CHEMICAL CONSTITUENTS AND ANTIOXIDANT CAPACITY OF EXTRACTS FROM *Elaeis guineensis* LEAVES

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FACULTY OF SCIENCE
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ABSTRACT

The present study investigated compounds present in *Elaeis guineensis* leaves and their potentials to act as natural antioxidants. Fresh leaves from *Elaeis guineensis* fronds were subjected to solvent extraction and ultrasonic-assisted methanol extraction. The chemical constituents were identified by gas chromatography-mass spectrometry (GC-MS). The crude methanolic extract (MeOH) obtained from the ultrasonic-assisted methanol extraction was then fractionated by repeated extractions, sequentially with solvents of different polarity into *n*-hexane (HEX), ethyl acetate (EA), water (WATER) and insoluble residue (INSOL) fractions. Major compounds identified in the methanolic crude extract which was further partitioned by *n*-hexane (MeOH-HEX) extract were phytol (42.94%), α-tocopherol (24.97%), squalene (14.95%), β-sitosterol (9.39%) and stigmasterol (5.84%). The same crude methanolic extract treated with activated carbon (MeOH-Activated C) extract yielded 2,4-di-*tert*-butylphenol (30.88%), phytol (23.80%), methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (13.49%), 16-heptadecenal (11.72%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (7.80%). Maceration using *n*-hexane as extracting solvent (Maceration-HEX) yielded α-tocopherol (30.51%), squalene (24.23%), arundoin (21.73%), 3-hexen-1-ol (5.31%) and β-sitosterol (4.77%). Maceration using ethyl acetate as extracting solvent (Maceration-Et_2O) yielded similar components in slightly different quantities with squalene (29.34%), α-tocopherol (24.35%), phytol (12.54%), arundoin (8.63%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (7.42%). The antioxidant activities of the crude methanolic extract and its fractions were evaluated by *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, lipid peroxidation (LPO) inhibition and β-carotene bleaching assays. The total phenolic contents (TPC) in the fractions were determined using Folin-Ciocalteu reagent. The results indicated that the INSOL fraction

showed the highest activity in DPPH radical-scavenging activity assay and β-carotene bleaching assay, while the EA fraction exhibited the highest activity through LPO inhibition. DPPH radical-scavenging activities of the fractions were negatively correlated (correlation coefficients = -0.896, -0.943 respectively, $P < 0.050$) with the TPC based on Pearson Product Moment Correlation and Spearman Rank Order Correlation. The findings of this present study suggests that *Elaeis guineensis* leaves have a high potential to be utilized as a natural source of antioxidants in palm oil producing countries such as Indonesia, Malaysia and Thailand, where *Elaeis guineensis* leaves are abundantly available.

ABSTRAK

Kajian ini mengkaji komponen kimia yang hadir dalam daun *Elaeis guineensis* dan potensinya untuk berfungsi sebagai antioksidan semula jadi. Daun segar *Elaeis guineensis* diekstrak dengan kaedah pengekstrakan pelarut and pengekstrakan ultrasonik berpelarut metanol. Komposisi kimia dalam hasil pengekstrakan telah dianalisa dan dikenal pasti dengan menggunakan gabungan kromatografi gas-spektrometer jisim (GC-MS). Ekstrak metanol tersebut (MeOH) telah diekstrak dengan pelarut-pelarut yang mempunyai polariti berbeza, ekstrak tersebut diasingkan mengikut urutan berikut: n-heksana (HEX), etil asetat (EA), air (WATER) dan baki tidak berketerlarutan (INSOL). Sebatian utama yang telah dikenalpasti dalam ekstrak metanol yang diekstrak lagi dengan *n*-heksana (MeOH-HEX) ialah fitol (42.94%), α-tokoferol (24.97%), skualin (14.95%), β-sitosterol (9.39%) dan stigmasterol (5.84%). Ekstrak metanol yang sama dilarutkan dalam metanol kemudian dikacau bersama karbon aktif dan ditapis telah dikenalpasti mengandungi 2,4-di-*tert*-butilfenol (30.88%), fitol (23.80%), metil-3-(3,5-di-*tert*-butil-4-hidroksifenil)propionat (13.49%), 16-heptadekenal (11.72%) and 3,7,11,15-tetrametil-2-heksadeken-1-ol (7.80%). Pengekstrakan dengan pelarut *n*-heksana (Maceration-HEX) memberi kandungan α-tokoferol (30.51%), skualin (24.23%), arundoin (21.73%), 3-heksen-1-ol (5.31%) and β-sitosterol (4.77%). Pengekstrakan dengan pelarut etil asetat (Maceration-Et_2O) dikenalpasti mengandungi sebatian utama yang sama dalam kuantiti yang berbeza iaitu skualin (29.34%), α-tokoferol (24.35%), fitol (12.54%), arundoin (8.63%) and 3,7,11,15-tetrametil-2-heksadeken-1-ol (7.42%). Aktiviti antioksidan ekstrak metanol dan pecahan-pecahan yang diekstrak ini telah diuji dengan pelbagai kaedah, iaitu esei penghapusan radikal bebas 2,2-difenil-1-pikrilhidrazil (DPPH), esei perencatan pengokisdaan lipid (LPO) dan esei pelunturan β-karotena. Jumlah kandungan fenolik (TPC) dalam ekstrak-ekstrak

turut dikaji dengan reagen Folin-Ciocalteu. Hasil ujian telah menunjukkan INSOL mempunyai aktiviti tertinggi dalam esei penghapusan radikal bebas DPPH dan esei pelunturan β-karotena, sementara EA memberi aktiviti ketara dalam esei perencatan pengoksidaan lipid. Aktiviti penghapusan radikal bebas DPPH ekstrak-ekstrak ini menunjukkan korelasi negative dengan TPC, berdasarkan *Pearson Product Moment Correlation* dan *Spearman Rank Order Correlation*, masing-masing dengan pekali korelasi = -0.896, -0.943, $P < 0.050$. Hasil kajian ini mencadangkan bahawa daun *Elaeis guineensis* berpotensi tinggi untuk dimanfaatkan sebagai sumber semula jadi antioksidan semula jadi di negara-negara keluaran minyak kelapa sawit seperti Malaysia, Indonesia dan Thailand, di mana daun kelapa sawit hadir dalam kuantiti yang besar.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	alpha
β	beta
ca	Circa
γ	gamma
λ	absorbance
%	percent
I	retention index
°C	degree celcius
µg	microgram
µL	microliter
µg/µL	microgram/microliter
µg/mL	microgram/milliliter
µM	micro molar
r	Pearson's correlation coefficient
AAPH	2,2'-azobis (2-amidinopropane) dihydrochloride
AUC	area under the curve
BHA	butylated hydroxyl-anisole
BHT	butylated hydroxyl toluene
cm	centimeters
DPPH	2,2'-diphenylpicrylhydrazyl
ET	electron transfer
etc.	et cetera
eV	electron volt
FDA	Food and Drug Administration
FRAP	ferric reducing ability of plasma

g	gram
GAE	gallic acid equivalent
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
HAT	hydrogen atom transfer
HPLC	high performance liquid chromatography
IC_{50}	50% inhibitory concentration
i.e.	id est
M	mol dm^{-3}
MDA	malondialdehyde
MeOH	methanol
mg	miligram
mg/mL	milligram / mililiter
mL	mililiter
mm	milimeter
nm	nanometer
ORAC	oxygen radical antioxidant capacity
PDMS	polydimethylsiloxane
SFE	supercritical fluid extraction
TBA	thiobarbituric acid
TLC	thin layer chromatography
TPC	total phenolic content

CHAPTER ONE: LITERATURE REVIEW

1.1 General Introduction

1.1.1 *Elaeis guineensis* and Malaysian Palm Oil Industry

The oil palms (*Elaeis*) consist of two species of the family Arecaceae. They are commonly used in commercial agriculture in the production of palm oil. The African Oil Palm *Elaeis guineensis* is native to West Africa, while the American Oil Palm *Elaeis oleifera* is native to tropical Central America and South America. *Elaeis guineensis* is widely cultivated in Malaysia for its oil producing fruits (Maclellan, 1983).

Mature oil palm trees are single-stemmed and grow to 20 m tall. The leaves are pinnate and reach 3-5 m long. A young tree produces about 30 leaves in a year. Matured trees over 10 years produce about 20 leaves in a year. The flowers are produced in dense clusters; each individual flower is small, consist three sepals and three petals.

Figure 1.1: The mature oil palm trees, *Elaeis guineensis*

The palm fruit is reddish, about the size of a large plum and grows in large bunches. Each fruit is made up of oily, fleshy outer layer (the mesocarp), with a single seed (the kernel), also rich in kernel oil. The palm fruit takes five to six months to mature after pollination.

Figure 1.2: The fruits of *Elaeis guineensis*

Palm oil is extracted from both the mesocarp of the fruit and the kernel. They are used for both food and non-food applications. Its increasing use in the commercial food industry is due to its economical pricing and the high oxidative stability of the refined products. In Malaysia, the oil palm cultivation area reached 5.74 million hectares as reported up to December 2016; produced 24.91 million tonnes of crude palm oil in the year to fulfil worldwide food and non-food demands (Malaysian Palm Oil Board, 2017). Total export of palm oil was 16.05 million tonnes in 2016, worth RM 43,369 million. Besides, there were also exports of other oil palm products such as palm kernel oil, palm kernel cake, oleochemicals, finished products, biodiesel and others, sum up to a total of 7.24 million tonnes, RM 21,219 million (Malaysian Palm Oil Board, 2017). The oil palm biomasses produced as by-products in the form of oil palm trunk, fronds and empty fruit bunch are about 80 million tonnes (dry basis). The oil palm fronds are collected during pruning and replanting activities with average generation rate of 9.8 and 14.9 tonnes (dry mass) per hectare plantation area per year, respectively. Out of that 80 million tonnes dry mass, a tremendous amount of about 45 million tonnes comes from the fronds (Ibrahim *et al.,* 2012). Beside the production of edible oil from its fruits, some studies have been done on the beneficial effects of extracts derived from various parts of *Elaeis guineensis*.

1.1.2 Phytonutrients in *Elaeis guineensis*

Crude palm oil is obtained from the fibrous mesocarp of the fruit while crude palm kernel oil is extracted from the kernel of the nut. Crude palm oil has a rich orange red hue while crude palm kernel oil is colourless (Pantzaris, 2000). At room temperature, crude palm oil is semi-solid in nature. The liquid fraction, palm olein is used mainly to produce cooking oils while the solid fraction, palm stearin is used in the manufacturing of bakery products such as shortenings and margarine.

Crude palm oil has a balanced ratio of saturated and unsaturated fatty acids which makes it suitable for a variety of food applications. It consists of more than 90% triacylglycerols, 2-7% diacylglycerols, less than 1% monoacylglycerols, 3-5% free fatty acids and 1% minor components, which are also known as phytonutrients. The palm oil phytonutrients are such as carotenoids, vitamin E, squalene, phytosterols, lecithin and coenzyme Q (Ooi, 1999; Goh *et al*., 1985; Chong & Jaais, 1995; Ooi, 1995; Choo & Basiron, 1996; Goh *et al*., 1987). Table 1.1 shows the concentration in parts per million (ppm) of some minor components in crude palm oil.

Table 1.1: Minor components in crude palm oil (Choo, 1990)

Minor Components	Concentration (ppm)
Carotenoids	500-700
Tocols	600-1000
Sterols	360-620
Phospholipids	5-130
Glycolipids	1000-3000
Triterpene alcohols	600-700
Methyl sterols	40-80
Squalene	200-500
Sesquiterpene & Diterpene	*ca* 30
Aliphatic alcohols	*ca* 100-200
Aliphatic hydrocarbons	*ca* 50
Methyl esters	*ca* 50

Apart from the above minor constituents, Neo *et al.* (2010) reported that the presence of phenolics from the by-products of palm oil milling and refining processes, that is from the fruits of oil palm. Balasundram *et al.* (2003) disclosed a phenolic-rich fraction isolated from oil palm fruits. This extract exhibits bioactive properties, in particular antioxidant effects. Tan *et al.* (2001) disclosed the presence of phenolic compounds in palm oil that is derived from oil palm fruit mesocarp and kernel. The phenolic compounds include gallic, chlorogenic, protocatechuic, gentisic, coumaric, ferulic and caffeic acids, as well as catechins, hesperidine, narirutin and 4-hydroxybenzoate, as shown in Figure 1.3.

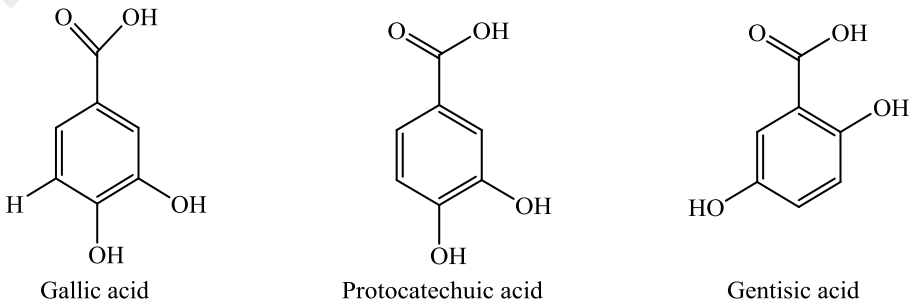
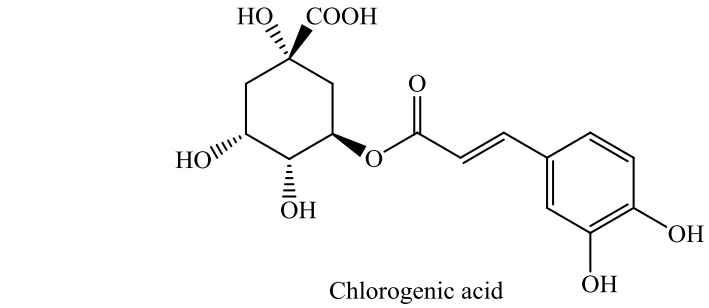
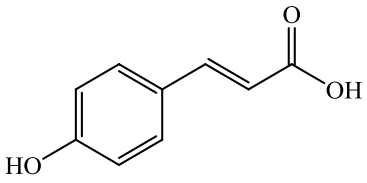


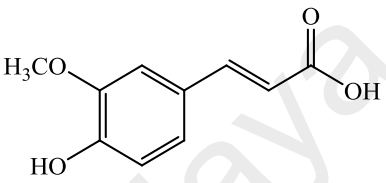
Figure 1.3: The phenolic compounds in palm oil



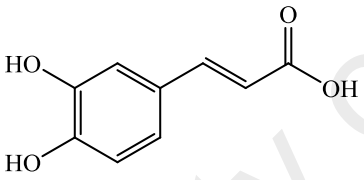
Chlorogenic acid



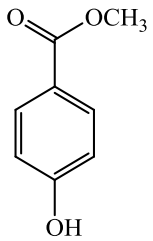
Coumaric acid



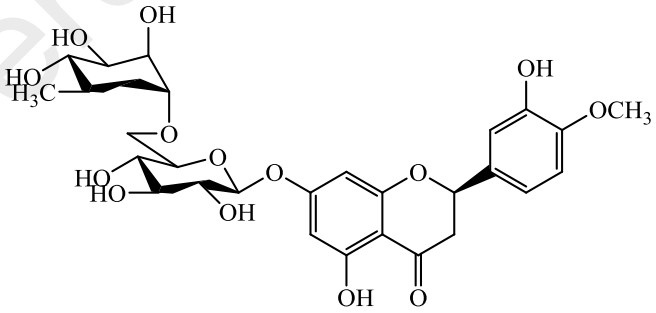
Ferulic acid



Caffeic acid

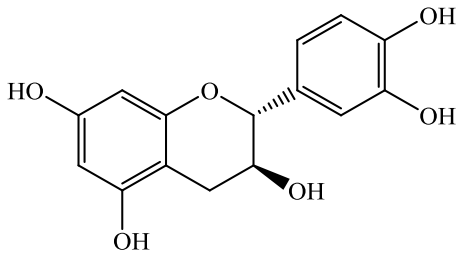


4-hydroxybenzoate

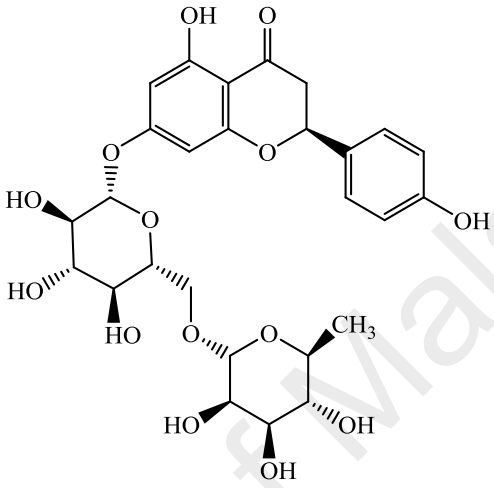


Hesperidin

Figure 1.3, continued: The phenolic compounds in palm oil



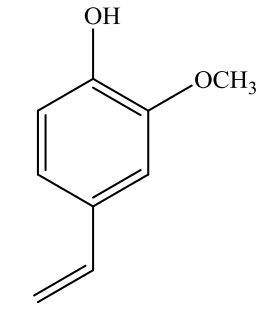
Catechins



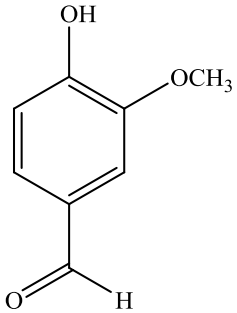
Narirutin

Figure 1.3, continued: The phenolic compounds in palm oil

Besides, Lau *et al.* (2007) recovered water-soluble compounds in fresh palm-pressed fiber by supercritical carbon dioxide extraction and 12 compounds were identified from the extract. These compounds are shown in Figure 1.4.

