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ANTIOXIDANT ACTIVITIES OF EXTRACTS FROM PLANTS IN MINOR USES: *Parkia speciosa, Vitex negundo,* AND *Etlingera elatior*

NORASYIDAH BINTI HARUN

Thesis submitted in fulfilment of the requirements
for the award of the degree of
Master of Science (Industrial Chemistry)

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I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged. This thesis has not been accepted for any degree and is not concurrently submitted for award of other degree.

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Dedicated to my beloved parents, husband, siblings and dear princess Airis

Ranea Abdul Rahman

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ABSTRACT

Our country is blessed with a huge number of plant species in our rainforest. According to World Wide Fund for Nature (WWF), the major types of forest in Malaysia are lowland dipterocarp forest, hill dipterocarp forest, upper hill dipterocarp forest and mangrove forest. Among of these abundance of plant sources, there are still a wide number of plants which can be classified as minor use, as they receive little research and therefore information on benefits of the plants are still limited. This current study is to evaluate the antioxidant activity of crude extracts of *Parkia speciosa* Hassk, *Vitex negundo* L., and *Etlingera elatior* Jack. The extraction were using reflux and maceration techniques in two different solubility which are 80% ethanol (aqueous ethanol) and total aqueous. Antioxidant was evaluated initially by thin layer chromatography with 2,2-Diphenyl-1-Picrylhydrazyl (TLC-DPPH) for screening, and quantitatively by DPPH radical scavenging assay and beta carotene bleaching assay with total phenolic and total flavonoid for additional information. Gas chromatography-mass spectrometry (GC-MS) was used for analyzing the volatile compounds existed in all extracts. TLC plate sprayed by DPPH solutions showed a positive reaction, where the yellowish white spot appeared on the purple background. *P. speciosa* (PSER) extracted in aqueous ethanol by reflux technique shows the best activity for both assays, with EC_{50} value for DPPH radical scavenging assay of 184.860 µg/mL and 57.595 % for antioxidant activity obtained from beta carotene bleaching assay. PSER also gave the highest value in total phenolic, which is 66.520 µg (GAE)/mg but low in total flavonoids content (TFC), which only 16.975 µg/mL as compared to the highest value showed by ethanolic *V. negundo* by reflux (VTER) with the value 50.060 µg/mL. These finding suggest that *P. speciosa* is a potential source of antioxidant that may be increasingly important for human consumption as well as assisting the antioxidant based industries.

ABSTRAK

Negara kita dianugerahkan dengan pelbagai jenis tumbuhan yang terdapat di dalam hutan hujannya. Menurut Tabung Hidupan Liar Antarabangsa (WWF), jenis-jenis hutan yang utama di Malaysia ialah hutan kayu keras tanah rendah, hutan kayu keras bukit, hutan kayu keras tanah tinggi dan juga hutan paya bakau. Di antara kepelbagaian jenis sumber tumbuhan ini, terdapat sebahagian besar daripadanya yang boleh dikategorikan sebagai tumbuhan dengan penggunaan kecil kerana penyelidikan tentang jenis tumbuhan ini belum meluas, justeru, maklumat tentang manfaat tumbuhan ini masih lagi terhad. Kajian ini dibuat adalah untuk menilai aktiviti antioksida daripada ekstrak mentah beberapa jenis tumbuhan, antaranya; *Parkia speciosa* Hassk, *Vitex negundo* L., and *Etlingera elatior* Jack. yang diekstrak menggunakan teknik refluks dan rendaman di dalam dua larutan yang berbeza iaitu 80% etanol (etanol akues) dan akues. Antioksida telah dinilai pada mulanya oleh kromatografi lapisan nipis dan 2,2-Diphenyl-1-Picrylhydrazyl (TLC-DPPH) untuk pengesanan awal dan seterusnya secara kuantitatif menggunakan ujian radikal bebas DPPH dan ujian pelunturan beta karotena serta jumlah kandungan fenolik dan flavonoid sebagai maklumat tambahan. Gas kromatografi dengan spektroskopi jisim (GC-MS) telah digunakan bagi menganalisa kandungan sebatian meruap yang terkandung di dalam setiap ekstrak. Plat TLC yang telah disembur dengan larutan DPPH telah menunjukkan tindak balas positif, yang mana bintik putih kekuningan kelihatan pada latar belakang yang berwarna ungu. *P. speciosa* (PSER) yang diekstrak di dalam pelarut campuran air dan etanol menggunakan teknik refluks telah menunjukkan aktiviti yang terbaik bagi kedua-dua ujian, iaitu nilai EC_{50} untuk ujian radikal bebas DPPH ialah 184.860 µg/mL dan 57.595 % bagi aktiviti antioksida yang diperolehi daripada ujian pelunturan beta karotena PSER turut menunjukkan nilai yang tertinggi dalam jumlah kandungan fenolik iaitu 66.520 µg (GAE)/mg tetapi rendah dalam jumlah kandungan fenolik (TFC) iaitu hanya sebanyak 16.975 µg/mL jika dibandingkan dengan nilai tertinggi yang ditunjukkan oleh *V. negundo* ekstrak etanol menggunakan kaedah reflux (VTER) iaitu dengan nilai sebanyak 50.060 µg/mL. Hasil ujian ini mencadangkan bahawa *P. speciosa* ialah sumber antioksida yang berpotensi dan bakal menjadi sumber penggunaan yang penting oleh manusia selain dapat membantu industri berasaskan antioksida.

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LIST OF SYMBOLS

%	Percent
µl	Microliter
µl/ml	Microliter per mililiter
g	Gram
h	Hour
m	Metre
cm	centimetre
mM	MiliMolar
nm	nanometer
ppm	part per million
M	Molarity
mg/ml	Miligram per milimeter
min	Minutes
ml	Mililiter
ml/min	mililiter per minute
mm	Milimeter
ºC	Degree celcius
s	Second
α	Alpha
β	Beta
λ	Lambda

LIST OF ABBREVIATIONS

AA	Antioxidant Activity
AlCl3	Aluminium trichloride
BH_4	Tetrahydrobioprotein
BHA	Butylated Hydroxyl Anisole
BHT	Butylated Hydroxyl Toluene
Ca	Calcium
CCl_4	Carbon tetrachloride
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DPPH-H	2,2-diphenyl-1-hydrazine
EC50	Effective concentration for 50% reaction
EEAM	*Etlingera elatior* aqueous maceration
EEAR	*Etlingera elatior* aqueous reflux
EEEM	*Etlingera elatior* ethanol maceration
EEER	*Etlingera elatior* ethanol reflux
Eq.	Equation
FAO	Food and Agriculture Organization
FCR	Folin Ciocaltaeu Reagent
FRAP	Ferric Reducing Activity of Plasma
GAE	Gallic acid equivalent
H_2O_2	Hydrogen peroxide
HAT	Hydrogen transfer
HIV	Human immunodeficiency virus
HOCl	Hydrochlorous acid
$HO^{\cdot}$	Hydroxyl radical
$HOO^{\cdot}$	Hydroperoxyl radical
K	Potassium

KAEMP	Kaempferol
Mg	Magnesium
MIC	Minimum inhibitory concentration
ND	Not detected
NGDA	Nordihydro guaretic acid
NO	Nitric oxide
$NO^{\cdot}$	Nitric oxide radicals
NRCS	Natural Resources Conservation Service
$O_2^{\cdot -}$	Superoxide anion
$ONOO^-$	Peroxynitrite
ORAC	Oxygen Radical Absorbance Capacity
P	Phosphorus
PG	Propyl Gallate
PSAM	*Parkia speciosa* aqueous maceration
PSAR	*Parkia speciosa* aqueous reflux
PSEM	*Parkia speciosa* ethanol maceration
PSER	*Parkia speciosa* ethanol reflux
$RO^{\cdot}$	Alkoxy radical
$ROO^{\cdot}$	Peroxyl Radical
ROS	Reactive Oxygen Species
RSC	Radical Scavenger Capacity
SET	Single Eletron Transfer
TBHQ	Tertiary butylhydroquinone
TCA	Thiazolidine-4-carboxylic acid @ Thioproline
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoids Content
TLC	Thin Layer Chromatoraphy
TPC	Total Phenolic Content
USDA	United States Department of Agriculture
UV	Ultraviolet
VTAM	*Vitex negundo* Aqueous Maceration
VTAR	*Vitex negundo* Aqueous Reflux

VTEM	*Vitex negundo* Ethanol Maceration
VTER	*Vitex negundo* Ethanol Reflux
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Practising a healthy diet with a healthy lifestyle is a major way to keep our body in the pink of health. In this growing world's age people are exposed to many dangerous elements that can harm their health. This approach is important in lowering the risk of many chronic diseases such as obesity, heart disease, diabetes, hypertension and cancer.

One part of healthy diet is to include an appropriate amount of essential nutrients in daily meals. World Health Organization (2004) had listed 5 recommendations in healthy diet which one of them is taking fresh fruits and vegetables. While in the report of joint WHO and Food and Agricultural Organization (FAO)(2003) expert consultation recommended the daily consumption of at least 400 g of fruits and vegetables for the prevention of heart disease, cancer, type 2 diabetes and obesity. Gan and Latiff (2011) stated in the report that diet rich in plant materials may solve various health problems such as constipation, diverticular disease, colon rectal disease, diabetes, obesity, gall stones and colon cancer.

Essential nutrients are nutrient required by the body functioning that either cannot be synthesized by the body at all or cannot be synthesized in amounts adequate for good health, and thus must be obtained from a dietary source. These essential nutrients can be obtained from natural sources such as herbs, vegetables and fruits and also synthetic products that have being commercialized in market. But taking the natural one seems to give more advantages to consumer in the sense of healthiness, as well as economical.

The flora of Malaysia comprises about 15000 species of higher plants and more than 1200 of these plants have been reported to possess medicinal values and potential to be utilized in pharmaceutical industry (Abdul Kadir, 1998).

The wealth of these medicinal plants in our rainforest is like a very valuable treasure not only because of their chemotherapic value in traditional health care, but also for their potential as a source of new chemical entities for drug discovery (Noor Rain et al., 2007). Discovery of new pharmaceutical compound from medicinal plants can contributed in fighting the infectious diseases in many countries especially in rural areas besides for the sake of economic reasons as well (Lachumy et al., 2010).

The most common bioactive compound that can be found in plants is phenolics compound. Several epidemiological studies prove that consumption of foods with high content of phenolics such as fruits, vegetables, legumes and wine will decrease the risk of diseases like cancer and cardiovascular disease (Saelim et al., 2008). Besides, polyphenols also beneficial for health as anticarcinogenic, antiatherogenic, anti-ulcer, anti-thrombotic, anti-imflammatory, immune modulating, anti-microbial and for its analgesic effect (Loganayaki et al., 2010). This fact drives the researcher interest to reveal the existence of polyphenols in plants.

1.2 PROBLEM STATEMENT

Oxidative stress is a condition where the production of reactive oxygen species and antioxidant defense system is imbalanced. This chemical phenomenon may cause the cells and tissues to injured or damaged.

Antioxidant is included as important nutrients for us since oxidation is an on going process that occurs inside and outside our body. It can be obtained from the natural sources like vegetables and fruits, and also there are many commercialized synthetic antioxidants available in markets. But, the use of synthetic antioxidants in foods and pharmaceutical preparations were restricted due to their potential health risks and toxicity. Synthetic antioxidants can accumulate in the body thus may result in liver damage and carcinogenesis (Deng et al., 2011). This has resulted in the initiation of

numerous laboratory studies on finding the natural sources of antioxidant.

1.3 RESEARCH OBJECTIVES

This study was undertaken to investigate the antioxidant properties of three local plants in Malaysia which considered as minor use plants. It has embarked on the following objectives:

1. To extract polyphenol compound of *Parkia speciosa*, *Etlingera elatior* and *Vitex negundo* using solvent and water extraction.

2. To determine polyphenol compound profiles using thin layer chromatography (TLC) technique.

3. To observe antioxidant activity of the extracts.

1.4 SCOPE OF STUDY

Three types of plants were chosen for this current work, which are *P. speciosa* (Hassk)., *V. negundo* L., and *E. elatior* (Jack). These plants may be considered as minor use plants because the utilization of these plants is limited, either only as food plants, or only certain parts were used while other parts were thrown as waste. Every part of a plant or tree usually has different uses and values. Part of plants used for this current work is pods for *P. speciosa*, leaves of *V. negundo* and buds of *E. elatior*. Two solvents used for extraction of samples which are 80% ethanol in ultrapure water (aqueous ethanol) and total ultrapure water for aqueous extract. The plants were extracted using reflux technique and maceration technique at certain temperature and time frame. For evaluation of antioxidative characteristic of extract, several assays have been used. One is thin layer chromatography (TLC) with DPPH solution as the spraying reagent. For quantitative evaluation, DPPH radical scavenging assay and beta carotene assay were used. Other tests carried out are total phenolic content (TPC) and total flavonoid content (TFC).

1.4 SIGNIFICANCE OF STUDY

P. speciosa is well known 'ulam' among Malaysians. They usually eat the seeds of *P. speciosa* either fresh or cooked for their high nutritional values. The seeds are bitter taste, with a stink smell. This makes many people refuse to take it as food. In certain places, with a very small division, they also consumed *P. speciosa* pods as they belief that the pods have its own medicinal function, as well as the seeds does. But in most places they considered *P. speciosa* pods as under-utilized agrowaste materials (Gan and Latiff, 2011).

E. elatior (Jack) also known as 'torch ginger' is a popular vegetable in Malaysia for their function as condiment or food flavouring. It is also used to enhance the taste of dishes. Young inflorescences are commonly used as the ingredients of spicy dishes (Chan et al., 2009). In some food in Malaysia, the inflorescence part of *E. elatior* was put in as the key ingredient (Wijekoon et al., 2011a). Recently many works has done on *E. elatior* providing mountain of information on the bioactive compound of the plant. Thus, this makes it as a potential source of basic ingredient in pharmaceutical as well as cosmeticeutical.

V. negundo from the family of *Lamiaceae* is also known as three leaves chaste tree. It has been used for various medicinal purposes in Ayurvedic and Unani systems of medicine (Goverdhan and Bobbala, 2009). In Malaysia the use of this plant is still very limited and the information of the benefits from every parts of the plants is little spread out.

This current work is done aiming to promote the use of antioxidant compound found in the plants, and providing knowledge of the compound, at the same time providing new source of antioxidant compounds. Besides, this will promote and widening the utilization of the plants. This information also useful to assist antioxidant-based industries such as foods, drugs and cosmetic industries.

CHAPTER 2

LITERATURE REVIEW

2.1 ANTIOXIDANT

Karou et al. (2005) has defined antioxidant as compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reaction.

Every living thing will go through an oxidation process. It is a natural process that occur internally or externally and this process will produce free radicals as the by product. Small amount of free radicals are essential to ward off diseases whereby out body may cope with. Excessive free radicals lead towards oxidative stress. Under normal conditions, nearly 2% of the oxygen consumed by the body is converted into O_2 through mitochondrial respiration, phagocytosis, etc (Kunwar and Priyadarsini, 2011). In this developed country there are many factors that cause oxidation or increased the rate of oxidation which is harmful to our health. Our defence mechanism may not able to combat this force without the help of external system. This external system is known as antioxidant. Antioxidant is a molecule that inhibit the formation of free radicals by removing the intermediates. Oxidation process involved induction, propagation and termination step. During induction, alkyl radicals are formed. These radicals will undergo reaction with oxygen molecules to form hydroperoxides and peroxides radicals during propagation phase. Termination step proceeds via association of two radicals to form a stable adduct (Williams et al., 1995). This is where antioxidant will play their roles where they will terminates chain reaction by removing free radicals intermediates (Kogje et al., 2010) and inhibit other oxidation reaction by oxidized themselves. Therefore, antioxidant usually are reducing agents such as thiols, ascorbic acid and

polyphenols. Antioxidants are mainly the monohydroxy or polyhydroxy phenol compound. It is stable compounds which have low activating energy to donate hydrogen. Therefore, the resulting antioxidant radicals do not initiate another free radicals (Hamid et al., 2010). Figure 2.1 shows structures of some antioxidants.

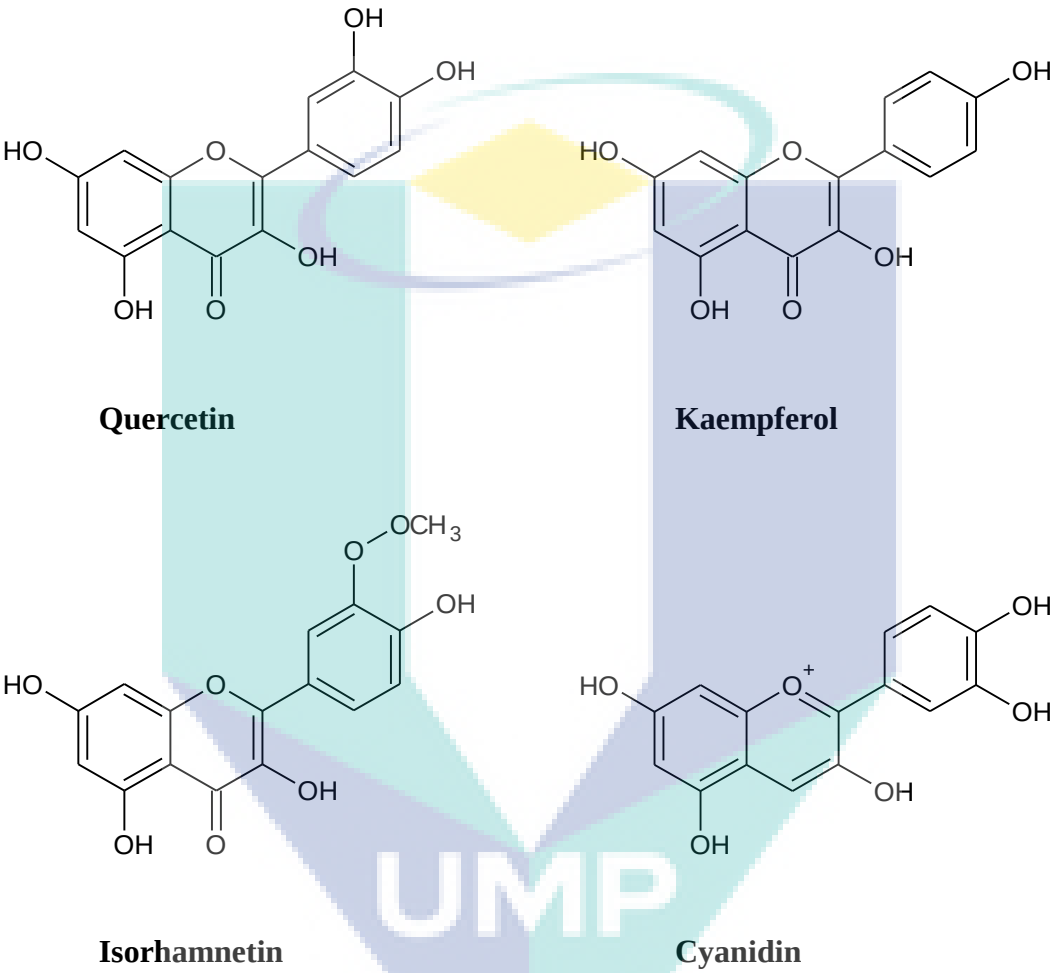


Figure 2.1: Structure of some antioxidant compounds

Source: Cartea et al. (2011)

Fruits, vegetables, nuts and grains are the main sources of antioxidant. Some examples are beta carotene which can be found in many foods that significantly orange in colour such as sweet potatoes, carrots, cantaloupe, squash, apricots, mangoes and also in some green leafy vegetables including collard greens, spinach and kale. Lutein can abundantly be found in collard greens, spinach and kale. Lutein is good for healthy

eyes. Tomatoes, watermelon, guava, papaya, apricots, pink grapefruit and oranges are rich of lycopene content. Antioxidant vitamins like vitamin A, C and E can be found in variety of foods and fruits. Vitamin A1 (retinol), A2 (3,4-didehydroretinol) and A3 (3-hydroxy-retinol) are rich in liver, sweet potatoes, carrots, milk, egg yolks and mozzarella cheese. Cereals, beef, poultry and fish are the source of vitamin C while α-tocopherol (vitamin E) is in almonds, in many oils including wheat germs, safflower, corn and soybean oils and in mangoes, nuts and broccoli (Hamid et al., 2010)

There are two types of antioxidant which were group based on how they work to inhibit oxidation process. One is preventive antioxidant and another one is chain breaking antioxidant. Preventive antioxidant will reduce the rate of chain initiation by scavenge the initiating radicals which will retard an oxidation chain from ever setting in motion and also by stabilizing transition metals such as copper or iron. Some examples of this type of antioxidant are metal chelators such as metallothionein, neuromelanin, transferin and antioxidant enzymes such as catalyse, superoxide dismutase and glutathione reductase. Chain breaking antioxidant will involve in the termination step of chain reaction. When a free radical releases or steals an electron, a second radical is formed. These molecules then turn around and does the same thing to a third molecule, continuing to generate more unstable product. The process continues until termination phase where the radical is stabilized by the chain breaking antioxidant. Examples of chain breaking antioxidant vitamin C, vitamin E, caretenoids and polyphenols. Polyphenols can be divided into hydrolysable tannins and phenylpropanoids such as lignins, flavonoids and condensed tannins. Free radical scavengers are belong to this type of antioxidants (Ciz et al., 2010). Chain reactions of free radicals are as follows.

