BIOACTIVE COMPOUNDS FROM *Hibiscus sabdariffa* SEED AND *Anacardium occidentale* NUT SHELL AS POTENTIAL NATURAL ANTIOXIDANT FOR BIODIESEL

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ABSTRACT

Fossil-fuel depletion and environmental pollution are two pressing issues leading to the focus on biodiesel as an alternative energy source. However, the oxidative stability of fatty acid methyl ester (FAME) is important to be maintained in order to keep biodiesel from deteriorating after long term storage. Additions of antioxidant in biodiesel can help prolong the shelf life by combating propagation of free radicals. In striving a sustainable green economy, the uses of natural antioxidants from renewable sources is preferred and have the same potential as synthetic antioxidant without the effect of being hazardous to environment. The two plants of choice, *Hibiscus sabdariffa* (Roselle) seed and *Anacardium occidentale* (Cashew) nut shells are common agriculture industry residues and normally left unconsumed. ABTS antioxidation test on both plant's part showed a significant result from all sample type regardless their polarity whereas β-Carotene test favoured more on nonpolar samples. Positive correlation of DPPH test and total phenolic content (TPC) test exhibit those phenolic compounds responsible for antioxidation activity of all samples type. A test in determining the total flavonoids using total flavonoid content (TFC) test also showed a positive results for all samples. Profiling of both plants' part using GCMS and LCMS machines revealed that all the samples contain phenolic compounds with an exception for Roselle seed extracted with hexane (RH) which contained mostly fatty acid. Oxidative stability test using Rancimat test showed that polar samples performed much better than nonpolar samples and pure extract (analytical grade) of 3-pentadecyl phenol showed a poor performance in the oxidative stability test.

ABSTRAK

Bahan api fosil yang semakin berkurangan dan pencemaran alam sekitar adalah dua isu penting yang mendesak untuk member lebih tumpuan kepada biodiesel sebagai sumber tenaga alternatif. Walau bagaimanapun, kestabilan pengoksidaan asid lemak metil ester (FAME) adalah penting untuk dikekalkan bagi mengelakkan biodiesel daripada terurai dalam simpanan jangka masa panjang. Dengan penambahan campuran antioksida dalam biodiesel dapat memanjangkan jangka masa simpanan biodiesel. Selaras dengan ekonomi hijau, kegunaan antioksida semulajadi daripada sumber yang boleh diperbaharui adalah diutamakan dan mempunyai potensi dalam menandingi kekuatan potensi antioksida sintetik yang membawa pencemaran kepada alam semulajadi. Dua tumbuhan pilihan iaitu biji benih *Hibiscus sabdariffa* (Roselle) dan cengkerang kacang *Anacardium occidentale* (Gajus) adalah bahan sisa industri pertanian dan biasanya dibiarkan sebagai bahan buangan. Ujian antioksida ABTS yang dilakukan pada bahagian kedua-dua bahagian tumbuhan itu menunjukkan perbezaan hasil yang ketara antara bahan uji kaji tanpa dipengaruhi sifat polar bahan ujikaji tersebut. Manakala ujian antioksida β-karotena lebih memihak kepada bahan ujikaji yang tidak polar. Ujian antioksida DPPH dengan korelasi ujian kandungan jumlah fenol (TPC) menunjukkan bahawa sebatian fenolik bertanggungjawab untuk aktiviti antioksida untuk semua bahan uji kaji. Satu ujian untuk menentukan jumlah flavonoid mengunakan ujian jumlah flavonoid (TFC) memberi hasil positif kepada semua bahan ujikaji. Ujian profil kedua-dua bahagian tumbuhan mengunakan GCMS dan LCMS mendedahkan bahawa semua bahan uji kaji mengandungi sekurang-kurangnya sebatian fenolik kecuali biji benih roselle yang diekstrak dengan heksana (RH) iaitu kebanyakannya mengandungi asid lemak. Ujian kestabilan oksidatif mengunakan Rancimat menunjukkan bahawa bahan uji kaji yang polar jauh lebih baik daripada bukan polar dan ekstrak tulen (gred analisis) 3-pentadecyl fenol pula tidak menunjukkan prestasi yang memuaskan dalam ujian kestabilan ini.

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ABBREVIATIONS

ABTS - 2, 2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid

AOC - Assessing antioxidant capacity

ArOH - hindered phenol

BHA - Butylated hydroxyanisole

BHT - Butylated hydroxytoluene

BTU - British thermal unit

CH - Cashew nut shell extracted with hexane

CM - Cashew nut shell extracted with methanol

CNSL - Cashew nut shell liquid

DPPH - 1,1-diphenyl-2-picrylhydrazyl

EU - European union

FAME - Fatty acid methyl ester

GAE - Gallic acid equivalents

GCMS – Gas chromatography-mass spectrometry

H_2O_2 - Hydrogen peroxide

HAT - Hydrogen atom transfer

HLB - Hydrophilic-lipophilic balance

HOCl - Hypochlorous acid

IC_{50} -Inhibition concentration (50%)

IP - Induction period

LCMS - Liquid chromatography mass spectrometry

O - Oxygen

O_2 - Superoxide anion

O_3- Ozone

OH - Hydroxyl

OOH - Hydroperoxyl

ORAC - Oxygen radical absorbance capacity

OSI - Oxidative stability index

PG - propyl gallate

PUFAs - Polyunsaturated fatty acids

RH - Roselle extracted with hexane

RM - Roselle extracted with methanol

RME - Rapeseed methyl ester

RO - Alkoxyl

ROO - Peroxyl

ROO· - Peroxyl Radical

ROS - Reactive oxygen species

SET - Single electron transfer

SPSS - Statistical package for the Social Science

TBHQ - *tert*-butylated hydroxyquinine

TEAC - Trolox® equivalent antioxidant capacity

TRAP - Total-radical trapping antioxidant parameter

VFA - Volatile fatty acid

VOCs - Volatile organic compound

$^1O_2$ - Singlet oxygen

CHAPTER 1

INTRODUCTION

1.1 General Introduction

With the ability to reinstate balance to damaged living cells, plant related products have been consumed by human since the last 2,000 years ago. Food rich in antioxidants such as polyphenols and carotenoids was taken to adversely combat the consequence of free radical scavengers (Katalinic *et al.,* 2006). These natural compounds which also inhibit and slow down oxidation rate of lipids and oils (Venskutonis *et al.,* 2005). These antioxidants are either in the form of chemical constituents or raw extracts and very efficient in preventing the destructive effects of oxidative stress (Saeed & Shabbir, 2012).

In manipulating the good properties of these bioactive compounds, the correct method of extraction must be used to avoid lost of bioactive compound and simultaneously maintaining the quality of the antioxidant. Extraction of antioxidant from plant usually done using either physical or chemical method such as supercritical-extraction, microwave-assisted extraction, ultrasound-assisted extraction and Soxhlet method (Wang & Weller, 2006). All these extraction methods could be utilized depending on the purposes of experiment and demand of the procedure because various plant species had been reported to exhibit antioxidant activity due to the high presence bioactive compounds such as flavones, flavonoids, catechins (Khalaf *et al.,* 2008).

All these bioactive compounds have their own particular polarity contributed by the different groups in the side chains. As there is no universal compound available in doing single extraction, using several solvent with different polarity to extract compound of interest is crucial in order to get the desired product. Fatty acid methyl

ester (FAME) which is the most common used biodiesel is mainly utilized from animals and plants due to their low cost properties. However biodiesel contained fatty acids, which are prone to be attacked by free radicals (Ghadge & Raheman, 2005). According to Dantas *et al.* (2011), peroxides could be formed through oxidation by initiators such as heat, light and air. These free radicals if not scavenged or suppressed by antioxidants, will lead to the formation of hydrogen peroxides in propagation steps in autoxidation. When the formation of hydrogen peroxides started to increase in concentration, it indicates the end of induction period (IP). According to Velasco (2009), the susceptibility against oxidation for edible fats and oils under accelerated conditions is mostly determined by oxidative index (OSI) of Rancimat method. In order to manipulate those useful properties of antioxidant in combating oxidative effect, the usage of industrial waste product such as Roselle seed and Cashew nut shell has been introduced as it shows promising capabilities to yield significant amount of antioxidant through laboratories extraction process. In long term, certainly will give added value to food processing industries, and at the same time give additional alternative energy for fossil fuel.