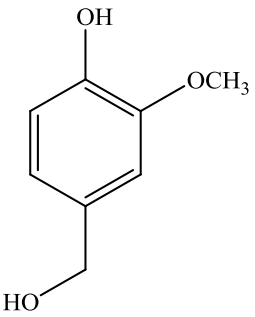


2-methoxy-4-vinylphenol

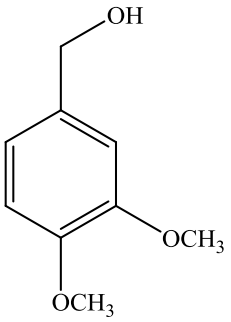


Vanillin

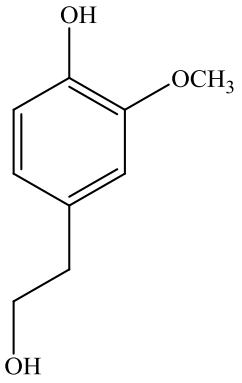
Figure 1.4: The water-soluble compounds in fresh palm-pressed fiber



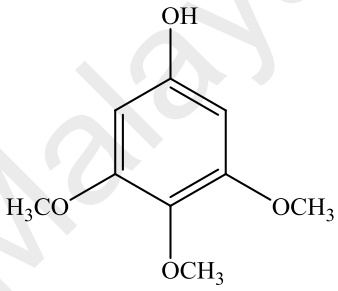
Vanillyl alcohol



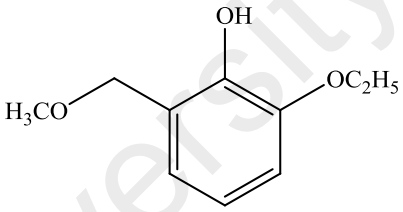
3,4-dimethoxy-benzenemethanol



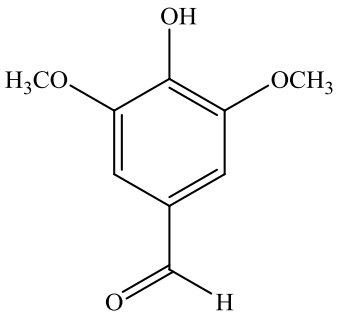
3-methoxy-4-hydroxy-phenylethanol



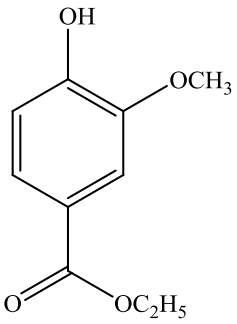
3,4,5-trimethoxyphenol



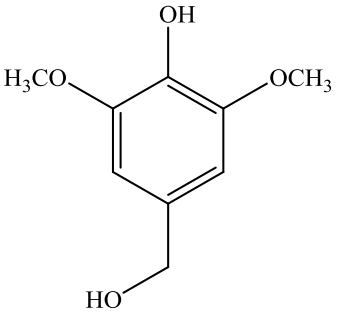
Methyl-(2-hydroxy-3-ethoxy-benzyl)-ether



4-hydroxy-3,5-dimethoxy-benzaldehyde

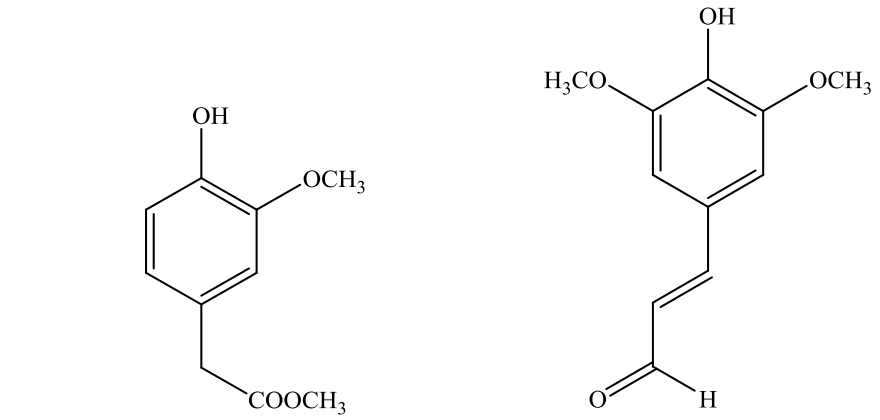


Benzoic acid, 4-hydroxy-3-methoxy-ethyl ester



3,5-dimethoxy-4-hydroxy-benzylalcohol

Figure 1.4, continued: The water-soluble compounds in fresh palm-pressed fiber



3-methoxy-4-hydrophenyl-methyl methanoate 3,5-dimethoxy-4-hydrocinnamaldehyde

Figure 1.4, continued: The water-soluble compounds in fresh palm-pressed fiber

Some researchers also discovered oil palm leaves have potential health benefits. Abeywardena *et al.* (2002) disclosed a polyphenol-enriched extract derived from *Elaeis guineensis* leaves. They reported that the said extract can be used to promote vascular relaxation *via* endothelium-dependent mechanisms. Mohamed *et al.* (2009) reported a method for preparing herbal extract from leaves of a plant of Arecaceae family (*Elaeis guineensis, Elaeis oleifera, Phoenix dactylifera and Cocos nucifera*) using polar solvent. The extract is reported to be incorporated into a daily diet for improving the cardiovascular system. Later, Mohamed *et al.* (2014) reported that oil palm leaf has 8% higher total phenols content than green tea extract and contains epigallocatechin, catechin, epicatechin and epigallocatechin gallate, as shown in Figure 1.5. They reviewed the health benefits of oil palm leaves extract on breast cancer, diabetes, hypertension, inflammation, oxidative stress, neurodegeneration, cognitive functions, dyslipidemia, cardiovascular, liver, kidney and neuroprotective properties.

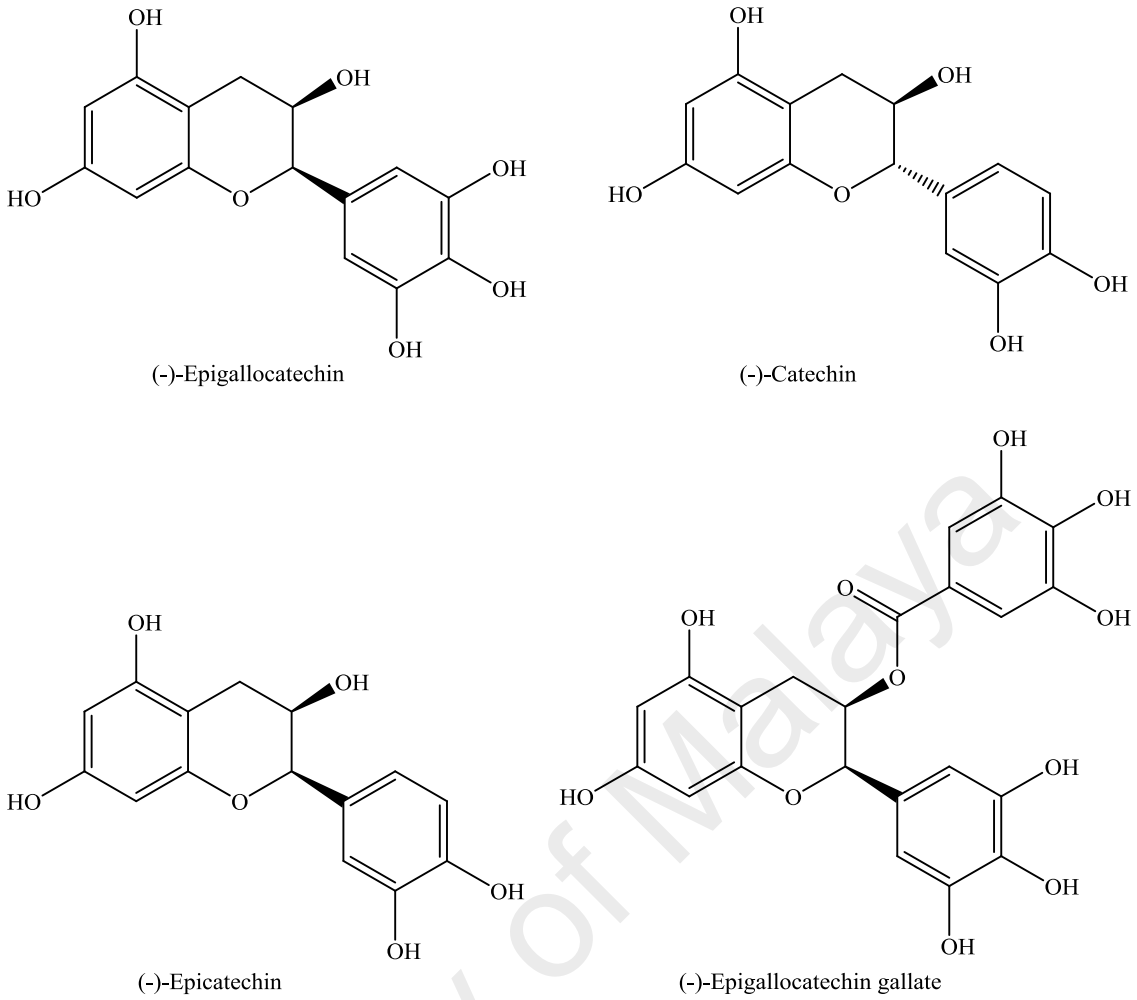


Figure 1.5: The phenolic compounds in oil palm leaves reported by Mohamed *et al.* (2014)

Kinnoudo (2007) disclosed extracts of *Elaeis guineensis* leaves that possess antimalarial properties. *Elaeis guineensis* leaves are also reported to be a potent source of antioxidants (Ng & Choo, 2010). Sun *et al.* (2001) discovered a new method of quantitative determination of hydroxycinnamic acids in oil palm leaves fiber. The analysis on other compounds was not included in the work done. Phang *et al.* (2009) characterized a composition of oil palm leaves extract comprises (-)-catechin gallate, ferulic acid and phenolic acids such as gallic acid and protocatechuic acid, as shown in Figure 1.6. A method of producing the said extract was claimed. Jaffri *et al.* (2011)

reported the effects of catechin-rich oil palm leaves extract on normal and hypertensive rats' kidney and liver.

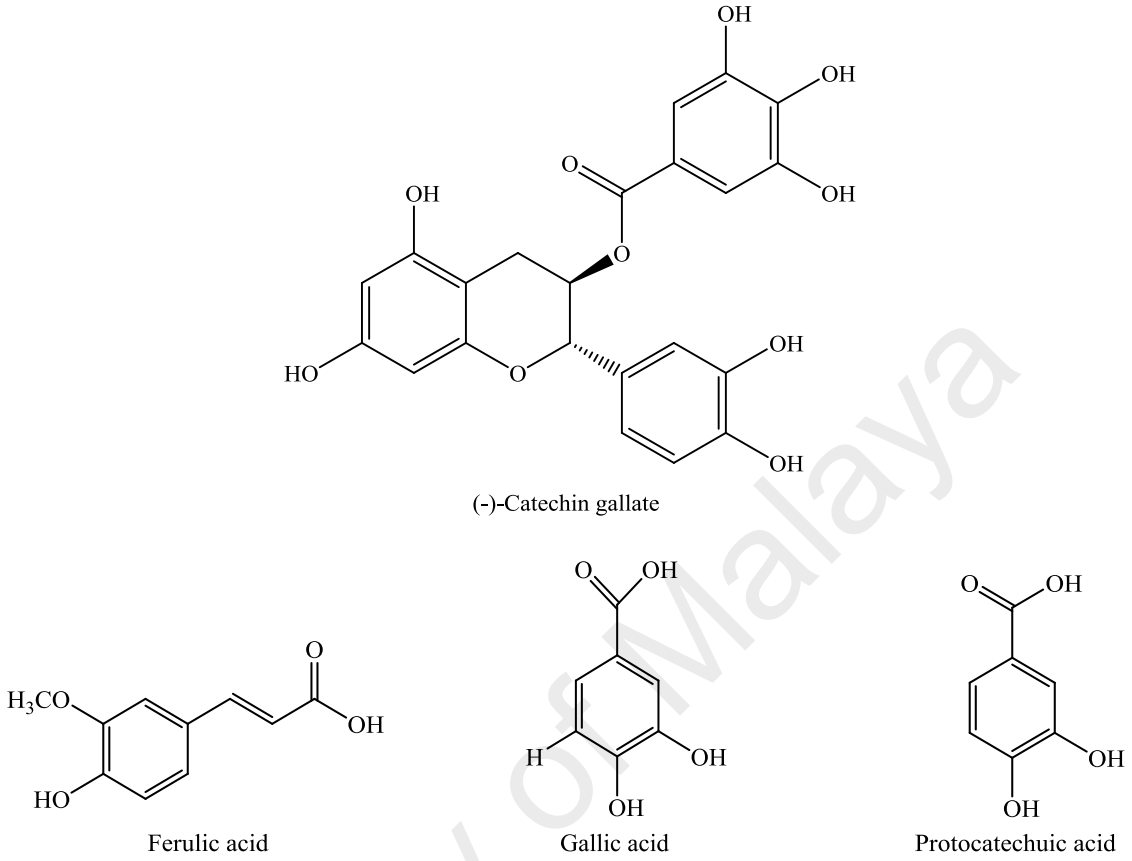


Figure 1.6: The phenolic compounds in oil palm leaves reported by Phang *et al.* (2009)

1.1.3 Antioxidants in Food and Their Benefits

Antioxidants are recognized for their potent uses in improving health by lowering the risk for cancers, hypertension and heart disease via their ability to scavenge free radicals, prevent oxidation of body cells. These antioxidants can be consumed directly as they are present in food or in the form of supplements. They can also be employed in the food industry as food additives to prevent oxidation and degradation of food during processing and storage. Natural antioxidants from a plant are attracting great interest from researchers worldwide due to some human health studies and scientific research about the safety of synthetic antioxidants in food (such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butyl hydroquinone) (Branen, 1975). The

naturally occurring antioxidant is focused more on edible plants, especially tea, fruits, vegetables, spices and herbs. They are an excellent source of phenolic compounds (secondary plant metabolites) such as flavonoids, phenolic acids, alcohols, stilbenes, tocopherols, tocotrienols, ascorbic acid and carotenoids which have been reported to show good antioxidant activity (Das & Pereira, 1990; Foti *et al.*, 1996; Santana-Méridas *et al.*, 2014; Tomas-Barberan *et al.*, 2000).

1.2 Methods of Extraction

The quantity and composition of an extract from plant materials are very much relying on the methods of extraction. Extraction of plants involves isolation of active components of a plant from the mixture of compounds by using various solvents of different degrees of polarity and different extraction parameters such as temperature, pressure, pH value and agitation. These extraction parameters are optimized, giving rise to variations of extraction to gain the desired products. Extraction yields can be in the form of liquids, paste, semisolids or powders.

1.2.1 Maceration

Maceration is a very simple and easy method where the parts of interest of a plant are soaked in a selected solvent at room conditions. After a period of time, the mixture is filtered to separate the solution portion and insoluble marc (Handa *et al*., 2008). Then the filtrate containing compounds of interest is subjected to further procedures such as isolation, analysis or bioactivity assays.

1.2.2 Soxhlet Extraction

In this method, different extraction solvents can be employed and the selection is based on the nature and polarity of the compounds of interest. Plant materials are placed in a

porous bag or extractive thimble made of strong filter paper which is placed in a chamber of the Soxhlet apparatus. The extracting solvent is heated and its vapors will travel upwards to a condenser, then the vapors will condense and drip into the chamber containing the plant materials. When the level of solvent in the chamber rises to the top of the siphon tube, the solvent in the chamber will flow through the siphon into the flask. This process is continuous and is carried out until the drops of solvent from the siphon tube do not leave any residue when evaporated. The advantage of this method is that large amounts of materials can be extracted with a much smaller quantity of solvent because clean solvent will be recycled in the extractor (Handa *et al*., 2008).

1.2.3 Hydrodistillation

Hydrodistillation is a method used to obtain essential oils or volatile components from plants. Plant materials are soaked in water and heated to carry over steam distillable components (Handa *et al*., 2008). Essential oils and volatiles form an azeotropic mixture with water in the liquid phase. Vapors of the volatiles will then be brought by steam to a condenser. Upon condensation, oil and aqueous layers are produced. They are separated by decantation process. The disadvantages of this method are the exposure of plant materials to high temperature around 100°C and unable to extract non-volatile compounds from plants. Prolonged heating and contact with water will cause hydrolysis of esters and aldehydes or decomposition of thermally sensitive components of plants (Denny, 1989).

1.2.4 Supercritical Fluid (SCF) Extraction

This method uses supercritical fluids as the extracting solvent. A supercritical fluid is any substance at a temperature and pressure above its critical point, where distinct liquid and gas phases do not exist. It can effuse through solids like a gas, and dissolve

materials like a liquid. In addition, close to the critical point, small changes in pressure or temperature result in large changes in density, allowing many properties of a supercritical fluid to be "fine-tuned". Supercritical fluids are suitable as a substitute for organic solvents in a range of industrial and laboratory processes. Extraction is usually from a solid matrix, but can also be from liquids. This extraction technique can be used as a sample preparation step for analytical purposes, on a larger scale to either strip unwanted material from a product (e.g. decaffeination) or collect the desired product (e.g. essential oils) (Handa *et al*., 2008).

Carbon dioxide (CO_2) is the most used supercritical fluid, sometimes modified by co-solvents such as ethanol or methanol. Extraction conditions for supercritical carbon dioxide are above the critical temperature of 31°C and critical pressure of 74 bars. Addition of modifiers may slightly alter this. The properties of a supercritical fluid can be altered by varying the pressure and temperature, allowing selective extraction (Chrastil, 1982).

The main advantages of this technique are time efficient and “cleanliness” of the extract. Extraction is a diffusion-based process, in which the solvent is required to diffuse into the matrix and the extracted material to diffuse out of the matrix into the solvent. Diffusivities are much faster in supercritical fluids than in liquids, and therefore extraction can occur faster. In addition, due to the lack of surface tension and negligible viscosities compared to liquids, the solvent can penetrate more easily into the matrix inaccessible to liquids. An extraction using an organic liquid may take several hours, whereas supercritical fluid extraction can be completed in 10 to 60 minutes. After the extraction, the supercritical fluids will be evaporated, hence there is no extra procedure for removal of solvents (Brunner, 1994).

The disadvantages of this method are its high running cost and limitation of compounds can be extracted. The requirement for high pressures increases the cost compared to conventional liquid extraction, so it will only be used where there are significant advantages. Carbon dioxide itself is non-polar and has somewhat limited dissolving power, so cannot always be used as a solvent on its own, particularly for polar solutes. The use of modifiers increases the range of materials which can be extracted. Food grade modifiers such as ethanol can often be used, and can also help in the collection of the extracted material, but reduces some of the benefits of using a solvent which is gaseous at room temperature (Chrastil, 1982).

1.2.5 Ultrasonic-assisted Methanol Extraction

Ultrasonic-assisted solvent extraction is a simple, relatively cheaper and efficient alternative to conventional extraction methods. Wang and Weller (2006) reported that application of ultrasound in their extraction procedure increased yield and extraction rate. The yield of oil extracted from soybeans also increased significantly when ultrasound was applied (Li *et al*., 2004). A study disclosed that employment of ultrasonic bath is an important pretreatment to gain high yields of oils from almond, apricot and rice bran (Sharma & Gupta, 2004). In another study, ginseng was subjected to ultrasonic-assisted extraction, the total yield and saponin yield increased by 15% and 30%, respectively (Li *et al.*, 1994).