Initiation step

(1) $RH \rightarrow R\cdot + H\cdot$

(2) $R\cdot \rightarrow R\cdot + O_2 \rightarrow ROO\cdot$

(3) $2ROOH \rightarrow ROO\cdot + RO\cdot + H_2O$

Propagation step

(1) $R\cdot + O_2 \rightarrow ROO\cdot$

(2) $ROO\cdot + RH \rightarrow ROOH + R\cdot$

(3) $RO\cdot + RH \rightarrow ROH + R\cdot$

Termination step

(1) $R\cdot + R\cdot \rightarrow R{-}R$

(2) $R\cdot + ROO\cdot \rightarrow ROOR$

(3) $ROO\cdot + ROO\cdot \rightarrow ROOR + O_2$

(4) Antioxidant + O_2 → oxidized antioxidant

Source: Hamid et al. (2010)

Antioxidant also can be classified into another two major groups which are the natural antioxidant and synthetic antioxidant. Primary or natural antioxidants are mainly consist of phenolic compound and can be subgroup into three types which are the antioxidant minerals, antioxidant vitamins and phytochemicals. Antioxidants are the co factors of antioxidant enzyme. They are aiding in the metabolism of macromolecules such as carbohydrate. Some examples are selenium, copper, iron, zinc and manganese. Antioxidant vitamins consist of vitamin C, E and B, while the phytochemicals are phenolic compounds that are neither vitamins nor minerals. As example, flavonoids which give colours to vegetables, fruits, grains, seeds, flowers, leaves and barks. Others are catechin, carotenoids, beta carotene, lycopene and zeaxanthin. Second group is secondary or synthetic antioxidant, which may capture free radicals and terminate chain reaction. Examples are butylated hydroxyl anisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tertiary butylhydroquinone (TBHQ) and nordihydro guaretic acid (NGDA) (Mukhopadhyay, 2006).

2.1.1 Medical Uses of Antioxidant

The brain is particularly vulnerable to oxidative stress because it generates more free radicals than other organ. Its high metabolic rate and elevated levels of polyunsaturated lipids make it as the target of lipid peroxidation (Reiter, 1995). Consequently, antioxidants are commonly used as medications to treat various forms of brain injury. Vitamin E (α-tocopherol) and vitamin C (ascorbate) play roles in protecting the brain from oxidative stress by direct scavenging of the toxic radicals. Reiter (1995) reported another important antioxidant in the brain which is the in dole melatonin. It may stimulates gluthathione peroxidase, the main antioxidant enzyme of the brain. Antioxidant is also important in combating other diseases apart from brain injuries. Lanthanides, lycopene and selenium derivatives were reported as potential and important cancer preventive agent. Epidemiology studies prove that high consumption of tomatoes which is known as lycopene rich vegetable is effectively lowers the risk of reactive oxygen species (ROS). Other than that, lycopene also shows other biological effects include cardio protective, anti-inflammatory, anti-mutagenic and anti-carcinogenic activities. Lipoic acid is an antioxidant that protect against aging stroke, heart attack and cataracts by suppressing the action of free radicals in the cells of the brain, heart and eyes. Lipoic acid can also boost up glutathione levels through the antioxidant network interactions (Hamid et al., 2010). Superoxide dismutase mimetics, sodium thiopental and propofol are used to treat reperfusion injury and traumatic brain injury. For the treatment of stroke, the experimental drug NXY-059 and obselen are being applied. These compounds may defence against oxidative stress in neurons and prevent apoptosis and neurological damage. Antioxidant also used for the treatments of neurodegenerative diseases such as Alzheimer's disease, Parkinson diseases and amyotrophic lateral sclerosis and as the way to prevent noise-induced hearing loss. Another role of antioxidant is in the treatment of fried Reich ataxia, a rare progressive condition that causes damage to the nervous system (Hamid et al., 2010)

2.1.2 Industrial Uses of Antioxidant

Apart from medicinal value, antioxidant also has other industrial and cosmetic uses. Oxidation also causes food spoilage. Therefore they also need antioxidant to

prevent the damaged of the foods. Antioxidant like ascorbic acid, Propyl Gallate (PG), tocopherols and tertiary butylhydroquinone (TBHQ) were used to prevent the spoilage of frozen or refrigerated food. In cosmeceutical, antioxidant is used to prevent rancidity. Furthermore these antioxidant also been used as stabilizers to prevent polymerisation of fuels and lubricants and to prevent degradation of rubber and gasoline. For this reason, small quantities of antioxidants such as phenols or amine derivatives have been added in such oils. In plastic industries, phenols or naphtol is used to prevent the decomposition of plastics by free radicals reaction and carbon black is used to protect low density polythene. Carbon black can absorbs the ultraviolet light which causes the radicals production (Hamid et al., 2010). Currently in industrialized countries, formulations of antioxidant are being sold as dietary supplements by neutraceutical and health food companies (Kogje et al., 2010).

2.2 OXIDATIVE STRESS AND FREE RADICALS

Oxidative stress is an increasing production of highly reactive free radical species or the decreasing of defence mechanisms to protect against biological damage by free radicals (Wang et al., 2011). It occurs when the production of harmful molecules of free radicals are beyond the protective capability of the antioxidant defence. Oxidative stress contributed to many cardiovascular diseases such as atherosclerosis, diabetes, heart failure and hypertension (Dusting and Triggle, 2005).

Free radicals can arise inside (endegeneous) and outside (exogeneous) our bodies. For endegeneous, free radicals were developed from various processes within our bodies such as aerobic respiration, metabolism and inflammation. For exogeneous, free radicals were formed from environmental factors such as pollution, sunlight, x-rays, smoking and alcohol consuming. There are many factors that may increase the production of these toxic radicals, hence expose our body to oxidative stress. Major cause is poor dietary and lifestyle like taken highly processed food, less exercise and physical activities and smoking. Other example is from the environmental contaminants such as emission from vehicles, factories or industries and cigarettes. Continuous usage of the same vegetable oils which are not even properly stored and reusing the oil (rancid) lead to generation of free radicals through lipid peroxidation (Hamid et al.,

2010). Common free radicals are includes superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), hydroperoxyl radical ($HOO^{\bullet}$), hydrogen peroxide (H_2O_2) and hydrochlorous acid (HOCl) (Kunwar and Priyadarsini, 2011).

2.2.1 Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) is a collective term used for oxygen containing free radicals depending on their reactivity and oxidizing ability. They participate in various chemical reactions with bio molecules that may cause oxidative stress (Kunwar and Priyadarsini, 2011). ROS mainly comprises superoxide (O_2) radicals and nitric oxide (NO) radicals. There are many ways how these free radicals or oxidants may be generated. The reduction of molecular oxygen to water will produce superoxide anion radicals, hydrogen peroxides, and hydroxyl radical in the intermediate of the process. Ground-state molecular (triplet) oxygen as a diradical can be electronically excited to singlet molecular oxygen which may occur as alkyl or peroxyl radicals. Peroxynitrite formed from the nitric oxide and superoxide anion radicals. Radicals also generated from radiations such as X-irradiation which formed hydroxyl radicals and ultrasound and microwave radiation will formed reactive oxygen species (ROS) (Sies, 1997).

Like other free radicals, the percentage of ROS also may increases during infections, exercises, exposure to pollutants, UV light and ionizing radiation. As report by Pollack and Leeuwenburgh (1999), many researchers have shown that oxygen utilization by mitochondria of aerobic organisms can generate several reactive radicals, such as superoxide (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and nitric oxide (NO). Superoxide (O_2) and nitric oxide (NO) radicals may undergo complex transformation reactions and will be converted to powerful oxidizing radicals like hydroxyl radicals ($HO^{\bullet}$), alkoxy radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and singlet oxygen (O_2).

Reactive free radicals can interrupt membrane fluidity, modification of protein nature, lipid peroxidation, changing in platelet function and oxidation of Deoxyribonucleic acid (DNA) in human body, which in futher will cause many chronic diseases such as cancers, inflammation and artheroclerosis. Excessive of $.O_2^-$ is harmful for our body because it will rapidly react with nitric oxide (NO), and will lead to many other bad effects such as loss of NO bioavailability, increasing of peroxynitrite ($ONOO^-$), reduction in the vascular effect of NO and reduction in the antiatherogenic effect of NO. Over production of O_2^- will also lead to oxidation of the important co-factor in the regulation of NO synthase, tetrahydrobioprotein (BH_4), which in further will lead to 'uncoupled eNOS' where it will synthesized $.O_2^-$ rather than NO. Reducing of NO level will weaken the cardiovascular function (Dusting and Triggle, 2005). The other reason is elevated production of $.O_2$- will cause plaque vulnerability. This may lead to plaque rupture which is the major trigger to myocardial infarction (Bennett, 2007).

Free radical from aerobic metabolism is one of the major cause of age related destruction of neuronal tissue (Reiter, 1995). Brain deterioration is extremely costly in terms of quality of life and longevity. Parkinson disease, Alzheimer's disease, multiinfarct dementia and amyotrophic lateral sclerosis are some examples of diseases that increasingly occurred during aging and were related to the damage inflicted by oxygen free radicals and their intermediates. During aging, free radicals formation may increases due to normal aging processes, exposure to toxins and UV light, while the defence system to fight these radicals attack may diminish. This will cause the accelerated rate of accumulated damage and associated pathophysiology in advanced age (Reiter, 1995)

2.3 *PARKIA SPECIOSA* HASSK.

2.3.1 Botanical Overview of *Parkia speciosa* Hassk.

P. speciosa Hassk., which is also known as stink beans or 'petai' by locals is one of the popular vegetables. This rainforest tree is from the family of *Fabaceae* (also place in *Leguminosae* and *Mimosaceae*), commonly found in Malaysia, Indonesia,

Thailand and Philippines. The common names are varied with places such as ‘upang’ by Filipino, ‘petai papan’, ‘pete’ and ‘peuteuy’ in Indonesia, ‘pete’ by Javanese and in Thailand they called it ‘sataw’, ‘sator’, ‘sator dan’, ‘sator kow’, ‘to dan’ and ‘to khao’. The seeds can be eaten raw as ‘ulam’ or put in local traditional delicacy. This tree can grow up to 40 m high and 100 cm in diameter. The bark is smooth and light coloured. The leaves are bipinnate in alternate arrangement. The flowers are very small, light bulb shapes that hang at the end of a long stalk. The upper portion has bisexual flowers while the lower portions are asexual or male flowers. The fruit of a matured tree is a cluster of long, twisted pods emerges at the end of each stalks and the bright green seeds are encapsulated in these pods. The pods measures are about 30-45 cm long and 3-6 cm wide, with 12-18 seeds, in the size and shape of almonds. Figure 2.2 shows the pod of *P. speciosa* Hassk. The flowering and fruiting season of the tree peaks between August and October each year (Abdullah et al., 2011)

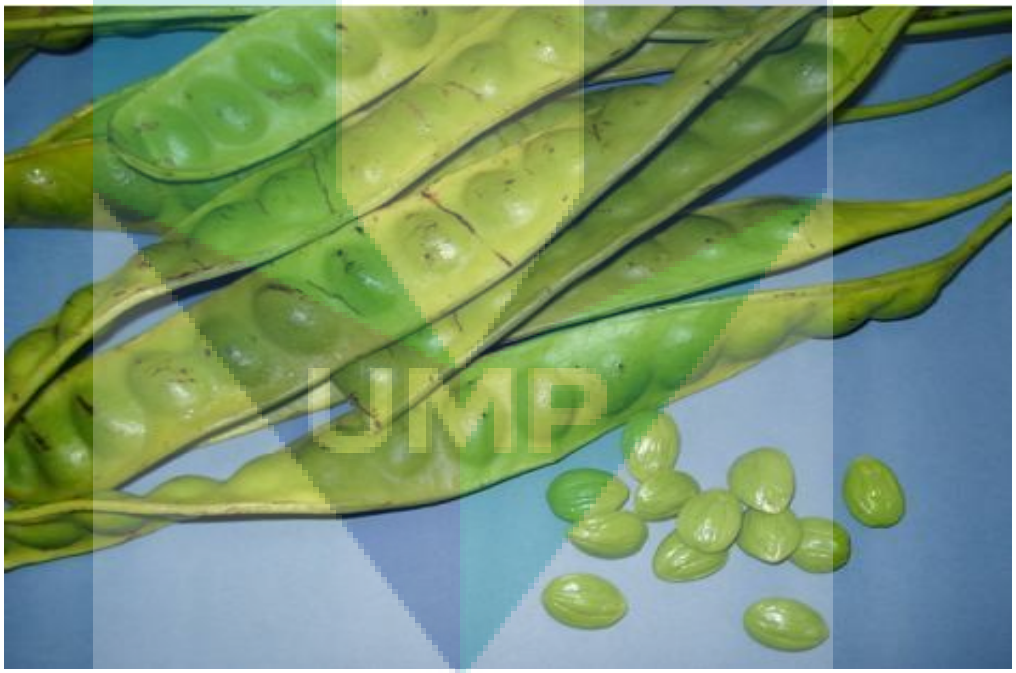


Figure 2.2: *Parkia speciosa* Hassk.

Source: Abdullah et al. (2011)

2.3.2 Botanical Classification of *Parkia speciosa* Hassk.

Kingdom : Plantae

Subkingdom : Tracheobiota

Superdivision : Spermatophyta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order : Fabales

Family : Fabaceae

Genus : Parkia R. Br.

Species : *Parkia speciosa* Hassk.

'

Source: Natural Resources Conservation Service (NRCS), (2014)

2.3.3 Traditional and Medicinal uses of *Parkia speciosa* Hassk.

Almost all parts of *P. speciosa* tree are very useful to human, including the seeds, pods, leaves, and also roots. The shoots, fruits and seeds are eaten raw or also can be boiled and taken with rice or as vegetable, since it possessed medicinal properties which is very good to our health. The seeds were used by locals as a remedy for diabetes, treating kidney pain, cancer, hepatalgia, oedema, nephritis, colic, cholera and as anthelmintic. It also has the carminative values to relieve flatulence. It can be applied externally to wounds and ulcers, for treating scabies and various types of pox. The decoction of roots can be use as diuretic to increase the excretion of urine, and to prevent many diseases. It is also can be taken to treat worms. In Malaysian decoctions of roots has been used in folk medicine to treat hypertension (Kamisah et al., 2013).

2.3.4 Phytochemical and Pharmacological Studies of *Parkia speciosa* Hassk.

P. speciosa is included in legumes and many research had proven that legumes contained nutritional values which gives many benefits in maintaining a good health. It provides macronutrients such as proteins, carbohydrates, and dietary fibre;

micronutrients such as vitamins, caretenoids and phenolic compounds. Kamisah et al. (2013) have reported the rich content of protein, fat, carbohydrate, minerals, vitamin C, α-tocopherol (vitamin E), thiamine and tannins in *P. speciosa* seeds. *P. speciosa* also possess the hypoglycaemic and hypocholesterolemic effects, prevent breast cancer, improved bone health and decrease the risk of cardiovascular diseases (Saelim et al., 2008). Many studies on phytochemical in almost all part of *P. speciosa* have been studied and Kamisah et al. (2013) had summarized the report as in Table 2.1.

Table 2.1 Phytochemical substances in *Parkia speciosa*

Parts	Alkaloid	Saponin	Terpenoids	Phenolic	Flavonoid	Tannin
Seeds	+	-	+	+	+	-
Barks	+	-	-	+	-	ND
Leaves	-	-	+	+	+	ND
Seed coats	+	+	-	-	+	+
Pods	ND	ND	ND	+	ND	+

Source : Yusof et al. (2013)

Previous research reported many kinds of medicinal properties of this plant. One of the important medicinal values in *P. speciosa* is antioxidant activity. Based on report tabulated in Table 2.1, generally *P. speciosa* is rich of the alkaloid, phenolic and flavonoid content. These compounds might be responsible for the antioxidant activity since many studies have reported the antioxidant properties of these compounds. Saelim et al. (2008) compared the total phenolic content (TPC) and antioxidant activity of two kinds of *P. speciosa*, for fresh samples and after storage for certain time and conditions. Amarnath, (2004) reported that both pods and seeds showing the antioxidant potential and the activity are much higher in the pods than the seeds. The major compound isolated and believes to be responsible for the values are polyphenolics. Gan and Latiff (2011) also revealed that *P. speciosa* pods are potential as the high antioxidant functional fibres and suggested to use the *P. speciosa* powder bakery products, replacing other commercial flour with addition of antioxidant content.

Suvachittanont et al. (1996) reported that *P. speciosa* seeds contain Thiazolidine-4-carboxylic acid (TCA) or the common name is thioproline, the condensation product of formaldehyde and cystein. TCA showing some anti-tumor effects when tested on patients with cancer and also anti-aging effects in Drosophila and mice. TCA also inhibit the endogenous formation of the carcinogenic N-nitroso compound, by acting as the nitrite-trapping agent. *P. speciosa* also possesses antibacterial potential. This was reported in the work done by Musa et al. (2008). *P. speciosa* shows antibacterial activity against *Aeromonas hydrophila*, *Staphylococcus aureus*, *Streptococcus agalatiae*, *Streptococcus aginosus* and *Vibrio parahaemolyticus*. *P. speciosa* contained several cyclic polysulfides which are responsible for the antibacterial activity on kidney, ureter and urinary bladder infections (Che Yunus et al., 2008). Al Batran et al. (2013) studied the antioxidant and antiulcer activity of ethanol extract of *P. speciosa* leaves against ethanol-induced gastric ulcers in rats. They had successfully proven that *P. speciosa* may roles as an antiulcer agent by enhancing the gastric mucosal defence or by inhibiting leukotriene synthesis. In the report made by Jamaluddin et al. (1995) stated that *P. speciosa* contained Stigmast-4-en-3-one which function as a new oral hypoglycaemic agent. The tests were done on alloxan-induced diabetes rats, where the chloroform extract of *P. speciosa* pods were orally consumed and a significant reduction in blood glucose levels were observed.

2.4 *VITEX NEGUNDO* LINN.

2.4.1 Botanical Overview of *Vitex negundo* Linn.

V. negundo also known as three chaste leaves have a wide range of varieties and species. *V. negundo* is known with varieties of common names in different places. Some examples are 'trifoliate chastetree', 'huang ping', 'gattilier incise', 'huang ching', and 'man jing'. In Malaysia they are known as 'lemuni', in Indonesia, 'legundi' and in Philipina as 'lagundi'. The genus *vitex* contains more than 270 known species include trees and shrubs all over the world with diverse medicinal active constituents and properties (Hernandez et al., 1999). Generally, *vitex* family is an aromatic shrubby tree with a sandy brown bark, smooth and variables leaves. For simpler trifoliate, the leaflets mostly sessile, elliptic, with blue or purple flowers in terminal. Figure 2.3 shows

the picture of *V. negundo* Linn.