1.2 Research Background

From the study of Oh *et al.* (2010), Malaysia's oil production has declined as much as 13% from 2006 to 2008 and it is estimated that crude oil reserved in Malaysia could be depleted in 20 years time. This will become a huge concern to the oil-dependent country as two-third of petroleum consumption in Malaysia is petrol and diesel while the other one-third are kerosene, fuel oil, gas and non-energy (Jayed *et al.,* 2011). Although the fossil fuel resources are declining, the demand for it kept increasing, thus, an alternative energy is needed. There are many ongoing active researches to complement the fossil fuel that can produce or give the same effect like petrol and diesel. Biodiesel has started to emerge as one of the alternative energy that can substitute diesel. However, there are multiple issues that have been recognized which currently hold biodiesel to function as fossil resources. One of the issue is the characteristic of biodiesel that tend to oxidize easily during storage compare to fossil fuel which is more stable. This will cause huge lost and considered uneconomical to use biodiesel as replacement of burn fuel. In order to overcome the deterioration problem of biodiesel, the use of antioxidant has been recommended due to their strong antioxidation properties. Plant's bioactive compounds come from industrial waste is the main focus in this study because choosing this type of plant part will avoid confliction of food sources. In this study, Roselle seed and cashew nut shell were utilized to be extracted to get the source of antioxidants.

1.3 Problem Statement

Although biodiesel possess a lot of potential benefit especially in reducing the carbon emission, the shelf life of biodiesel is limited, due to the threat of oxidation. This will reduce the effectiveness and commercial value of biodiesel because of incapability for long term storage of product. Industrial players are seeking a new, effective, cost conscious ways to extend the biodiesel shelf life. One of the notion is by adding antioxidants which known to suppressed the formation of free radical causing oxidative insult to the biodiesel compound. The use of synthetic antioxidants in biodiesel is more preferable in the market because of the lower price. However synthetic antioxidant such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) respectively producing higher carbon emission and higher nitrogen oxide (Rizwanul *et al.,* 2014). Excess production of hydrocarbons is toxic to all type of life forms (Abha & Singh, 2012). It play a role in forming health and environmental hazard compounds such as nitrogen dioxide ($NO_{2)}$ and ozone (O_3) while nitrogen oxide lead to the formation of nitric acid which is the main constituent of acid rain. Because of this reasons, it is advisable to use antioxidant from natural product so that adverse effect of synthetic antioxidant can be reduce in providing better environmental condition.

Normally, raw plants that will be used to extract antioxidant usually come from the plant that can be consumed. Using this type of plant as main material of antioxidant may cause conflict with the food supply-demand chain. In order to overcome this issue, usage of unconsumed part of a plant remaining such as Roselle and Cashew nut shell is the best alternative choice to extract the antioxidant compound. At has been suggested in beginning, two type of plants part is used because from the study of Lomonaco *et al.* (2012), production of *Anacardium occidentale* (cashew) nut shell was around 3,300,000 tonnes in the year of 2009. From

that amount, almost 800,000 tonnes is in the form of byproduct of cashew nut shell. These unused parts are suitable as a feedstock for antioxidants, preventing it from merely become waste. While in the case of *Hibiscus sabdariffa* (Roselle), seed part of this plant is left unconsumed although other parts of this plant are used for food products (Ilham *et al.,* 2013).

1.4 Objectives of the Proposed Study

The general objective of this study is to determine the potential of bioactive compounds derived from *Hibiscus sabdariffa* (Roselle) seed and *Anacardium occidentale* (Cashew) nut shell as potential natural antioxidants for biodiesel. Specific objectives of this study are as follows:

i) To identify the antioxidant capacity of *Hibiscus sabdariffa* (Roselle) seed and *Anacardium occidentale* (Cashew) nut shell.

ii) To determine the bioactive compound that is responsible in giving the antioxidant properties of the plant species.

iii) To examine the concentration of the antioxidants that will improve the oxidation stability of biodiesel.

1.5 Scope of Work

This study will focus on the utilization of bioactive compound of *Hibiscus sabdariffa* (Roselle) seed and *Anacardium occidentale* (Cashew) nut shell as potential antioxidant for biodiesel. The extract of bioactive compounds from these plants will be tested with antioxidation test to get a better understanding of their natural polarity related to the effectiveness of the compounds. Antioxidation test which favor either polar or nonpolar extract were used to evaluate these compounds. This study will also evaluate the bioactive compound activity towards oxidation stability of biodiesel by using several designated tests. The identification of compound responsible for antioxidant capacity will be done to get a clear view of which compound and side chain group of the compound is responsible for antioxidation. At the same time, the study will also study the effectiveness of the yielded antioxidant. The concentration of the antioxidants is evaluate using oxidative stability test to determine the optimum concentration needed to surpassed the standard hour of biodiesel stability. As been noted at the beginning both *Hibiscus sabdariffa* (Roselle) seed and *Anacardium occidentale* (Cashew) nut shell are usually left unused as waste in the industry. This will create an alternative use of waste product if the proposed study showed high potential through a series of positive outcome of results.

1.6 Thesis structure

There are 5 chapters in this thesis which begin with Chapter 1, introducing the background, problems, purposes and aims of this study. Chapter 2 introduces the key background of plant extraction associated with the antioxidant and a brief introduction on biodiesel oxidative stability.

Chapter 3 describes methods and properties necessary for extraction continued with methods of testing the antioxidants with biodiesel. Chapter 4 discusses the result of this study together with reference of previous findings and justifications of phenolic determination. The summaries of results are presented in Chapter 5, together with recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 Biodiesel

Increasing environmental issues due to the accumulation of waste forced global measures to solve these problems. Several international agreements including European Union (EU) regulations and directives have been adopted to regulate emission of greenhouse gases in order to promote the usage of renewable energy sources, and to ensure effective management and utilization of waste. One major issue is the atmospheric pollution caused by transportation sector. European Union member states are promoting the production and use of biofuel in order to reduce the impact of this problem (Sendzikiene *et al.,* 2005).

Biodiesel originates from the word "bio", which brings the meaning of life and the word "diesel" referring to Rudolf Diesel, a famous German that invented diesel engine in 1893 (Nitske & Wilson, 1965 ; Demirbas, 2008; Jayed *et al.* 2011). In general, it means a biomass derived fuel as an alternative to the conventional petrol-diesel According to Howell (2007), biodiesel is produced by transesterification reaction of renewable fats and oils with alcohol to produce long chained fatty acid alkyl esters. As biodiesel is naturally produced by chemically reacting vegetable oil or animal fat, carbon in the oils or fats commonly originated from carbon dioxide, absorbed by the plant biomass during photosynthesis, thus making it zero carbon cycle (Gerpen, 2005). Oxidation stability of vegetable oil is more stable compared to animal oil although large amount of polyunsaturated fatty acid is present. This is because of the absence of natural antioxidant in fatty acid methyl ester of animal fat (Sendzikiene *et al.,* 2005). Production of biodiesel started from conversion of biomass to

biochemical rather than thermo-chemical conversion that will produce syn-oil, bio-syngas and biochemical. Biomass that undergoes biochemical conversion processes produces bio ethanol through fermentation and biodiesel through the process of transesterification (Jayed *et al.,* 2011).

Generally transesterification is a process of alcohol displacement from an ester by another alcohol. In other words, the organic group of ester is replaced with an organic group of alcohol in similar manner of hydrolysis except alcohol is used instead of water. Specifically, the organic group of triglyceride are replaced by an organic group of methanol and vice versa. As a result of this exchange, triglyceride will become glycerol and methanol will become fatty acid methyl ester (FAME) (Srivastava & Prasad, 2000). For each triglyceride, three monohydric alcohol are needed to produce methyl or ethyl ester with the help of catalyst to increase the yield and reaction rate of ester (Jayed *et al.,* 2011). This conversion is visualized in the Figure 2.1

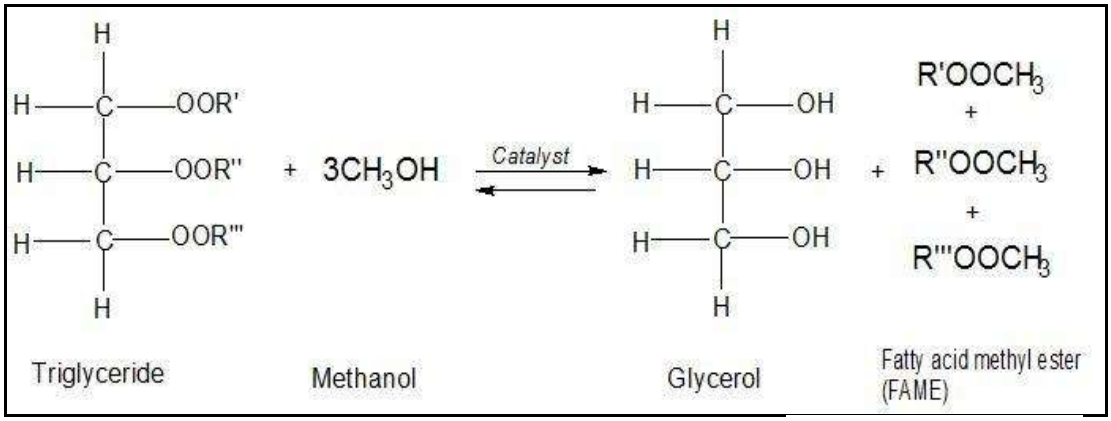


Figure 2.1: Stoichiometric of transesterification reaction (Srivastava and Prasad, 2000).