These can be explained by looking into propagation of electromagnetic waves through the extractive solvent which resulting in cavitation phenomena. The controlling mechanism of ultrasonic-assisted extraction is associated with mechanical, cavitation,

and thermal aspects which may lead to disruption of plant cell walls, increasing total surface area to volume ratio and facilitating movement of substances across cell membranes. These lead to compounds of interest being released from plant materials into the solvent, hence increasing extraction yield and rate simultaneously (Shirsath *et al*., 2012).

1.3 Overview of Antioxidant Activities

An antioxidant is a chemical species that prevents the oxidation process of other molecules. The term "antioxidant" is commonly used in two different areas: chemicals which are added to industrial products to prevent degradation due to oxidation, and natural products found in plant or animal bodies which are reported to have beneficial health effects.

In various sectors of industry, antioxidants have been used widely, such as food and cosmetic preservatives and to prevent rubber, plastics or gasoline deterioration. (Dabelstein *et al*., 2007). Oxidation is a reaction that generates free radicals, causing various chain reactions that may harm body cells. To oppose the oxidative state, plants and animals form complex biological systems of antioxidants in their bodies, such as glutathione, enzyme catalase and enzyme superoxide dismutase which are produced *via* metabolism or through food intake to gain dietary antioxidants, such as Vitamin A, Vitamin C, Vitamin E, polyphenols and β-carotene. These antioxidants act as a terminator to end the possible chain reactions caused by free radicals (Stanner *et al.*, 2004). They can be consumed directly as they are present in food or in the form of supplements.

There is an increasing interest in search for safe and effective natural antioxidants from plant origins. In recent years, many different methods have been used for the evaluation of antioxidant power. Most of them are based on the measurement of the relative abilities of antioxidants to scavenge radicals in comparison with the antioxidant potency of a standard antioxidant compound. Several chemical assays have been used to estimate antioxidant capacities in various plants and their products and foods for clinical studies including 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), oxygen radical absorption capacity (ORAC), total radical-trapping antioxidant parameters (TRAP), lipid peroxidation (LPO) inhibition, β-carotene bleaching and the tetrabenzo-[b,f,j,n][1,5,9,13]-tetraazacyclohexadecine copper(II) complex immobilized on silica gel (CuTAAB-SG) (Zaporozhets *et al*., 2004; Gubbuk *et al.*, 2009).

According to their mechanistic pathways, antioxidant assays can be categorized into two main groups: (a) hydrogen atom transfer (HAT) reaction based assays and (b) single electron transfer (ET) reaction based assays. The single ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally involve a synthetic free radical initiator, an oxidizable molecular probe and an antioxidant. HAT- and single ET- based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample (Huang *et al.*, 2005). The antioxidant assays are categorized as shown in Figure 1.7.

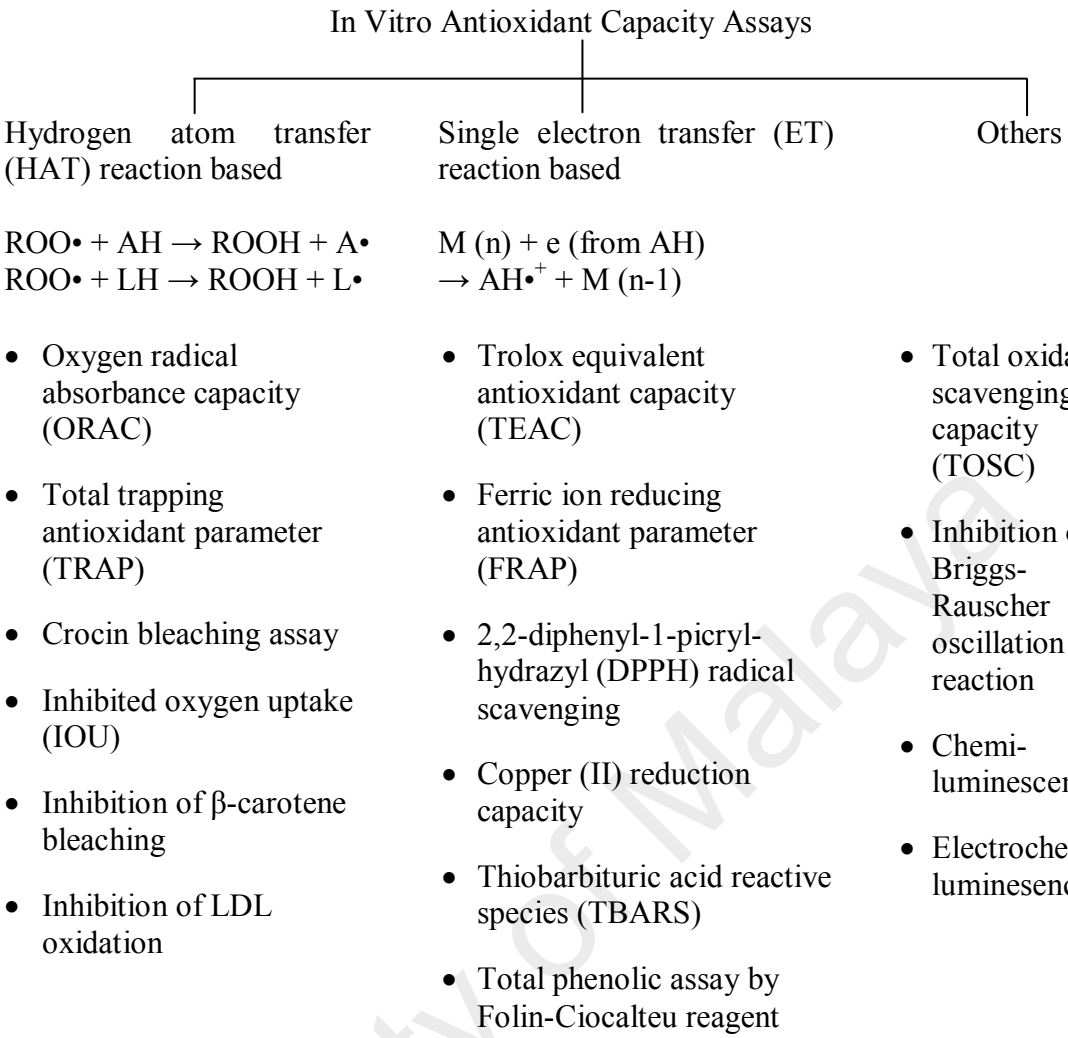
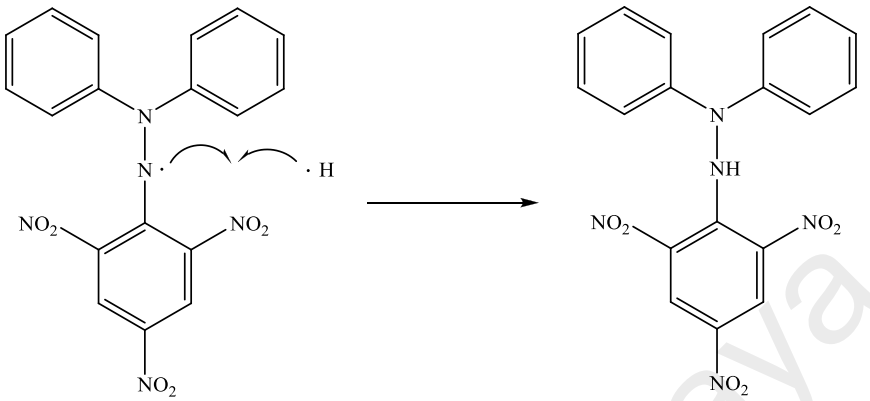


Figure 1.7: *In vitro* antioxidant capacity assays according to their mechanistic pathways

1.3.1 DPPH Radical-scavenging Activity Assay

The molecule of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) is a stable free radical which has the ability to delocalise extra electrons over the whole molecule due to the presence of three benzene rings per molecule in its structure. Therefore, DPPH molecules do not dimerise which occur to most other free radicals. The delocalisation also creates the deep violet colour which portrays an absorption band in methanol solution centred at about 520 nm. As soon as DPPH is mixed with a substance that can donate a hydrogen atom, reduction occurs and the violet colour disappears. However,

the presence of the picryl group will result in a residual pale yellow colour (Brand-Williams *et al.*, 1995). This mechanism is demonstrated in Figure 1.8.



DPPH free radical
Deep violet colour in methanol
Strong absorption at wavelength 517 nm

Reduced DPPH free radical
Pale yellow colour in methanol
Weak absorption at wavelength 517 nm

Figure 1.8: Mechanism of action of DPPH radical scavenging assay

1.3.2 Lipid Peroxidation (LPO) Inhibition Assay

This assay was first proposed in 1958 for the purpose of testing rancidity of food substances as a result of oxidation of lipids (Sinnbhuber, Yu & Yu, 1958). It is currently the most widely used method to measure lipid peroxidation of a certain sample. This assay can be performed by standard method using malondialdehyde (MDA). MDA and other aldehydes have been identified as secondary lipid peroxidation products that react with thiobarbituric acid (TBA) to give a pink coloured product that absorbs spectrophotometrically at 532 nm. There are also several minor variations to the main components used for this assay. Despite its wide use as a test to measure lipid oxidation in food industries, this assay is not specific for lipid peroxidation products. Other than aldehydic products which are produced from lipid peroxidation, many other substances such as alkanals, proteins, sucrose and urea may react with TBA to develop coloured species (Jardine *et al.*, 2002). As a result, this could contribute to an overestimation of

the extent of lipid peroxidation of a particular sample (Jardine *et al.*, 2002). In food samples, ketones, ketosteroids, acids, esters, sugars, proteins, pyridines, pyrimidines and vitamins are some compounds which can react with TBA (Devasagayam *et al*., 2003). However, inorganic phosphate buffer used in this assay, up to 40 mM do not affect the production of colour (Devasagayam *et al.,* 2003).

Autoxidation of unsaturated aldehydes can cause further changes and produce other volatile compounds. Therefore, hydroperoxy aldehydes undergo cleavage to give shorter chain aldehydes with other chemical groups. Among these groups, MDA is of interest. Many precursors of MDA have been proposed, but the most feasible biochemically would be the monocyclic peroxides formed from fatty acids with three or more double bonds (Devasagayam *et al.,* 2003).

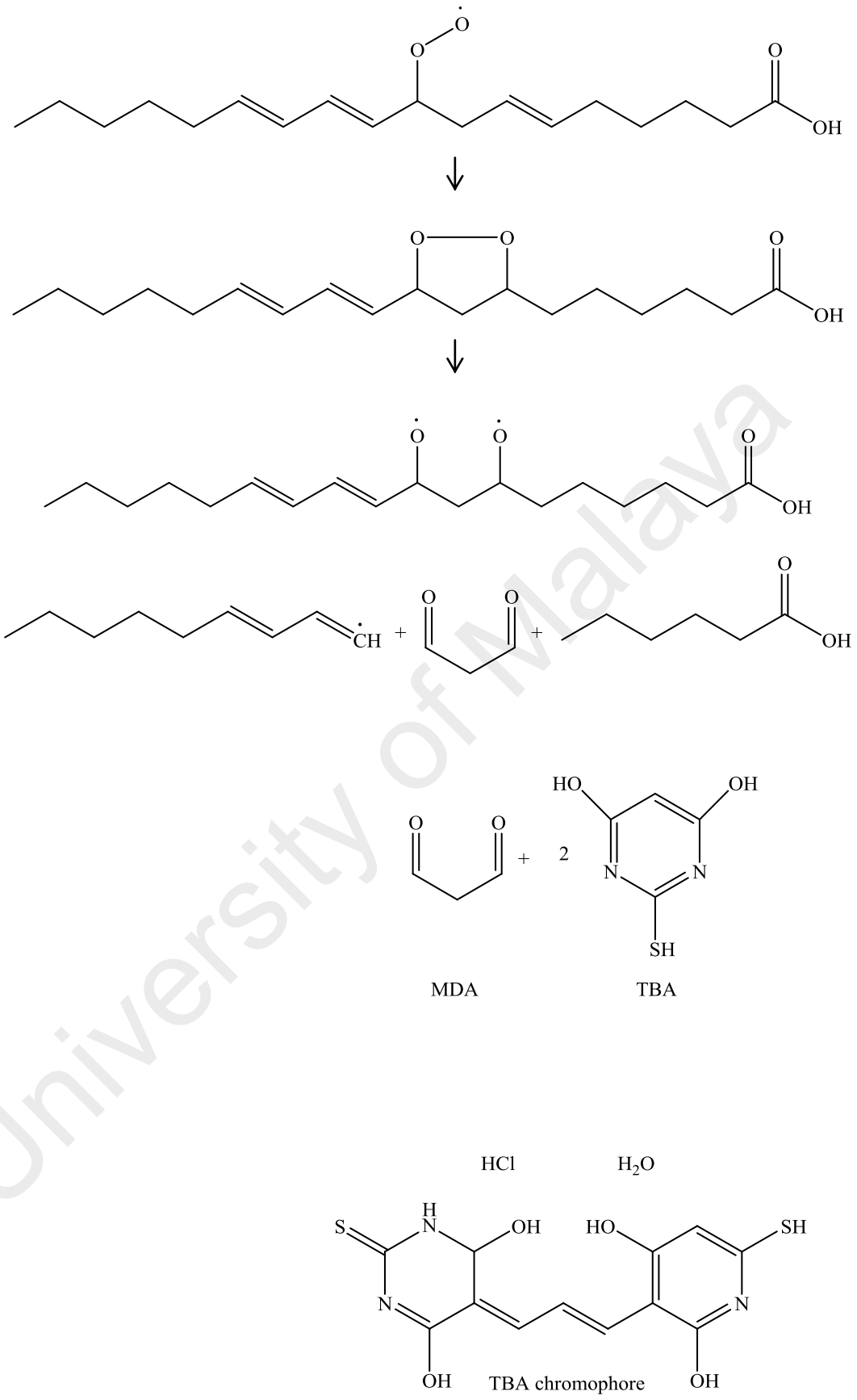


Figure 1.9: Mechanistic action of LPO inhibition assay

1.3.3 β-carotene Bleaching Assay

The β-carotene bleaching assay was first described by Miller (1971) and is one of the antioxidant assays suitable for plant samples. This technique tests the ability of an antioxidant to inhibit lipid peroxidation. This method measures the loss of orange coloration of β-carotene due to its reaction with radicals. These free linoleic acid radicals are formed by the oxidation of linoleic acid due to an abstraction of a hydrogen atom from diallylic methylene groups of linoleic acid (Kumaran & Joel, 2006). These free radicals will then oxidize the highly unsaturated β-carotene. As a result, the orange coloured chromophore of β-carotene would be degraded and the result could be observed spectrophotometrically. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants and was measured by the difference between the initial reading in spectral absorbance at 470 nm at 0 min and after 120 min. The antioxidant activity was expressed as inhibition percentage relative to the positive control.

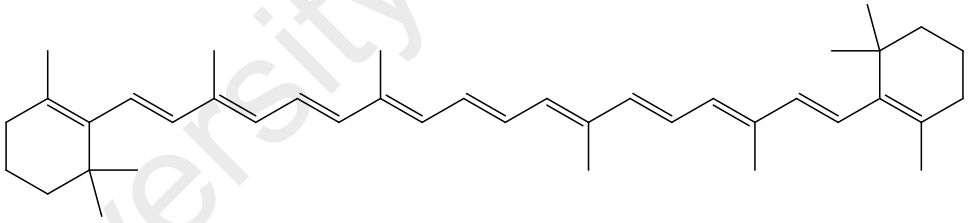


Figure 1.10: β-carotene

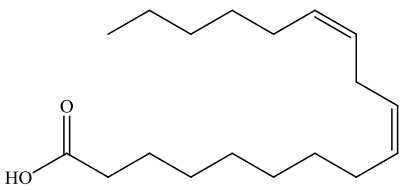


Figure 1.11: Linoleic acid

Although this is one of the most common methods used for assessing antioxidant activity, it has been criticized for many different reasons. Firstly, it has been discussed

that the β-carotene reaction is not representative of the lipid oxidation in food, where the fatty acids are mainly present as triacylglycerides and whose crude extracts can contain interfering materials (Frankel, 1991) and other oxidation modifying agents, which could cause synergistic or antagonistic interactions. These interactions have yet to be studied in depth. Secondly, β-carotene is sensitive to oxygen and temperature even in the absence of linoleic acid (Gloria *et al.*, 1993; Laguerre *et al.*, 2007). In a lipid emulsion, the hydrophobic repulsion phenomena favour the activity of apolar oxidation modifiers against the polar ones (polar paradox) (Frankel *et al*., 1994; Naguib, 2000; Tsuchihashi *et al*., 1995). Finally, the assay reproducibility can be low due to (a) the reagent complexity (Koleva *et al*., 2002), (b) the non-specific conditions of heat induction (Laguerre *et al.*, 2007; Roginsky & Lissi, 2005) which would suggest the use of free radicals (*e.g.* 2,2'-azobis(2-amidinopropane)dihydrochloride (AAPH) or Fe^{2+}) as initiators, (c) the frequent use of a single reaction time which does not guarantee the reliability of the results (Frankel & Finley, 2008; Roginsky & Lissi, 2005) and (d) factors such as pH, solvent ratios, the presence of metals and its effects on the reaction (Dawidowicz & Olszowy, 2010) which could be especially relevant in complex systems.

1.3.4 Total Phenolic Assay by Folin-Ciocalteu Reagent

Folin-Ciocalteu reagent was originally used for the analysis of proteins due to the reagent's activity towards protein tyrosine (containing a phenol group) residue (Folin & Ciocalteu, 1927). Recently, some researchers (Singleton *et al*., 1999) broadened the range of this assay for the analysis of total phenolic content in wine and since then the assay has found many applications (Singleton *et al.,* 1999).

The Folin-Ciocalteu reagent based assay is commonly known as the total phenols (or phenolic) assay. Recent studies show excellent linear correlations between the total

phenolic profiles and antioxidant activity. This was done using total phenolic assay by Folin-Ciocalteu reagent and an electron transfer (ET) based antioxidant capacity assay (*e.g.*, FRAP, TEAC, *etc.*). This was expected considering the similar chemistry between the two assays.

In the present study, the Folin-Ciocalteu reagent was purchased. However, it can be prepared by first boiling a mixture of sodium tungstate ($Na_2WO_4.2H_2O$, 100 g) for 10 hours, sodium molybdate ($Na_2MoO_4.2H_2O$, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL) and water (700 mL). After boiling, lithium sulfate ($Li_2SO_4.4H_2O$, 150 g) is added to the mixture to give an intense yellow colour to the Folin-Ciocalteu reagent solution. Contamination of reductants leads to a green colour, and the addition of oxidants such as bromine can restore the desired yellow colour.

