465**	-0.464**	-0.518**	-0.624**	0.553**	1		
ABTS	-0.376**	-0.401**	-0.427**	-0.396**	0.219**	0.545**	1	
FRAP	0.528**	0.582**	0.561**	0.834**	-0.553**	-0.772**	-0.463**	1

** Correlation is significant at $p<0.01$
* Correlation is significant at $p<0.05$
TFC= total flavonoid content; TPC= total phenolic content; TAC= total anthocyanin content; TCC= total chlorophyll content; TC= total carotenoid content; DPPH= 2, 2-diphenyl-1-picrylhydrazyl; ABTS= 2, 2-azino-bis (3-ethylbenzotiazoline-6-sulfonic acid; FRAP= ferric reducing antioxidant power.

CHAPTER 5: DISCUSSION

5.1 Phytochemical Screening

The phytochemical screening was carried out with the crude methanol extract of *C. nutans* for all treatment groups which include no fertilizer (CC), chemical fertilizer (CF), vermicompost (VC), and the combination of vermicompost and chemical fertilizer (MF) according to the standard phytochemical screening methods by Solihah *et al.* (2012). In the present study, phytochemical screening indicated the presence of phenolic compounds, flavanoids and saponins. No alkaloid and tannins were detected. This result is in agreement with studies by Abdul Rahim *et al.* (2016) and Yang *et al.* (2013). In addition, the methanolic extract of *C. nutans* also showed the presence of steroids and triterpenes (Abdul Rahim *et al.*, 2016).

5.2 Determination of Pigment Content

5.2.1 Total Chlorophyll Content and Total Carotenoid Content

The methanolic extracts of *C. nutans* were found to contain three natural pigments namely chlorophyll, carotenoid and anthocyanin. In the present study, the highest chlorophyll content was observed in plant extracts supplied with chemical fertilizer (CF), followed by VC, MF and CC. The pigment levels for plants supplied with vermicompost were higher or at least not significantly different than control. This results are in agreement with previous study by Coria-Cayupán *et al.* (2009) who obtained higher total chlorophyll reading for Lettuce plant that was supplied by vermicompost than the control. In the present study, the total chlorophyll content also decreased gradually with the advancement of storage duration at different storage temperatures. Similar findings were documented by Raya *et al.* (2015) who found that

there was reduction in total chlorophyll content by 35, 62 and 78% after 2, 3 and 4 days of storage respectively. The reduction of total chlorophyll content is associated with several factors such as the breakdown of chloroplast, degradation of chlorophyll, and environmental factors including temperature, light, humidity, and other physiological processes such as senescence and ripening (Meir *et al.*, 1992; Roura *et al.*, 2000). Giusti & Wrolstad (2001), on the other hand, opined that changes in chlorophyll content are the consequence of different physicochemical changes during post-harvest processing and storage. According to Matile *et al.* (1997), there is one enzyme that initiates the degradation of chlorophyll, known as chlorophyllase, resulting in the senescence of the leaves. Results from this study also showed higher concentration of chlorophyll b in all plant etxracts as compared to chlorophyll a, similar to findings by Ali *et al.* (2018) and Ling *et al.* (2009). Chlorophyll b is exclusively located in the pigment antenna system while chlorophyll a can be found in the reaction centres of photosystems I and II in the pigment antenna (Lichtenthaler *et al.*, 1981). The high concentration of chlorophyll b resulted in lower chlorophyll a to chlorophyll b (Ca/Cb) ratio. Engel & Poggiani (1991) reported that the value of Ca/Cb ratio could be lower in leaves exposed to direct sunlight because chlorophyll *a* has the tendency to degrade faster in conditions of high irradiance than chlorophyll *b* due to the little protection of the photosynthetic mechanism. Goodwin (1980) and Wolf (1956) also agreed that chlorophyll a deteriorate faster than chlorophyll b. Due to this fact, sun exposed leaves showed lower chlorophyll levels in comparison with shade leaves (Boardman, 1977). Furthermore, the enlargement of the pigment antenna system of photosystem II could also resulted in the decreased value of Ca/Cb ratio (Lichtenthaler & Buschmann, 2001). However, this result is contrary to the results by Guzman *et al.* (2012) who obtained 4 times higher level of chlorophyll a to chlorophyll b for *B. oleracea* vegetables which comprised of four broccoli varieties.

