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

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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

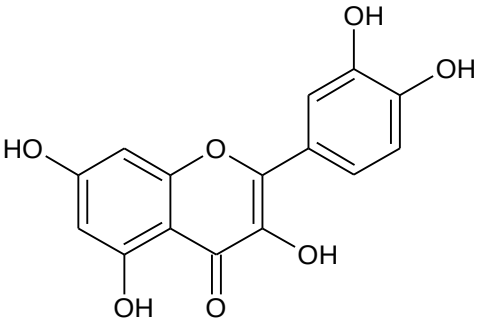
LITERATURE REVIEW

2.1 ANTIOXIDANT

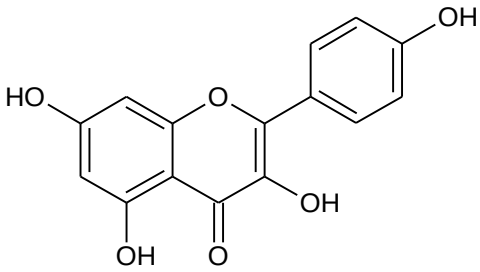
Karou et al. (2005) has defined antioxidant as compounds that can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reaction.

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

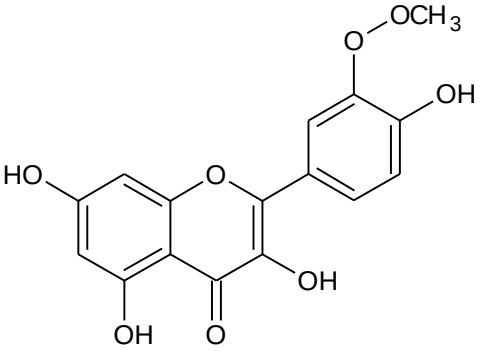
polyphenols. Antioxidants are mainly the monohydroxy or polyhydroxy phenol compound. It is stable compounds which have low activating energy to donate hydrogen. Therefore, the resulting antioxidant radicals do not initiate another free radicals (Hamid et al., 2010). Figure 2.1 shows structures of some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



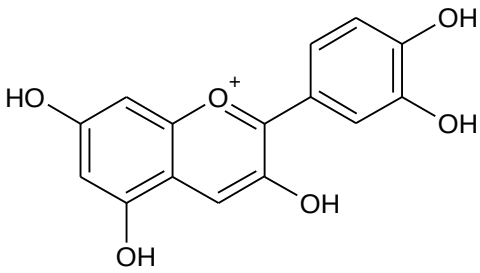
Quercetin



Kaempferol



Isorhamnetin



Cyanidin

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.,