The exact chemical nature of the Folin-Ciocalteu reagent is not known, but it is known to contain heteropoly phosphotungstates-molybdates. Sequence of reversible one- or two-electron reduction reactions will result in a blue species, possibly $(PMoW_{11}O_{40})^{4-}$ (Huang *et al.*, 2005). The molybdenum is easier to reduce in the complex form and electron-transfer reaction occurs between reductants and Mo(VI) (Huang *et al.*, 2005):

$$Mo(VI) + e \rightarrow Mo(V)$$

Folin-Ciocalteu reagent is not specific to phenolic compounds only as it can be reduced by many non-phenolic compounds (*e.g.*, vitamin C, Cu(I), *etc.*) (Huang *et al.*, 2005). Phenolic compounds react with Folin-Ciocalteu reagent only under basic conditions (adjusted to pH ~10 using a sodium carbonate solution). Dissociation of a phenolic proton leads to phenolate anion, which is capable of reducing Folin-Ciocalteu reagent. This supports the concept that the reaction occurs through the electron transfer mechanism. The blue compounds formed between phenolate and Folin-Ciocalteu

reagent are independent of the structure of phenolic compounds, ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds (Huang *et al.*, 2005). Regardless of the undefined chemical nature of Folin-Ciocalteu reagent, this assay is a convenient, simple, and reproducible method to measure the phenolic content of a sample. Therefore, it has become a regular assay in studying phenolic antioxidants.

1.4 Problem Statement

Apart from bacterial contamination, oxidative reactions are the major cause of deterioration of food quality and shelf-life reduction which can easily occur during the processing and storage (Buckley *et al*., 1995). Antioxidants, both natural and synthetic, are used by the food industry as food additives to help prolong the shelf life and appearance of many foodstuffs. In order to reduce this quality loss, the usage of antioxidants is one of the major strategies for preventing lipid oxidation and may be effective in controlling and reducing off-odour development in food products. Current food industries employ synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyl anisole (BHA) and tertiary butyl hydroquinone (TBHQ) which work as preservatives to prevent lipid peroxidation and prolong the storage stability of food. However, side effects of BHT include cell division and allergic reactions which may trigger hyperactivity and asthma. There were also serious concerns over carcinogenic and estrogenic effects when research found that if large doses of BHT and BHA were used, tumours developed in lab animals. Government authorities and consumers are concerned about potential adverse effects of synthetic preservatives on health. According to the U.S. Food and Drug administration (FDA), BHT was patented in 1947 and approved by FDA in 1954 for use in food products such as vegetable oils, lard, fat, margarine, carbonated drinks, cheese spreads, chewing gum, ice cream and dry

breakfast cereal. Due to the side effects, Europe has restricted its use since 1987. BHT was also banned in Japan in 1958 while experts in the UK suggested banning BHT but due to industry pressure, it was not carried out (The UK Food Guide, 2014). Consumer demand is currently in favour of food products that are natural and additive-free which leads to the use of natural antioxidants as an effective preservative to replace synthetic antioxidants. These natural antioxidants can control the rancidity of lipids and the production of off-odour which can then increase the acceptability of food products to consumers. This study extracts the compounds present in *Elaeis guineensis* leaves by using solvent maceration and ultrasonic-assisted solvent extraction methods, then characterizes the compounds by gas chromatography-mass spectroscopy (GC-MS) analysis. This study suggests that *Elaeis guineensis* leaves have a high potential to be utilized as a natural source of antioxidants in palm oil producing countries such as Indonesia, Malaysia and Thailand, where *Elaeis guineensis* leaves is abundantly available.

1.5 Research Objectives

The objectives of this study are:

a) To extract using solvent maceration and ultrasonic-assisted solvent extraction methods then characterize chemical constituents of *Elaeis guineensis* leaves by gas chromatography-mass spectroscopy (GC-MS) analysis
b) To evaluate antioxidant activity of *Elaeis guineensis* leaves extracts by *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, lipid peroxidation (LPO) inhibition and β-carotene bleaching assays
c) To determine total phenolic content (TPC) in *Elaeis guineensis* leaves extracts

CHAPTER TWO : EXPERIMENTAL

2.1 Plant Materials and Chemicals

Fresh fronds of *Elaeis guineensis* were collected from three 10-year old trees in Sime Darby East Plantation, Carey Island, Selangor, Malaysia. On the same day, the middle leaflets of all the plant materials were separated from rachis and subjected to pretreatments prior to extraction. Green and matured leaves were selected, washed and rinsed with distilled water to remove dust and contaminants, then air-dried under shade to evaporate surface moisture. The treated plant materials were cut into small pieces (~2 cm) and pulverized into a coarse powder (≤ 2 mm diameter).

Figure 2.1: Collection point of fresh *Elaeis guineensis* fronds

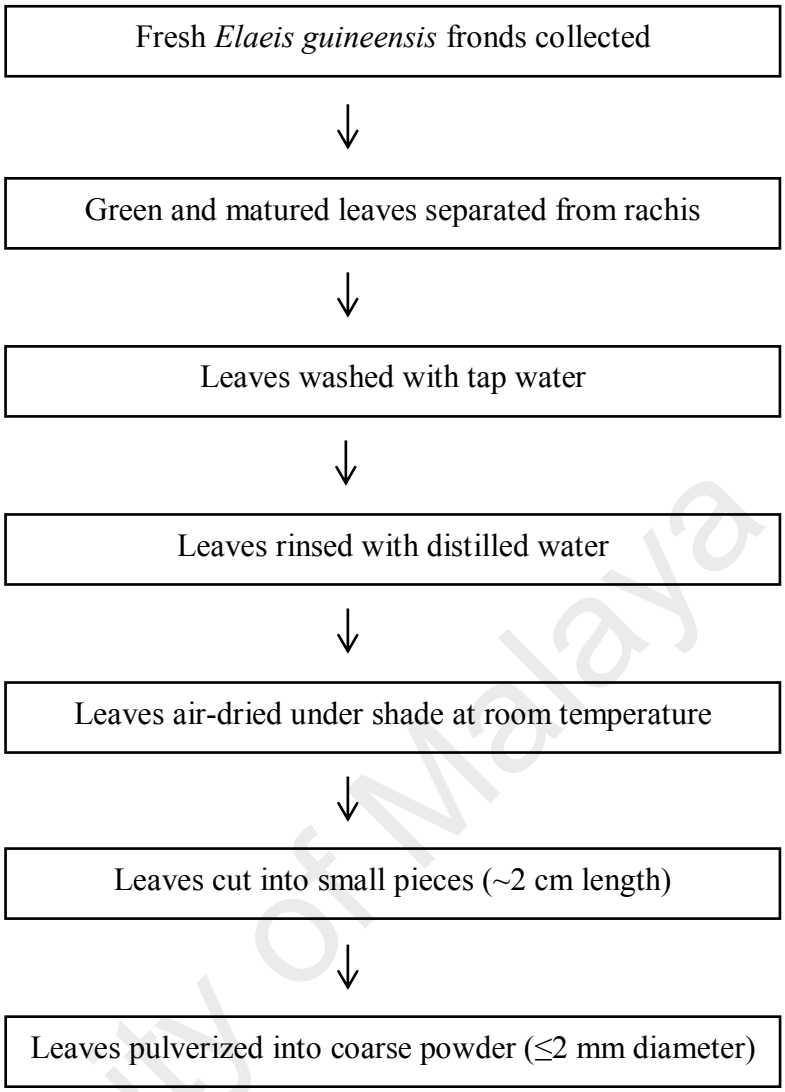


Figure 2.2: Pretreatment of *Elaeis guineensis* leaves

Figure 2.3: Small pieces (~2 cm length) of *Elaeis guineensis* leaves

2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), Tween 80, Folin-Ciocalteu reagent and β-carotene were purchased from Sigma-Aldrich, Co. (St. Louis, USA). Butylated hydroxytoluene

(BHT), iron (II) sulfate ($FeSO_4$), 2-thiobarbituric acid (TBA), trichloroacetic acid (TCA) and HPLC-grade solvents, namely, methanol and n-hexane, were purchased from Merck (Darmstadt, Germany). Sodium sulfate (Na_2SO_4) anhydrous, and analytical grade methanol, *n*-hexane and ethyl acetate used for extraction and partitioning were purchased from Systerm (Shah Alam, Malaysia).

2.2 Extraction of *Elaeis guineensis* Leaves

In the present study, *Elaeis guineensis* leaves were extracted by ultrasonic-assisted extraction and solvent extraction.

2.2.1 Ultrasonic Extraction with Methanol

The pulverized *Elaeis guineensis* leaves (1 kg) were subjected to ultrasonic-assisted extraction (16 kHz, 30 min) using Ultrasonic bath (WiseClean, DAIHAN Scientific Co., Ltd., Korea) with methanol (1L × 1, 500 mL × 3) (Luque & Silva, 1997). The methanolic extract was collected and combined while trace of water was removed using anhydrous sodium sulfate, filtered and the filtrate was evaporated under reduced pressure using a rotary evaporator at 40°C to a dark green and sticky crude methanolic extract (MeOH, 10.9%).

Figure 2.4: Appearance of the crude methanolic extract (MeOH)

2.2.2 Partition of Crude Methanolic Extract

The crude methanolic extract (10.02 g) was then extracted and concentrated in *vacuo* to obtain a brownish green hexane fraction (MeOH-HEX). The hexane insoluble residue was further partitioned using ethyl acetate and water (ratio 1:1) to give a dark greenish brown ethyl acetate fraction (MeOH-EA) and a brown sticky water fraction (MeOH-WATER) after evaporated to dryness under vacuum. The blackish brown insoluble solid remained after partitioning was collected as an insoluble fraction (MeOH-INSOL). The crude methanolic extract (MeOH) and all its fractions were subjected to antioxidant assays. The MeOH-HEX fraction was dissolved in methanol and analyzed using gas chromatography-mass spectrometry (GC-MS).

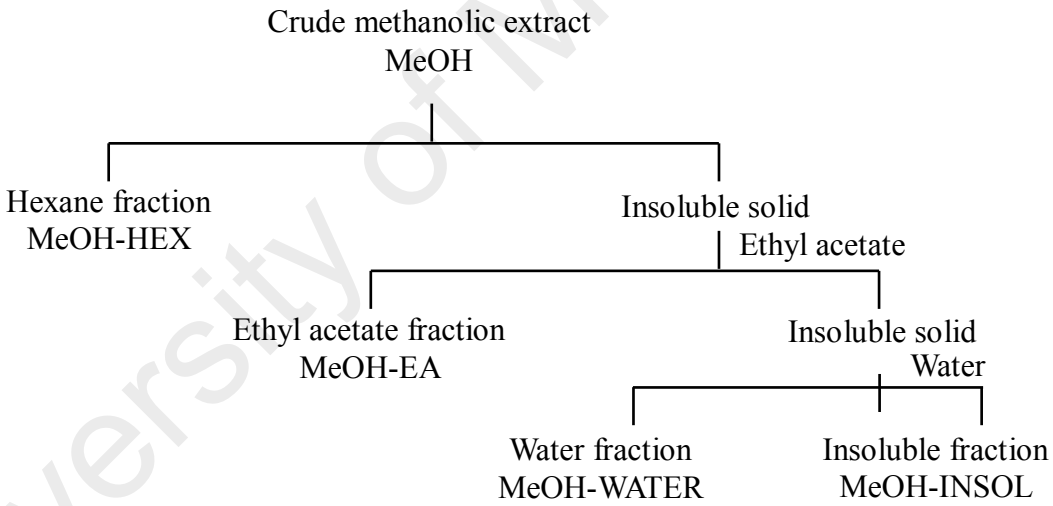


Figure 2.5: Partitioning scheme of the crude methanolic extract (MeOH)

Another portion of the crude methanolic extract (10.64 g) was dissolved in 50 mL methanol and stirred with activated carbon for 5 minutes, and then the mixture was filtered by suction. The filtrate was concentrated in *vacuo* to obtain a dark brown solid (MeOH-Activated C). This solid was dissolved in methanol and analyzed using GC-MS.

2.2.3 Maceration

The pulverized *Elaeis guineensis* leaves (10.20 g and 10.45 g respectively) were extracted by maceration with 50 mL of 99.8% *n*-hexane and ethyl acetate respectively, at room temperature for 72 hours. Maceration with *n*-hexane produced Maceration-HEX and maceration with ethyl acetate produced Maceration-Et_2O. The mixture was then filtered through cotton wool, dried over sodium sulfate and passed through a short colum silica gel column (10 cm length × 0.5 cm diameter). Then the eluate was analysed using GC-MS.

2.3 Preliminary Biological Screening Test

The extracts and fractions were spotted on TLC plates and developed in appropriate solvent systems, dried and sprayed with 0.2% DPPH in methanolic solution. The plates were examined for 5 minutes after spraying. Active extracts and fractions appeared as yellow spots against the purple background.

2.4 Biological Activity Evaluation

Several antioxidant activity assays were carried out to evaluate the antioxidant capacity of methanolic extract (MeOH) from *Elaeis guineensis* leaves and its partitioned fractions. These assays include *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, lipid peroxidation (LPO) inhibition and β-carotene bleaching assays. Total phenolic contents (TPC) in the fractions were determined with Folin-Ciocalteu reagent.

2.4.1 DPPH Radical-scavenging Activity Assay

Samples were dissolved in methanol and diluted to various concentrations. DPPH radical-scavenging activities were determined using spectrometric method as described

(Brand-Williams *et al.*, 1995) with some modifications on volumes in proportion. 96-well microplate was used for this assay. BHT was used as a standard reference. 50 µL of sample solution was mixed with 50 µL of 200 µM DPPH solution in methanol. The DPPH free radical was prepared freshly before analysis and protected from light throughout the analysis. Absorbance was measured during analysis to confirm the stability of the free radical. The mixture was incubated for 30 min at room temperature in dark. The absorbance was measured in microplate-reader at 517 nm, by using OASYS UVM 340, Austria. The DPPH radical-scavenging activity was calculated using the formula as follow:

$$\text{DPPH radical-scavenging activity (\%)} = [(A_C - A_S)/A_C] \times 100 \quad (2.1)$$

where A_C is the absorbance of the control at t = 30 min and A_S is the absorbance of the sample at t = 30 min.

2.4.2 Lipid Peroxidation (LPO) Inhibition Assay

Thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed using egg yolk homogenates as lipid-rich media, as described (Ruberto & Baratta, 2000). The samples were dissolved in methanol and diluted to various concentrations. Egg homogenates were prepared by adding 1.25 g egg yolk in 100 mL phosphate buffer in water, which consists of 1.52 g Na_2HPO_4, 0.58 g NaH_2PO_4, 0.85 g NaCl and 1 L distilled water. The phosphate buffer was adjusted to pH 7.4 prior to addition of egg yolk. 0.1 mL of the sample solution was added to 1 mL buffered egg yolk in glass centrifuge tube, and then 0.2 mL 3 mM $FeSO_4$ solution was added to induce lipid peroxidation. The reaction mixture was kept in dark for 1 h at room temperature. After incubation, 0.5 mL of 15% TCA aqueous solution was added

immediately to terminate lipid peroxidation and prevent any further reaction. Then, 1 mL of 1% TBA aqueous solution was added to form a pinkish complex as malondialdehyde was formed. The mixture was stirred with a vortex mixer and incubated in water bath at 95°C for 10 min to ensure completion of the reaction. After cooling to room temperature, the mixture was centrifuged at 3,500 rpm for 10 min. 200 µL of the supernatant was transferred into a 96-well plate and absorbance at 532 nm was measured.

$$\text{LPO inhibition activity (\%)} = [(A_C - A_S)/A_C] \times 100 \quad (2.2)$$

where A_C is the absorbance of the control at t = 1 h and A_S is the absorbance of the sample at t = 1 h.

2.4.3 β-carotene Bleaching Assay

The coupled oxidation of *beta*-carotene and linoleic acid was determined using the method described (Chew *et al.*, 2008). An emulsion was prepared by adding 0.02 mL linoleic acid and 0.2 mL Tween 80 to the 1 mL *beta*-carotene solution in chloroform (100 µg mL^{-1}). The chloroform was then evaporated and oxygen-saturated ultrapure water was added to the residue. The *beta*-carotene/linoleic acid emulsion was shaken vigorously and 200 µL of this emulsion were added to 8 µL sample solutions of various concentrations in 96-well plate. The absorbance was read at 470 nm immediately after the emulsion was prepared (t = 0 min), the mixture was incubated at 50 °C and absorbance was measured every 20-min interval for 2 h.

$$\beta\text{-}carotene \text{ bleaching inhibition activity (\%)} = [(A_{S(120)} - A_{C(120)})/(A_{C(0)} - A_{C(120)})] \times 100 \quad (2.3)$$

where $A_{S(120)}$ is the absorbance of the sample at t = 2 h, $A_{C(120)}$ is the absorbance of the control at t = 2 h and $A_{C(0)}$ is the absorbance of the control at t = 0 min.

2.4.4 Total Phenolic Content (TPC) by Folin-Ciocalteu Reagent

TPC in fractions was determined following the Folin-Ciocalteu procedure (Kähkönen, *et al.,* 1999). All the fractions and BHT were dissolved in 50% methanol and prepared in various concentrations. In each well of a 96-well microplate, the sample (10 µL, in triplicate) was mixed with 150 µL of water and 30 µL of Folin-Ciocalteu reagent. After 6 minutes, 75 µL of 7% (w/v) sodium carbonate was added. The mixture was kept in the dark for 2 h before measuring the absorbance at 765 nm in microplate-reader, using TECAN Infinite® M200, Switzerland. A calibration curve was constructed using gallic acid and results were expressed as mg gallic acid equivalents (mg GAE/g).

2.4.5 Statistical Analysis

The tests were performed in triplicate for each independent sample to be analyzed. All data were expressed as mean ± standard deviation. Correlation between the TPC and the antioxidant activities was determined using Pearson Product Moment Correlation and Spearman Rank Order tests. $P < 0.050$ was considered statistically significant. Microsoft Excel 2007 (Roselle, IL, USA) was used for the statistical and graphical evaluations.

2.5 Identification of Compounds from *Elaeis guineensis* Leaves

A Hewlett Packard HP 6890 series mass selective detector linked to GCMS-QP 2010 Plus Shimadzu gas chromatograph was used for the identification of chemical constituents. A sample volume of 2 µL was injected in a splitless mode into the gas chromatograph fitted with a DB-5ms column coated with 5% phenyl 95% dimethyl arylene siloxane with film thickness of 0.25 µm, length 30.0 m and a diameter of 0.25

mm. Helium was used as a carrier gas with a flow rate of 58.2 mL per minute. The injector temperature was set at 250°C. An electron impact mass spectrum was recorded in the 50-600 amu mass range. An electron ionization system was adopted with ionization energy of 70 eV.

For Maceration-HEX and Maceration-Et_2O extracts, the initial oven temperature was set at 40°C and held for 1 minute. The temperature was then increased to 280°C at 5°C per minute and held for 20 minutes.