V. negundo grows in humid places or along water courses in wastelands and mixed open forest in Afghanistan, India, Pakistan, Sri Lanka, Thailand, Malaysia, Eastern Africa and Madagascar. It is grown commercially as a crop in parts of Asia, Europe, North America and West Indies (Vishwanathan and Basaraju, 2010).

Figure 2.3: Picture of *Vitex negundo* L.

Source: Raji (2013)

2.4.2 Botanical Classification of *Vitex negundo* Linn.

Kingdom : Plantae

Subkingdom : Tracheobionta

Superdivison : Spermatophyta

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Lamiales

Family : Verbenaceae

Genus : Vitex Linn.

Species : *Vitex negundo* L.

Source: Ladda and Magdum (2012)

2.4.3 Traditional and Medical uses of *Vitex negundo* Linn.

There were a wide use of *V. negundo* in traditional medical practise. Vishwanathan and Basavaraju (2010) have describe the use of *V. negundo* in Ayurvedic, Unani and Chinese medicines. In Ayurvedic, people sleep on pillows stuffed with *V. negundo* leaves to dispel catarrh and headache. Crushed leaf poultice is applied to cure headaches, neck gland sores, tubercular neck swelling and sinusitis. For treatment of syphilitic skin disorders, they use the essential oil of the leaves. In Unani medicines, one way of using this plant is by administered the seeds internally with sugarcane vinegar for removal of swellings. Chinese people use the fruit of *V. negundo* for treatment of reddened eyes, headache and arthritic joints.

Almost all parts of *V. negundo* plants have its own effects in medicinal applications. Decoction of leaves is used externally for flatulence, fever, headache, toothache, cough and asthma (Raji, 2013). It also being used for treatment of inflammation, eye-disease, toothache, leucoderma, enlargement of spleen, ulcers, cancers, catarrhal fever, rheumatoid, arthritis, gonorrhoea, sinuses, scrofulous sores and bronchitis. In Malaysia, young leaves were traditionally used as a remedy for postnatal women (Keerti and Padma, 2012). Seeds are boiled in water and eaten or the water

drunk to prevent the spread of toxin from bites of poisonous animals and infusion of seeds are used for disinfecting wounds and ulcers. Roots are used as tonis, febrifuge, expectorant, dyspepsia, colic, rheumatism, worms and leprosy. Flowers are used for treatment of diarrhea, cholera, fever and disease of liver (Raji, 2013)

2.4.4 Phytochemical and Pharmacological Studies of *Vitex negundo* Linn.

Many researchers have reported the presence of bioactive compounds in *V. negundo* and experimentally proved the biology activities of the plant. Generally, this precious plant contained flavonoids, flavones glycosides, volatile oil, triterpenes and tannins and possess pharmacological activities like anti-inflammatory, anti-rheumatic, antibiotic, hepatoprotective, antioxidant, anticonvulsant, oxidative stress, anti-androgen, snake venom neutralization and anti-allergic activities (Ladda and Magdum, 2012).

Sahayaraj and Ravi (2008) reported the presence of phenols, saponins, xanthoproteins, triterpenoids, tannins and flavonoids in the extract of *V. negundo*. Identification of chemical compound were done using GC-MS which resulted the contained of 1H-indene, cyclododecanol, patchoulane, 1,2-dihexylcyclopropane-3-carboxylic acid, 2-heptenoic acid, aromadendrene, trans-caryophyllene, 7-oxabicyclo[4.1.0]heptanes, cyclohexane, farnesol, pentadecane and 1-octanol.

Kannan et al. (2012) evaluated the activity of *V. negundo* leaf extracts against HIV-1 Reverse Transcriptase (RT) with recombinant HIV-1 enzyme, using a non-radioactive HIV-RT colorimetric ELISA kit. Phytochemical studies were done using HPLC analysis and reported the content of seven flavonoids which are rutin, luteolin, myricetin, quercetin, kaempherolisorhamnetin and quercetagetin. They claimed that *V. negundo* leaf extract possess the anti-RT substances and probably the flavonoids act as its anti-virus agents.

Keerti and Padma (2012) studied the antimicrobial activity of flavonoids extracted from flower buds of *V. negundo* using some multidrug resistant pathogenic bacteria. Using the disc diffusion method, the extracts showed positive responses against *Bacillus subtilis, Raoutella planticola, Agrobacterium tumifaciens*.

Das and Kanodia (2013) reported the anti-inflammatory and antioxidant properties of *V. negundo* leaves. By taking albino rats as the sample, they evaluated the effect of *V. negundo* leaves in experimentally induced inflammatory bowel disease (IBD) and investigated the probable mechanism for the activity. They found out that the extract showed positive effect on experimentally induced colitis, which may contributed to its anti-inflammatory and antioxidant activities.

Devi et al. (2007) studied the effect of the oral administration of *V. negundo* leaf extracts on the levels of enzymic and non enzymic antioxidant. Adjuvant induced arthritic (AIA) rats were used as the test samples. Their findings shows that levels of antioxidant enzymes such as SOD, CAT, GPx, G6PD, GSH and vitamin C were significantly low in the liver of experimentally rats as compared to normal rats. Thus he suggested that the leaf of *V. negundo* to possess antioxidant activity.

2.5 *ETLINGERA ELATIOR* (Jack) R. M. Sm.

2.5.1 Botanical Overview of *Etlingera elatior* Jack.

E. elatior Jack from the family of *verbenaceae* is a rhizomatous perennial herb that grows up to 6m in heights. It consists of large leafy shoots up to 85 cm long, flushed pink when young, leathery with a central groove and produced a pleasant sour scent when crushed. Inflorescences grow at end of stalk, bracts spreading, deep pink with paler margin, floral bracts smaller, pink and fleshy. Figure 2.4 shows the buds of *E. elatior*. As it have colourful and attractive *E. elatior* also known with a variety of common names such as ‘torch ginger’, ‘ginger flower’, ‘red ginger lily’, ‘combrang’, ‘bunga kantan’, ‘Philippine wax flower’, ‘Xiang Bao Jiaing’, ‘ Indonesian tall ginger’, ‘Boca de Dragon’, ‘Rose de Porcelaine’ and ‘Porcelaine Rose’. This plant is native to Indonesia, Malaysia, South Thailand and widely cultivated and naturalized in South East Asia. A total of 15 *Etlingera* species have been record in Peninsular Malaysia (Chan et al., 2007).

Figure 2.4: Buds/ inflorescences of *Etlingera elatior* Jack

Source: Wijekoon et al. (2011b)

2.5.2 Botanical Classification of *Etlingera elatior* Jack.

Kingdom : Plantae

Subkingdom : Tracheobionta

Superdivision : Spermatophyta

Division : Magnoliophyta

Class : Liliopsida

Subclass : Zingiberidae

Order : Zingiberales

Family : Zingiberaceae

Genus : Etlingera Giseke

Species : Etlingera elatior (Jack) R.M.Sm.

Source : NRCS (National Resource Conservation Service), (2014)

2.5.3 Traditional and Medical uses of *Etlingera Elatior* Jack.

In most places, *E. elatior* are generally use as spices, condiments, flavours, vegetables, in traditional medical, ornamentals and in cosmetic. Young inflorescence is an essential ingredient of curries and rice dishes. Heart of young shoots, inflorescences and fruits of *E. elatior* are used as condiment and consumed raw or cooked as vegetables. In Malaysia, fruits of *E. elatior* are used to treat earache while the decoctions of leaves are used for cleaning wounds. The leaves also used by post-partum women and can be mix with other aromatic herbs in water for bathing to remove body odour (Chan et al., 2009).

Presence of saponins, tannins and flavonoids in *E. elatior* contribute to other medical uses of these plants. Saponins are medically used as expectorants and for treatment of excessive salivation, chlorosis and migrains. Tannins have been used as antibacterial, antiviral and antiparasitic and flavonoids and tannins are among the phenolic compounds that may act as the primary antioxidant (Lachumy et al., 2010).

Flowers of *E. elatior* are applied externally to relieve itching and treat skin problems. Daily intake of raw inflorescence of this plant can reduce diabetes and hypertension, while taken it together with the bitter leaves may relieve flatulence in post-partum women (Wijekoon et al., 2011a).

2.5.4 Phytochemical and Pharmacological Studies of *Etlingera Elatior* Jack.

Study of the nutritional and anti-nutritional contents of the inflorescence part of the torch ginger have been done by Wijekoon et al. (2011a) and revealed a wider range of potential medicinal properties of the plant. They reported that *E. elatior* contained protein, fat, fiber, fatty acids (palmitoleic acid, linoleic acid, oleic acid), amino acids, minerals and antinutrients (saponin and phytic acid). Amino acids and minerals found in *E. elatior* extract are as in Table 2.2.

Table 2.2 Amino acids and minerals present in *E. elatior* inflorescence extract

Amino Acids		Minerals
Essential	Non essential	Potassium
Lysine	Glutamic acid	Calcium
Leucine	Aspartic acid	Magnesium
Valine	Arginine	Phosphorus
Threonine	Alanine	Manganese
Isoleucine	Serine	Sodium
Phenylalanine	Cysteine	Zinc
Histidine	Glycine	Boron
Methionine	Proline	Iron
	Tyrosine	Fluorine
		Copper
		Selenium
		Cobalt
		Chromium
		Molybdenum

Source: Wijekoon et al. (2011a)

Presence of dietary fibre may reduce serum cholesterol level, reduce risk of coronary heart disease and lower the risk of hypertension and constipation while fatty acids may act as functional foods by affecting various physiological processes and provide protection against cardiovascular diseases, hypertension, inflammation and autoimmune disorders. Essential minerals (Ca, K, Mg, and P) are needed by our body for a normal extracellular and intracellular body function and also as components in body structure. Potassium are beneficial for hypertension and magnesium and zinc are important to prevent cardiomyopathy, muscle degeneration, growth retardation, dermatitis, gonadal atrophy, impaired spermatogenesis, congenital malformations and bleeding disorders (Wijekoon et al., 2011a).

In short report by Jackie et al. (2011), suggested that flower extract of *E. elatior* possess a powerful antioxidant effect against lead-induced oxidative stress and it may also cure lead toxicity. Many research was done proving that lead may induced oxidative stress by disturbing the balanced of generation and removal of reactive oxygen species. Thus reducing the possibility of lead acetate interacting with cellular

metabolic biomolecules significantly decreased the risk of damaging tissue by oxidative stress (Jackie et al., 2011).

Chan et al. (2007) evaluated the total phenolic compound (TPC) antioxidative and antibacterial activity in different parts of torch ginger which are the leaves, inflorescence and rhizomes and they have made conclusion that TPC and antioxidant activity are most in the leaves following by the inflorescence part and the least is in the rhizomes. They also found out that altitudinal variations affect the TPC and antioxidative behaviour of the plants whereby the leaves of highland populations displayed higher values as compared to lowland counterpart. For the antibacterial activity, the leaves of this plant positively react towards Gram positive bacteria but failed to inhibit Gram negative bacteria (Chan et al., 2007)

Aqueous ethanol extract of *E. elatior* flower shoots possess antimicrobial and cytotoxicity activity against HeLa cells and anti tumor promoting activity while DCM and methanol extract of the rhizomes shown a comparable antioxidant activity as compared to α-tocopherol in ferric thiocyanate method (Mohamad et al., 2005). In their work, they have isolated eight compounds from the rhizomes of *E. elatior* consist of six known compound and two new compound which isolated for the first time from the natural resource. The new compounds are 1,7-bis(4-hydroxyphenyl0-2,4,6-heptatrione (**1**) and 16-hydroylabda-8(17),11,13-trien-15,16-olide (**2**). The known compounds are as follows ; demethoxycurcumin (**3**), 1,7-bis(4-hydroxyphenyl)-1,4,6-he[tatrien-3-one (**4**), stigmast-4-en-3-one (**5**), stigmast-4-en-3,6-dione (**6**), stigmast-4-en-6β-ol-3-one (**7**), and 5α, 8α-epidioxyergosta-6,22-dien 3β-ol (**8**). Antioxidant activity also being report on the compounds **1**,**3** and **4** in terms of their ability to inhibit lipid peroxidation in a more potent manner than α-tocopherol. Compound **5** have once been found in *P. speciosa* empty pods and proved to possess hypoglycaemic effect (Jamaludin et al., 1995).

Abdelwahab et al. (2009) investigated essential oils of *E. elatior* for their antioxidant and antibacterial properties as well as their phytochemical properties. They have reported that essential oils in *E. elatior* contained about 73 compounds and exhibit high total phenolic and total flavonoids content thus made them considerable as natural source of antioxidant. These essential oils have antibacterial effect against Gram

positive bacteria but failed to inhibit Gram negative bacteria. Phytochemical and pharmacological activity of torch ginger flowers methanolic extract also being investigated by Lachumy et al. (2010). The pharmacological study included the determination of antimicrobial activity of extract using minimum inhibitory concentration (MIC) method. The flower extracts also being tested for their toxicity using brine shrimp method and result shows that the extract exhibit no significant toxicity. Thus this plant can be classified as biologically safety compounds with pharmaceutical properties as they possess a great in vitro potential for antimicrobial and antifungal activity. The presence of flavonoids, terpenoids, saponin, tannins and carbohydrate has been report (Lachumy et al., 2010).

2.6 ANTIOXIDANT CAPACITY ASSAY

There are a wide numbers of assay that have been used since antioxidant evaluation captured an interest of many researchers. Prior et al. (2005) have listed eight points of ideal requirements in selecting method for the evaluation of antioxidant capacity which are; measure chemistry actually occurring in potential application, utilizes a biologically relevant radical sources, simple, uses a method with a defined endpoint and chemical mechanism, instrument is readily available, good within run or between day reproducibility, adaptable for assay of both hydrophilic and lipophilic antioxidant and use of different radical sources and the last one is adaptable to 'high-throughput' analysis for routine quality control analysis.

They also have listed few factors that need to be considered in the standardization of method, which are; analytical range, recovery, repeatability, reproducibility and recognition of interfering substances (Prior et al., 2005).

Antioxidant capacity assay was divided into two systems which are based on hydrogen transfer (HAT) and single electron transfer (SET). For SET, it involved one redox reaction with two components which is the oxidant as the probe and antioxidant. Examples of SET system is Folin Ciocaltaeu assay which is for total phenolic content, DPPH radical scavenging assay, trolox equivalent antioxidant capacity (TEAC) and ferric reducing activity of plasma (FRAP). For HAT, it measure competitive kinetics

which compose of synthetic radical generator, oxidisable molecular probe and an antioxidant. It is more relevant to radical chain-breaking antioxidant. Some examples of HAT system is oxygen radical absorbance capacity (ORAC) and β-carotene bleaching assay (Ndhlala et al., 2010).

2.6.1 DPPH Radical Scavenging Assay

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) is a popular method, introduced for the first time by Marsden Blois in 1958 and recently revised method by Brand Williams in 1995 (Molyneux, 2004). It is a simple and inexpensive method to measure the antioxidant capacity of food, plants, or compounds involves the use of the free radical DPPH. It can be used for solid and liquid samples and not specific to any particular antioxidant and was used on the basis of ability of sample to act as free radical scavengers or hydrogen donors. DPPH is abbreviation for 2,2-diphenyl-1-picrylhydrazyl, the dark colour crystalline powder composed of free radical molecules. DPPH is characterised as a stable free radical by virtue of the delocalization of the spare electron over the molecules as a whole, so the molecules do not dimerise. Delocalization gives rise to the deep purple colour, characterized by an absorption band at about 517 nm. When a solution of DPPH mixed with a substance that can donate a hydrogen atom, it will be reduced to a non-radical 2,2-diphenyl-1-picrylhydrazine with the loss of the purple colour (become colourless or pale yellow). The resulting decolourization is stoichiometric with respect to number of electrons captured. The reaction is as in Figure 2.5.

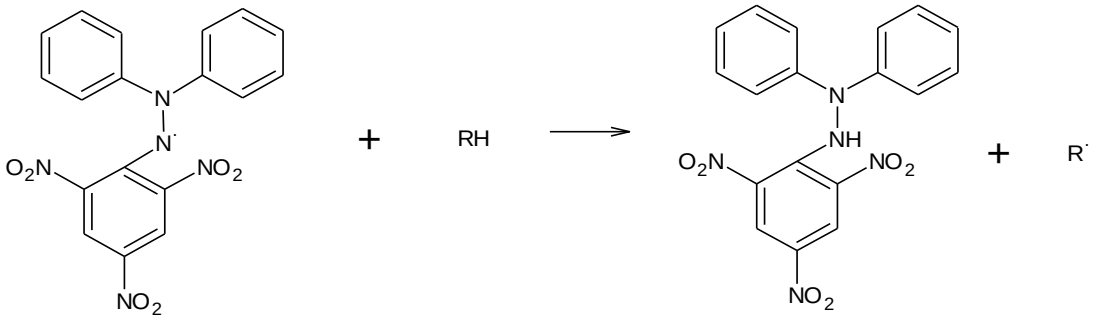


Figure 2.5: Reaction of DPPH molecules against antioxidant compound

Source: Dureja and Dhiman (2012)

$R^{\cdot}$ is a free radical produced in this first step that will undergo further reactions which control the overall stoichiometry (the number of molecules of DPPH reduces by one molecule of reductant). This method have been used extensively by many researchers and being one of the most applied because of the accuracy and repeatability results, and valid to quantify samples with hydrophilic and lipophilic antioxidants, as well as its simple procedures (Ndhlala et al., 2010). Usually, antioxidant activity of sample derived from DPPH assay is presented in terms of Effective Concentration (EC50) value. EC50 is a value of concentration of substrate that causes 50% loss of DPPH activity. This parameter was introduced by Brand Williams and his colleagues in 1995 (Williams et al., 1995).

2.6.2 Beta Carotene Linoleate Bleaching Assay

Beta carotene bleaching assay is one of the method in HAT reaction mechanism. It measures the inhibition of the production of volatile organic compound and the formation of conjugated diene hydroperoxides due to linoleic acid oxidation which bleach the β-carotene in the emulsion. This assay involved the incubation of reaction solution in 50°C water bath and the heat will induce oxidation that cause the bleaching of caretenoids. The existence of antioxidant compounds will inhibit or diminish the discolourization by donating the H atom to quench the radicals. Few advantages had been listed for this assay such as its applicability in both lipophilic and hydrophilic

environments, and ability to detect either the antioxidant or pro oxidant action of the compound under investigation. It is also adaptable to high throughput technology such as the use of microplates (Ndhlala et al, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 GENERAL

3.1.1 REAGENTS AND SOLVENTS

Organic solvents used for extraction, isolation and purification were ethanol absolute and methanol from Fisher. Meanwhile Trizma Base, acetic acid, 2,2-diphenyl-1-picrylhydrazyl, Tween 20, linoleic acid and Beta Carotene were from Sigma-Aldrich, St Louis. The solvents used were of analytical grade.

Thin Layer Chromatography (TLC) was performed on Merck pre-coated silica gel 60 F254 for normal phase.

3.1.2 APPARATUS AND INSTRUMENTS

A Buchi Rotavapor R-11 system was used together with an Eyela A-1000 S Vacuum pump and Buchi R-11 heating bath as unit components for rotary evaporator. Thermo Scientific Genesys 20 Spectrophotometer was used for the measurement of absorbance for bioassay samples. The UV light (UVGL-58 Handheld UV Lamp) at 254 and 365 nm was used to visualize TLC plates.

3.1.3 PLANT MATERIALS

Fresh *P. speciosa* and *E. elatior* were obtained from wet market around Kuantan, Pahang. *V. negundo* was collected from Dungun, Terengganu. All samples

were validate by Institute of Bioscience University Putra Malaysia with the specimens voucher no. SK 2393/14, SK 2394/14 and SK 2395/14 for *P. speciosa*, *E. elatior* and *V. negundo* respectively (Appendix A).