The weight ratio of chlorophylls a and b to total carotenoid ((a+b)/(x+c)) was also measured in the present study to analyze the greenness of plants (Lichtenthaler & Buschmann, 2001). Initially before storage, the ratio for all plant samples was high but decreased with storage. The lower values for the ratio (a+b)(x+c) could be due to several factors which include senescence, stress, and the damage to plant and the photosynthetic apparatus which is characterised by a rapid degradation of chlorophylls as compared to carotenoids (Lichtenthaler & Buschmann, 2001). Besides that, chromoplast development also contributed to continuous decrease of the (a+b)(x+c) ratio which can be seen by the yellowing colour of the plant leaves (Giusti & Wrolstad, 2001).

The highest total carotenoid content was shown by CC followed by MF, CF and VC. Total carotenoid content increases after prolonged storage at -20ºC and 4ºC. Noseworthy *et al.* (2008) obtained similar findings in which the total carotenoid content for *Cucurbita maxima* squash increases after 30 days of storage at 15ºC. Ali *et al.* (2018) also reported the increase of total carotenoid content in the extracts of the *Orthosiphon stamineus* coloured calli after 4 weeks of storage at -20ºC. The author suggested that at low temperature, carotenoids were less prone to degradation as compared to chlorophylls (Ali *et al.*, 2018). The increase in carotenoids during storage can also be referred to as post maturation or after-ripening (Noseworthy *et al.*, 2008). Results from this study indicate that the carotenoid synthesis in *C. nutans* continues to function long after harvest, and the system is enhanced by storage at low temperature. The extract of *C. nutans* was also subjected to HPLC for the analysis of carotenoid composition, where it was screened against 8 different types of carotenoids; neoxanthin, violaxanthin, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene and lutein. Results showed presence of violaxanthin, lutein, α-carotene, and β-carotene for CF, VC,

and MF plant extracts while CC plant extract only showed presence of violaxanthin and lutein. Englert *et al.* (1995) reported that the pigments contained within green leafy vegetables are constituted by the four major carotenoids which are β-carotene, lutein, violaxanthin, and neoxanthin.

5.2.2 Total Phenolic Content, Total Flavonoid Content and Total Anthocyanin Content

The initial total phenolic content and total flavonoid content of *C. nutans* plant extract supplied with vermicompost (VC) were lower with respect to control plant extract that was not supplied with fertilizer (CC). This finding is in agreement with that of Coria-Cayupán *et al.* (2009) who reported that almost all vermicompost additions reduce the total phenolic content of Lettuce (*Lactuca sativa* L.). These results are also parallel to studies by Pant *et al.* (2009) and Luján-Hidalgo *et al.* (2016) who obtained lower total phenolic and flavonoid for vermicompost-treated plants. This indicate that there are some factors present in vermicomposts that negatively interfere with the phenolic compound synthesis produced through the phenylpropanoid pathway (Coria-Cayupán *et al.*, 2009). The accumulation of these various secondary metabolites has been shown to be affected by interactions between plant genotype (species and variety within species) and environmental factors, including cultivation technique, season, abiotic and biotic stress, and nutrient status (Downey *et al.*, 2013; Ksouri *et al.*, 2007).

The nutritional status of plants plays a very important role. It is evident from various studies that vermicompost provides all nutrients to the plants in readily available form and enhances nutrient uptake by plants (Adhikary, 2012). Besides that, soil available nitrogen also increased significantly with increasing levels of vermicompost. Thus, the production of phenolics and flavonoids are low when plants are not nutrient limited. On

the contrary, the biosynthesis of these compounds was found to be increased under environmental stresses such as nutrient deffiencies, so the compounds are synthesized and accumulated (Kang & Saltveit, 2002). Nutrient stress also has a marked effect on phenolic levels in plant tissues (Chalker-Scott & Fuchigami, 1989). The concentrations of various secondary plant products are strongly dependent on the growing conditions and have impact on the metabolic pathways responsible for the accumulation of the related natural products. It has been demonstrated previously that stress, particularly low Nitrogen content, can induce greater concentrations of phenolics in plant tissue. Nutrient stresses can reduce growth more than photosynthesis. Deficiencies in nitrogen and phosphate directly influence the accumulation of phenylpropanoids (Dixon & Paiva, 1995). Potassium, sulfur and magnesium deficiency are also reported to increase phenolic concentrations. Low iron level can cause increased release of phenolic acids from roots (Chalker-Scott & Fuchigami, 1989). Calcium levels have been implicated in plant response to many abiotic stresses including cold, drought and salinity. Similarly, anthocyanin accumulation is stimulated by various environmental stresses, such as UV, blue light, high intensity light, wounding, pathogen attack, drought, sugar and nutrient deficiency (Winkel-Shirley, 2001). Therefore, an increased concentration of total phenolics and flavonoids can be observed in control plants as compared to vermicompost-treated plants in response to the nutrient stress. It can also be observed that the total phenolic contents of *C. nutans* extracts were lower as compared to total flavonoid. This result is in agreement with previous study by Sepahpour *et al.* (2018) who also obtained higher total flavonoid content for the methanolic extract of turmeric (*Curcuma Longa*).