For MeOH-HEX and MeOH-Activated C extracts, the initial oven temperature was set at 100°C. The temperature was then increased to 290°C at 3°C per minute and held for 10 minutes.

The compounds were identified by comparing their mass spectra with NIST library. The calculation of peak area percentage was carried out by comparing the Total Ion Chromatograph (TIC).

CHAPTER THREE : RESULTS AND DISCUSSION

According to the preliminary screening test results of scavenging effects on DPPH radical of *Elaeis guineensis*, the methanolic crude extract of the leaves of this plant showed impressive activity. Further investigations were conducted to analyse its chemical constituents and bioactivities.

3.1 Extraction and Partition Yields

The leaves were subjected to pre-treatment then methanol extraction facilitated with ultrasonic bath as described in Chapter Two. The methanolic crude extract (MeOH) obtained is a dark green and sticky solid. Then, the MeOH was partitioned using appropriate solvents of different polarities according to the procedure described in Chapter Two. The results of extraction and partition are presented in Figure 3.1. The colour and appearance of the extract and fractions are presented in Table 3.1.

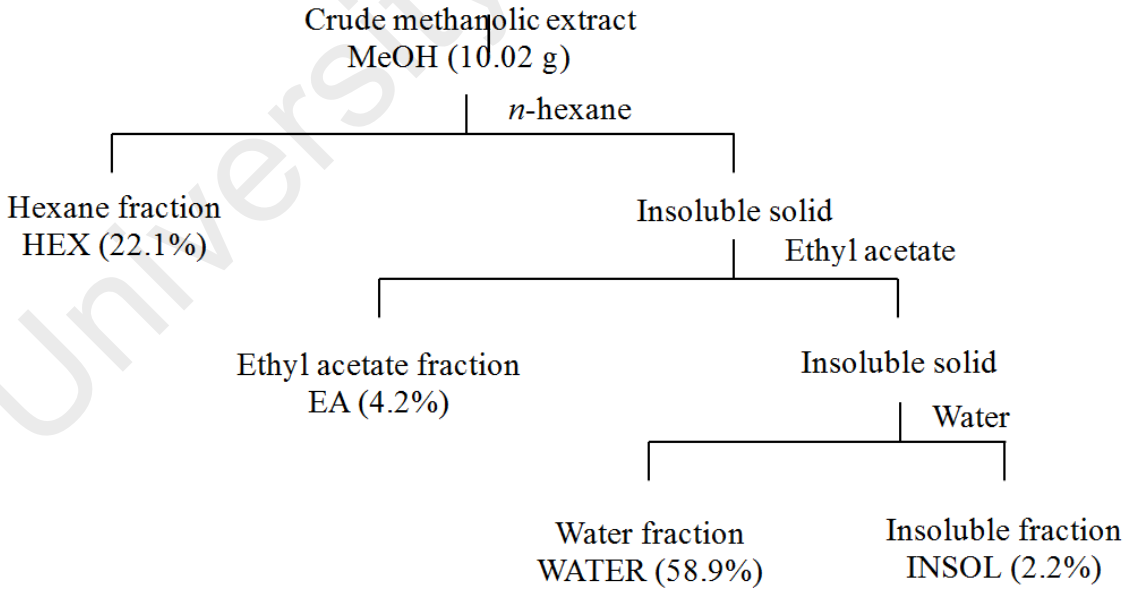


Figure 3.1: Extraction and partition yield of methanolic crude extract

Table 3.1: Colour and appearance of fractions partitioned from crude methanolic extract

Partitioning solvent	Fraction name	Colour and appearance
n-Hexane	HEX	Dark green oily solid
Ethyl acetate	EA	Greenish black sticky solid
Water	WATER	Brownish black sticky solid
Insoluble	INSOL	Black solid

3.2 Preliminary biological screening test

Radical-scavenging ability of the crude extract and its fractions was observed using TLC autographic assay described in Chapter Two. The preliminary results of radical-scavenging effects are presented in Table 3.2.

Table 3.2: DPPH radical-scavenging effect of *Elaeis guineensis* leaves methanolic crude extract and its fractions

Fractions	DPPH radical-scavenging activity based on TLC autographic
MeOH	+++
HEX	+
EA	++
WATER	++
INSOL	+++

Note:

+++: strong activity (colour changed immediately)

++: moderate activity (colour change detected after 1 to2 minutes)

+: weak activity (colour change detected after 2 to 5 minutes)

The fractions were dissolved in methanol (~10 mg/mL), then they were spotted on TLC silica gel $60F_{254}$. The plates are developed in different mobile phase systems:

- Chloroform-methanol-ethyl acetate-water (16.2:18.8:52:3 v/v)
- 2-propanol-acetone-water (70:10:20 v/v)
- Benzene-acetic acid (1:1 v/v)

Then the plates were air-dried and sprayed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) methanolic solution (200 μM). The active substances were seen as yellow spots on a purple background. Table 3.2 demonstrates that all the fractions exhibited radical-scavenging effect against DPPH. Hence, all the fractions were subjected to biological activity evaluations for further investigations.

3.3 Biological Activity Evaluation

3.3.1 DPPH Radical-scavenging Activity Assay

DPPH in its radical form has a deep violet colour with a strong absorbance at 517 nm. In the presence of antioxidant compounds, electron or hydrogen atom will transfer from the antioxidant to DPPH radical and a pale brownish yellow solution is formed. The change of absorbance values at 517 nm is proportional to the reduction level of DPPH radicals. In this assay, the methanolic DPPH solutions were incubated with palm leaves fraction at different concentrations to determine its antioxidant activity. Figure 3.2 and Figure 3.3 shows the DPPH radical-scavenging ability decreasing in the order INSOL > BHT > MeOH > EA > WATER > HEX. The INSOL fraction was the most potent radical scavenger among these fractions and showed stronger radical-scavenging ability than the positive control BHT. The DPPH assay is usually categorized as an electron transfer reaction; the free radical in fact may be reduced either by electron transfer or by H atom transfer. Actual mechanisms are difficult to interpret without detailed information about the composition and structures of antioxidants being tested in the present study. Figure 3.2 and Figure 3.3 show that oil palm leaves MeOH and its

fractions possess comparable radical-scavenging power with that of the synthetic antioxidant BHT.

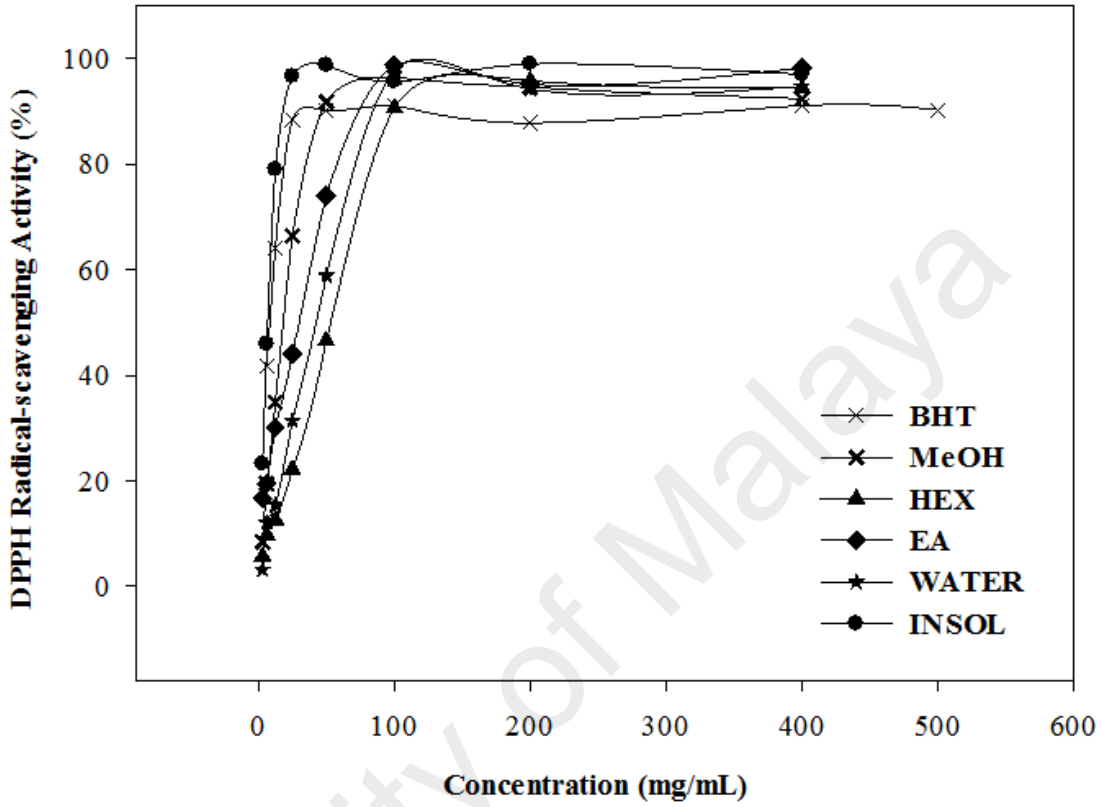


Figure 3.2: Scavenging effect of various extracts at a range of concentrations on the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), in comparison to standard reference BHT

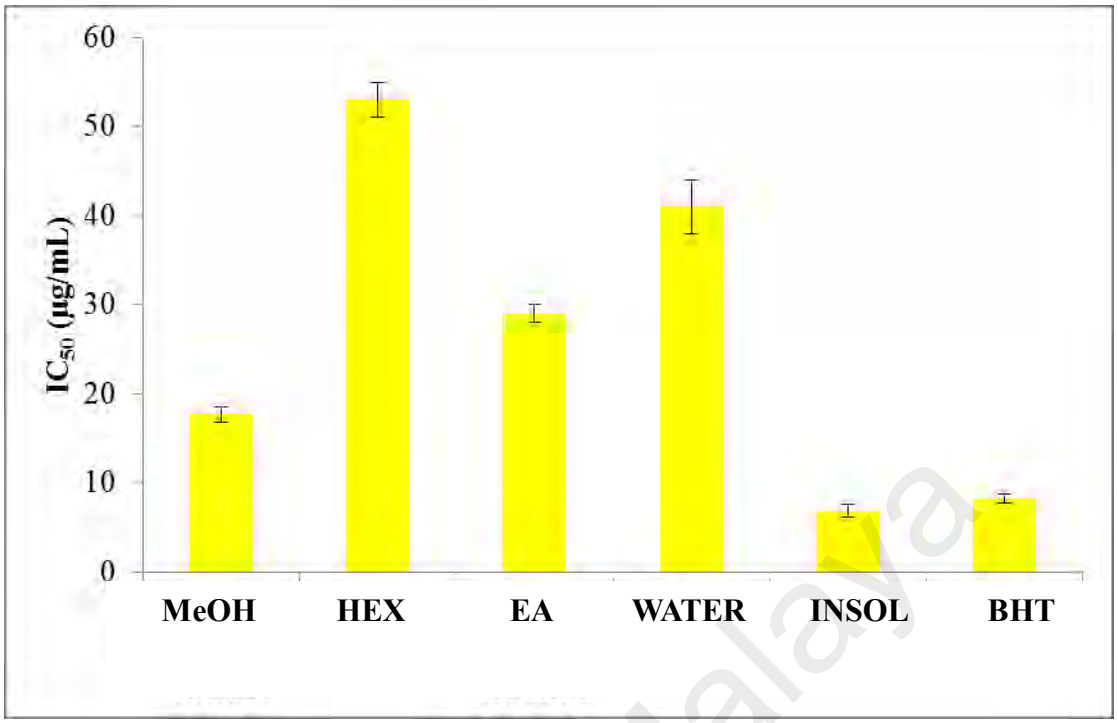


Figure 3.3: IC_{50} value from scavenging effect of methanolic extract and its fractions on the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), in comparison to standard reference BHT

3.3.2 Lipid Peroxidation (LPO) Inhibition Assay

This method is an in vitro LPO induced by Fe^{2+} from $FeSO_4$ aqueous solution. Transition metal ions Fe^{2+} provoked LPO by reductive cleavage of endogenous lipid peroxides (ROOH) of phospholipids in plasma membrane (Repetto, Ferrarotti & Boveris, 2010). ROOH are unstable and decompose to form various compounds, which include MDA, a reactive carbonyl compound, naturally occurring product of LPO. It is frequently used as an indicator of oxidative stress at cellular level. In our study, MDA produced from Fe^{2+}-induced oxidation of polyunsaturated fatty acids in egg homogenates, reacted with two molecules of thiobarbituric acid (TBA) to yield a pinkish red complex with strong absorption at 532 nm. This method measures the intensity of pink chromophore generated in reaction of TBA with MDA. In the presence of antioxidant compounds, rate of polyunsaturated fatty acids oxidation can be

diminished, and hence less MDA being produced, generating less pinkish red complex, gave rise to low absorbance at 532 nm. The absorbance values at 532 nm provide a measure of the extent of LPO. As shown in Figure 3.3, the EA, MeOH, HEX and WATER fractions have similar inhibitory power. The LPO inhibition decreased in order BHT > EA > MeOH > HEX > INSOL > WATER. This result indicates that the polar water fraction has the lowest LPO inhibition among all the fractions and BHT tested. We believe this is due to compounds extracted using water have low solubility in the egg homogenates system, creating a hindrance to intimate contact between polar antioxidant compounds and less polar polyunsaturated fatty acids.

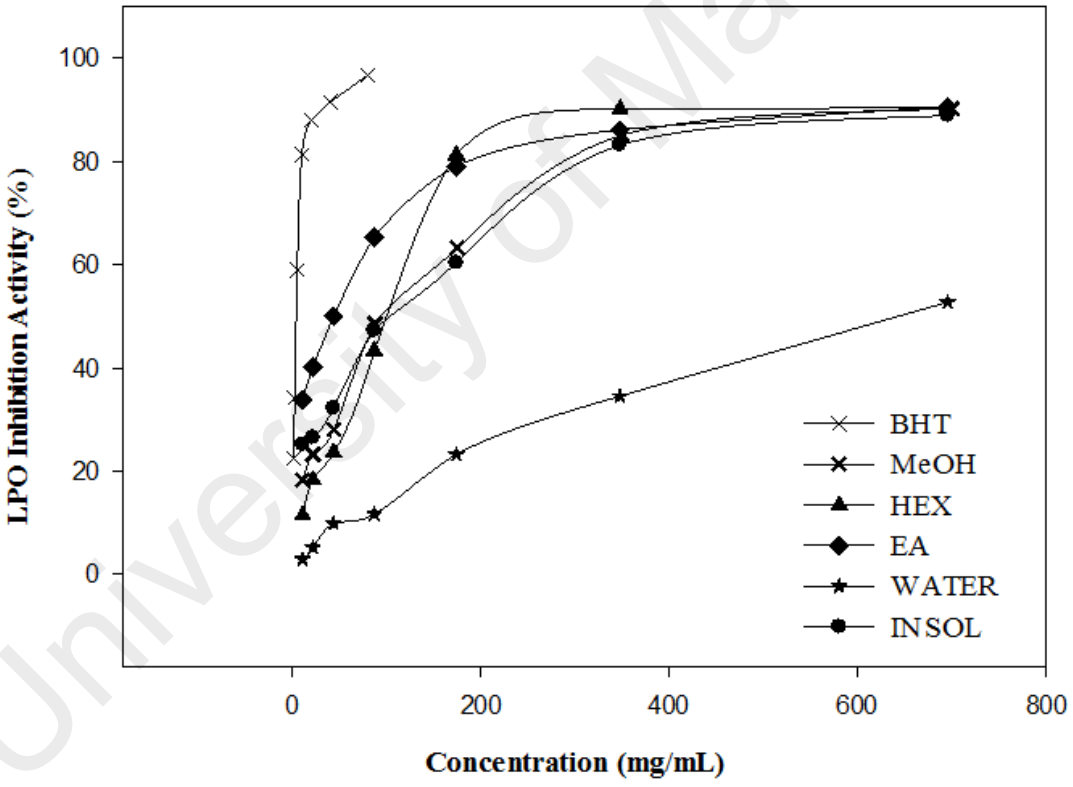


Figure 3.4: Inhibition of Fe^{2+} induced lipid peroxidation by various extracts at a range of concentrations compared to standard reference BHT

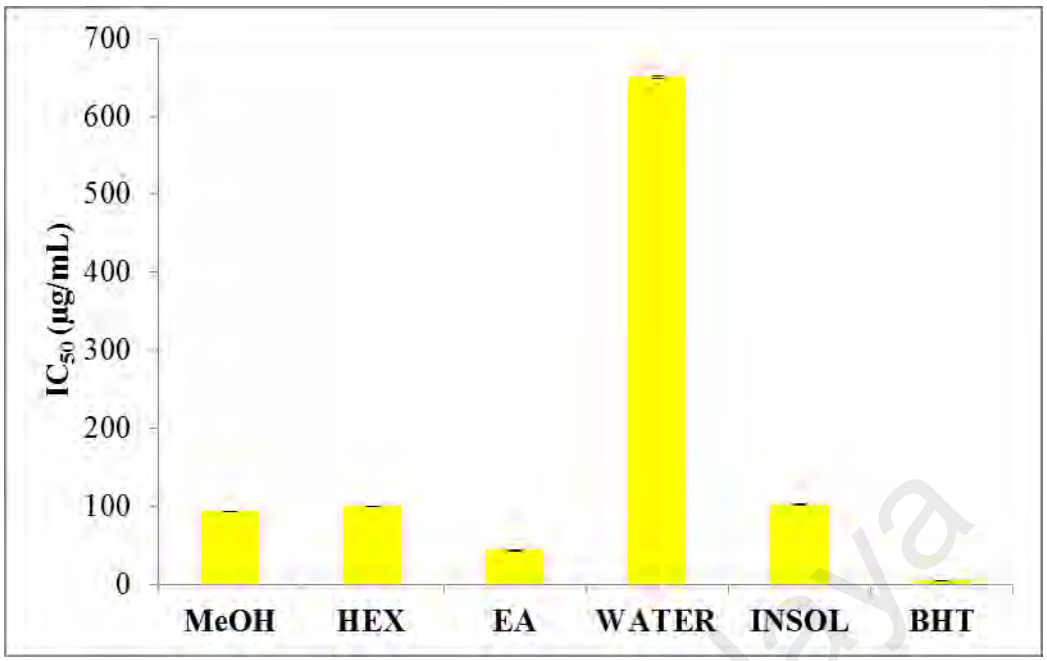


Figure 3.5: IC_{50} value from inhibition of Fe^{2+} induced lipid peroxidation of methanolic extract and its fractions, in comparison to standard reference BHT

3.3.3 β-carotene Bleaching Assay

In this assay, an emulsion of linoleic acid was oxidized by oxygen-saturated ultrapure water to form radicals. The radicals produced upon the abstraction of a hydrogen atom from one of its methylene groups attacked the β-carotene molecules, which lost the double bonds hence its characteristic orange colour at 470 nm. The presence of antioxidants can slow down the rate of β-carotene bleaching. The rate of β-carotene bleaching was measured by the difference between the initial absorbance reading in 470 nm at time 0 and after 2 h. The antioxidant activity was expressed as percent inhibition relative to the control. It can be observed from Figure 3.5 that the β-carotene antioxidant activity increased with increasing concentration of the samples used. The control without addition of antioxidant decolourized at the highest rate and decreasing rate were observed in the presence of WATER > HEX > MeOH > EA > INSOL > BHT. Similar to LPO inhibition assay, this result shows that the polar water fraction has the lowest activity among all the fractions and BHT tested. Linoleic acid used is polyunsaturated

omega-6 fatty acid, which is also susceptible for LPO. Water-soluble polar antioxidant compounds in water fraction are of lower efficiency in protecting β-carotene of low polarity as compared to antioxidant compounds in other fractions.