3.2 METHOD

The flow of this work was summarized as shown in Figure 3.1

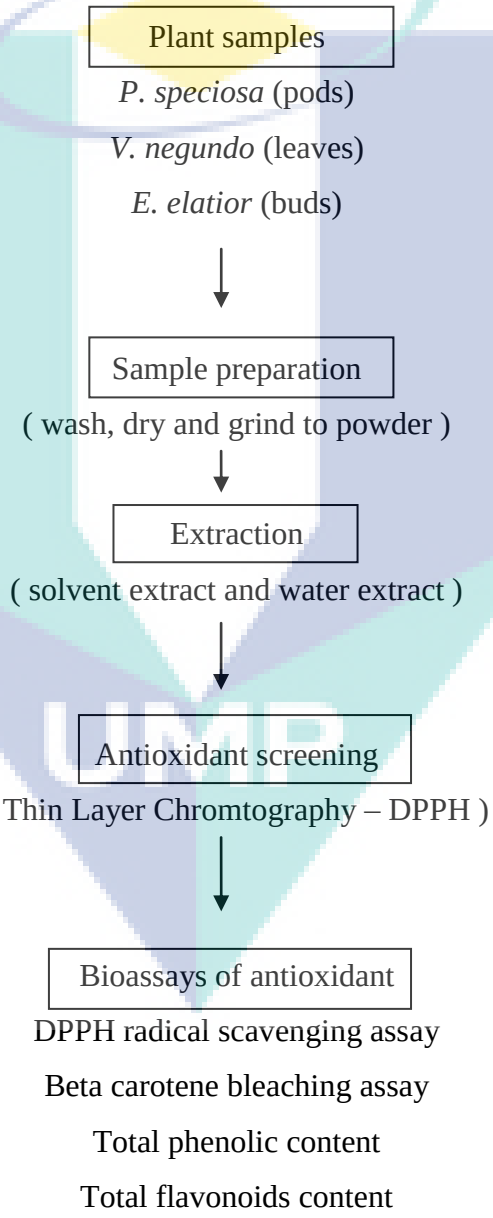


Figure 3.1: Flow chart of extraction, and antioxidant evaluation of plant samples

3.2.1 SAMPLE EXTRACTION

Test plants consist of pods of *P. speciosa*, matured leaves of *V. negundo* and buds of *E. elatior*. All plants were rinsed thoroughly with tap water to get rid of debris then were cut into small pieces. For drying purpose, samples were put in oven at temperature 40-50 °C for 24 hours. The dried plants were powdered using grinder.

3.2.1.1 REFLUX

About 5.0 g of each powdered sample were reflux separately in ethanol (50 mL, 80%), at 65 ^{0}C for 2 hours. Then it was filtered using Whatman filter paper no 1. The filtrates were kept in refrigerator for further use. For aqueous extract, 50 ml ultrapure water was added to 5.0 g of each sample powder and was reflux up to 2 hours. The temperature was maintained in the range of 40-50 ^{0}C. Then it was filtered and keep in refrigerator for further analysis.

3.2.1.2 MACERATION

About 5.0 g of each powdered sample was soaked separately in ethanol (50 mL, 80%) for ethanol extract and ultrapure water for aqueous extract. All plant samples were left for seven days with occasional shakes, then were filtered using Whatman filter paper no 1. The filtrates were kept in refrigerator for further use.

3.2.2 THIN LAYER CHROMATOGRAPHY (TLC)

TLC was used initially for screening purpose of the crude sample and was carried out on Merck pre-coated silica gel 60 F254 supported on aluminium plates. The components were detected by visualization under UV light at λ 254 and 365 nm and/or sprayed with 0.05% methanolic DPPH reagent. DPPH reagent was used to detect antioxidant compound that may exist in the sample. Antioxidative compound will appear as yellow spot on the purple background. DPPH reagent was prepared by dissolving 5.0 mg of DPPH reagent in 10 ml methanol. Sample was spotted on the TLC plate, then it was developed in a solvent system consist of acetic acid and chloroform in

the ratio of 1:9, respectively. The plate was left to dry for a while before spraying with reagent. After spraying, the TLC plate was heated for 30 s and the colour produced was recorded.

3.2.3 DPPH· RADICAL SCAVENGING ASSAY

To determine the free radical scavenging activity of the extracts, DPPH• free radical scavenging assay reported by Zang et al. (2006) was used with minor modification. Various concentrations of test materials for both ethanol and aqueous extract were prepared through serial dilutions. About 1 mL of sample extract and 4 mL of 0.3 mM of DPPH reagent were mixed and vigorously shaken followed by incubation in darkness at room temperature for 1 hour. After incubation, the absorbance of the reaction mixture was measured spectrophotomectrically at 517 nm against the blank. The scavenging effect of DPPH• free radical was calculated by using the Eq. (1.0). EC50 value was used to evaluate the antioxidant capacities of the samples which is the effective concentration at which the scavenging effect were 50%. Samples were analyzed in triplicates.

$$\text{Scavenging effect (\%)} = \left(1 - \frac{\text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}} \times 100\right) \quad (1.0)$$

3.2.4 BETA CAROTENE BLEACHING ASSAY

The β-carotene bleaching assay was determined based on the method described by Ismail and Hong (2002) with slight modification. β-carotene solution (0.2 mg in 1 ml chloroform), was pipetted into a round bottom flask containing 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20. The mixture was evaporated at 40 °C for 10 mins using rotary evaporator to remove the chloroform. About 100 ml distilled water was added slowly to the mixture and shaken vigorously to form an emulsion. Five millilitre aliquots of the emulsion were pipette out and transferred into test tubes containing 0.2 ml samples.

The samples were prepared in 80% methanol at the concentration of 1.0 mg/ml. All test tubes were incubated for 2 hours in 50 °C water bath. The absorbance of samples was measured using spectrophotometer at 470 nm for 2 hours against a blank which is an emulsion without addition of β-carotene. The control consisted of 5 ml of β-carotene solution with 0.2 ml of 80% methanol. Readings taken at zero time (t=0) and at every 20 mins intervals. All determinations were performed in triplicates and the total antioxidant activity was calculated based on the following Eq. (2.0).

$$\text{Antioxidant activity (\%)} = [1-(A_0 - A_t) / (A'_0 - A'_t)] \times 100 \quad (2.0)$$

where AA is antioxidant activity, A0 and A'0 are the absorbance values measured at initial time (t=0) of the incubation of the samples and control respectively, while At and A't are the absorbance of the samples and control at t = 120 mins.

3.2.5 TOTAL PHENOLIC CONTENT (TPC)

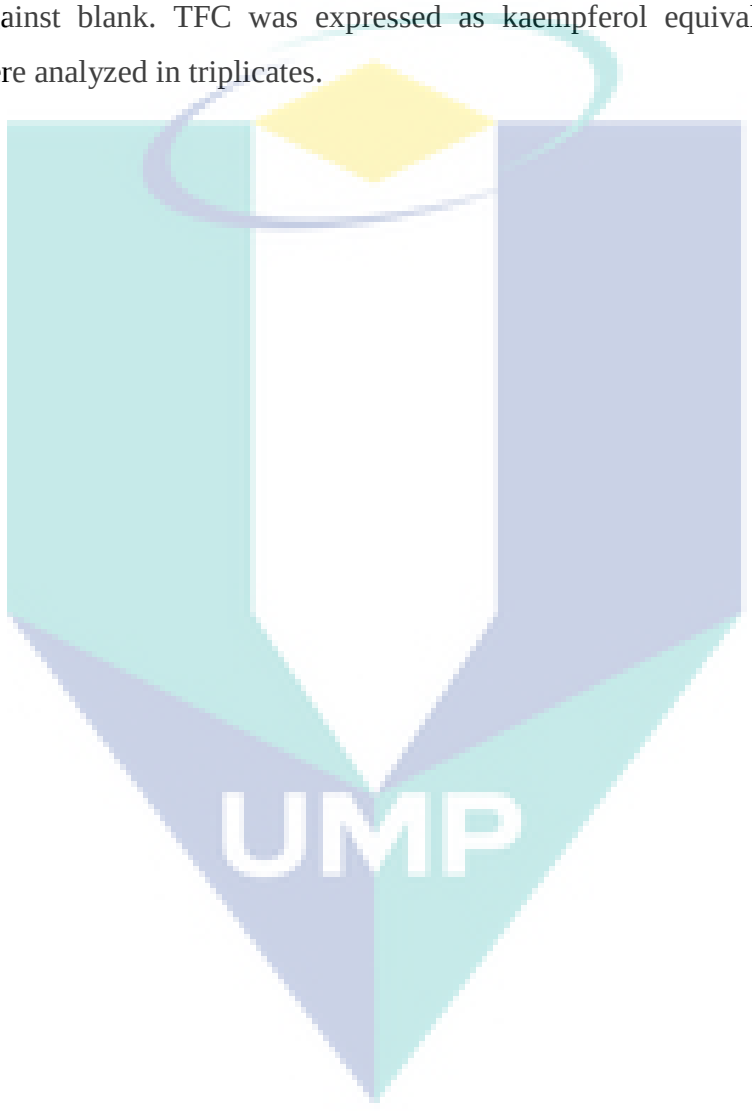
Total phenolic content of plant sample was done following the method by Liu et al. (2009). About 1000 ppm sample were prepared by taking 0.1 ml of stock solution (100 000 ppm) and diluted up to 10.0 ml in volumetric flask using either ethanol or ultrapure water while 10% Folin Ciocalteu Reagent (FCR) was prepared by diluting 1.0 ml FCR in 9.0 ml ultrapure water.

Sample (0.5 ml) was added to Folin Ciocalteu Reagent and the test solutions were kept for incubation at 30 ℃ for 5 min in the dark. A saturated solution of sodium carbonate (2 ml) was added to the test solutions and stand for 1 hour. Absorbance was read at 747 nm against blank. TPC was expressed as mg gallic acid equivalent (μg GAE)/ mg of sample and calculated using Eq. (3.0). Samples were analyzed in triplicates.

$$\text{Total Phenolic Content} = \frac{\text{Concentration of sample (μg GAE/mL)}}{\text{0.1 mg/mL}} \quad (3.0)$$

3.2.6 TOTAL FLAVONOIDS CONTENT (TFC)

Dowd method (Ramamoorthy and Bono, 2007) was adopted for the analysis of total flavonoids content of plant extracts with slight modifications. Five millilitre of 2% aluminium trichloride prepared in methanol were added to extract (5 ml). The test solutions were kept for incubation for 10 mins and the absorbance were measured at 415 nm against blank. TFC was expressed as kaempferol equivalent/g of sample. Samples were analyzed in triplicates.



CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

The purpose of this study is to investigate the antioxidant properties of three different types of minor used plants namely *P. speciosa*, *V. negundo* and *E. elatior*. Two types of extraction had been used, which are reflux and maceration method with two different solvents, (ethanol and water). For qualitative screening of antioxidant properties of each plant, TLC has been used. Bioassays for the comparison of the antioxidant values are DPPH Radical Scavenging Assay and Beta Carotene Bleaching Assay.

4.2 THIN LAYER CHROMATOGRAPHY (TLC)

TLC had been adopted for the screening purpose of antioxidant compound in the sample extracts. According to report by Kannan et al. (2010) TLC with DPPH as the visualization reagent can be used to detect the existence of antioxidant in sample. The positive antioxidant compound will appear as yellowish white spot on the purple background. Figure 4.1 shows the bioautography of *P. speciosa*, *V. negundo* and *E. elatior* extracts spotted on the TLC plate and sprayed by methanolic DPPH. It can be seen that all extracts are positive towards DPPH reagent.

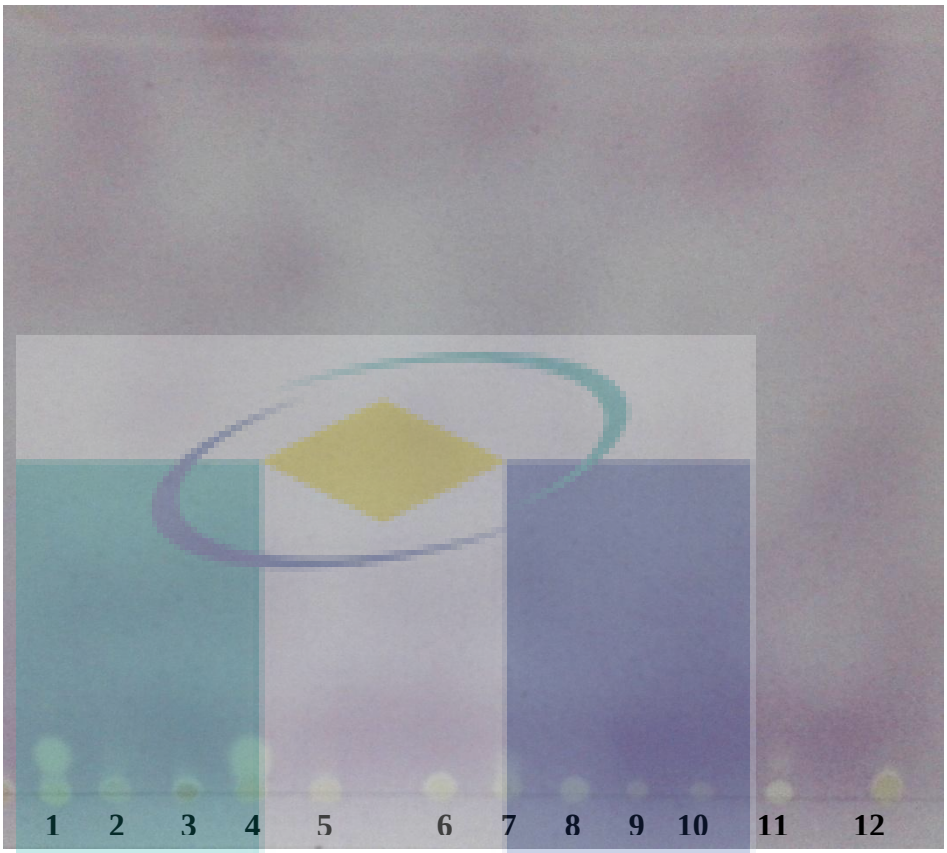


Figure 4.1: Bioautography showing positive antioxidant compound appear as yellowish white spots on purple background of DPPH reagent.

(1: PSER, **2**: VTER, **3**: EEER, **4**: PSEM, **5**: VTEM, **6**: EEEM, **7**: PSAR, **8**: VTAR, **9**: EEAR, **10**: PSAM, **11**: VTAM, **12**: EEAM.)

4.3 DPPH RADICAL SCAVENGING ASSAY

The DPPH radical scavenging assay is a simple method to evaluate the antioxidative activity of test sample besides comparing the extraction methods and solvents. This method is widely use in the quantification of radical scavenging capacity (RSC). The amount of sample needed to scavenge the DPPH radical can be expressed as its measurement of antioxidant ability (Deng et al., 2011).

DPPH is a well-known radical and a trap (scavenger) for other radicals. This radical is very stable, to be compared to *in vitro* generated free radicals such as the hydroxyl radical and superoxide anion. DPPH radical is unaffected by certain side

reactions, such as metal ion chelation and enzyme inhibition, brought by various additives (Stankovic et al., 2010). A freshly prepared DPPH solution exhibits a deep purple colour with the maximum absorption at 517 nm. The purple colour of the solution will fades when the reaction between antioxidant molecules and DPPH free radical occurred. The purple colour of the reagent will disappear with the presence of radical scavenger in the sample and when the odd electron of the nitrogen in the DPPH was paired. The chemical reaction was showed in Figure 4.11.

The antioxidant molecules may quench DPPH free radicals either by providing hydrogen atoms or by electron donation and convert them into a colourless solutions which is a substituted analogous hydrazine or 2,2-diphenyl-1-hydrazine , and cause the absorption to decrease. The absorbance was monitored spectrophotometrically at wavelength 517 nm and the scavenging effect is presented in term of EC_{50}. EC_{50} is the concentration of antioxidant necessary to reduce the original amount of radical by 50%. Figure 4.2 shows the antioxidant value (scavenging effect) for each sample. It can be seen that ethanolic extract of *P. speciosa* gives the lowest value of EC_{50} which indicate that it bears the highest antioxidant activity.

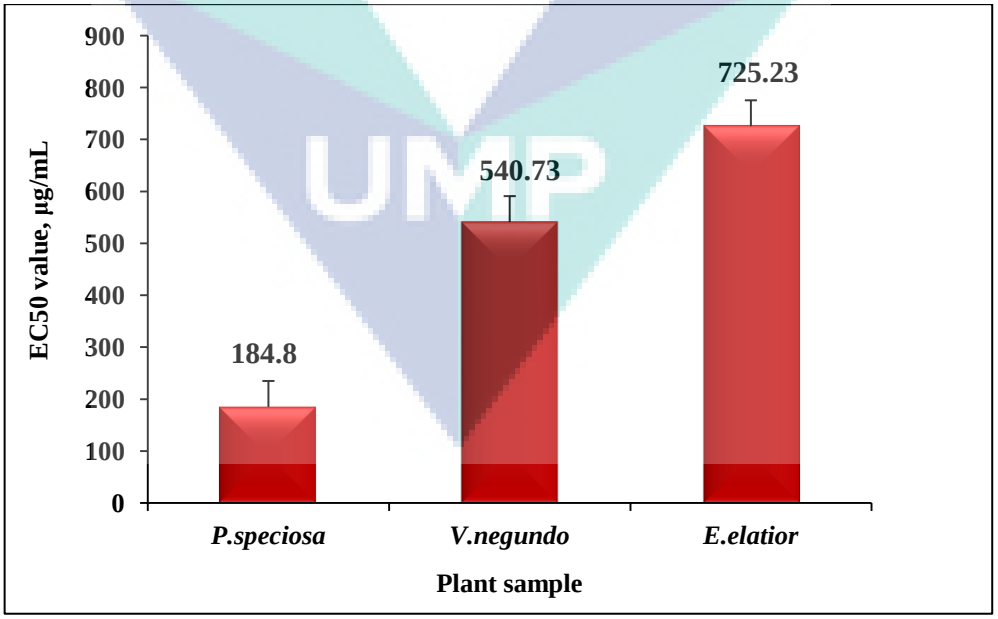


Figure 4.2: EC_{50} value of *P. speciosa*, *V. negundo* and *E. elatior*.

4.4 BETA CAROTENE BLEACHING ASSAY

Another method to evaluate antioxidative potential of extract is β-carotene Bleaching Assay. In this method, the antioxidant capacity of the extract was expressed as the ability of the extract to inhibit oxidation of β-carotene for a certain incubation time. Linoleic acid will produce hydroperoxides as free radicals during incubation at 50 °C through oxidation process. These free radicals will attack the highly unsaturated β-carotene molecules causing the decreasing in absorbance at 470 nm and bleaching of the yellow colour of β-carotene solution.

The presence of antioxidant in the extract can retard the β-carotene bleaching by neutralizing the hydroperoxides formed in the system. From the data obtained, the percentage of antioxidant activity had been calculated and showed in Figure 4.3. Some researcher also determined the degradation rate of the sample for the expression of the antioxidant activity in the particular sample. Degradation rate bears an inverse relationship with the antioxidant activity index where the extract with the lowest degradation rate exhibit the highest antioxidant activity. This means that sample which may retain the yellow colour of β-carotene solution for a longer time (less bleached) contained the highest antioxidant compound.