Transesterification is a catalytic method that can be either homogeneous or heterogeneous. Alkaline such as sodium hydroxide, sodium methoxide and potassium hydroxide and acid such as sulfuric acid, hydrochloric acid and sulfonic acid are types of homogenous catalyst in nature. On the other hand, example of heterogeneous catalyst are enzymes, titanium silicates, alkaline earth metal compounds, anion

exchange resins and guanadines heterogenized on organic polymers. There are three types of catalyst based namely acid, alkaline and enzyme and the most effective and widely used in industries is alkaline based catalyst because of its less corrosive characteristic (Jayed *et al.,* 2011).

2.1.1 Advantages of Biodiesel

The advantages of biodiesel over fossil-fuel are higher boiling point and lower emission, contributed by the existence of oxygen. This is supported by the study of Masjuki *et al.* (1999), which stated that palm oil, mineral and palm oil based lubricant effectively reduces carbon monoxide and hydrocarbon emission levels. In addition, with the higher specific gravity, higher boiling point, higher kinematic viscosity and short ignition delay due to higher cetane number reduces the NOx emission during initial combustion (Ryu, 2009). Other than that, hydrocarbon, particulate matter, polycyclic aromatic hydrocarbon, sulfur dioxide (SO_2) and carbon monoxide (CO) emissions are lower compared to mineral diesel (Ramadhas *et al*., 2005; Ryu, 2009). According to Aluyor & Ori-Jesu (2008), biodiesel from the source of vegetable oils have low volatilities manifested with high flash points. In facts vegetables oil exhibit double the viscosity indices than mineral diesel thus proved that it has low viscosity-temperature variations. With the good lubricity and good British Thermal Unit (BTU) content compared to petrol diesel, biodiesel also produce virtually no sulfur or aromatic content (Howell, 2007). According to Ryu (2009), as biodiesel consists of monoacylglycerol or free fatty acids which compose of hydrogen carbon only with polarity-imparting oxygen atoms, it is used as an addictive to petroleum fuel to improve lubrication properties. This statement is proved by the study of Knothe & Steidley, (2005) which stated that the low lubricity ultraflow-sulfur diesel can be improved by adding biodiesel.

2.1.2 Disadvantages of Biodiesel

Apart from being environmental friendly, biodiesel is susceptible to oxidation, especially when the feedstock is high in unsaturated fatty acids. These unsaturated fatty acids could be degraded by the existence of free radicals, reducing the storage time of biodiesel. Free radical is defined as any atom or molecule possessing unpaired electrons. Example of primary oxygen derived free radical are oxygen derived free radicals are superoxide anion ($O_2\cdot$), hydroxyl ($OH\cdot$), hydroperoxyl ($OOH\cdot$), peroxyl ($ROO\cdot$) and alkoxyl ($RO\cdot$) radicals while hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($1O_2$) are non-free radical. These reactive intermediates are collectively termed as reactive oxygen species (ROS) (Umamaheswari & Chatterjee, 2008).

According to Velasco (2009), the two main cause of biodiesel degradation are thermal stability and oxidative stability. Thermal stability is cause by temperature and usually temperature above 250°C wills deterioration the biodiesel. For oxidative stability, it is cause by oxygen either in the gaseous form or that had been dissolved that come in contact with the fuel during a sufficiently long period of time. Oxidative stability is also known as storage stability because of the reason of oxygen-air interaction with the fuel under storage that is likely to interfere with the fuel stability (Dunn, 2008). Other minor factors of biodiesel degradation are interference of other agents such as light, water and the presence of metals in the fuel that will speed up the oxidation process (Jain & Sharma, 2010). Light interference cause photo-oxidation mechanism, however it is unlikely to happen in biodiesel because it requires exposure to ultraviolet and the presence of a photo-sensitizer. Likewise photo-oxidation, auto-oxidation normally occurs in biodiesel (Lapuerta, *et al.,* 2010; Knothe, 2007). According to Waynick (2005), auto-oxidation mechanism occurs when oxygen in the

atmosphere reacts with unsaturated methyl esters especially with those with two or more double bonds in its molecule that initiates free-radical chain reaction (Waynick, 2005). Thus according to Lapuerta, *et al.* (2012), more saturated biodiesel fuels showed better stability but it does not sufficiently explain the unsaturation degree of the biodiesel itself.

In primary oxidation mechanism, initiation step start with peroxidation. In this stage hydrogen is removed from carbon to form free radical carbon of peroxide. Then propagation reaction is proceed when free radical carbon reacts with diatomic oxygen to form peroxy radical (Church & Pryor, 1985). This radical is not reactive if to be compared with carbon free radical but is strong enough to attract hydrogen and form hydroperoxide and another carbon free radical. The new carbon radical form will react with other diatomic oxygen to continue the propagation cycle. This is the stage of induction period (IP) where there is low concentration of hydrogen peroxide. However when the concentration of peroxide is at its peak, induction period will end, indicating the onset of the overall oxidation process. Termination of the reaction happened when the two same free radical of carbon or hydroperoxide react with each other to form a stable product (Jain & Sharma, 2010).While in secondary oxidation, fatty acid oil hydroperoxide formed decomposed to form another chain of aldehyde such as hexenals (Refaat, 2009) and other by-product. Shorter formation of fatty acids due to oxidation lead to acidity increase decomposition of hydroperoxide causing viscosity increase dueto oxidative linking of fatty acid to form species with higher molecular weight (Jain & Sharma, 2010).

2.1.3 Oxidative Stability Test of Biodiesel

From the American standard ASTM-D6751 which stated that "Biodiesel is a fuel comprised of mono-alkyl ester of long-chain fatty acids derived from animal fat or vegetable oils". According to Valle *et al.* (2014), the nature of biodiesel obtained from natural renewable sources yield significant energy security and better environmental benefits rather than petroleum diesel. Storage stability is defined as the ability of biodiesel to resist chemical and physical changes due to the interaction with environment. This is very important because usage of degraded oil could cause operational problems in engines, thus hampering biodiesel commercialization. According to Jain & Sharma, (2010), increase in the number of chain length of carbon atoms has been found to increase the viscosity of the oils due to the increase in the degree of saturations. The configuration of double bonds influence the viscosity, for example the *trans*-double bond configuration results in higher viscosity than *cis*-double bond configuration while the position of double bond affect the viscosity a lesser extent. Oxidation processes causes the double bond of free fatty acid to isomerizes from *cis* to *trans* and formation of high molecular weight products causing the viscosity to increase corresponding to oxidation increase. Thus free fatty acids are concluded to be responsible for higher viscosity.

Several methods are used to determine oxidative stability and oxidation products such as by using various peroxide analytical procedures, measurement of aldehyde and acid formation, conjugated dienes formation which are rearrangement products that result from peroxide formation by using UV spectroscopy, iodine peroxide, acidity indexes, physico-chemical techniques and viscosity measurement (Larson & Marley, 2011; Dantas *et al.*, 2011). However all of these methods have a huge drawback in terms of determination of oil stability (Dantas *et al.*, 2011). However there has an easier method of measuring rates of oxygen uptake to measure

oxidative stability by detecting volatile acids produced in a closed and heated system at 110°C such as Rancimat method (Larson & Marley, 2011). According to Farhoosh, (2007), Rancimat was developed by Hadorn & Zurcher (1974) and since then it has become most common accelerated technique used to access oxidative stability of oils and fats. There are no periodic analytical determinations and no organic solvent needed using this method. Furthermore, this method can operate under different parameters of interest such as airflow, temperature and oil sample size. Furthermore this continuous measurement method is reproducible and easy to use. It works by measuring the time needed to maximize the oxidation rate of change of deionized water that was bubbled in volatile acid in the form of dry air from heated sample. The rate of change is known as oxidative stability index (OSI) that is correlated with lipid oxidation. Figure 2.2 showed a schematic diagram of Rancimat apparatus.