The total phenolic and flavonoid content of *C. nutans* methanolic extracts for all treatments decreased after 4 weeks of storage at -20^{0}C and 4^{0}C. These results were

consistent with the findings of Moldovan *et al.* (2016) who reported the decrease in total phenolic contents of Cornelian cherries extracts after being kept at 2 °C for up to 60 days of storage. There was also a drastic decrease in the total phenolic and flavonoid content of *Anemopsis californica* extracts after being stored at 4°C and −20°C (Del-Toro-Sánchez *et al.*, 2015). Results from the present study is in agreement with that reported by Karabegović *et al.* (2012), which showed that the total flavonoids in freshly prepared herbal liquor were greatly reduced by storage duration. Oxidation by polyphenol oxidase (PPO), degradation of compounds, the polymerization of these compounds with proteins, and the conversion between free and bound phenolic substances are among the factors affecting the decrease of total phenolics and flavonoids after prolonged storage (Ferrante & Maggiore, 2007; Varela-Santos *et al.*, 2012). Besides that, processing could also cause disruption in cell structure which will result in the loss of total phenolics and flavonoids (Kim & Padilla - Zakour, 2004).

On the other hand, the decrease in total phenolic content may be due to the action of enzymes such as glycosidase, phenolase and PPO activity (Kchaou *et al.*, 2014). PPO is an enzyme that when it reacts with oxygen, it can cause the oxidation of phenolic compounds to quinones (Lee *et al.*, 1990) and it has been reported that polyphenols were used as substrates for the PPO enzyme (Janovitz-Klapp *et al.*, 1990). In addition, non-enzymatic reactions also contributed to the loss of total phenolic and flavonoid contents after storage. The hydrogen peroxide produced as the by-product from the oxidation of ascorbic acid to dehydroascorbic acid is one of the factors that adversely affect the production of phenolic compounds (He, 2008). Shen *et al.* (2000) reported that chilling stress could increase oxygen radicals and the production of ascorbic acid and dehydroascorbic acid. Prasad *et al.* (2010) also mentioned that light intensity and temperature during storage are among the factors that might be directly related to the

reduction in total flavonoid content. However, other studies showed an increase in the total phenolic and flavonoid content during storage as evident by Samad *et al.* (2016) who observed an increase in the total phenolic content of date fruit extracts after 8 weeks of storage at 4^0C.

Results from the present study also revealed that extracts from plants supplied with vermicompost showed better stability as compared to other extracts when stored for a longer period at different temperatures. This is evident by the less percentage of total phenolic and flavonoid loss calculated. At present, no published scientific literature was found on the exact mechanisms behind this observation. However, it could be postulated that the compounds present in the extracts from plants supplemented with vermicompost may undergo less oxidation, less degradation and less polymerization compared to the compounds present in plants supplemented with chemical fertilizer and control plants. In addition to that, according to Malik *et al.* (2012), soil and fertilizer application has been found to affect protein composition and concentration in plants, which can interact with plant polyphenols. Various scientific literature has reported that the covalent interaction between plant polyphenols and proteins can further influence the content of free polyphenols as well as their antioxidant capacity during processing, transportation and storage (Le Bourvellec & Renard, 2012; Ozdal *et al.*, 2013; Trombley *et al.*, 2011). These could attribute to the observations recorded in this study.

5.3 Antioxidant Activities of *C. nutans* Extract

Antioxidant radicals or oxidants react differently with the different types of antioxidant compounds present in the plant extract through several mechanism of actions. The scavenging activity of the free radicals and lipid peroxidation inhibition by the antioxidants are evaluated by appropriate antioxidant assays (Marathe *et al.*, 2011). In the present study, three different types of antioxidant assays were used which

comprised of DPPH, ABTS and FRAP assays to increase the accuracy in measuring the antioxidant capacities of the plant extract.