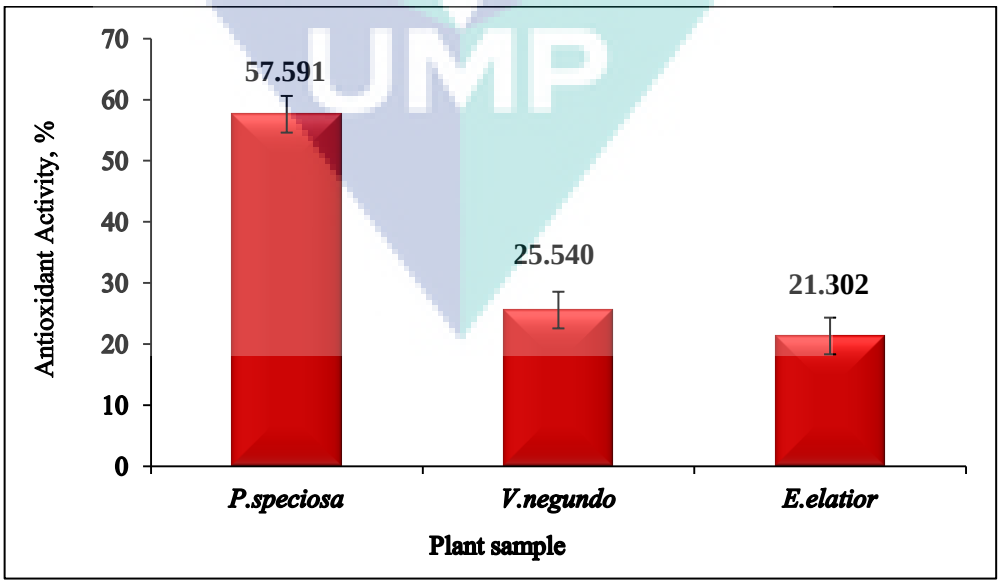


Figure 4.3: Antioxidant activity of each plant using β-carotene Bleaching Assay.

Another way to express the degree of bleaching of β-carotene by the extract is by calculating the percentage of remaining β-carotene in the solution. The amount of remaining β-carotene, relative to the initial amount is a reciprocal relation with the oxidation inhibition. In other words, the percentage showed the unoxidized state of β-carotene. The extract with the highest percentage at the end of incubation time bears the highest antioxidant capacity. As shown in Figure 4.4, the percentage of unoxidize β-carotene in *P. speciosa* extract is the highest which is in agreement with the calculated value that *P. speciosa* sample contained the highest antioxidant activity among the tested plant.

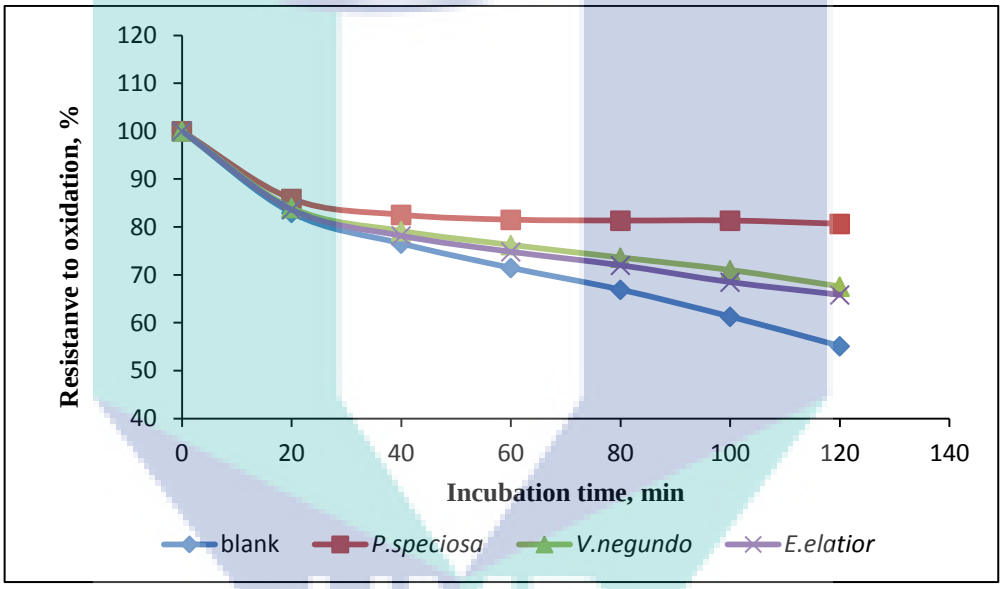


Figure 4.4: Antioxidant activity of extract in terms of resistance to oxidation.

4.5 COMPARISON OF ANTIOXIDANT ACTIVITY FOR SAMPLES EXTRACTED USING DIFFERENT SOLVENTS AND METHODS.

There are various methods of extraction that have been used by researchers in natural products field either by traditional method or modern method with the help of instrument or external system. Some examples are reflux system, maceration, percolation, soxhlet, ultrasound assisted solvent extraction and microwave assisted extraction method. The variations in different extraction method that will effect quantity, activity and secondary metabolite composition of an extract are type of extraction, time of extraction, temperature, nature of solvent, concentration of solvent

and solvent polarity (Tiwari et al., 2011). Several factors need to be considered when choosing method of extraction. Amirah et al. (2012) pointed out that a desirable extraction technique should be simple, inexpensive, efficient, selective, environmentally friendly and compatible with various analytical techniques.

Different solvent used for extraction definitely will give a different activity of the extract. This is because every solvent and chemical compound has different solubility and polarity. Therefore, choosing a suitable solvent is a critical step in plant extraction. Few characteristics of a good solvent are low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action and inability to cause the extract to complex or dissociated (Tiwari et al., 2011).

In this current work, reflux and maceration methods were chosen as the variables for extraction methods and comparison of extraction solvent was done between aqueous ethanol and total aqueous.

Figure 4.5 and Appendix B1 until B8 show the antioxidant value in terms of EC_{50} of *P. speciosa* by DPPH radical scavenging effect and the values for beta carotene bleaching assay were presented in Figure 4.6 and Appendix C1 until C4. Generally, *P. speciosa* extracted with aqueous ethanol by reflux technique seems to give the highest antioxidant activity for both assays with an exception for aqueous extract of maceration method tested by DPPH assay. In this case, EC_{50} value of aqueous extract by maceration method slightly higher than ethanol extract, but the difference is very small so the value of activity can be considered as similar. Antioxidant values for *V. negundo* L. were shown in Figure 4.7, Figure 4.8 and Appendix B9 until B16. It is clearly seen that reflux method with ethanol as the extraction solvent gives the highest antioxidant activity based on its EC_{50} value in DPPH assay and also the percentage of antioxidant determined by beta carotene assay. The same trend also can be seen for *E. elatior* sample. Figure 4.9, Figure 4.10 and Appendix B17 until B24 presented the values for DPPH radical scavenging assay and beta carotene bleaching assay respectively and it can be seen that EC_{50} value for ethanolic extract by reflux technique is the lowest and percentage of bleaching activity is the highest, which indicated the better antioxidant ability among the tested samples.

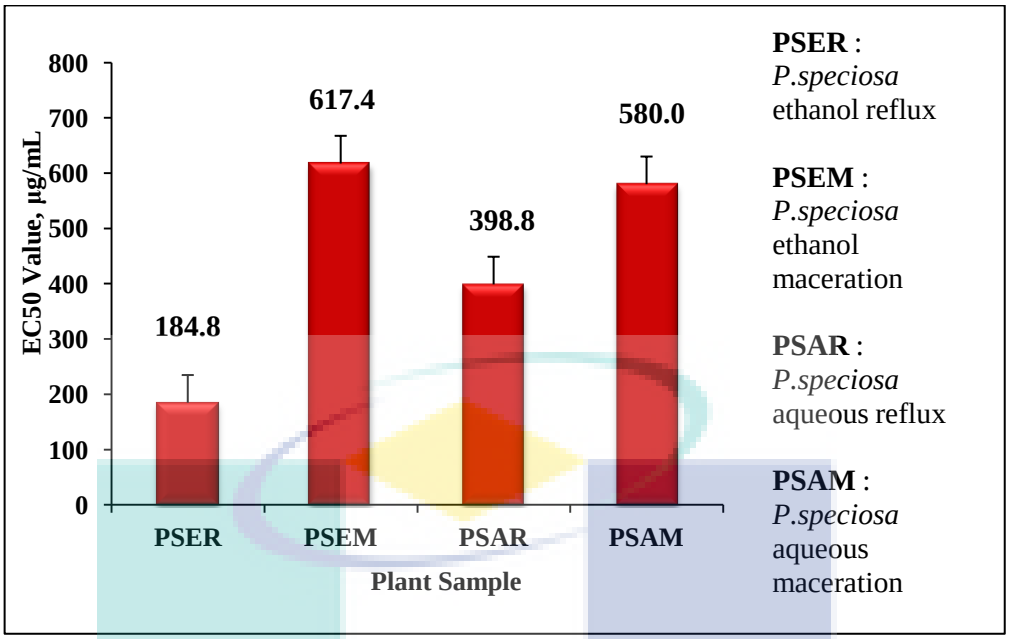


Figure 4.5: EC_{50} values for the extract of *P. speciosa* by DPPH radical scavenging assay.

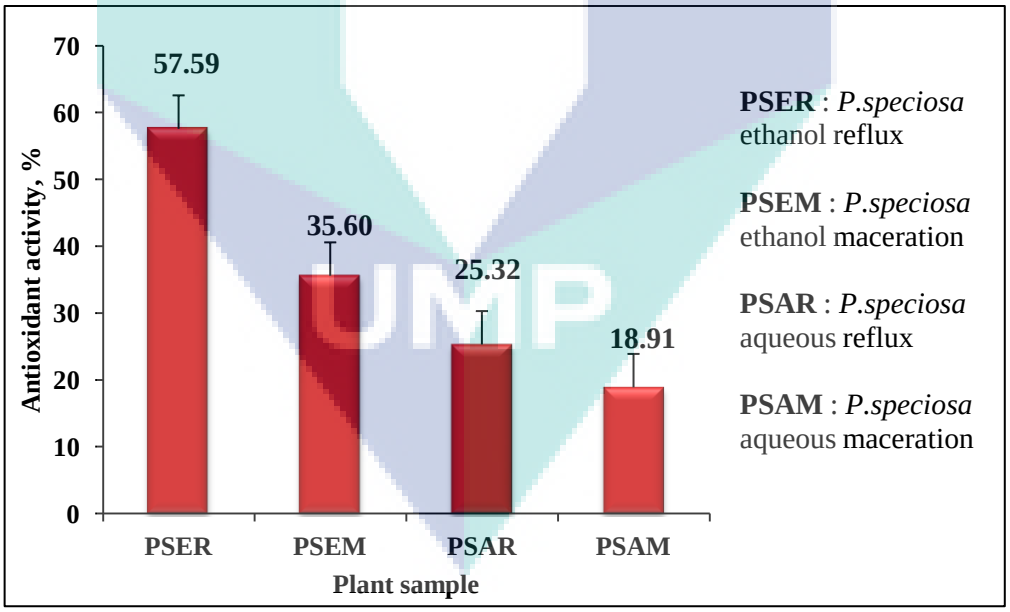


Figure 4.6: Antioxidant activity of *P. speciosa* by beta carotene bleaching assay.

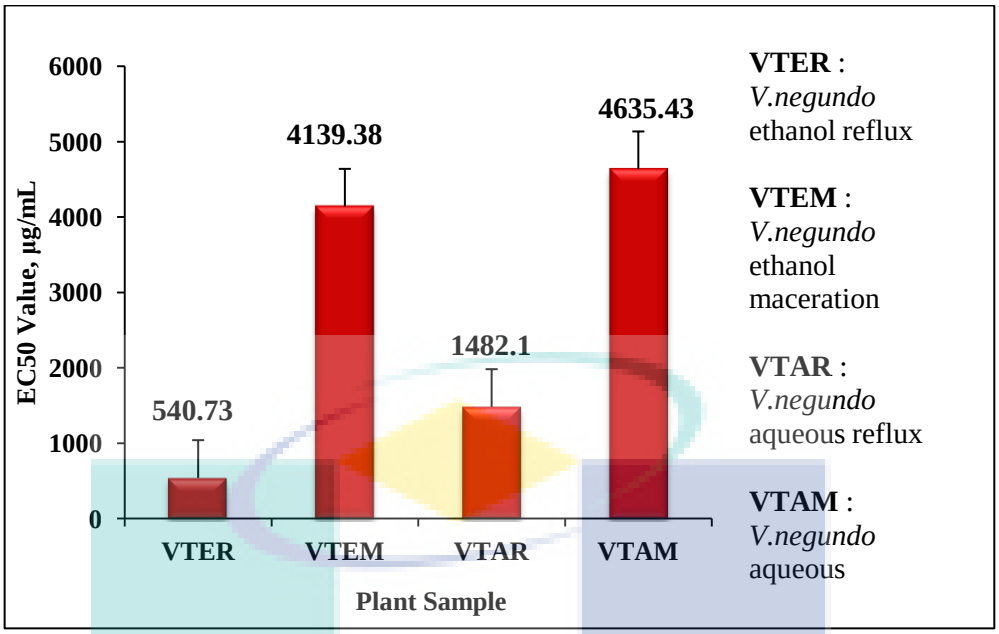


Figure 4.7: EC_{50} values for the extract of *V. negundo* by DPPH radical scavenging assay.

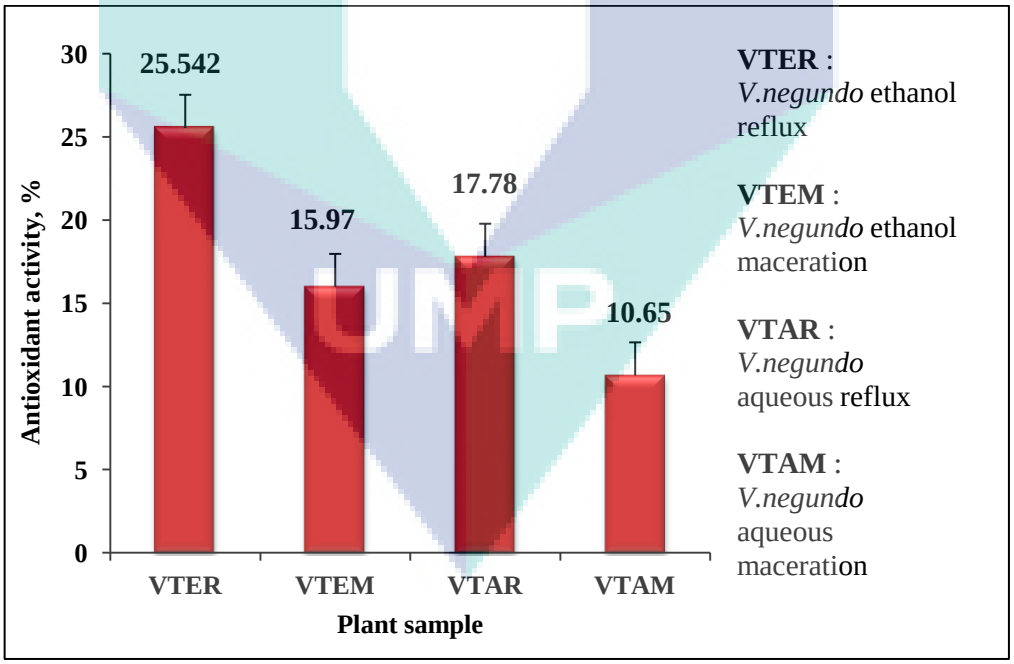


Figure 4.8: Antioxidant activity of *V. negundo* by beta carotene bleaching assay.

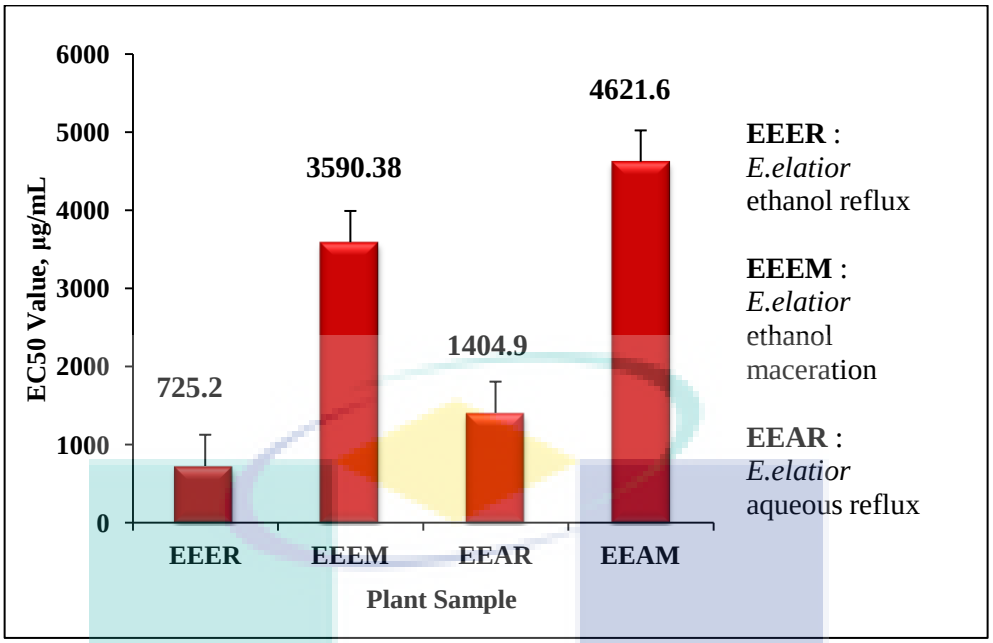


Figure 4.9: EC_{50} values of *E. elatior* by DPPH radical scavenging assay.

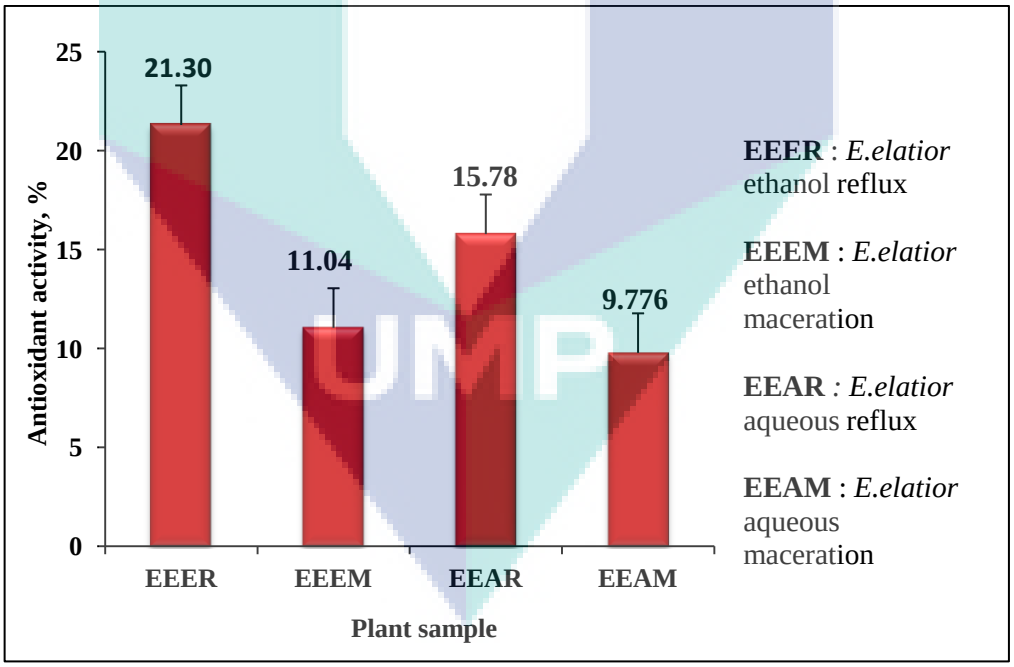


Figure 4.10: Antioxidant activity of *E. elatior* by beta carotene bleaching assay.

Among the samples tested, ethanolic extract, extracted using reflux technique gives the lowest value of EC_{50}. This means that aqueous ethanol has the better ability in extracting antioxidant compounds from plant extract and reflux gives rise to the values

of antioxidant activity of the samples. The higher antioxidant activity found in ethanolic extract may be because the presence of higher amount of polyphenols as compared to aqueous extract. Polyphenols are a common chemical compound that can be found in most plants and this compound was prove to play role as an antioxidant compound (Karou et al., 2005).

Ethanol is more efficient in cell walls which have unpolar character and cause polyphenols to be released from cells. Another reason for the higher activity in ethanolic extract is because in aqueous extract enzyme polyphenol oxidase exist that may degrade polyphenols in water extract. This enzyme is inactive in ethanol. Instead of using total ethanol, in this work we use 80% aqueous ethanol based on report by Spigno et al. (2007) where they suggested that addition of small quantity of water to organic solvent usually creates more polar medium which facilitate the extraction of polyphenols. They also stated that mixture of alcohol and water is more efficient to extract phenolic constituents as compared to mono component solvent system.