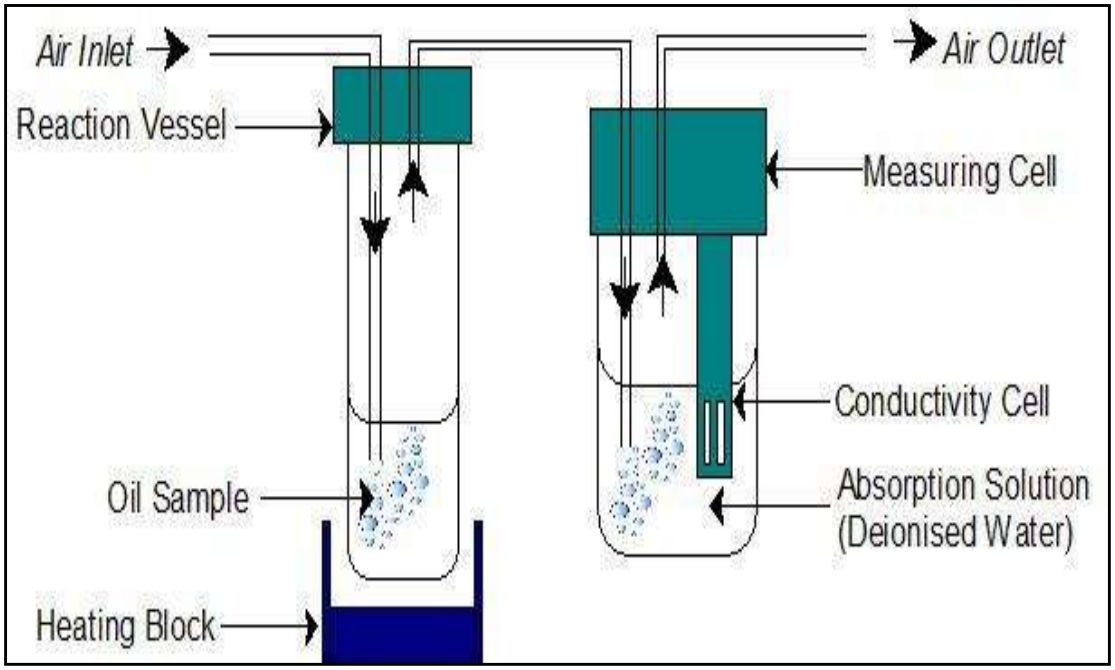


Figure 2.2 : Schematic diagram of Rancimat equipment.

According to Jain & Sharma, (2010), in Rancimat system, oxidations are induced by passing the biodiesel samples with air stream at 10 L/h that is kept at constant temperature of 100 ° C, 110°C or 120°C. Air and vapor released during the process of oxidation are passed through the flask that contained 60 ml of

demineralized water with fitted electrode for conductivity measurement. The end of the induction period is detected by the electrode when the conductivity increase due to disassociation of volatile carboxylic acids produced from the oxidation processes that were absorbed in the mineralized water. If a graph is plotted by using the data of conductivity (μS/cm) against induction time (hours), induction period can be obtained by calculation of two intersection points of two tangents. By plotting the OSIs logarithm against elevated temperature, one can extrapolate shelf-life of an oil at ambient, room temperature or any temperature of interest. Moreover the slope or gradient of the curve represent the temperature coefficients of oil. Other than that, acceleration factor Q_{10} also can be determined based on oxidative increment starting at 10°C from the curve (Farhoosh, 2007).

The Rancimat method is widely used for the determination of the oxidative stability of natural fats and oils under high temperatures and constant aeration. The induction period is characterized by the change in conductivity of deionized water due to oxidation-generated products (Tiveron *et al.,* 2012). There are two European standards for determination of oxidative stability test which are EN 14214 for automotive diesel fuel and EN 14213 for home heating fuel. Both of these standards meet the requirement stability based on the Rancimat test of using fatty acid methyl ester as fuel at the temperature of 110°C. The differences of these two method is the standard induction period needed to run through. For EN 14214, the minimum IP must be 6 hours and for EN 14213 only for 4 hours (Jain & Sharma, 2010). ASTM D6751-07 includes an oxidation stability standard of a 3 hours minimum induction period (IP) as measured by using the Rancimat test (EN14112). The European Committee for standardization adopted a 6 hours minimum IP as the specification (Tang *et al.,* 2008). The difference of the Rancimat IP test between Europe and the United States is because due to the B100 produced in United States is made from yellow grease or

soybeans. Thus because they have composition that consist of higher polyunsaturated content, the biodiesel produced have a slightly lower Rancimat IP compared to B100 produced in the Europe that is produced from rapeseed oil. Biodiesel from rapeseed methyl ester (RME) have significantly lower polyunsaturated content resulting in the higher IP (Jain & Sharma, 2010).

2.2 Antioxidants

Nowadays antioxidants related research is growing and had nearly quadrupled in the past decade (Huang & Prior, 2005). From *Webster's Third New International Dictionary*, antioxidant is stated as "a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of the substances being used as preservatives in various products such as in gasoline to retard the development of rancidity, in petroleum product to retard the gum formation and in rubber to retard the aging process". In order for a compound to act as antioxidant, it must have the ability to stabilize the formed phenoxylradical after reaction with lipid radicals and formed delocalized unpaired electrons. This allows the molecule to act as hydrogen donor, singlet oxygen donor and reducing agents (Matthaus, 2002). Lee *et al.* (2004), has classified antioxidants into two major classes of non-enzymatic and enzymatic. Enzymatic antioxidants are those produced endogenously while non-enzymatic are those produce exogenously. The four main general sources of antioxidants are listed in Table 2.1.

Table 2.1: General sources of antioxidants.

Source of Antioxidant	Classes	Examples
Enzymes	Enzyme	Superoxide dismutase, glutathione peroxide and catalase.
Large molecules	Non-Enzyme	Albumin, ceruloplasmic, ferritin and other proteins.
Small molecules	Non-Enzyme	Ascorbic acid,glutathione, uric acid, tocopherol, carotenoids and phenols.
Hormones	Enzyme & Non-Enzyme	Estrogen, angiotensin and melatonin.

(Prior & Schaich, 2005).

From the study of Hue *et al.* (2012), antioxidants are divided into two categories known as primary and secondary antioxidant that differ in term of action mechanism. Primary antioxidant stabilizes the free radical by scavenging it and donates a hydrogen atom or electron. However secondary antioxidant suppresses the formation of free radicals and prevent the oxidative damage (Prior & Schaich, 2005). Two methods of primary antioxidant mechanism are hydrogen atom transfer (HAT) and single electron transfer (SET). Based on the name implied, HAT-based method measures the ability of the antioxidant to suppress free radicals by donating hydrogen (Prior & Schaich, 2005). This assay is kinetic based which antioxidant and substrate compete for peroxyl radical that thermally generated by decomposition of azo compound (Apak *et al.,* 2007). Likewise HAT, SET-based method is slightly different because it measure the capability of antioxidant to donate one electron to reduce any compound (Prior & Schaich, 2005). Examples of HAT assay are ORAC and TRAP while for SET are TEAC, DPPH, CUPRAC, Folin-Ciocalteu and FRAP method. Regardless of different name and mechanism involved, both have the same outcome to prevent oxidation. (Prior & Schaich, 2005; Apak *et al.,* 2007).

Although several *in vitro* detection methods are available to allow rapid screening and analyzing antioxidant activity but their limitations and advantages are still being discussed and no consensus has been reached to set a standard encompassing all the distinctive features of different classes of antioxidants because each method provide different concept and way of expressing the result. Indirect *in vitro* method such as ABTS, DPPH, and FRAP that involve electron transfer reaction are simple to apply but have some limitations. For example, free radical scavenging ability of antioxidant compounds that are evaluated by these indirect methods are not necessarily reflected the real oxidative degradation even though in some extent the donation of hydrogen atoms or electrons correlates with antioxidant activity (Tiveron

et al., 2012). While respective to Becker *et al.* (2004), direct *in vitro* method such as β-Carotene will assess the characteristic of antioxidant activity by their ability to inhibit or halt lipid oxidation in model systems. It will base on measuring changes in the concentration of compounds being oxidized, on oxygen depletion, or on formation of oxidation products. Thus, there is a need to use several different methods to assess antioxidant activity since these chemically distinct methods are based on different reaction mechanisms.

2.2.1 Antioxidants Polarity

Antioxidants are categorized into hydrophilic and lipophilic properties. Example of hydrophilic antioxidants are ascorbic acid and phenols while lipophilic antioxidant is carotenoids (Cano *et al.,* 2002). Ascorbic acid generally used as calibration reagent for hydrophilic while Trolox® is used for lipophilic to assessing antioxidant capacity (AOC) (Prior & Schaich, 2005). Compound that exhibit different polarity causing antioxidant activity to be differ depending on solvent used for extraction (Pyrzynska & Pękal, 2013). For example by the study of Pyrzynska & Pękal, 2013, on seaweed extract by using DPPH radical scavenging gave different result of antioxidant activity by using different solvent of extraction such as water and ethanol. This might be due to the differences in polarities of each antioxidants compound group present in the seaweeds.

There is also a correlation between the phenolic content and polarity of the extraction solvent. More than one functional group of various solutes consists in various parts of plant such as stem, bark and root. Most of this solute is bioactive compounds of molecules with medium size. Hence it is not easy to identify the solubility of the solute in a particular solvent after extraction. The high polarizability of this solute is susceptible to specific interaction with polar solvent such as

protonation and hydrogen bonding due to the presence of aromatic delocalized μ-electrons. Thus other way of considering solubility is to apply the concept of polarity (Kumoro & Singh, 2009). According to Snyder & Glajch, (1982), polarity is defined as the capability of a molecule to engage in strong interactions with other polar molecule not particularly with the presence in a molecule of a large dipole moment. Thus polarity represents the capability of a molecule to enter the interaction of all kinds and relative polarity is the sum of all possible interactions (Kumoro *et al.,* 2009).