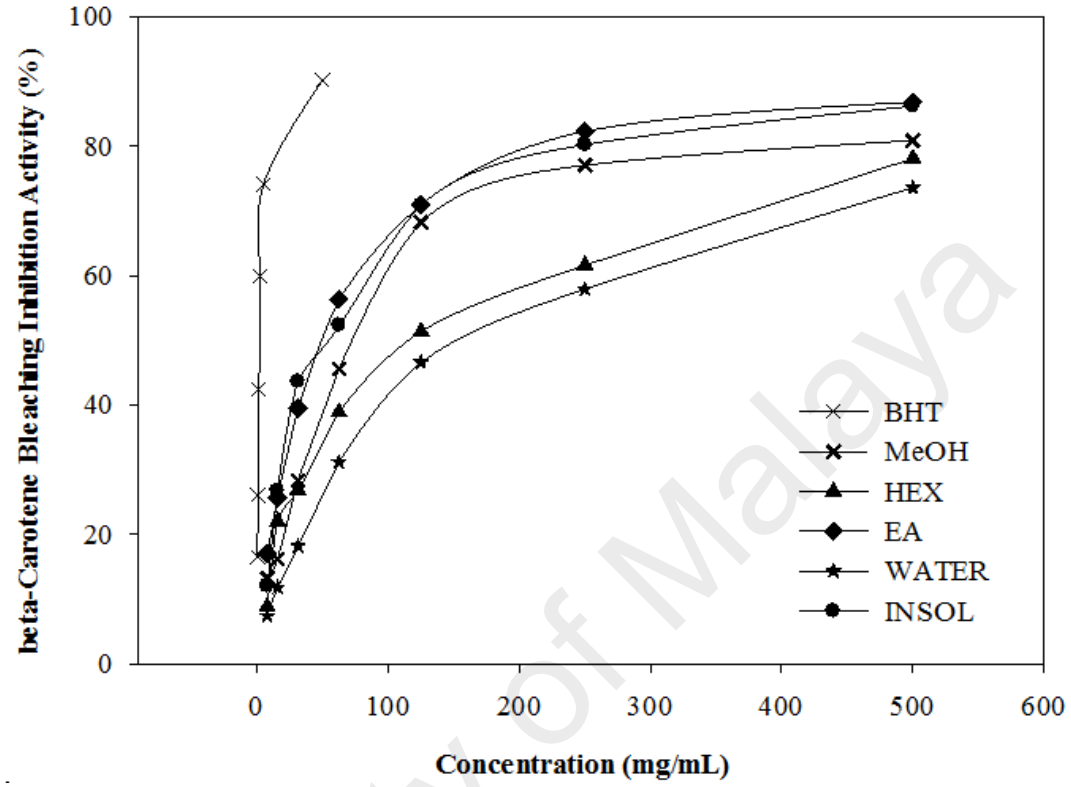


Figure 3.6: Inhibition of β-carotene bleaching by various extracts at a range of concentrations and standard reference BHT

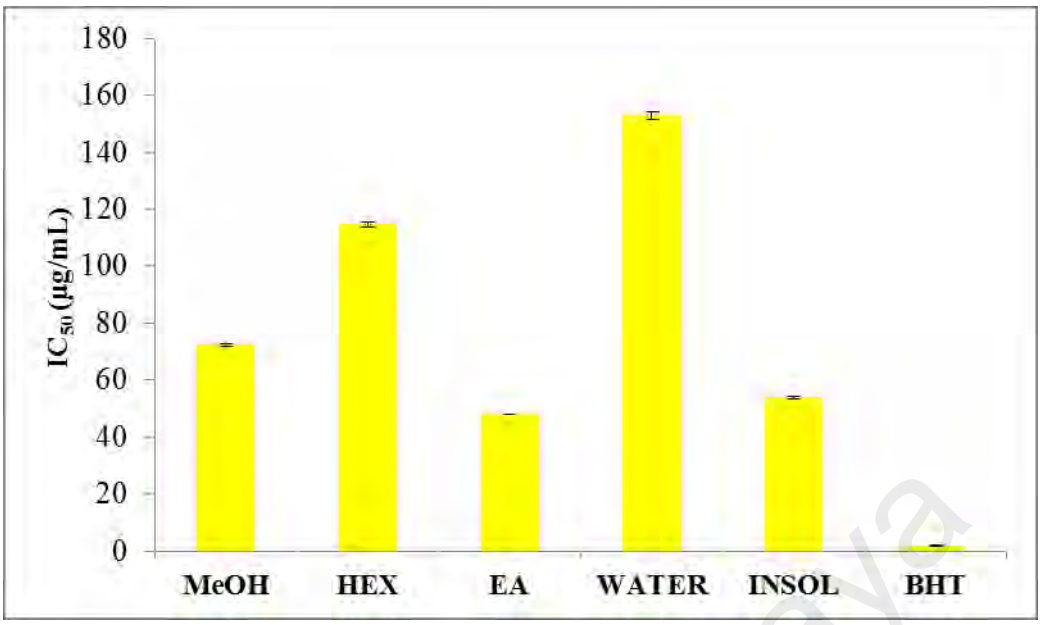


Figure 3.7: IC_{50} value inhibition of β-carotene bleaching of methanolic extract and its fractions, in comparison to standard reference BHT

3.3.4 Total Phenolic Content (TPC) by Folin-Ciocalteu Reagent

A calibration curve of gallic acid as shown in Figure 3.8 was used to determine total phenolic content in the extracts. Naturally occurring phenolic compounds are secondary metabolites in the plants and have received great attention as potential natural antioxidants in terms of their abilities to act as radical scavengers, reducing agents, hydrogen donors, singlet oxygen quenchers, hydroxyl radical quenchers and metal chelators. They are synthesized by plants to resist external stress. It has been disclosed that light induces the synthesis of flavonoids (Dixon & Paiva, 1995) and they are strongly believed to protect plants against UV-B damage and cell destruction by preventing DNA from dimerization and breakage (Strack, 1997). Being a maritime country close to the equator, Malaysia naturally has abundant sunshine and thus solar radiation. On average, Malaysia receives about 6 hours of sunshine per day. This partially explains why *Elaeis guineensis* leaves have generally increased accumulation of antioxidants and phenolic compounds as tabulated in Table 3.3.

Figures 3.2, 3.4 and 3.6 suggest that the antioxidant activities of all the fractions and standard reference BHT was dose-dependent in DPPH, LPO inhibition and beta-carotene bleaching assays. In our study, we find that the DPPH assay is the fastest and least tedious way to evaluate radical-scavenging ability and estimate antioxidant activity. In the present study, all samples and DPPH were completely miscible in methanol. We suggest that this assay is the most suitable and representative for evaluating the antioxidant activity of our extract, fractions and BHT. It also showed high reproducibility which is in agreement with an earlier report (Thaipong, *et al.,* 2006).

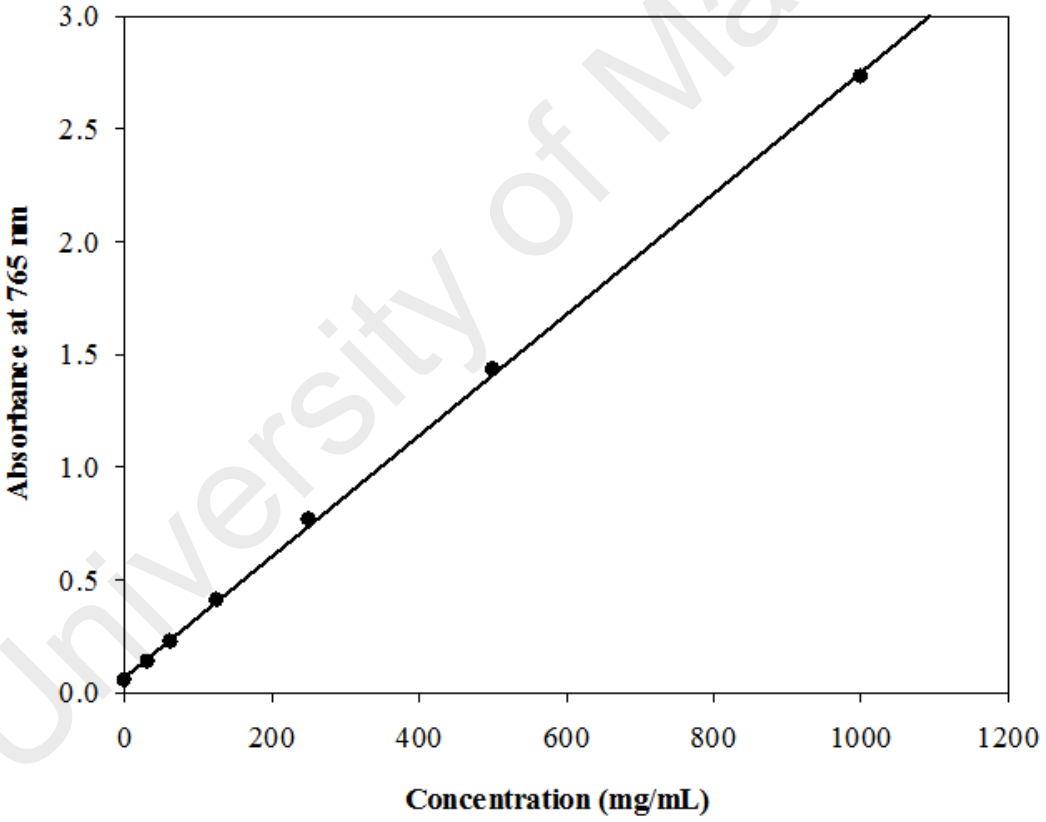


Figure 3.8: Calibration curve of gallic acid to determine total phenolic content in the extracts ($R^2 = 0.9996$)

Table 3.3: Half maximal inhibitory concentration, IC_{50} values and total phenolic contents of the fractions and BHT

Fractions	Half Maximal Inhibitory Concentration, IC_{50} (µg/mL)			TPC (mg GAE/g)
	DPPH	LPO	BCB	
MeOH	17.73 ± 0.04	93.20 ± 0.39	72.38 ± 0.55	63.67 ± 0.14
HEX	53.12 ± 0.18	100.34 ± 0.10	114.72 ± 0.82	16.96 ± 0.13
EA	29.05 ± 0.22	43.24 ± 0.45	47.86 ± 0.02	44.03 ± 0.03
WATER	41.22 ± 0.36	650.01 ± 1.29	152.96 ± 1.26	61.13 ± 0.28
INSOL	6.87 ± 0.11	101.45 ± 0.82	53.93 ± 0.49	118.44 ± 0.09
BHT	8.33 ± 0.07	3.91 ± 0.21	1.78 ± 0.33	116.22 ± 0.04

Note:

Results are means of three independent samples analysed in triplicate ± standard deviation

Results are expressed as mg of gallic acid equivalents/g sample

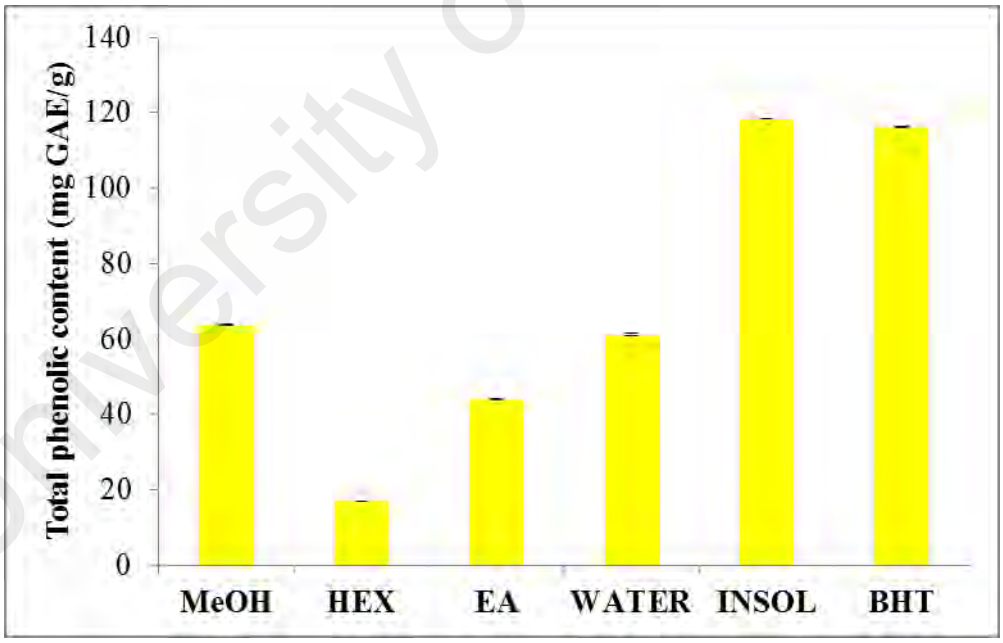


Figure 3.9: Total phenolic contents of methanolic extract and its fractions, in comparison to standard reference BHT

3.3.5 Comparison of Antioxidant Activity Evaluation Assays

Total phenolic contents of methanolic extract and its fractions, in comparison to standard reference BHT are presented in Figure 3.9. Statistical analyses on the correlation between TPC and antioxidant activities showed good negative correlation between TPC with DPPH based on both Pearson Product Moment Correlation and Spearman Rank Order Correlation as shown in Table 3.4 and Table 3.5 respectively. The pairs of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases. For pairs with P values greater than 0.050, there is no significant relationship between the two variables. These data suggest that phenolic compounds in *Elaeis guineensis* leaves are powerful radical scavengers. A similar trend was observed in previous studies (Kumar *et al.*, 2008). Analysis on the TPC with LPO inhibitory activity and TPC with beta-carotene bleaching inhibitory activity had very poor correlations, implying that different mechanisms of antioxidant actions in OPF fractions. The difference in the correlations between TPC and antioxidant assays suggests there are different groups of phenolic compounds corresponding to different assays for the determination of the antioxidant properties, this concurs with previous studies (Khoudja *et al.*, 2014; Prior *et al.*, 2005; Stagos *et al.,* 2012).

Table 3.4: Correlation coefficients and P values for bioassays and TPC of the extracts and BHT based on Pearson Product Moment Correlation

Pairs of variables tested		IC_{50} (µg/mL)			TPC (mg GAE/g)
		DPPH	LPO	BCB	
IC_{50} (µg/mL)	DPPH	-	0.444[a] 0.378[b]	0.789[a] 0.0624[b]	-0.896[a] 0.0158[b]
	LPO	-	-	0.809[a] 0.0511[b]	-0.161[a] 0.761[b]
	BCB	-	-	-	-0.605[a] 0.203[b]
TPC (mg GAE/g)		-	-	-	-

[a]Correlation coefficient

[b]P value

The pairs of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases. For pairs with P values greater than 0.050, there is no significant relationship between the two variables.

Table 3.5: Correlation coefficients and P values for bioassays and TPC of the extracts and BHT based on Spearman Rank Order Correlation

Pairs of variables tested		IC_{50} (µg/mL)			TPC (mg GAE/g)
		DPPH	LPO	BCB	
IC_{50} (µg/mL)	DPPH	-	0.257[a] 0.658[b]	0.657[a] 0.175[b]	-0.943[a] 0.0167[b]
	LPO	-	-	0.829[a] 0.0583[b]	-0.0286[a] 1.000[b]
	BCB	-	-	-	-0.429[a] 0.419[b]
TPC (mg GAE/g)		-	-	-	-

[a]Correlation coefficient

[b]P value

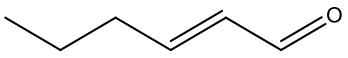
The pairs of variables with positive correlation coefficients and P values below 0.050 tend to increase together. For the pairs with negative correlation coefficients and P values below 0.050, one variable tends to decrease while the other increases. For pairs with P values greater than 0.050, there is no significant relationship between the two variables.

3.4 Analysis of Chemical Constituents of *Elaeis guineensis* Leaf

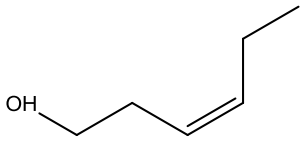
In this study, the leaves of *Elaeis guineensis* were investigated for their chemical constituents. The chemical constituents of *Elaeis guineensis* obtained by various extraction techniques were identified using GC-MS. In this study, only fraction obtained from ultrasonic-assisted methanolic extraction, followed by *n*-hexane partitioning was subjected to GC-MS analysis, the MeOH crude extract and its other fractions (EA, WATER and INSOL) were not analysed due to the presence of highly polar large molecules which are not suitable to be analysed by GC-MS.

3.4.1 Gas-Chromatography-Mass Spectrometry (GC-MS) analysis

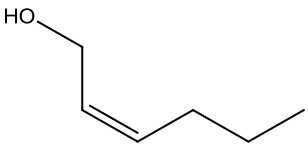
There are 16 compounds in total identified from the extracts of *Elaeis guineensis* leaves. Ultrasonic-assisted methanolic extraction, followed by *n*-hexane solvent partitioning, yielded MeOH-HEX extract. The main components in MeOH-HEX are phytol (42.94%), *alpha*-tocopherol (24.97%), squalene (14.95%), *beta*-sitosterol (9.39%) and stigmasterol (5.84%) (see Table 3.6). The MeOH-Activated C extract yielded 2,4-di-*tert*-butylphenol (30.88%), phytol (23.80%), methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (13.49%), 16-heptadecenal (11.72%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (7.80%) as shown in Table 3.7. Maceration-HEX yielded *alpha*-tocopherol (30.51%), squalene (24.23%), arundoin (21.73%), 3-hexen-1-ol (5.31%) and *beta*-sitosterol (4.77%) as tabulated in Table 3.8. Maceration-Et_2O yielded similar components with Maceration-HEX in slightly different quantities as shown in Table 3.9, with squalene (29.34%), *alpha*-tocopherol (24.35%), phytol (12.54%), arundoin (8.63%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (7.42%). The structures of the chemical components from extracts of *Elaeis guineensis* leaves are shown in Figure 3.10 and Figure 3.11.