Reflux method gave a higher activity of the extracts for both solvent used. This might be due to the rising of temperature during the process. Temperature affects many physical properties including viscosity, diffusivity, solubility and surface tension (Jayathi et al., 2013). An increased temperature will allows the solvent to have higher capacity to solubilise analytes and also may improves sample wetting and matrix penetration by decreasing the surface tension and solvent viscosity (Amirah et al., 2012).

Better yield of extract might give better activity of the extracts. Regarding to Sultana et al. (2009), yields of extract were better when extraction was done under reflux, regardless of the plant material and solvent used. Hot solvent systems in reflux technique are more efficient for the recovery of antioxidant components, thus offering higher extracts yield. This is one of the reasons why sample extracted under reflux possess a higher antioxidant activity as compared to maceration.

In addition, report made by Dutra et al. (2008) in his work on Vogel seeds, they found out that among the different extraction techniques namely reflux, maceration,

ultrasound and heating plate, extraction made under reflux using ethanol : water (70 : 30) offered the highest polyphenol levels in the sample. This maybe because reflux leads to higher release of some bounded phenolic. Another advantage of reflux is its offer a milder heating as it have the continuous supply of water to the vessel. A high temperature may cause of the loss of natural antioxidant and sometimes the heat will accelerate their oxidation and cause other degenerative reactions. Report by Dutra et al. (2008) suggested that the temperature during the drying and heating process affects compound stability due to chemical and enzymatic decomposition losses by volatization and thermal decomposition, and this may have been the main mechanisms causing the reduction of polyphenol contents. While in the other hand, Spigno et al. (2007) stated that extraction of total phenolic compounds will increase with the increasing temperature, due to increasing both solubility of solute and diffusion coefficient, but after a certain temperature, phenolic can be denatured and subjected to loss.

4.6 TOTAL PHENOLIC CONTENT (TPC)

Evaluation of total phenolic content was done with expectations that antioxidant activity of the plant is responsible of phenolic contents since phenolic is the most abundance chemical group in plants. Many reports highlighted the contribution of these phenolics compounds to the antioxidant properties of plant samples. The presence of hydroxyl groups makes them able to scavenge free radicals (Gnanaraj and Iqbal, 2012). Phenolics compound able to donate hydrogen atoms to free radicals and have ideal structure for free radicals scavenging properties (Sulaiman et al., 2011). Mechanism of reaction of phenolics towards DPPH radical as proposed by Ancerewicz et al. (1998) is as in Figure 4.11.

Total content of phenolics in extracts was evaluated by gallic acid equivalent (GAE). Standard curve of GA has been plotted as in Figure 4.12.

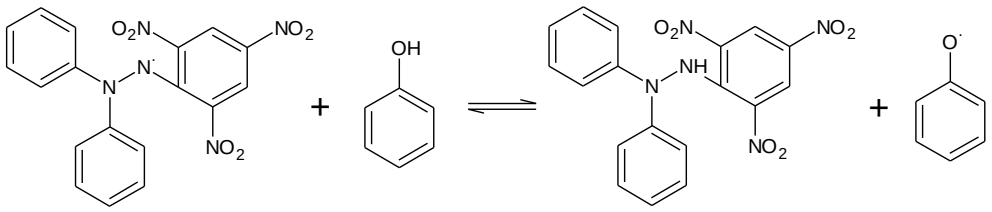


Figure 4.11: Mechanism of reaction between phenolic compound against DPPH radical

Source: Ancerewicz et al. (1998)

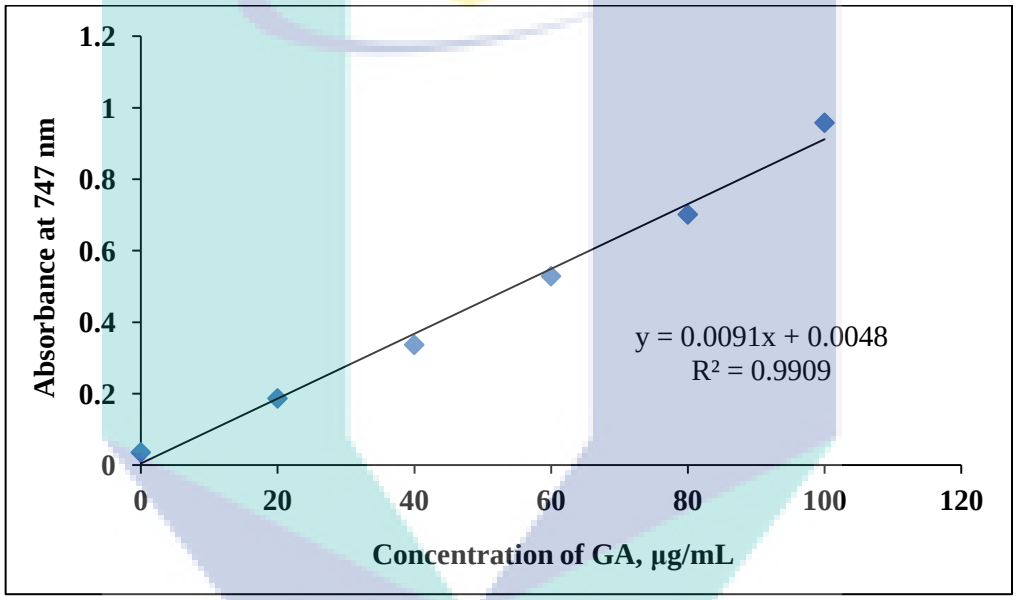


Figure 4.12: Standard curve of Gallic acid.

Figure 4.13, Appendix D1 and D2 presented the total phenolics values for all extracts and it can be seen that *P. speciosa* possess the highest value for all kinds of extracts, which is in agreement with the trend of antioxidant activities by DPPH radical scavenging assay and beta carotene bleaching assay. Here we can conclude that there is some correlations between antioxidant properties and total phenolic content of the extracts.

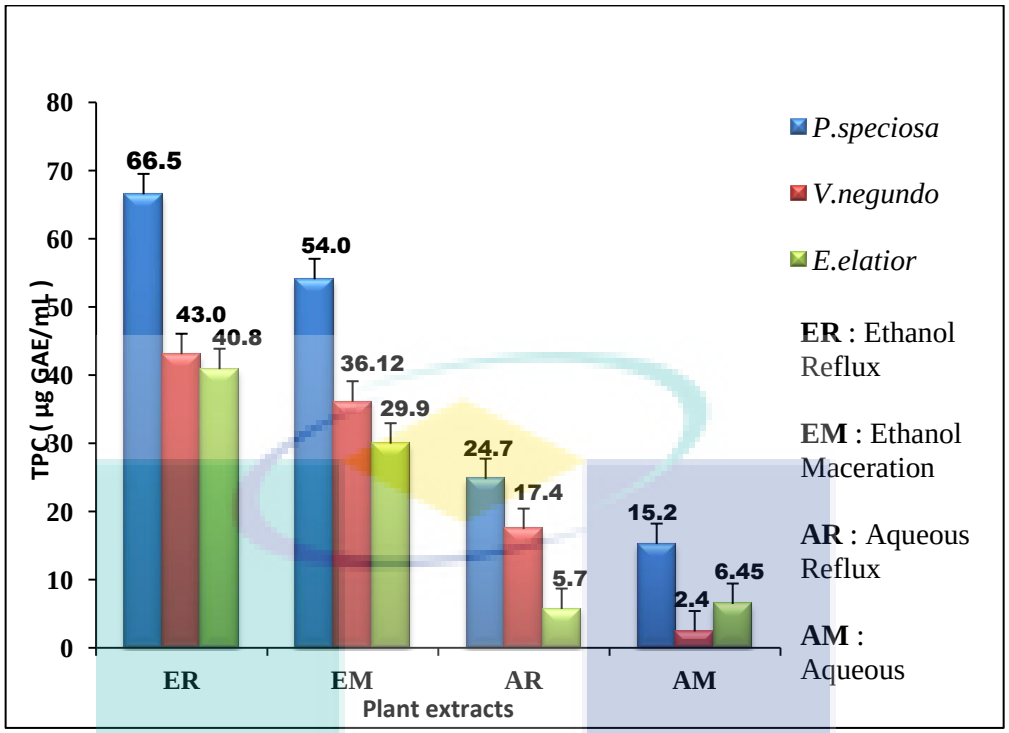


Figure 4.13: Total phenolic content expressed in µg GAE/mg of sample extracts.

4.7 TOTAL FLAVONOIDS CONTENT (TFC)

Flavonoids are the largest group of plant phenolic. These compounds also possess many medicinal properties including antioxidant, antimicrobial (Cushnie and Lamb, 2005), anti allergic, anti inflammatory and antitumor activity. They existed in plants in many forms such as flavanonones, flavones and flavonols. Figure 4.14 shows the mechanism of reaction by kaempferol as the flavonoids against 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical. Kaempferol donate one of the hydrogen atoms to DPPH radical yielding a non radical 2,2-Diphenyl-1-Picrylhydrazine (DPPH-H) and kaempferol quinone.

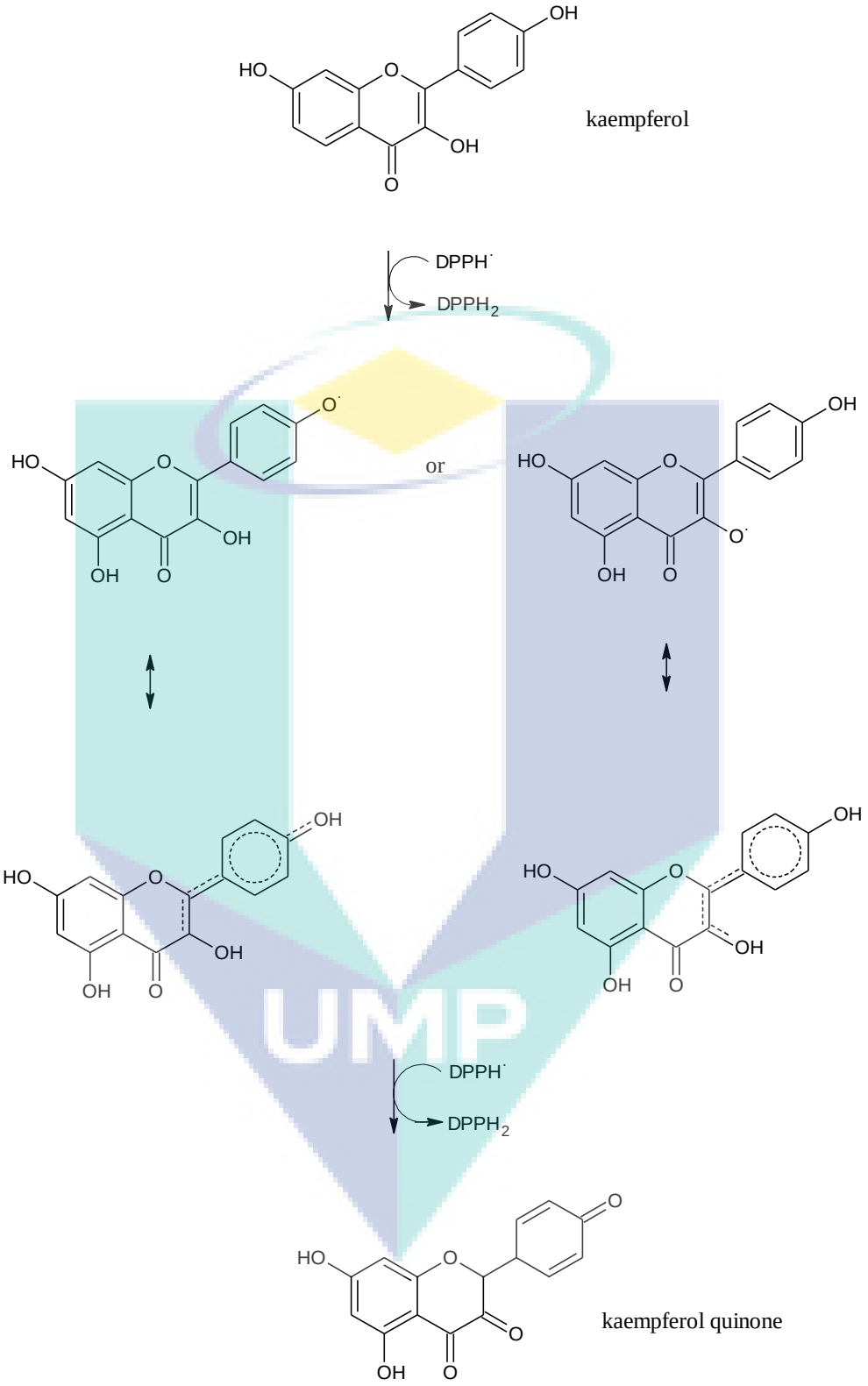


Figure 4.14: Reaction mechanism of kaempferol against DPPH radical

Source: Isimogiannis and Oreopoulou (2006)

Figure 4.15, Appendix E1 and E2 graphically present the content of flavonoids in all tested plant extracts and shows that result for TFC varies among the extracts. The highest flavonoids content was found in *V. negundo* for both ethanol and aqueous extracts, and both extraction techniques. As comparison, *P. speciosa* contains high in phenolics but low in flavonoids. This may be due to the method of measuring the total phenolic itself, whereas other chemical groups like amino acids and protein may also react with Folin Ciocaltaeu Reagent (FCR) thus contributing to the value of total phenolics (Meda et al., 2005). On the other hand, Gnanaraj and Iqbal (2012) stated that free radical scavenging activity is not only due to phenolic compounds but it also comes from the presence of other antioxidant secondary metabolites in the extracts which directly or indirectly contribute to the activity.

The function of flavonoids in plants is to provide colours attractive to plant pollinators. One special features of *V. negundo* is having the three chaste violet foliages which is very attractive to butterflies. This might be the reason lies behind the high value of flavonoids as compared to other plant tested in this work. Meda et al. (2005) suggested that in order to get the real contents of total flavonoids in the plants, aluminium chloride ($AlCl_3$) method must be done, together with 2,4-dinitrophenylhydrazine method because the former is only for flavones and flavonols while the latter is specific for flavonones. The sum values from both methods are the total flavonoids content of the plants. The use of only one method may cause the underestimation of potential values of flavonoids in plants and maybe that is the cause of resulted values for some tested plants in this current work are low.

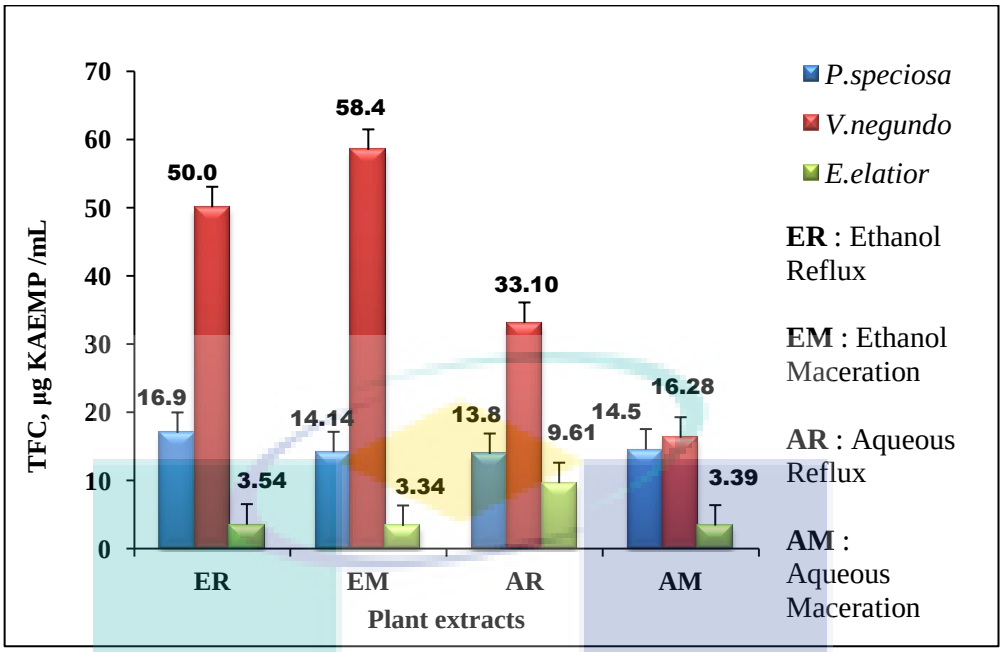


Figure 4.15: Total flavonoids content in all sample extracts expressed in µg kaempferol/mg of extract.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The crude of *P. speciosa*, *V. negundo* and *E. elatior* have been successfully extracted by reflux and maceration, yielding the aqueous ethanol and total aqueous extracts, and evaluation of their antioxidant properties were investigated through several bioassays. The result shows that *P. speciosa* possesses the highest antioxidant behaviour compared to other plant tested as it have lowest EC_{50} value and highest degree of bleaching. The extraction procedure does affect the total phenolic content and antioxidant activity of the extracts. For the comparison of extraction techniques and extraction solvents, aqueous ethanol presented the best activities and reflux offered the best separation of the antioxidant compounds. The result of TPC and TFC shows that *P. speciosa* is high in phenolics compound but low in flavonoids content. This is in contra with *V. negundo* where it contained higher flavonoids than phenolic compounds. This shows that the content of phenolic and flavonoids are variables in different plant extracts and they may have no correlations with the degree of antioxidative properties of the extracts. Some previous study successfully correlate between TPC and TFC with the antioxidant activity where they concluded that high content of phenolic and flavonoids will resulted in high antioxidant activity (Meda et al., 2005) and in contrast, several studies stated that there is no correlations between phenolic and flavonoids content and antioxidant properties of the particular plants (Conforti et al., 2009 and Kahkonen et al., 1999). In conclusion, all tested plants possess a promising value of antioxidant.The crucial matter is to find the best method to extract them out efficiently.

5.2 RECOMMENDATIONS

Further work should be done as rationale for the widespread use of these plants is expected to exist. As example, for the *V. negundo* L., there are still a wide range of study on the phytochemistry and pharmacological properties of this plant since very less work have been done on this species variety. One of the traditional uses of this species is for its antibacterial properties, and considerable content of flavonoids found in this current work might contributed to the features as reported by Cushnie and Lamb (2005) that flavonoids is a potential antibacterial. Better and modern extraction techniques that give high recovery of extract should be apply in order to receive better activity of the plants. Further work on the isolation of chemical compound that responsible for the activity is also suggested as these plants seems to be a potential source of natural antioxidant since they can be found abundantly in our country.