Polar antioxidants which pose higher antioxidation activity such as ascorbic acid, Trolox® and gallic acid have high efficiencies in bulk oil but lower efficiencies in emulsion while the reverse is true for nonpolar alkyl esters. However there are exception for synthetic lipophilic antioxidants of BHA and BHT that poses high efficiencies in emulsions than in vegetable oil. For other synthetic antioxidant such δ-tocopherols, it showed a vice versal trend in bulk oil and liposomes (Shahidi & Zhong, 2011). Antioxidant of complex natural extract exhibit a function of degree of polarity, thus to counteract this problem, the extraction method mostly involve solvents such as water, dimethyl sulfoxide, methanol, dichloromethane, chloroform, acetone or hexane which is different in polarity depending on the compound of interest. Antioxidant activity of extract is tested with different analytical method to evaluate the capabilities of the extract as certain compound presence in the extract also dependent on the degree of polarity. Methods that commonly used to test the antioxidant of different degree of polarity are DPPH, ABTS and β-Carotene (Lage *et al.,* 2013).

2.2.2 Synthetic Antioxidants

Although been reported as carcinogenic, synthetic antioxidant being used in industry are butylated hydroxyanisole (BHA),butylatdhydroxytoluene (BHT), *tert*-butylated hydroxyquinine (TBHQ) and propyl gallate (PG) to combat the adverse effect of lipid oxidation caused by free radicals (Abdullah *et al.,* 2011; Shahidi & Zhong, 2005). Each of antioxidant has their own advantages and drawbacks in terms of thermal stability, synergism and effective concentration (Shahidi & Zhong, 2005). Main drawbacks in using synthetic antioxidants is restriction of being used in food industry because it is suspected to have some toxic properties that are highly volatile and unstable in elevated temperature (Matthaus, 2002). This is very crucial because in order to be used in biodiesel, the antioxidant must be stable in high temperature.

2.2.2.1 Tert-Butylhydroquinone (TBHQ) and Gallates

Hydroxyphenol compound is known as the most popular antioxidants found in market. Tertiary Butyl Hydroquinone (TBHQ), Butylated Hydroxy Toluene (BHT), Butylated Hydroxy Anisole (BHA), and Propyl Gallate (PG) are some of example of hydroxyphenol antioxidants. They are characterized by how low activation energies for the hydrogen donation to process (Aluyor *et al.,* 2009). As one of diphenolic compound, TBHQ is very soluble in alcohol compared to fat and water while gallate is a compound practically dissolved easily in fats and oils but not in water and it is reported to be one of the effective inhibitor of cholesterol oxidation (Shahidi & Zhong, 2005).

Alkyl gallates act as antioxidants in a variety way. It includes inhibiting various prooxidant enzymes that involved in the production of the reactive oxygen speicies, quenching reactive oxygen species, and chelating divalent metal ions such as Fe^{2+} and Cu^{2+}. Due to this special characteristic, alkyl gallates also has been defined

as multifunctional antioxidants. Their diverse antioxidant activities are directly or indirectly multifunctional antioxidants and explained by their head together with their tail structure. Furthermore, alkenyl side chain is largely associated with the activity (Kubo *et al.,* 2010). Epigallocatechin-3-gallate (EGCG), the main and most noteworthy polyphenol found abundantly in green tea, has shown numerous health promoting effects action in multiple different pathways. Besides acting as antioxidant, anti-inflammatory and anti-atherogenic agent, it also have capability to show gene expression activity, functioning through growth factor-mediated pathways, the mitogen-activated protein kinase-dependent pathway. At the same time, this compound also works in the ubiquitin/proteasome degradation pathway, as well as eliciting an amyloid protein remodeling activity (Mereles & Hunstein, 2011).

2.2.2.2 Butylated Hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT)

Phenolic and polyphenolic are compound that contain aromatic ring carbon attached with alkyl group with the only exception for phenol. The variation of alkyl group directly affects their physical properties and one of the factors making this compound an effective antioxidant (Shahidi & Zhong, 2005). Butylatedhydroxyanisole (BHA) and butylated hydroxytoluene (BHT) exhibit these characterization and widely used in industry (Abdullah *et al.,* 2011). Close up view of chemical structure of BHA, one can indentify that this compound is a mixture of two isomer of 2-tertiary-butyl-4-hydroxyanisole and 3-tertiary-butyl-4-hydroxyanisole that have physical structure of a white and waxy solid with the characteristic of highly soluble in fat (Shahidi & Zhong, 2005). Quiet similar to the function of BHA, 3,5-di-tert-butyl-4-hydroxytoluene (BHT) exhibit a physical feature of white crystalline solid. Unfortunately, BHT as not stable as BHA when treated with high thermal treatment. However both BHA and BHT exhibit a common feature of a monophenolic compound with poses an effect as antimicrobial agent (Shahidi & Zhong, 2005).

2.2.3 Natural Antioxidant

Identification of phenolic antioxidants in plant sources has boost up a recent year of research focusing on isolation of effective antioxidants from natural origin. Three major classes of natural antioxidants such as tocopherols, carotenoids and ascorbic acid are used commercially. Other natural substances such as phospholipids, flavonoids, sterols, organic acids, enzymes and protein hydrolyzates have been identified to exhibit an antioxidant activity although having different mechanism (Shahidi & Zhong, 2005). According to Matthaus, (2002), oilseeds contain a compound with antioxidant activity such as flavonoids, hydroxylated derivatives of benzoic and cinnamaric, lignins and caumarins.

2.2.3.1 Tocopherols and Tocotrienols

Tocopherols and tocotrienols are collective known as tocols which can be classes as monophenolic and have a lipophilic characteristic. Main source of natural tocopherols is soybean while for tocotrienols is commonly present in palm oil, rice bran oil, cereals and legumes. These compound are classified into α-, β-, γ- and δ- based on their chemical structure. Generally tocotrienols poses a stronger antioxidant effect on lipid oxidation compared to tocopherols. The antioxidant activity of tocopherols is highest in the form of δ- structure followed by γ-, β- and the lowest is in α- form.

Other than structure, temperature is one of dependent factor determining antioxidant activity of tocopherols. Tocols act synergistically with citric acid, ascorbic acid and phospholipids with function as terminator of free radical in autoxidation reaction. It is insoluble in water but soluble in oil, which will give benefit to the biodiesel compound. However, excessive use of tocopherol may cause pro-oxidation effects (Shahidi & Zhong, 2005).

2.2.3.2 Ascorbic Acid and Ascorbate Salt

Characterize by white or slightly yellow crystalline powder, *L*-ascorbic acid and its salts such as sodium ascorbate and calcium ascorbate has an antioxidant function of reducing agent of free radicals, quenching various form of oxygen, regeneration of primary antioxidants and produce a good synergy with other antioxidants such as α-tocopherol However, ascorbic acid can be easily deteriorated by pH, oxygen, heat light, water activity and acrid smoke and it is insoluble in lipids therefore its application is limited in fats and oils only (Shahidi & Zhong, 2005).

2.2.3.3 Carotenoid

Carotenoids can be widely found in plants with physical properties of yellow, red and orange color. This compound quenching singlet oxygen and trapping free radicals in the absence of singlet oxygen to prevent oxidation thus acting up as secondary antioxidant by forming a good synergist with tocopherols. Varies in structures, there are 40-carbon of isoprenoids that can be classified as carotenes and xanthophylls. Certain carotenoids namely β-Carotene, α-carotene and β-cryptoxanthin are also referred as pro-vitamins. β-Carotene with a structure of hexagonal prism is highly reactive and poorly soluble in most solvents has an unstable characteristic to pH, oxygen, light and heat (Shahidi & Zhong, 2005).

2.2.3.4 Phenolic Compound

Since the ability of this compound was discovered in 1940s, phenolic compound has boost up the application of synthetic antioxidant in food industry (Matthaus, 2002). According to Puttaraju *et al.* (2006), tannicacid, gallic acid, protocatechuic acid and genstisic acids are common phenolics detected in mushrooms. The ability to undergo photosynthesis by absorbing sunlight and synthesis secondary metabolites and antioxidants make plant a potential source of natural bioactive compounds. Example

of secondary metabolite is phenolic acids that assist plant growth and reproduction, defend the plant against infections and responding to environmental cues (Ghasemzadeh & Ghasemzadeh, 2011).