DPPH assay is typically based on the scavenging of free radicals and converting them to colorless products. When methanolic extract of *C.nutans* reacts with DPPH solution, the free radicals are reduced by hydrogen donation and then this gives rise to the reduced form of 1,1-diphenyl-2-picryl hydrazine (non-radical) with the changes of violet color to pale yellow (Molyneux, 2004). ABTS (2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) on the other hand is a decolorization assay, in which the stable radical is generated directly through the reaction of ABTS with potassium persulfate which resulted in the production of blue or green ABTS chromophore, prior to reaction with antioxidants. DPPH and ABTS scavenging activities are expressed by IC_{50} value which is defined as the concentration of antioxidants required to scavenge 50% of DPPH or ABTS free radicals in the extract.

Based on the results from the present study, antioxidant capacity of *C. nutans* methanolic extract evaluated by ABTS method was higher than that evaluated by DPPH method. Before storage, the ABTS IC_{50} values were 0.98, 1.15, 1.41 and 1.67 mg/ml for CC, CF, VC and MF respectively while DPPH IC_{50} values were 1.18, 1.41, 1.67 and 2.23 mg/ml for CC, CF, VC and MF respectively. The DPPH assay underestimated the antioxidant capacity of *C. nutans* methanolic extract in about 18.44-33.53%, as compared to ABTS assay. This result is in agreement with previous study by Almeida *et al.* (2011) who obtained higher antioxidant capacity for the fresh Braziliian exotic fruits evaluated by ABTS assay than that evaluated by DPPH method. Three main factors that may be responsible for this are firstly, for DPPH assay, the absorbance reading was measured at the wavelength of 515 nm, while 734 nm was selected for ABTS assay.

Arnao (2000) reported that the underestimation by the DPPH free radicals are due to the overlapping of the visible region for some colored compounds, such as carotenoids and anthocyanins that are present in the extract may have the spectra that overlaps with DPPH at 515nm and thus, interfering with the absorbance reading. Second possible explanation may be due to the structural conformation of the antioxidants that influenced the reaction mechanisms of free radical scavengers and DPPH. Bigger molecules that have less access to the radical site will have a lower antioxidant activity for this test as compared to smaller molecules (Prior *et al.*, 2005). The third possible reason may be due to the reactions of certain phenols such as eugenol and its derivatives, being reversible when reacted with DPPH, resulting in low readings for the antioxidant capacity (Bondet *et al.*, 1997). However, DPPH does have an advantage over ABTS assay in which the DPPH free radical can be directly acquired without preparation as it is ready to dissolve whereas ABTS radical cation must be produced by enzymatic (peroxidase and myoglobin) or chemical (manganese dioxide and potassium persulfate) reactions (Arnao, 2000). ABTS and DPPH assays are among the popular spectrophotometer methods for determining the antioxidant capacity in foods and chemical compounds (Kim *et al.*, 2003; Kuskoski *et al.*, 2005).

FRAP assay on the other hand, measures the reducing potential of antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ). In this assay, the free radical chain breaking takes place through donating a hydrogen atom which results in the reducing of ferric (Fe^{3+}) to ferrous (Fe^{2+}). The reducing power is determined by the ability of the antioxidants present in the extract to reduce the Fe^{3+}-TPTZ complex to blue colored Fe^{2+}-TPTZ (Wong *et al.*, 2006). The greater amount of Fe^{3+} reduced to Fe^{2+}, the higher the total antioxidant activity observed. Benzie and Strain (1996) reported that FRAP is

an antioxidant assay that is inexpensive, simple, results are highly reproducible and the procedure is straightforward. FRAP values of *C. nutans* methanolic extract varied from the lowest, 10.10 ± 0.07 mg/ml (MF plant extract) to the highest, 12.16 ± 0.18 mg/ml (control plant extract). Results for FRAP assay from the present study closely resembles to that of DPPH assay in which the plant extract supplied with vermicompost showed lower antioxidant capacity as compared to control. These results are in agreement with previous study by Coria-Cayupán *et al.* (2009) who obtained higher level of antioxidant activity in control plants as compared to plants supplied with vermicompost.

Luján-Hidalgo *et al.* (2016) also reported that the addition of vermicompost had negative effects on antioxidant activity. The lower antioxidant activity shown by plant extract supplied with vermicompost may be due to the low expression of total phenolics and flavonoids from the plant. Antioxidant activity has been attributed to total phenolic and flavonoid content which have been found to be strongly correlated (Nisar *et al.*, 2015). Antioxidant capacity of *C. nutans* methanolic extract for all treatments decreased after 4 weeks of storage at 4^0C and -20^0C. The decrease in antioxidant activity can be due to degradation of total phenolic compounds and vitamin C during storage which was reported by Zhou *et al.* (2014).