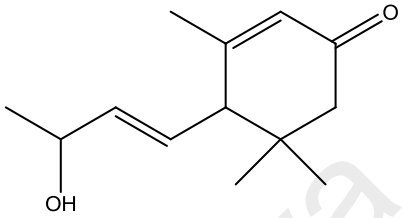
2-hexenal
#1



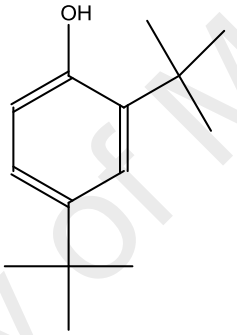
3-hexen-1-ol
#2



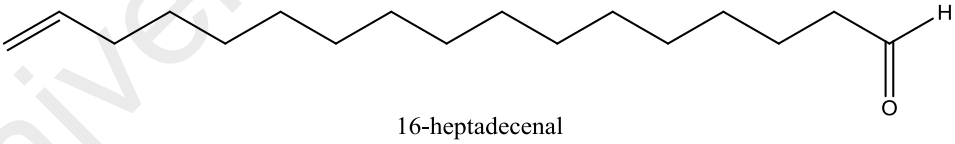
2-hexen-1-ol
#3



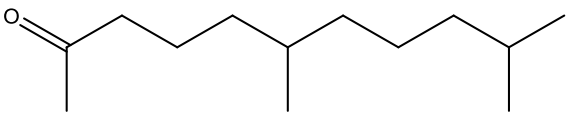
3-oxo-alpha-ionol
#4



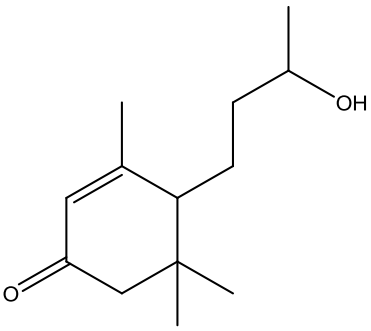
2,4-di-tert-butylphenol
#5



16-heptadecenal
#6

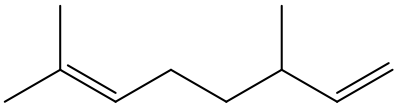


6,10-dimethyl-2-undecanone
#7

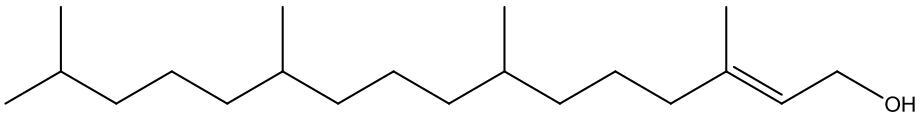


Blumenol C
#8

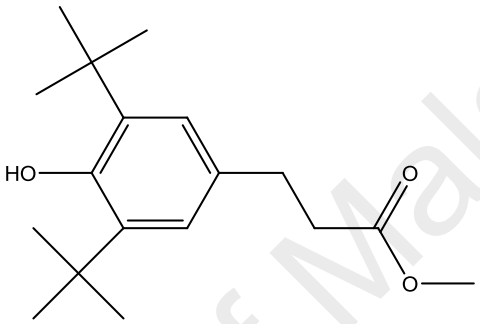
Figure 3.10: Chemical components identified in extracts of *Elaeis guineensis* leaves



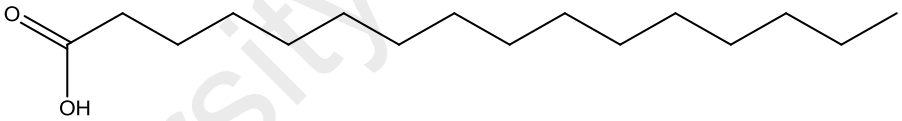
3,7-dimethyl-1,6-octadiene
#9



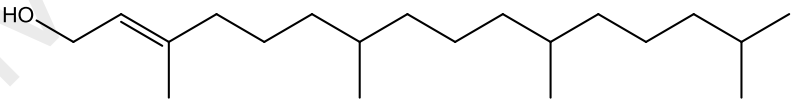
3,7,11,15-tetramethyl-2-hexadecen-1-ol
#10



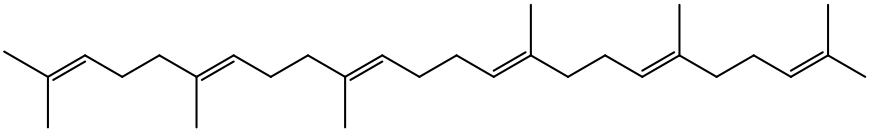
Methyl-3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate
#11



Palmitic acid
#12

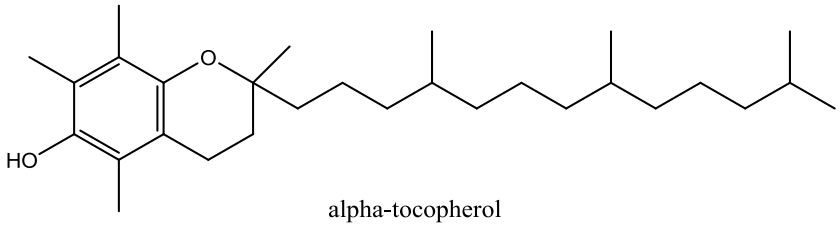


Phytol
#13

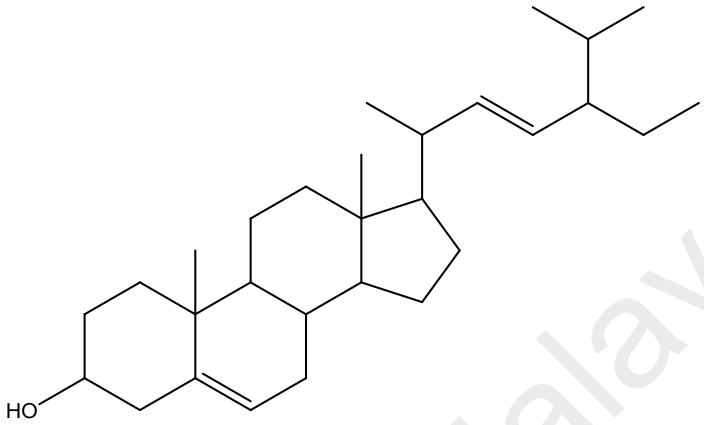


Squalene
#14

Figure 3.10, continued: Chemical components identified in extracts of *Elaeis guineensis* leaves



alpha-tocopherol
#15



Stigmasterol
#16

Figure 3.10, continued: Chemical components identified in extracts of *Elaeis guineensis* leaves

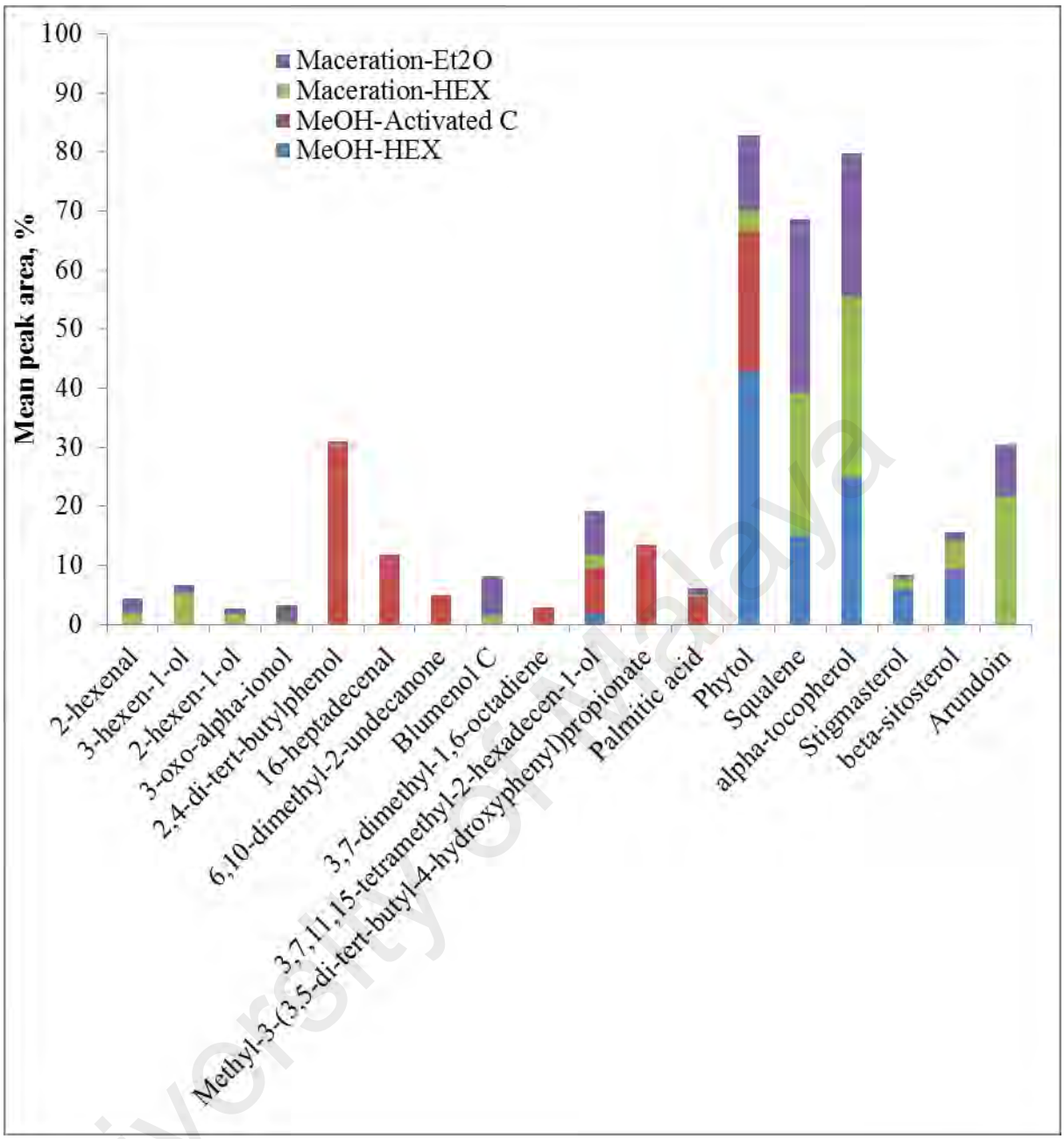


Figure 3.11: Percentage compositions of compounds identified from extracts of *Elaeis guineensis* leaf obtained by four different extraction methods

Both macerations using n-hexane (Maceration-HEX) and ethyl acetate (Maceration-Et_2O) yielded 2-hexenal (2.01% and 2.30%), 3-hexen-1-ol (5.31% and 1.39%), 2-hexen-1-ol (1.81% and 0.89%) and 3-oxo-alpha-ionol (0.39% and 2.88%) (see Figure 3.14 and Figure 3.15) which are not detected in the ultrasonic-assisted crude methanolic extract followed by *n*-hexane partitioning (MeOH-HEX) (see Figure 3.12) and the ultrasonic-assisted crude methanolic extract followed by activated carbon treatment (MeOH-

Activated C) (see Figure 3.13). This variation may arise due to the elevated temperature during solvent removal; the components with low boiling points were removed together with the solvent.

MeOH-HEX contains mostly large apolar molecules with high boiling points. This may due to the nature of the compounds which have higher solubility in *n*-hexane with polarity index 0. It yielded phytol (42.94%), *alpha*-tocopherol (24.97%), squalene (14.95%), *beta*-sitosterol (9.39%) and stigmasterol (5.84%). It is also worth mentioning that there might be chemical changes or decompositions to the compounds arose due to the reduced pressure and elevated temperature during solvent removal. The total phenolic content of the MeOH-HEX extract, calculated from the calibration curve in Figure 3.8 (R^2 = 0.9996), was 16.96 mg GAE/g as shown in Table 3.3. Phenolic compounds have redox properties, which allow them to act as antioxidants (Balasundram *et al*., 2003). As their free radical scavenging ability and antioxidant capacity are facilitated by their phenol groups, the high phenolic content is responsible for the bioactivity of this extract. The presence of phenolic compounds such as 2,4-di-*tert*-butylphenol, methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate and *alpha*-tocopherol in the MeOH-HEX extract contributes to radical scavenging activity and antioxidant capacity, depicted by IC_{50} values of 53.12 µg/mL, 100.34 µg/mL and 114.72 µg/mL in DPPH radical scavenging activity, LPO inhibition and β-carotene bleaching assays respectively (see Table 3.3).

After the crude methanolic extract dissolved in methanol, it was stirred with activated carbon and filtered to yield MeOH-Activated C extract. From Table 3.7, we can clearly observe that this extract does not contain the large apolar molecule such as squalene, α-tocopherol, stigmasterol, β-sitosterol and arundoin. This can be explained by adsorption

of these large apolar molecules on the surface of activated carbon and being removed by filtration. However, the use of activated carbon effectively concentrated 2,4-di-*tert*-butylphenol (30.88%) in this extract. This suggests that treatment with activated carbon was able to produce an extract with a high percentage of phenolic compounds. In this study, although the MeOH-Activated C extract was analysed to contain high percentage of 2,4-di-*tert*-butylphenol (30.88%), but its antioxidant activities were not evaluated due to low yield of extract and insufficient amount for bioassay tests.

In this study, the 16 compounds identified in *Elaeis guineensis* leaves extracts were obtained through ultrasonic-assisted methanol extraction followed by solvent partitioning, maceration using *n*-hexane and ethyl acetate as extractive solvents respectively, then analysed using GC-MS. The results on TPC and bioassay activities are in agreement with the findings reported by other researchers (Abeywardena *et al*., 2002; Mohamed *et al*., 2009; Balasundram *et al*., 2003; Kinnoudo, 2007; Ng & Choo, 2010) as reviewed in Chapter 1, on various health benefits of *Elaeis guineensis* leaves extracts. However, the compounds identified in this study are varied from the compounds reported by other researchers (Mohamed *et al.*, 2014; Phang *et al.*, 2009; Sun *et al.*, 2001; Jaffri *et al.*, 2011). This variation is due to the different extraction methods and analytical tools employed. The compounds present in *Elaeis guineensis* leaves extracts reported earlier such as epigallocatechin, catechin, epicatechin, epigallocatechin gallate, ferulic acid, gallic acid, hydroxycinnamic acid and protocatechuic acid were isolated and analysed by high performance liquid chrmotography (HPLC) and liquid chromatography-mass spectrometry (LC-MS).

3.4.2 Chemical Constituents in MeOH-HEX Extract

Table 3.6: Compounds identified from MeOH-HEX extract of *Elaeis guineensis* leaves

Compounds	**#**	**Mean Peak Area, % (from GC-MS data)**
2-hexenal	1	ND
3-hexen-1-ol	2	ND
2-hexen-1-ol	3	ND
3-oxo-alpha-ionol	4	ND
2,4-di-*tert*-butylphenol	5	0.11 ± 0.02
16-heptadecenal	6	ND
6,10-dimethyl-2-undecanone	7	ND
Blumenol C	8	ND
3,7-dimethyl-1,6-octadiene	9	ND
3,7,11,15-tetramethyl-2-hexadecen-1-ol	10	1.81 ± 0.33
Methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate	11	ND
Palmitic acid	12	ND
Phytol	13	42.94 ± 1.07
Squalene	14	14.95 ± 0.85
alpha-tocopherol	15	24.97 ± 2.24
Stigmasterol	16	5.84 ± 0.09
beta-sitosterol	17	9.39 ± 1.6
Arundoin	18	ND

***#: compound number**

***ND: not detected**

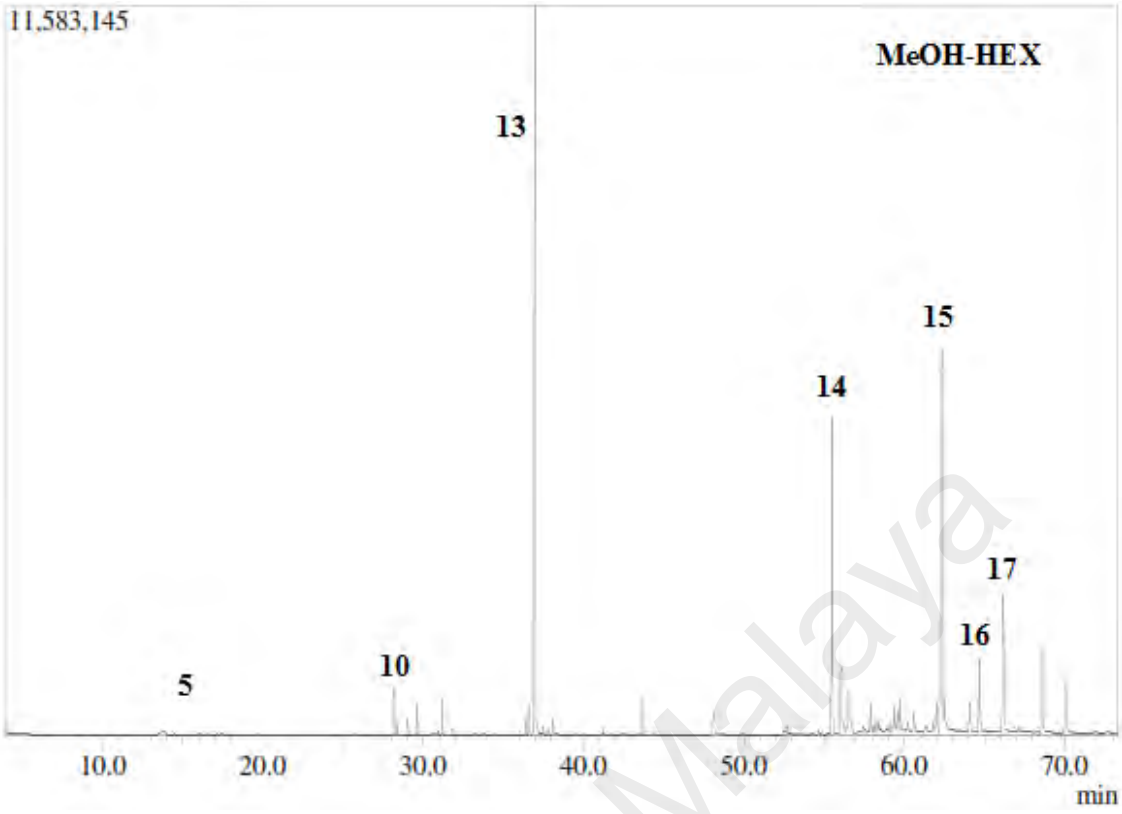


Figure 3.12: GC-MS chromatogram profiles of MeOH-HEX extract of *Elaeis guineensis* leaves