According to Stalikas (2007), plant phenolics encompasses of simple phenols, phenolic acids, flavanoids, hydrolysable and condensed tannins, lignins and lignans, stilbenes, and coumarins. The term of phenolic acids generally refer to phenols that consist of at least one carboxylic acid functionality but when describing about plant metabolites, it refer to diverse group of organic acids which is not phenols alone. With diversification in term of structure, phenolic compounds being characterized by their hydroxylated aromatic rings of hydroxycinnamic and hydroxybenzoic. This carbon skeleton can be found inside or outside of the cell walls dependent on their solubility. For example soluble phenolics is comparted inside plant vacuole while insoluble phenolics is localized in cell walls. However there are exception for minor fraction of phenolics as some of the compounds exist in the form of free acid being linked through ether, ester or acetal bond to cellulose, proteins, terpenes and glucose (Ghasemzadeh & Ghasemzadeh, 2011; Stalikas, 2007). Other than these compounds, proanthocyanidins (a condensed tannins) and lignins also present in plant but being polymerized into larger molecules. Moreover some phenolic compound such as glycosides or ester may arise together with other natural compound of alcohols, glucosides, hydroxyfatty acids and sterols (Ghasemzadeh & Ghasemzadeh, 2011).

2.2.3.5 Flavonoid

According to Matthaus, (2002), more attention have been given to the use of flavonoid as natural antioxidant since several years ago, this type of compound can be ubiquitously found in food plants as it participate in establishing a normal growth for the plant plus it also responsible in defending the plants against infection and injury.

There are about 6000 flavonoids compound in higher plants (Ghasemzadeh & Ghasemzadeh, 2011) which are belong to the class of polyphenols which contain at least two phenols subunits of hydrolysable and non-hydrolysable compounds (Stalikas, 2007). Variation of flavonoids glycosides are based on the glycosylation level, number of type of sugar and number of position on the flavonoids involve in glycosylation (Ghasemzadeh & Ghasemzadeh, 2011). There are six subgroups of flavonoids listed in Table 2.2 below.

Table 2.2 : List of compounds in flavonoid.

Subgroups	Examples
Flavones	Luteonin, apigenin and tangeritin.
Flavonols	Quercetin, kaemferol, myricetin, isorhamnetin and pachypodol.
Flavanones	Hesteretin, naringenin and eriodictyol.
Flavan-3-ols	Catechins and epicatechenis.
Isoflavones	Genistein, daidzein and glycitein.
Anthocyanidins compounds	Cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin.

(Ghasemzadeh and Ghasemzadeh, 2011).

Glycosides are the flavonoids compound that accumulated and a can be found in the vacuoles of plant cells that are either linked with oxygen or carbon (O- or C-). Its chemical component build up with skeleton of C_6-C_3-C_6 made of two aromatic six carbon rings with a heterocyclic ring that consist of one oxygen atom. Flavonoids, which one of phenolic acids are secondary metabolite product of a plant but being synthesized from slightly different pathway of polypropanoid with starting precursor of phenylalanine molecule. Common flavonoids known are condensed tannins, xanthones and aurones while catechins and leucoanthocyanidins have a similar structure but seldom exist as their glycosides (Ghasemzadeh & Ghasemzadeh, 2011).

2.3 Effects of Phenol Substituents

The reducibility of phenolic and flavonoid compounds would be strengthen by steric hindrance which depends on the number of hydroxyl groups in their molecular structures. However flavonoids alone has a function to maintain plant physiology and structure such as plant-microorganism communications, plant protection and stimulation, contribution in plant colors and pigmentation, and taste and flavoring (Ghasemzadeh & Ghasemzadeh, 2011).

Phenol compound has the basic benzene structure with a hydroxyl group attached directly to the ring. The alkyl group such as methyl, *tert*-butyl or methoxy on the ring is known as substituent. The nature of electron-donating substituents can enhance the electron density of phenol compound at the oxygen of hydroxyl group. The position attachment of this substituents on the ring is called o*rtho*, *meta-* or *para-* depending on which carbon the attachment is. Figure 2.3 show the structure of the phenol ring with the position name of the substituents. R1, R2 and R3 positions is known respectively as *ortho*, *meta* and *para* (Ghasemzadeh & Ghasemzadeh, 2011; Stalikas, 2007; Kajiyama & Ohkatsu, 2002; Kajiyama & Ohkatsu, 2001; Matsuura & Ohkatsu, 2000).

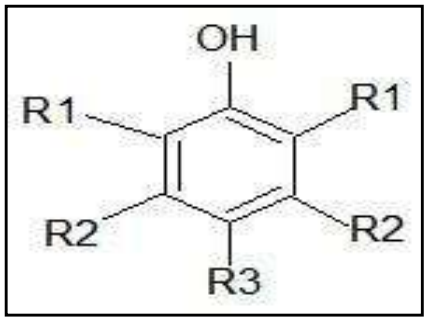


Figure 2.3: Phenol structure.

2.3.1 Steric Effect

The number of substituent is not the only factor affecting antioxidant activity because the position of substituent located will affect the potential of the substituent to scavenge free radicals. From the study of *ortho*-alkoxyphenols and *para*-alkoxyphenols, result showed that antioxidation is depends on the position of a substituent. In this study all the substituents are similar for both *ortho-* and *para-* but been located at different position, the result showed that the IP are double for *para-* position while almost no effect for *ortho-* position (Kajiyama & Ohkatsu, 2001). This study was supported by Lawandy *et al.* (1996), stated that strength of hydroxyphnyl antioxidant activity is following the order of *para- >ortho- >meta-* position. Even *ortho- position* for methoxy alkoxy group show no induction period thus no antioxidant activity. However with the addition of alkoxy substituents into *para-* position showed significant increase of induction period.

The *para*-alkoxy with the characteristic of high electron donating nature donate electron to the hydroxyl group of the phenol. The free electron dissociate hydrogen atom from the oxygen and break the bonding between them. Such electron transfer resulting in the breakage of the intramolecular hydrogen bonding with alkoxy group at *ortho- position*. The dissociated hydrogen will bind to free radical available. It can be concluded that the presence of *para-* constituent causing the distance of oxygen and hydrogen in hydroxyl group to be farther apart thus reduce the strength of hydrogen bond between them so that antioxidant activity developed (Kajiyama & Ohkatsu, 2001; 2002).

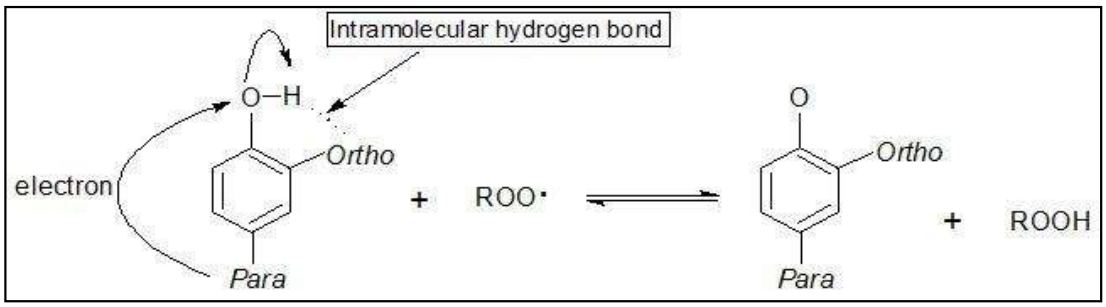


Figure 2.4 : Mechanism of steric effect (Matsuura and Ohkatsu, 2000 ; Bendary *et al.*, 2013; Ma *et al.*, 2011).

Figure 2.4 showed the mechanism of steric effect of the substituents that increase the number of the peroxy radical (ROO·) trapped and preventing it from coupling (Matsuura and Ohkatsu, 2000). This happened because phenoxyl radical generated after scavenging activity stabilized through intramolecular hydrogen bonding and resonance effect of the benzene ring. As the hydrogen atom is extracted from hydroxyl group and causing phenoxyl radical to form, *para*- substituent will donate an electron by localization to the hydroxyl group. A lone electron on the hydroxyl group will formed intermolecular hydrogen bond with *ortho*- substituent thus stabilizing the compound (Bendary *et al.*, 2013; Ma *et al.*, 2011).

2.3.2 Polar Paradox

In designing antioxidants for particular experiment, relevant factors must be taken into consideration as antioxidant was found to behave in particular manner in different media. This condition is called polar paradox theory as nonpolar antioxidants tend to be more effective in more polar media such as liposome or oil-in-water emulsion whereas polar antioxidants are more effective in less polar media such as bulk lipids (Shahidi & Zhong, 2011). The polar paradox was studied as early as 1980 when Porter (1980), stated in his study that amphiphilic antioxidants with low hydrophilic-lipophilic balance to be more active in polar emulsion of high surface-to-volume ratio while polar primary antioxidants or amphiphiles with high hydrophilic-lipophilic balance tend to be more active bulk oils with low surface-to-volume ratio. In bulk

lipids there are three essential parts namely water droplets, water-lipid interface and bulk lipids. Antioxidants in bulk lipids which is nonpolar media, with the presence of water droplet in the bulk lipids, hydrophilic antioxidants which is polar will distribute at water-lipid interface and forming a protective shell around the water droplet. Thus when free radical from the lipids media interfering the water droplet, they will be scavenged by the polar antioxidants present at the interface layer while for antioxidants in emulsions counteracting in the bulk lipids.