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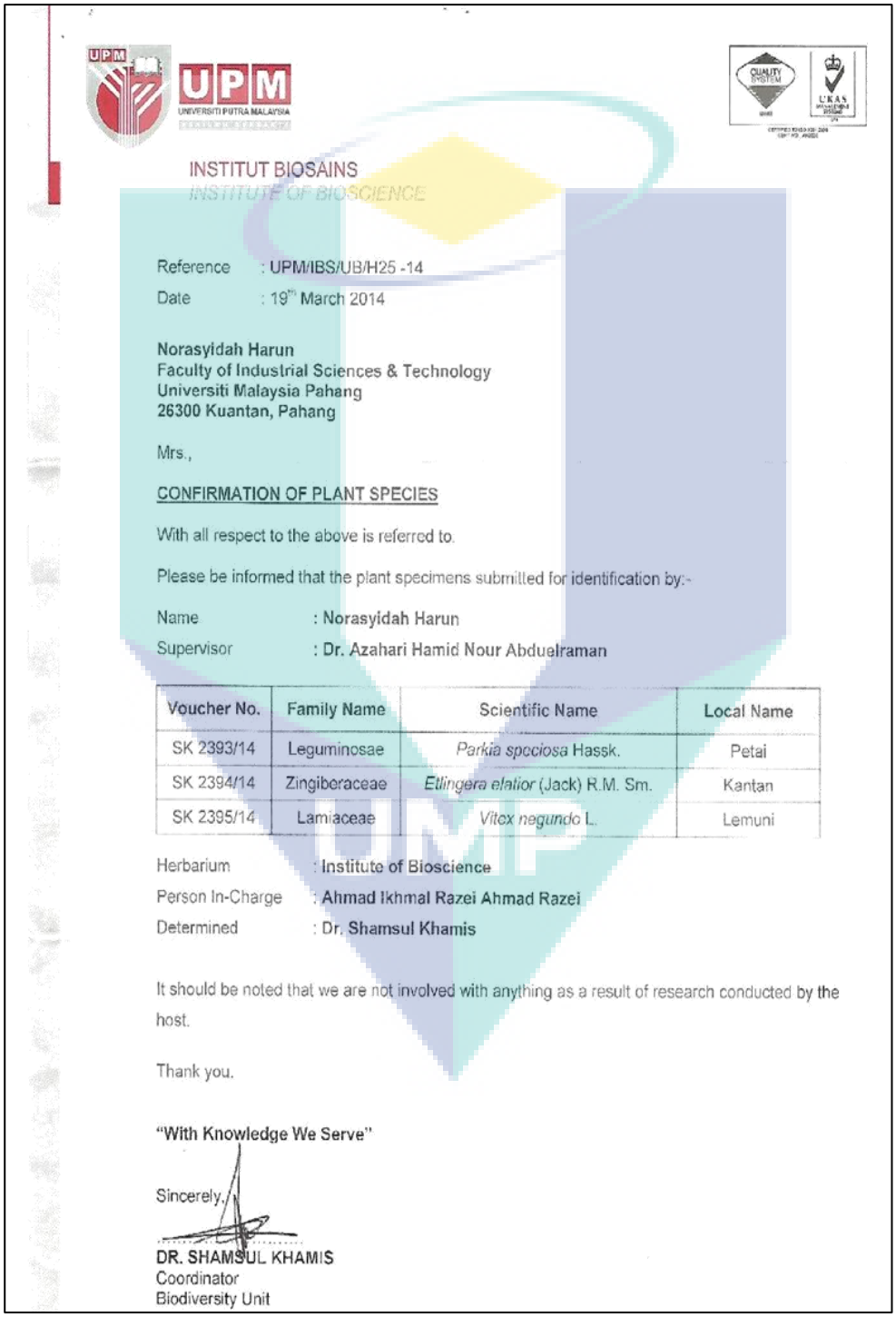
APPENDICES

APPENDIX A



UPM
UNIVERSITI PUTRA MALAYSIA

INSTITUT BIOSAINS
INSTITUTE OF BIOSCIENCE

Reference : UPM/IBS/UB/H25 -14
Date : 19th March 2014

Norasyidah Harun
Faculty of Industrial Sciences & Technology
Universiti Malaysia Pahang
26300 Kuantan, Pahang

Mrs.,

CONFIRMATION OF PLANT SPECIES

With all respect to the above is referred to.

Please be informed that the plant specimens submitted for identification by:-

Name : Norasyidah Harun
Supervisor : Dr. Azahari Hamid Nour Abduelraman

Voucher No.	Family Name	Scientific Name	Local Name
SK 2393/14	Leguminosae	*Parkia speciosa* Hassk.	Petai
SK 2394/14	Zingiberaceae	*Etlingera elatior* (Jack) R.M. Sm.	Kantan
SK 2395/14	Lamiaceae	*Vitex negundo* L.	Lemuni

Herbarium : Institute of Bioscience
Person In-Charge : Ahmad Ikhmal Razei Ahmad Razei
Determined : Dr. Shamsul Khamis

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you.

"With Knowledge We Serve"

Sincerely,

DR. SHAMSUL KHAMIS
Coordinator
Biodiversity Unit

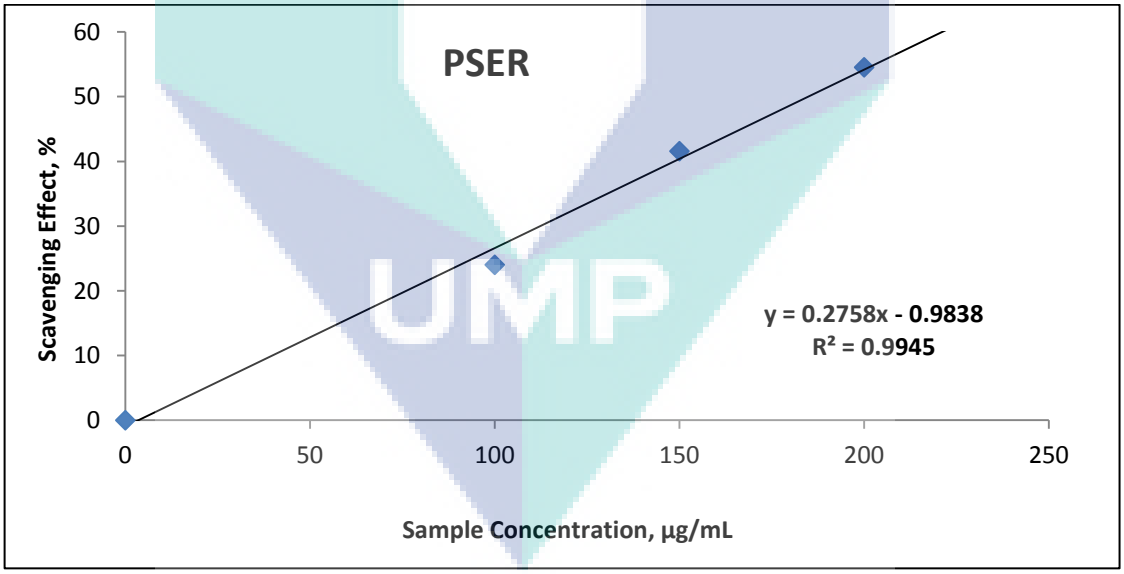
Appendix A: Letter of Confirmation of Plant Species

APPENDIX B

DPPH RADICAL SCAVENGING ASSAY

Appendix B1: Scavenging Effect of PSER

Conc.(ppm)	Absorbance					
	I	II	III	AVE	SCA	SD
400	1.136	1.135	1.134	1.135	55.9565	0.0010
200	1.171	1.172	1.171	1.171	54.5466	0.0006
150	1.505	1.506	1.505	1.505	41.5858	0.0006
100	1.958	1.958	1.958	1.958	24.0202	0.0000
50	2.696	2.697	2.696	2.696	4.6308	0.0006
0	2.576	2.577	2.577	2.577	0.012	0.0006
					AVE	**0.001**

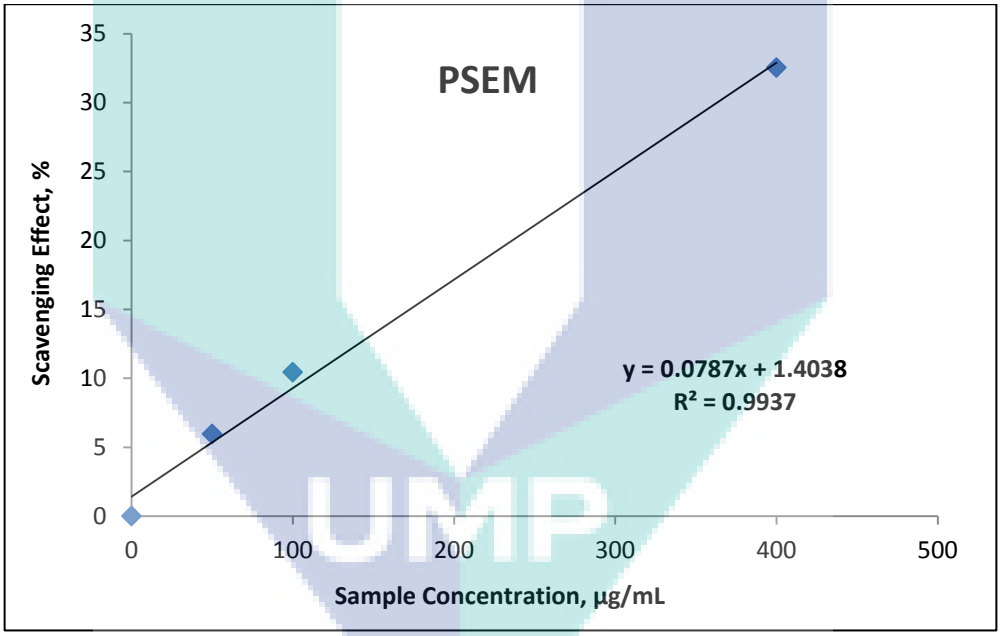


Appendix B2: Graph of Scavenging Effect of PSER

EC_{50} Value: 184.858 µg/mL

Appendix B3: Scavenging Effect of PSEM

	Absorbance					
Conc.(ppm)	**I**	**II**	**III**	**AVE**	**SCA**	**SD**
400	1.575	1.577	1.574	1.575	32.5339	0.002
200	1.777	1.777	1.777	1.777	23.8972	0.000
150	1.926	1.925	1.925	1.925	17.5446	0.001
100	2.092	2.091	2.091	2.091	10.4354	0.001
50	2.197	2.195	2.196	2.196	5.9529	0.001
0	2.337	2.334	2.334	2.335	0.0000	0.002
					AVE	**0.001**

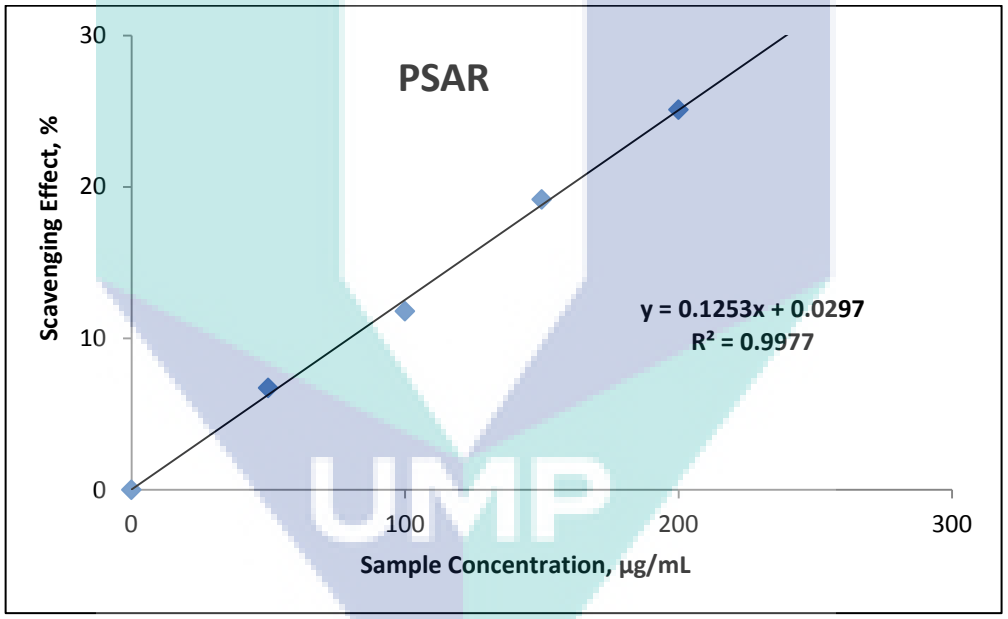


Appendix B4: Graph of Scavenging Effect of PSEM

EC_{50} Value: 617.49 µg/mL

Appendix B5: Scavenging Effect of PSAR

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	1.54	1.541	1.539	1.5400	31.4031	0.001
200	1.682	1.682	1.681	1.6817	25.0927	0.001
150	1.815	1.815	1.814	1.8147	19.1685	0.001
100	1.98	1.981	1.98	1.9803	11.7891	0.001
50	2.094	2.095	2.093	2.0940	6.7260	0.001
0	2.245	2.244	2.246	2.2450		0.001
					AVE	**0.001**

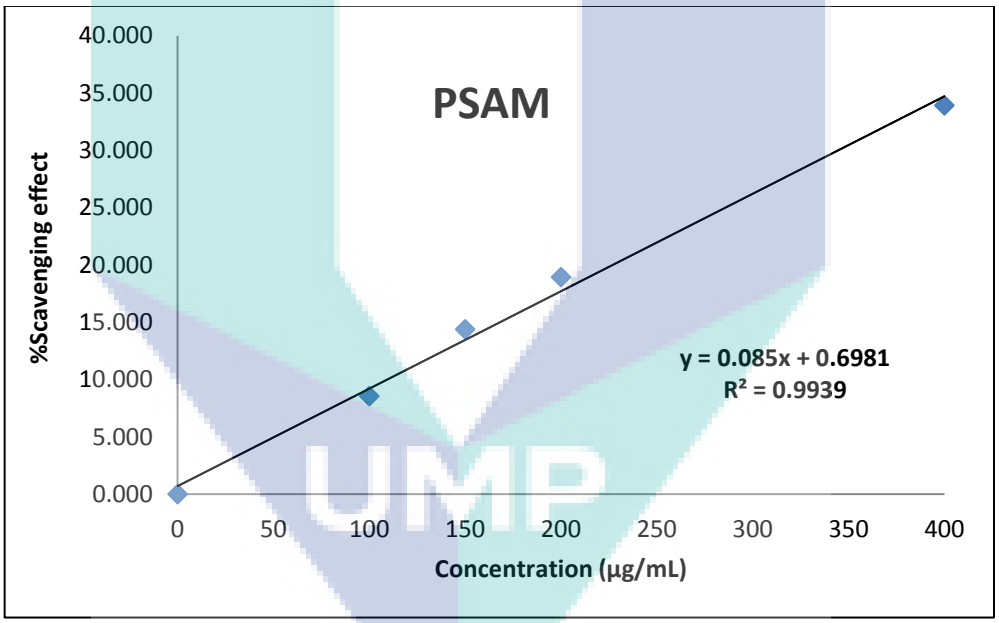


Appendix B6: Graph of Scavenging Effect of PSAR

EC_{50} Value: 398.81 µg/mL

Appendix B7: Scavenging Effect of PSAM

	Absorbance					
Conc.(ppm)	**I**	**II**	**III**	**AVE**	**SCA**	**SD**
400	1.486	1.485	1.485	1.485	33.897	0.001
200	1.822	1.822	1.822	1.822	18.914	0.000
150	1.926	1.925	1.922	1.924	14.360	0.002
100	2.052	2.058	2.056	2.055	8.530	0.003
50	2.032	2.033	2.031	2.032	9.568	0.001
0	2.249	2.247	2.246	2.247		0.002
					AVE	**0.001**

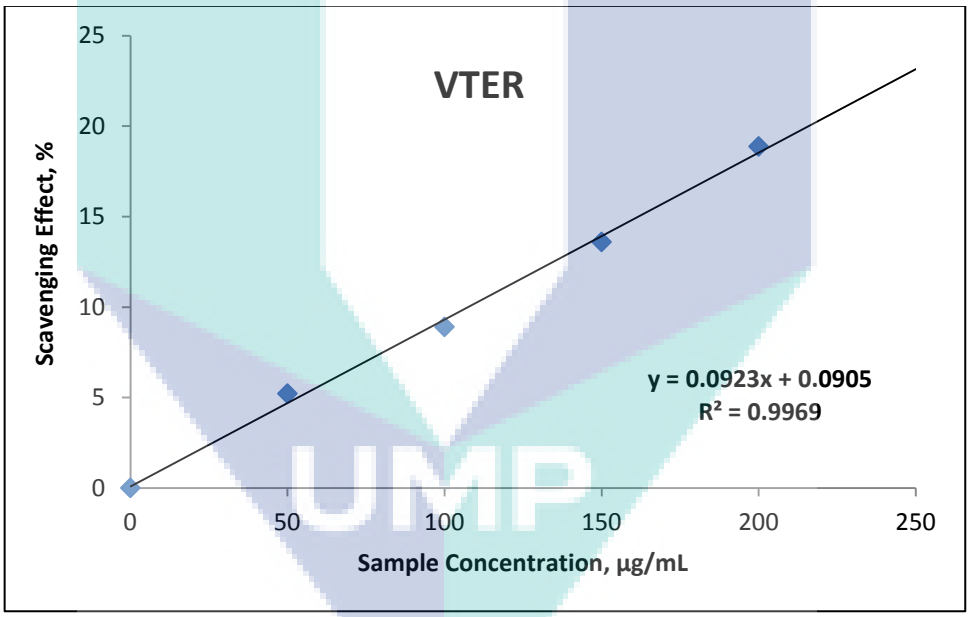


Appendix B8: Graph of Scavenging Effect of PSAM

EC_{50} Value: 580.022 µg/mL

Appendix B9: Scavenging Effect of VTER

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	1.989	1.989	1.989	1.989	22.8172	0.000
200	2.091	2.09	2.091	2.091	18.8721	0.001
150	2.23	2.22	2.23	2.227	13.5946	0.006
100	2.348	2.348	2.347	2.348	8.8992	0.001
50	2.442	2.443	2.443	2.443	5.2128	0.001
BLANK	2.576	2.577	2.577	2.577		0.001
					AVE	**0.002**

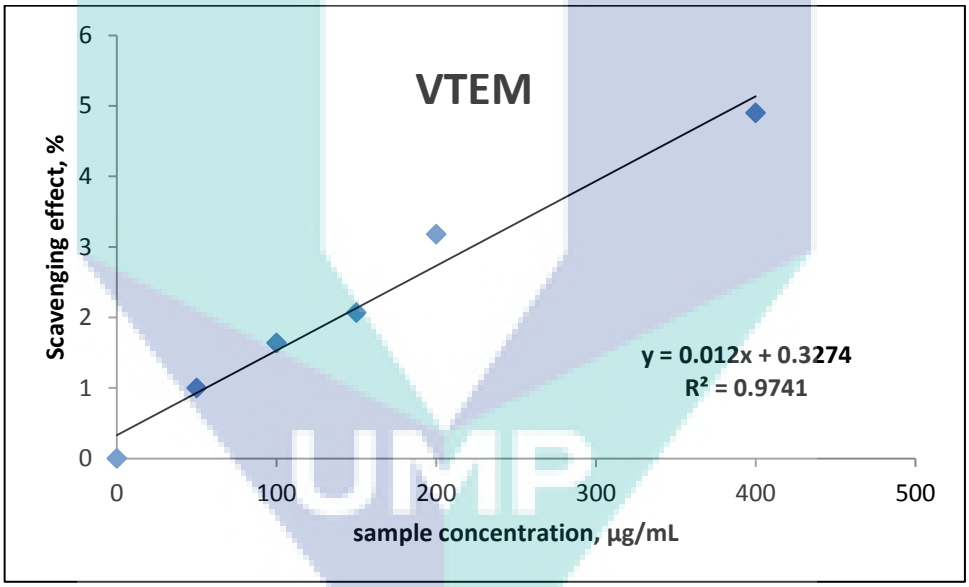


Appendix B10: Graph of Scavenging Effect of VTER

EC_{50} Value: 540.73

Appendix B11: Scavenging Effect of VTEM

Conc.(ppm)	Absorbance					
	I	II	III	AVE	SCA	SD
400	2.285	2.284	2.282	2.284	4.887	0.002
200	2.326	2.325	2.324	2.325	3.165	0.001
150	2.353	2.351	2.351	2.352	2.055	0.001
100	2.363	2.362	2.361	2.362	1.624	0.001
50	2.378	2.378	2.376	2.377	0.986	0.001
0	2.403	2.401	2.400	2.401		0.002
					AVE	**0.001**