3.4.3 Chemical Constituents in MeOH-Activated C Extract

Table 3.7: Compounds identified from MeOH-Activated C extract of *Elaeis guineensis* leaves

Compounds	**#**	**Mean Peak Area, % (from GC-MS data)**
2-hexenal	1	ND
3-hexen-1-ol	2	ND
2-hexen-1-ol	3	ND
3-oxo-alpha-ionol	4	ND
2,4-di-*tert*-butylphenol	5	30.88 ± 1.09
16-heptadecenal	6	11.72 ± 0.04
6,10-dimethyl-2-undecanone	7	5.00 ± 0.81
Blumenol C	8	ND
3,7-dimethyl-1,6-octadiene	9	2.79 ± 0.21
3,7,11,15-tetramethyl-2-hexadecen-1-ol	10	7.80 ± 0.20
Methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate	11	13.49 ± 1.04
Palmitic acid	12	4.53 ± 0.49
Phytol	13	23.80 ± 1.83
Squalene	14	ND
alpha-tocopherol	15	ND
Stigmasterol	16	ND
beta-sitosterol	17	ND
Arundoin	18	ND

***#: compound number**

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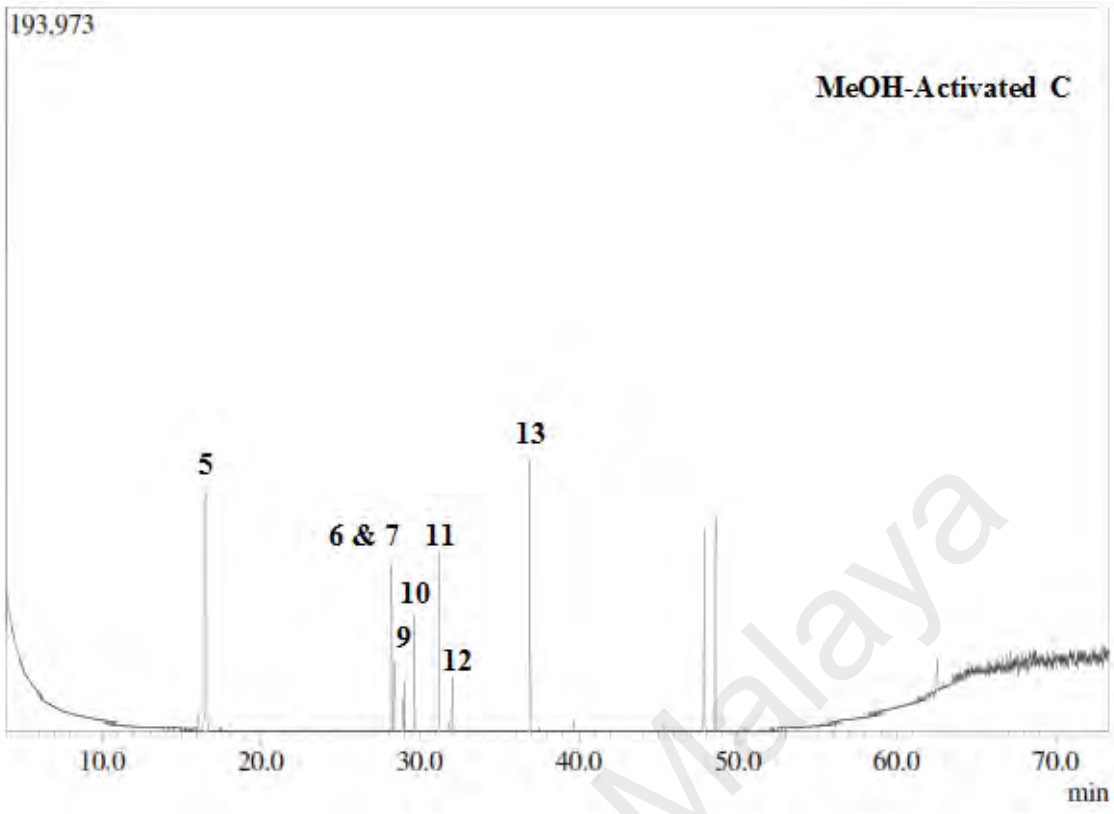

Figure 3.13: GC-MS chromatogram profiles of MeOH-Activated C extract of *Elaeis guineensis* leaves

3.4.4 Chemical Constituents in Maceration-HEX Extract

Table 3.8: Compounds identified from Maceration-HEX extract of *Elaeis guineensis* leaves

Compounds	**#**	**Mean Peak Area, % (from GC-MS data)**
2-hexenal	1	2.01 ± 0.03
3-hexen-1-ol	2	5.31 ± 0.19
2-hexen-1-ol	3	1.81 ± 0.11
3-oxo-alpha-ionol	4	0.39 ± 0.06
2,4-di-*tert*-butylphenol	5	ND
16-heptadecenal	6	ND
6,10-dimethyl-2-undecanone	7	ND
Blumenol C	8	1.61 ± 0.23
3,7-dimethyl-1,6-octadiene	9	ND
3,7,11,15-tetramethyl-2-hexadecen-1-ol	10	2.21 ± 0.14
Methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate	11	ND
Palmitic acid	12	0.43 ± 0.08
Phytol	13	3.49 ± 0.31
Squalene	14	24.23 ± 2.39
alpha-tocopherol	15	30.51 ± 1.85
Stigmasterol	16	1.50 ± 0.29
beta-sitosterol	17	4.77 ± 0.94
Arundoin	18	21.73 ± 1.22

***#: compound number**

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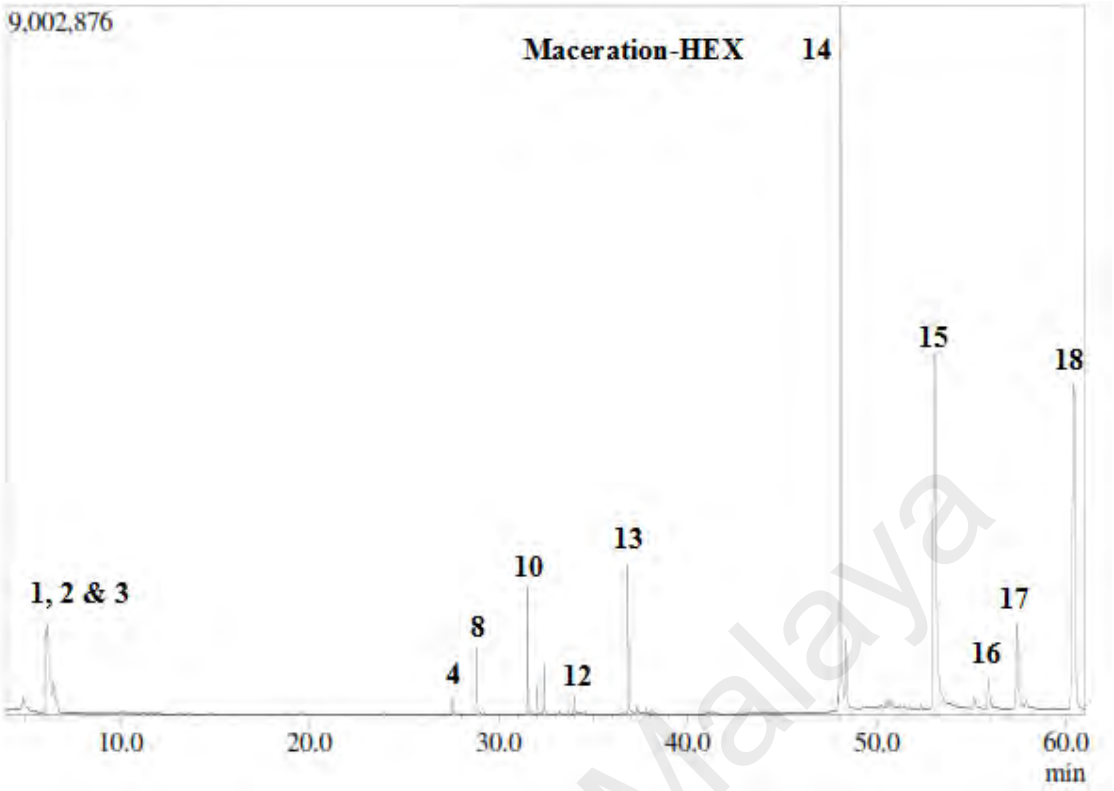

Figure 3.14: GC-MS chromatogram profiles of Maceration-HEX extract of *Elaeis guineensis* leaves

3.4.5 Chemical Constituents in Maceration-Et_2O Extract

Table 3.9: Compounds identified from Maceration-Et_2O extract of *Elaeis guineensis* leaves

Compounds	**#**	**Mean Peak Area, % (from GC-MS data)**
2-hexenal	1	2.30 ± 0.58
3-hexen-1-ol	2	1.39 ± 0.06
2-hexen-1-ol	3	0.89 ± 0.06
3-oxo-alpha-ionol	4	2.88 ± 0.72
2,4-di-*tert*-butylphenol	5	ND
16-heptadecenal	6	ND
6,10-dimethyl-2-undecanone	7	ND
Blumenol C	8	6.61 ± 0.13
3,7-dimethyl-1,6-octadiene	9	ND
3,7,11,15-tetramethyl-2-hexadecen-1-ol	10	7.42 ± 0.40
Methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate	11	ND
Palmitic acid	12	1.08 ± 0.01
Phytol	13	12.54 ± 0.96
Squalene	14	29.34 ± 1.41
alpha-tocopherol	15	24.35 ± 0.57
Stigmasterol	16	1.08 ± 0.25
beta-sitosterol	17	1.51 ± 0.01
Arundoin	18	8.63 ± 0.14

***#: compound number**

***ND: not detected**

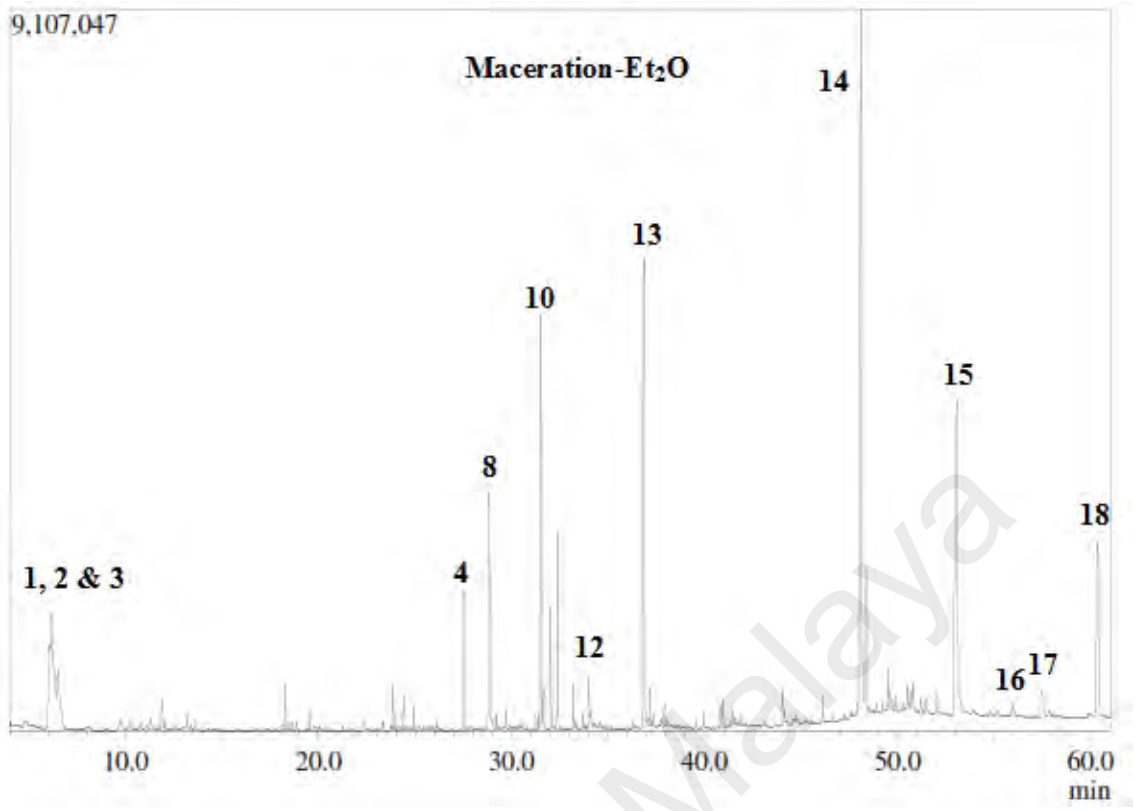

Figure 3.15: GC-MS chromatogram profiles of Maceration-Et_2O extract of *Elaeis guineensis* leaves

3.4.6 Mass Spectral Fragmentation for Identification

The major components were identified by GC-MS analysis and by comparison of their mass spectral data. The mass spectral data of stigmasterol and beta-sitosterol were used as examples to illustrate the identification.

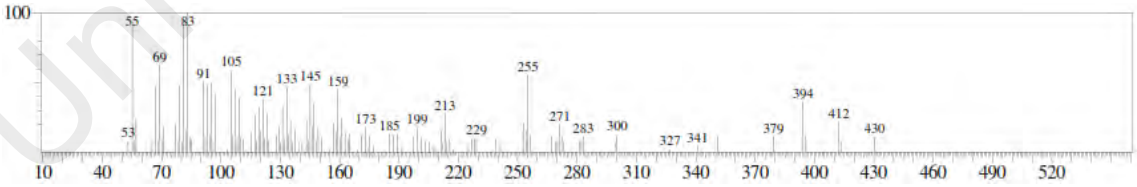

Figure 3.16: The mass spectrum of stigmasterol

Figure 3.16 shows the mass spectrum of stigmasterol. EI-MS *m/z* (%): 412 (16, [M^+]), 394 (4), 369 (2), 351 (6), 271 (16), 255 (22), 229 (5), 55 (100). The mass spectral data was also in agreement with reported data by Gutierrez (2006).

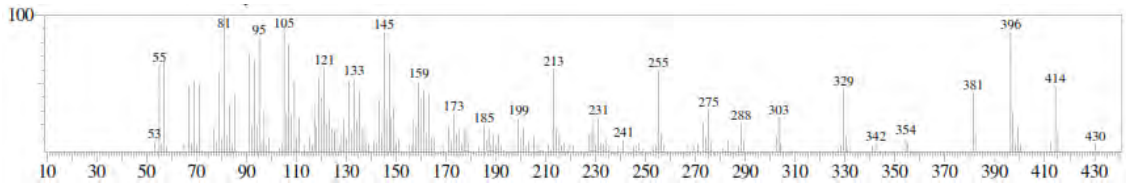

Figure 3.17: The mass spectrum of ß-sitosterol

Figure 3.17 shows the mass spectrum of ß-sitosterol. EI-MS *m/z* (%): 414 (100, [M^+]), 396 (57), 381 (43) was compared to reported data (Nes *et al.*, 1992).

3.5 Conclusion

The profile of the methanolic crude extract further partitioned by *n*-hexane (MeOH-HEX) extract is mainly phytol (42.94%), α-tocopherol (24.97%), squalene (14.95%), β-sitosterol (9.39%) and stigmasterol (5.84%). The same crude methanolic extract treated with activated carbon (MeOH-Activated C) extract yielded 2,4-di-*tert*-butylphenol (30.88%), phytol (23.80%), methyl-3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (13.49%), 16-heptadecenal (11.72%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (7.80%), significantly increasing the concentration of 2,4-di-*tert*-butylphenol in the extract. Maceration-HEX yielded *alpha*-tocopherol (30.51%), squalene (24.23%), arundoin (21.73%), 3-hexen-1-ol (5.31%) and β-sitosterol (4.77%). Maceration-Et_2O yielded similar components in slightly different quantities with squalene (29.34%), α-tocopherol (24.35%), phytol (12.54%), arundoin (8.63%) and 3,7,11,15-tetramethyl-2-hexadecen-1-ol (7.42%). The presence of these compounds might have contributed to the health benefits of *Elaeis guineensis* leaves reported by the researchers as reviewed in Chapter One (Abeywana *et al*., 2002; Mohamed *et al*., 2009; Mohamed *et al*., 2014; Kinnoudo, 2007; Ng & Choo, 2010; Jaffri, *et al*., 2011)

Different extraction techniques were employed in order to obtain compounds of interest. It was proven that maceration without involving reduced pressure and elevated

temperature in the solvent removal step was able to extract and retain small molecules with low boiling points. However, this technique of extraction does not allow the investigation of its antioxidative potential as the extracts were not evaporated to dryness; hence their concentrations were not quantified for IC_{50} value calculation.

Several antioxidant activity assays were carried out to evaluate the antioxidant capacity of the methanolic extract (MeOH) from *Elaeis guineensis* leaves and its partitioned fractions; the *in vitro* 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, lipid peroxidation (LPO) inhibition and β-carotene bleaching assays. Total phenolic contents (TPC) in the fractions were determined with Folin-Ciocalteu reagent. The results of this study show that the antioxidant capacity of a sample is dependent on the concentration and the measured parameter. Similar samples exhibit different antioxidant activity in each assay as the mechanistic pathways of the assays are different. Nevertheless, these results clearly show there is significant antioxidant activity in *Elaeis guineensis* leaves and it may have potential to be used as natural antioxidants to substitute the use of synthetic antioxidants that have adverse effects on our health.

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LIST OF PUBLICATIONS

Journal Article

Ang, C. H., Cheng, S. F., & Chuah, C. H. (2017) Antioxidant activities of *Elaeis guineensis* leaves. *Journal of Palm Oil Research, 29*(3): 343-351.

Conference Papers

Ang, C. H., Cheng, S. F., & Chuah, C. H. (2012). Water-soluble Compounds from *Elaeis guineensis* (Oil Palm) Leaf and their Antioxidant Activities. Oral presentation in 17th Malaysian Chemical Congress (17MCC), 15 – 17 October, Putra World Trade Centre (PWTC), Kuala Lumpur, Malaysia.

Ang, C. H., Cheng, S. F., & Chuah, C. H. (2014). Antioxidant Potential of Tannin-rich *n*-Butanol Extract from *Elaeis guineensis* Leaf. Poster presented at The 9th Mathematics Physical Science Graduate Congress (MPSGC) 2014, 8 January 2014 to 10 January 2014, Kuala Lumpur, Malaysia.

Ang, C. H., Cheng, S. F., & Chuah, C. H. (2014). Isolation and Identification of Tannins from Antiradical *n*-Butanol Extract of *Elaeis guineensis* Leaves. Poster presented at the 12th Euro Fed Lipid Congress, 14 September 2014 to 17 September 2014, Montpellier, France.