There are three crucial parts in oil-in-water namely continuous aqueous phase, lipid droplets and the water interface. Both lipid and non-lipid were partitioned according to solubility, surface activity, chemical structure and polarity. Due to differences of the two system of bulk oils and oil-in-water emulsions, antioxidants in this two system exhibit different effectiveness (Zhong & Shahidi, 2011; Shahidi & Zhong, 2011). In polar paradox theory, oil-in-water emulsion are protected better by nonpolar antioxidant compared to polar one from oxidation because of the reason of nonpolar antioxidants have great affinity for the oil-in-water interface. Nonpolar antioxidants with low hydrophilic-lipophilic balance (HLB) forming a layer of protective membrane around the lipid droplet due to high concentration at the oil-water interface whereas the polar antioxidants commonly dissolved in the aqueous phase. This state of distribution will prevent free radical from penetrating the lipid phase because free radical will be scavenged by nonpolar antioxidant at the interface layer (Zhong & Shahidi, 2011; Shahidi & Zhong, 2011).

2.4 Antioxidant Tests

There are several ways to improve the oxidative stability of biodiesel and one of the method is to decrease unsaturation degree of the oil by hydrogenation. However this method increase freezing point of this natural fuel. Other alternative to prevent the degradation of the fuel is by adding antioxidants (Luo *et al.,* 2012). Thus to investigate the degree of antioxidants activity, DPPH, TEAC and ABTS are the most widely procedure used (Marinova & Batchvarov, 2011).

2.4.1 DPPH Free Radical Scavenging Activity Assay

The most precise name used in literature review describing the mechanism of reaction for DPPH method are free radical scavenging activity rather than antioxidant activity (Marinova & Batchvarov, 2011).Based on the original method of Blois and Brand-William in the year of 1958 and 1995 respectively (Marinova & Batchvarov, 2011), 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay is one of the most accepted methods in doing evaluation of the free radical scavenging activity. It is one of the stable free radical with maximum absorption of 517 nm and appears purple in methanol. The purple color will change to yellow due to antioxidant which are reacted with DPPH because of the stable free radical is paired with hydrogen donated from the antioxidant and then reduced to DPPH causing the absorbance to be decreased (Patel and Patel, 2011).

One drawback of this method is the effect of stearic inaccessibility that cause antioxidants that react fast with peroxyl radicals may react slowly or even worst it inert to DPPH. For example interpretation of test compound such as carotenoid that overlap the wave length that 515 nm is quite complicated as it interfered the absorbance reading (Prior & Schaich, 2005). It is been recommended by the study of

Molyneux (2004), to load the sample and DPPH solution in the ratio of 1:1 into the cuvette in order to get the optimum result. Other than above recommendation, the use of methanol or ethanol is preferable rather than acetone. Although this assay is widely used, there are significant differences of the method and literature review about DPPH antioxidant activity. For example of common differences in studies are the type of equations used to calculate the antioxidant activity. Most of the studies used the equation of:

$$\left(\frac{\text{Control} - \text{Sample}}{\text{Control}}\right) \text{X } 100 \quad \text{AND} \quad \left(\frac{1 - (\text{Sample} - \text{Blank})}{\text{Control}}\right) \text{X } 100$$

Other common conflict of variation used in this assay is the type of standard used. Most of the study used ascorbic acid and only a few study use Trolox® and α-tocopherol. The last variation of the method is the wavelength used which prefer 517 nm than 515 nm (Marinova & Batchvarov, 2011). According to Molyneux (2003), there are two types of stoichiometry of DPPH that are 1:1 or 2:1 ratio of DPPH with other antioxidants. For example in the case of cystein, this molecule exhibit 1:1 stoichiometry ratio because two molecules of cystein will reduce two molecule of DPPH in a reaction. However for the case of ascorbic acid, by two-step mechanism, one molecule of DPPH will be reduced by a molecule of ascorbic acid that lead to 2:1 ratio. This happened because ascorbic acid has internally connected two adjacent site of hydrogen abstraction.

2.4.2 β-Carotene Bleaching Assay

β-Carotene bleaching method is a combination study of different researchers in different year. β-Carotene method was a study by Marco in the year of 1968 while crocin bleaching was studied by Bors, Michel, & Saran, in the year of 1984. According to Prieto *et al.* (2012), β-Carotene bleaching assay is one of the most used

antioxidant test. The reaction of antioxidant works in aqueous emulsion of linoleic acid and β-Carotene. As fatty acid is prompted to undergo spontaneous oxidation by thermal induction normally at 50°C and produce free radical, β-Carotene that decayed in order to stabilize the free radical will be discolored. The evaluation of antioxidant activity at 470 nm is proportional to the quantification of β-Carotene decayed.

According to Jayaprakasha *et al*. (2001), β-Carotene bleaching mechanism started from formation of hydroperoxides free radical from linoleic acid. Hydroperoxide, a free radical that lose a hydrogen atom from one of its diallylic methylene groups then attacks the highly unsaturated β-Carotene molecules. When β-Carotene molecules lose their double bonds by oxidation, the compound loses its chromophore and orange color thus decolorization happened and can be detected by using spectrophotometer. The presence of antioxidants in different extracts can protect β-Carotene by neutralizing the peroxide product which is formed from linoleic acid. Thus decolorization is absent and high absorbance reading indicate highest antioxidant activity (Mammadov *et al.,* 2011).In quantifying antioxidant and pro-antioxidant activities, β-Carotene and crocin bleaching reactions is one of two methods extensively used. This method is widely used for testing hydrophilic and lipophilic matrices in term of providing information to study complex natural extract that exhibit variable degree of polarity. β-Carotene is a compound that will oxidize substrate of lipophilic and exhibit hydrophobic repulsion. Thus this compound is able to lipidic micelles the system and corresponding oxidation is accomplished in lipidic environment (Lage *et al.,* 2013).

2.4.3 ABTS Radical Scavenging Activity Assay

First reported by Miller and Rice-Evans, (1993), this method using radical anion ABTS activity toward antioxidants. Antioxidant capacity is measured by the capability of antioxidants to reduce ABTS anion to it stable state resultant in color reduced. Intensity of the color reduced will then expressed relative to Trolox® (Prior & Schaich, 2005). Besides being one of the fastest method, ABTS provides good solubility, which allows the analyses of both lipophilic and hydrophilic compounds (Tiveron*etal.,* 2012). This is also supported by the study of Prior *et al.* (2005), stated that ABTS is soluble in both organic and aqueous solution and not affected by ionic strength. However, the pitfall of this method is does not suitable to assess antioxidant that reacted slowly because the reading taken is not correlates the completed reaction of the antioxidant of interest.

2.5 Plants Extract

Plants such as vegetables, fruits, leaves, oilseeds, barks and roots, spices and herbs contained a lot of potential natural antioxidants such as flavonoids and other common phenolic compounds. These compound are pivotal in maintaining the normal growth and involve in defensive mechanism (Kähkönen *et al.,* 1999). These mechanisms happened due to the redox properties of these compounds which allow them to become either reducing agent, singlet oxygen quenchers or hydrogen donator (Rice-evans *et al.,* 1995).

2.5.1 Roselle

Roselle has many names depending on locality (Bamgboye & Adejumo, 2009). In tropical region such as in Middle Eastern countries, Roselle known as Sorrel Mesta (Tounkara *et al.,* 2013) while in other places such as Nigeria it is known as Zoba in the northern part and as Isapa pupain the southern part of the country (Bamgboye & Adejumo, 2009). Despite of the many names Roselle has, scientifically it is known as *Hibiscus sabdariffa* belong to the family of Malvacea. This plants exhibit lot of medicinal value and frequently use in the kitchen as a food product such as beverages, making jams, herbal drinks and jellies (Tounkara *et al.,* 2013). For every calyces of fruit capsule contain for about 5 grams of seed (Sánchez-Mendoza *et al.,* 2008) and for every seeds its moisture content is 6.48% and the crude oil content is 21.85% (Nzikou *et al.,* 2011). Although the origin is not fully known, *Hibiscus sabdariffa* is believed to be native to tropical Africa. Reported to have seeds bigger than pearl millet varieties, Roselle seed having the average dimension of 5.21 mm and 2.81 mm with a bitter taste (Cissouma *et al.*, 2013).