5.4 Correlation between Bioactive Compounds and Antioxidant Activities

In this study, the correlation between bioactive composition and the antioxidant capacity of *C. nutans* methanolic extract assesed by DPPH, ABTS and FRAP assays were performed by Pearson's correlation analyses. The results from the analyses showed that polyphenols and flavonoids may play a role in the antioxidant activity of *C. nutans* extract as evident by the significant correlation shown. Soobrattee *et al.* (2005) suggested that both of the compounds contribute to the antioxidant activity due to their

redox properties which allow them to act as reducing agents, hydrogen donators, metal chelators and single oxygen quenchers. Pereira *et al.* (2009) explained that there are a number of ways on how phenolics worked as antioxidants. The structure of phenolics which contained hydrophobic benzenoid rings and hydrogen-bonding potential of the phenolic hydroxyl groups give it the ability to strongly interact with proteins. This enable phenolics to act as antioxidants by inhibiting some enzymes such as various cytochrome P450 isoforms, lipoxygenases, cyclooxygenase and xanthine oxidase that are involed in the generation of radicals (Cos *et al.*, 1998; Parr & Bolwell, 2000). Furthermore, hydroxyl groups of phenolics are good hydrogen donors and its interaction with the π-electrons of the benzene ring gives the molecule the ability to generate free radicals where the radical is stabilized by delocalization and the radicals formed are able to modify radical-mediated oxidation processes (Parr & Bolwell, 2000).

Besides that, the capacity of flavonoids to act as antioxidants was also explained by Pietta (2000) in which flavonoids was reported to have the ability to suppress cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase and NADH oxidase which are involved in the formation of reactive oxygen species. Flavonoids also inhibit the enzymes responsible for superoxide anion production, such as xanthine oxidase39 and protein kinase C. The 3-hydroxyl group in the heterocyclic ring of flavonoids helps to increase the stability of the aroxyl radical and enhance the antioxidant capacity of the parent flavonoid.

Besides phenols and flavonoids, anthocyanins also contribute to the antioxidant capacity of the plant extract. DPPH and FRAP assays showed strong significant correlation with TAC while ABTS assay exhibited moderate significant correlation with TAC, indicating that anthocyanin may contribute to the *C. nutans* antioxidant ability.

Among the factors that are responsible for the antioxidant effects include the number of sugar residues, oxidation state of carbon ring, hydroxylation and methylation pattern of anthocyanin (Jordão & Correia, 2016). These results were in agreement with that reported by Neill *et al.* (2002), whereby anthocyanin was found to be the most active components of the extractable phenolic compounds in *Elatostema rugosum* and also contributed to its high antioxidant activity.

Strong correlations were also observed between TCC and the antioxidant capacities of the extracts. These results suggested that in addition to TFC, TPC and TAC, the chlorophyll content in the samples also contributed to the high free radical-scavenging activity of the extracts. This is parallel to previous studies by Rungruang & Chanthachum (2009) who found that the antioxidant capacity of *Gnetum gnemon* assessed by the three antioxidant assays was influenced by TCC in the plant extract. In other studies by Ferruzzi *et al.* (2002) and Silva-Beltrán *et al.* (2015), it was also reported that chlorophyll exhibited high free radical-scavenging activity and has the ability to inhibit free radicals of DPPH and ABTS. It has been reported that chlorophylls are able to act as H^+ donor to break the chain reaction that resulted in cellular oxidation caused by the free radicals (Endo *et al.*, 1985). However, the results obtained from this study were in contrast to that reported by Santos *et al.* (2013), who found that there was no correlation between the antioxidant substances with chlorophyll in the extracts of cassava leaves.

CHAPTER 6: CONCLUSION

6.1 Conclusion

As a conclusion, the usage of vermicompost on *C. nutans* produced plants with just as high content of bioactive compounds as the control and the plant supplied with chemical fertilizer. Phenols, flavonoids, anthocyanin and chlorophyll may contribute to the antioxidant activity of *C. nutans* methanolic extracts. Different storage condition was found to affect the stability of bioactive compounds present in the extract. The compounds present in VC (plant grown with vermicompost) extract showed better stability when stored at different storage conditions.

6.2 Future Recommendation

Further studies involving multi-omics platform, such as through metabolomics and proteomics are essential to better understand the dynamics of protein and compound synthesis in relation to fertilization practices, as well as their interactions.

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LIST OF PUBLICATIONS

1. **Yusof, Z**., Ramasamy, S., Mahmood, N. Z., & Yaacob, J. S. (2018). Vermicompost Supplementation Improves the Stability of Bioactive Anthocyanin and Phenolic Compounds in Clinacanthus nutans Lindau. *Molecules, 23*(6), 1-13.