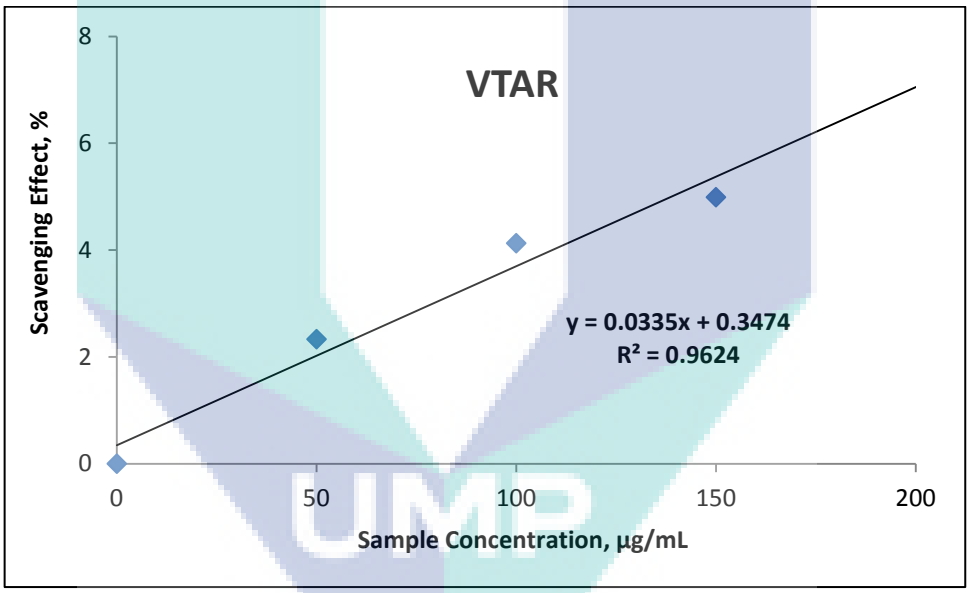


Appendix B12: Graph of Scavenging Effect of VTEM

EC_{50} Value: 4139.38 µg/mL

Appendix B13: Scavenging Effect of VTAR

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	2.064	2.065	2.064	2.0643	8.0475	0.001
200	2.121	2.123	2.123	2.1223	5.4639	0.001
150	2.136	2.131	2.132	2.1330	4.9888	0.003
100	2.15	2.151	2.156	2.1523	4.1276	0.003
50	2.192	2.193	2.193	2.1927	2.3311	0.001
0	2.245	2.244	2.246	2.2450		0.001
					AVE	**0.002**

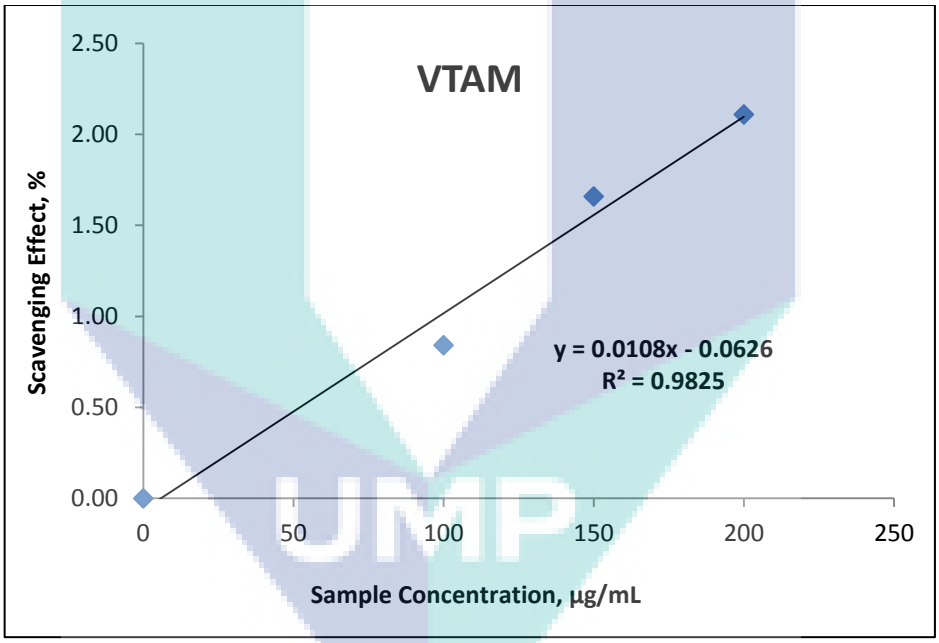


Appendix B14: Graph of Scavenging Effect of VTAR

EC_{50} Value: 1482.17 µg/mL

Appendix B15: Scavenging Effect of VTAM

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	2.615	2.616	2.615	2.615	7.027	0.001
200	2.757	2.751	2.753	2.754	2.109	0.003
150	2.767	2.766	2.766	2.766	1.659	0.001
100	2.792	2.789	2.787	2.789	0.841	0.003
50	2.859	2.857	2.859	2.858	-1.612	0.001
0	2.814	2.813	2.812	2.813	0.000	0.001
					AVE	**0.001**

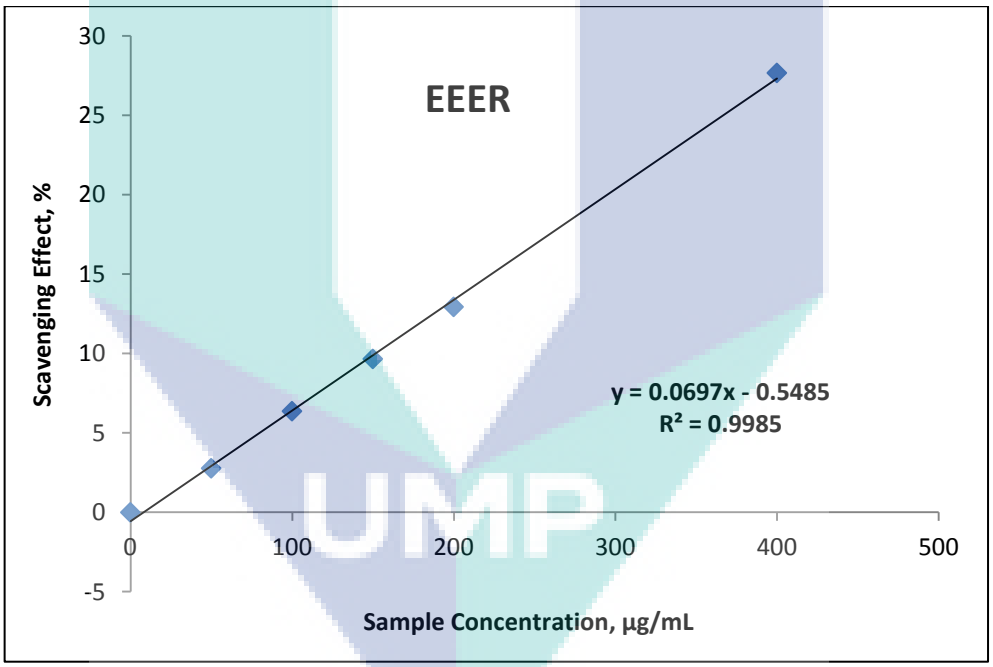


Appendix B16: Graph of Scavenging Effect of VTAM

EC_{50} Value: 4635.43 µg/mL

Appendix B17: Scavenging Effect of EEER

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	1.737	1.738	1.736	1.737	27.655	0.001
200	2.092	2.090	2.090	2.091	12.925	0.001
150	2.17	2.17	2.168	2.169	9.649	0.001
100	2.249	2.248	2.248	2.248	6.358	0.001
50	2.336	2.335	2.333	2.335	2.763	0.002
0	2.402	2.402	2.400	2.401		0.001
					AVE	**0.001**

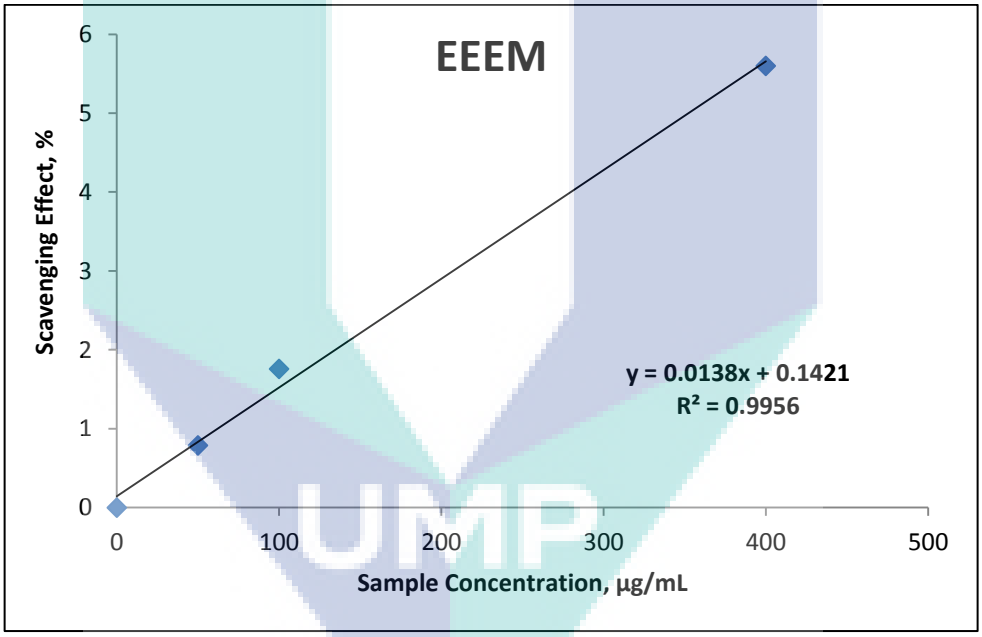


Appendix B18: Graph of Scavenging Effect of EEER

EC_{50} Value: 725.25 µg/mL

Appendix B19: Scavenging Effect of EEEM

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	2.433	2.432	2.433	2.433	5.6008	0.001
200	2.45	2.46	2.46	2.457	4.6695	0.006
150	2.499	2.5	2.499	2.499	3.0138	0.001
100	2.531	2.532	2.532	2.532	1.7592	0.001
50	2.56	2.56	2.55	2.557	0.7890	0.006
0	2.574	2.576	2.575	2.575		
					AVE	**0.003**

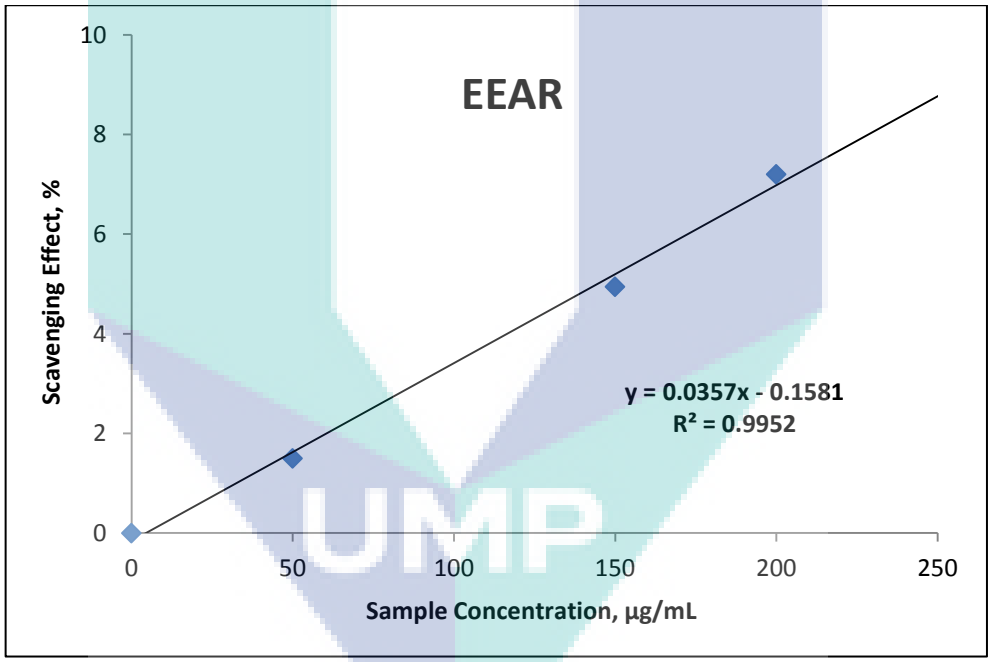


Appendix B20: Graph of Scavenging Effect of EEEM

EC_{50} Value: 3590.38 µg/mL

Appendix B21: Scavenging Effect of EEAR

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	2.03	2.031	2.03	2.0303	9.5620	0.001
200	2.084	2.083	2.083	2.0833	7.2012	0.001
150	2.133	2.134	2.135	2.1340	4.9443	0.001
100	2.193	2.194	2.196	2.1943	2.2569	0.002
50	2.211	2.212	2.211	2.2113	1.4996	0.001
0	2.246	2.245	2.245	2.2453		0.001
					AVE	**0.001**

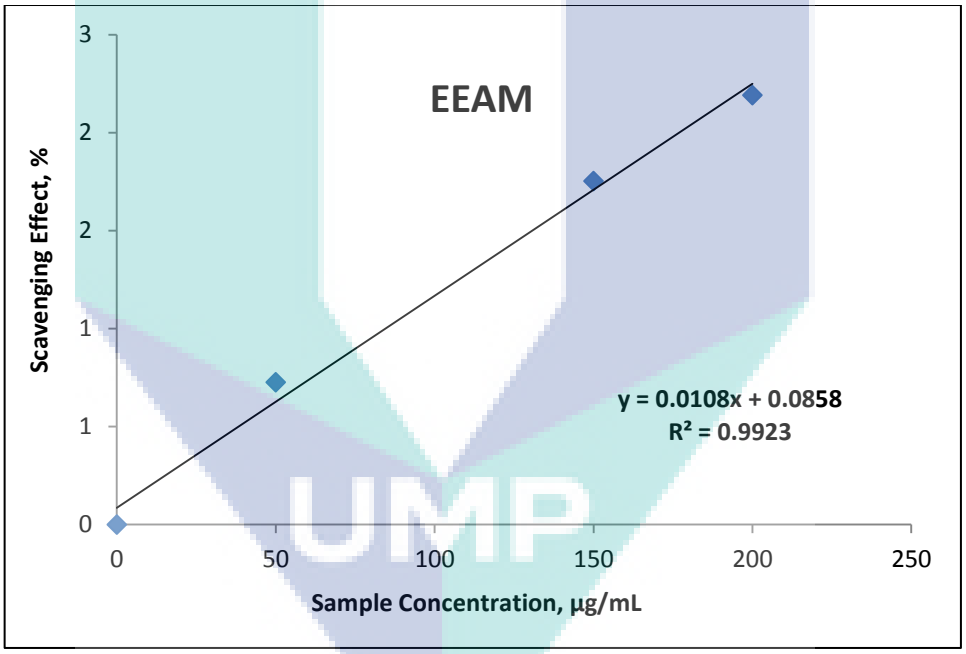


Appendix B22: Graph of Scavenging Effetc of EEAR

EC_{50} Value: 1404.99 µg/mL

Appendix B23: Scavenging Effect of EEAM

Conc.(ppm)	Absorbance					
	I	**II**	**III**	**AVE**	**SCA**	**SD**
400	2.596	2.598	2.595	2.596	2.467	0.002
200	2.604	2.606	2.602	2.604	2.179	0.002
150	2.617	2.614	2.616	2.616	1.741	0.002
100	2.623	2.622	2.621	2.622	1.503	0.001
50	2.644	2.643	2.642	2.643	0.714	0.001
0	2.664	2.661	2.662	2.662		0.002
					AVE	**0.001**



Appendix B24: Graph of Scavenging Effect EEAM

EC_{50} Value: 4621.69 µg/mL

APPENDICES C

BETA CAROTENE BLEACHING ASSAY

Appendix C1: Ethanol Reflux

Sample	Absorbance							
Time(min)	**0**	**20**	**40**	**60**	**80**	**100**	**120**	**AA**
blank	2.259	1.874	1.729	1.614	1.511	1.384	1.245	0.00
ps	2.227	1.912	1.838	1.815	1.811	1.811	1.796	57.4951 ± 0.155
vt	2.327	1.954	1.842	1.774	1.713	1.652	1.572	25.5424 ± 0.251
ee	2.334	1.951	1.824	1.746	1.68	1.598	1.536	21.3018 ± 0.277

Appendix C2: Ethanol Maceration

Sample	Absorbance							
Time(min)	**0**	**20**	**40**	**60**	**80**	**100**	**120**	**AA**
blank	2.259	1.874	1.729	1.614	1.511	1.384	1.245	0.00
ps	1.785	1.651	1.593	1.451	1.325	1.267	1.132	35.6016 ± 0.232
vt	1.516	1.455	1.278	1.115	1.086	0.987	0.664	15.9763 ± 0.292
ee	1.412	1.278	1.121	0.965	0.878	0.653	0.51	11.0454 ± 0.325

Appendix C3: Aqueous Reflux

Sample	Absorbance							
Time(min)	**0**	**20**	**40**	**60**	**80**	**100**	**120**	**AA**
blank	2.235	2.188	1.769	1.568	1.264	1.198	0.987	0.00
ps	2.321	2.167	1.997	1.654	1.462	1.417	1.389	25.321 ± 0.385
vt	2.351	2.298	2.187	1.974	1.547	1.433	1.325	17.789 ± 0.432
ee	2.336	2.198	1.985	1.778	1.615	1.366	1.285	15.785 ± 0.402

Appendix C4: Aquoeus Maceration

Sample	Absorbance							
Time(min)	**0**	**20**	**40**	**60**	**80**	**100**	**120**	**AA**
blank	2.235	2.188	1.769	1.568	1.264	1.198	0.987	0.00
ps	2.981	2.789	2.512	2.469	2.357	2.298	1.969	18.91 ± 0.331
vt	2.865	2.779	2.698	2.365	2.171	1.867	1.75	10.657 ± 0.446
ee	2.513	2.298	1.789	1.655	1.562	1.426	1.387	9.776 ± 0.437

APPENDICES D

TOTAL PHENOLIC COMPOUND

Appendix D1: Ethanol Extracts

ETHANOL		REPLICATIONS I	II	III	MEAN	STD	TPC
REFLUX	PS	0.065	0.065	0.066	0.065	0.001	66.52 ± 0.001
	VT	0.045	0.044	0.043	0.044	0.001	43.08 ± 0.001
	EE	0.04	0.041	0.045	0.042	0.003	40.88 ± 0.003
MACERATION	PS	0.053	0.055	0.054	0.054	0.001	54.07 ± 0.001
	VT	0.037	0.037	0.039	0.038	0.001	36.12 ± 0.001
	EE	0.027	0.029	0.032	0.029	0.003	29.96 ± 0.003

Appendix D2: Aquoeus Extracts

AQUEOUS		REPLICATIONS I	II	III	MEAN	STD	TPC
REFLUX	PS	0.025	0.029	0.028	0.027	0.002	24.76 ± 0.002
	VT	0.019	0.021	0.022	0.021	0.002	17.44 ± 0.002
	EE	0.009	0.01	0.011	0.010	0.001	5.71 ± 0.001
MACERATION	PS	0.019	0.017	0.02	0.019	0.002	15.24 ± 0.002
	VT	0.007	0.008	0.006	0.007	0.001	2.42 ± 0.001
	EE	0.01	0.011	0.011	0.011	0.001	6.45 ± 0.001

APPENDICES E

TOTAL FLAVONOIDS CONTENT

Appendix E1: Ethanol Extracts

ETHANOL		REPLICATIONS			MEAN	TFC
		I	II	III		
REFLUX	**PS**	0.116	0.116	0.116	0.116	16.9254 ± 0.228
	VT	0.338	0.338	0.339	0.338	50.0597 ± 0.149
	EE	0.026	0.025	0.025	0.025	3.3433 ± 0.228
MACERATION	**PS**	0.096	0.096	0.097	0.096	13.9403 ± 0.228
	VT	0.393	0.394	0.393	0.393	58.2687 ± 0.228
	EE	0.024	0.024	0.024	0.024	3.194 ± 0.299

Appendix E2: Aquoeus Extracts

AQUEOUS		REPLICATIONS			MEAN	TFC
		I	II	III		
REFLUX	**PS**	0.096	0.096	0.096	0.096	13.9402 ± 0.086
	VT	0.223	0.223	0.224	0.223	32.8955 ± 0.228
	EE	0.069	0.069	0.067	0.069	9.9104 ± 0.299
MACERATION	**PS**	0.09	0.09	0.09	0.09	13.0448 ± 1.493
	VT	0.11	0.111	0.111	0.111	16.1791 ± 0.172
	EE	0.025	0.025	0.024	0.025	3.3433 ± 0.228