2.5.2 Cashew Nut

The most conventional method use to process raw nut is by drying under open sun followed by steaming and kernel drying with electricity. The moisture content of the cashew nut shell was found to be 6.47% (Mohod *et al.,* 2011) and the oil content is about 20 -25%. Most cashew nut shell liquid (CNSL) is used as fungicide, pesticide and insecticide (Rodriques *et al.*, 2006). Contained natural source of unsaturated long-chain phenols, CNSL can be obtained cheap from industry as CNSL is by-product and discarded as waste. (Rodriques *et al.*, 2006). CNSL can be employed as starting material for organic synthesis and replace phenols in a lot of equivalent occurrence, resin from CNSL also being used in several miscellaneous application such as surface coating and laminates (Rodriques *et al.*, 2006). There are two basic methods of cashew nut shell extraction namely solvent-extracted CNSL and technical CNSL by roasting the shell at 180°C to 200°C (Rodriques *et al.*, 2006). Although there is no preferential *ortho-* or *para-* position in phenolic substitution, CNSL contained mixture of phenols with long alkyl substitution at the meta-position (Rodriques *et al.*, 2006).

2.6 Soxhlet as Solid-liquid Extraction

The term "Soxhlet" is applied to the method of extraction to honor the German chemist, Franz Von Soxhlet as its inventor. Although it is an ages-old method but it is widely used since the year of 1879 when it was first invented. He devised a liquid/solid extraction by placing the sample in the cellulose thimble so that the sample is solubilized and the solvent is siphoned back into the distillation flask (Anderson & Luthria, 2004).Extraction of solid samples is normally known as solid-liquid extraction and also referred as leaching or lixiviation. In conventional Soxhlet, sample is placed in the extraction thimble-holder and once the solvent reached the overflow level, siphon aspirates the whole content into the distillation flask carrying the extracted solute in solvent content below. The assembly of the solvent that soaked the thimble filter can be considered as a batch system but the circulating solvent through the whole system is considered continuous. This system makes Soxhlet a continuous-discontinuous technique in sample extraction (De Castro *et al.,* 2000). Solid/liquid extraction commonly uses to isolate compound from natural products. The resultant extract are typically in homogeneous liquid mixture thus separation is necessary to separate the desire product from mixture of by product and residual matters.

Solvent is one of the important criteria that need to be carefully chosen depending on the compound need to be analyzed. Example of polar solvent is methanol followed with ethanol and isopropanol. While most common nonpolar solvent used are hexane, petroleum ether and pentane (Williamson and Master, 2011). Solvent coupled with the use of heat and sometimes agitation is one of the classic techniques of extraction. However the choice of solvent used often decrease the molecular affinity between the solvent and the solute resulting in less recovery of bioactive compound. The cost of using alternative solvent is sometimes higher than

casual solvent used (Wang & Weller, 2006). Thus according to Li & Weiss (2004), using a co-solvent such as isopropanol together with conventional such as hexane is reported to increase the yield and kinetics of extraction. After the extraction process, solvent is evaporated to recover bioactive compounds. The compound recovered is significantly affected by the temperature used in the evaporation process which is supported by the study of Mamidipally & Liu (2004), stated rice bran oil extracted with different temperature gave a different appearance in terms of color which might be due to higher extraction and evaporation temperature. As the solvent is heated, a decomposition of the bioactive compound might happened thus this will affect the study (Wang & Weller, 2006). In order to decrease the boiling temperature to retain a good quality of the oil, solvent recovery by evaporation coupled with vacuum or membrane separation is a good practice (Mamidipally & Liu, 2004).

According to Wang & Weller (2006), advantage of Soxhlet extraction is that there is no need for filtration after leaching and high temperature can be maintain with heat from the distillation flask. Other than that, solid sample can be repeatedly extracted by displacing the solvent with a fresh one. However this method is not applicable for the sample that need short interval of time to be extracted and sample with a small volume. Plus agitation cannot be imposed throughout the extraction process in order to accelerate the extraction and the need of large amount of solvent for the Soxhlet to operate.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

Hibiscus sabdariffa (Roselle) was bought from a local supplier in Shah Alam, Selangor while *Anacardium occidentale* (Cashew nut) was collected from a plantation in Padong nearby Bukit Kayu Hitam, Kedah. Separation of Roselle seed was done sooner because Roselle seed tend to be infected by fungus if it is not properly dried. However for Cashew nut shell, the shell can be kept in room temperature as the shell is hard and high in resistance to spoilage. Extra precaution must be kept as the shell nut oil can cause skin irritation.

The plant materials collected were then extracted with different solvent polarity of hexane and methanol. Then, all the extracted samples were tested with 4 different tests of antioxidant activities, phenolic determination, profiling of extracted compound and oxidative stability of the biodiesel added with the extracted samples. All tests are separately done and not in continuous from the previous test because each sample for each individual test is consumable. The tests were done in triplicates to get accurate reading while avoiding machinery errors. Figure 3.1 showed the overall flow of methodology that has been simplified for better understanding.

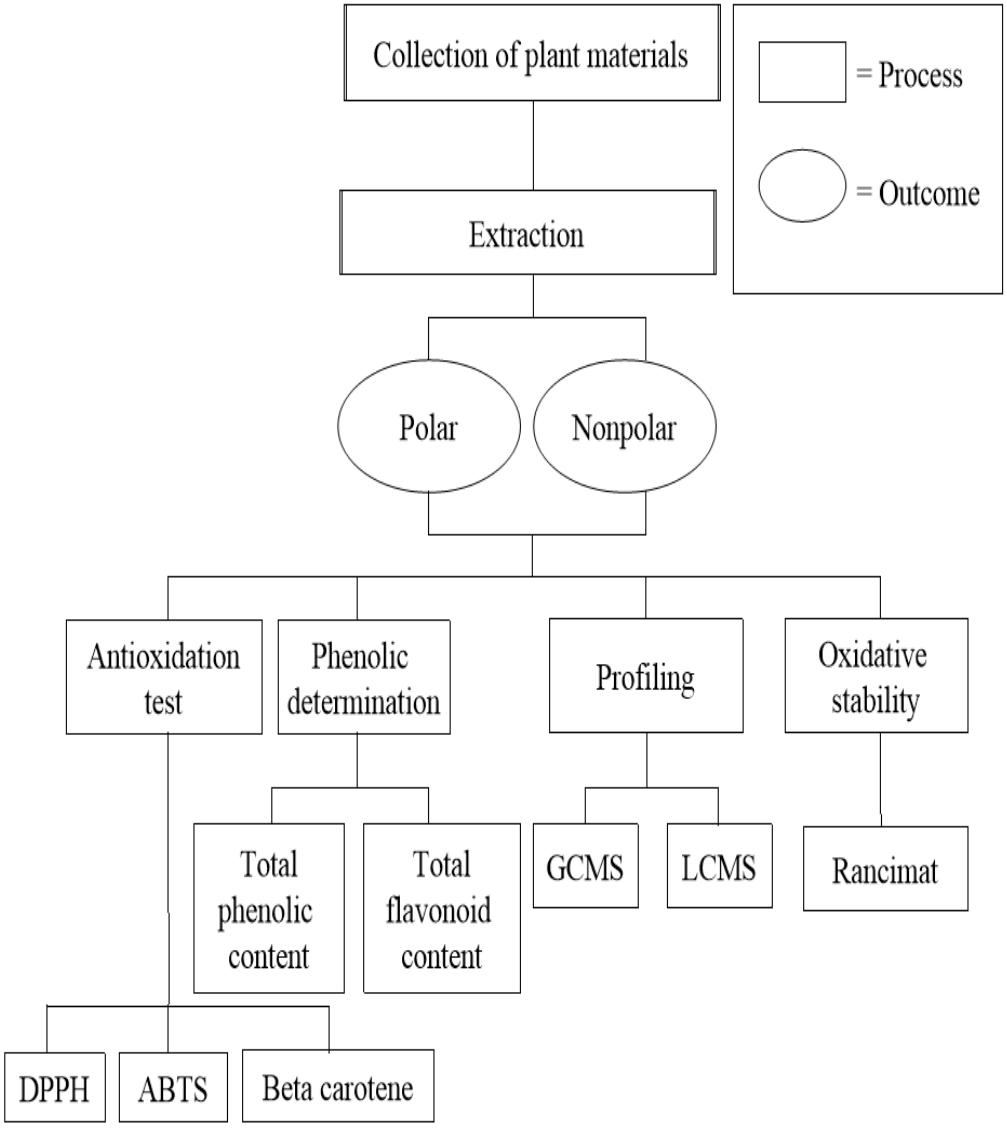


Figure 3.1 : Overall flow of methodology

3.2 Reagents and Apparatus

Potassium acetate, quercetin, Folin–Ciocalteu reagent, sodium bicarbonate, gallic acid, acetonitrile, formic acid, ammonium formate, methanol, chloroform, hexane and butylated hydroxylanisole were purchased from Merck Chemical Co. (Malaysia). Trolox®, 3-pentadecyl phenol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium peroxodisulfate, phosphate buffer, β-Carotene and linoleic acid were purchased from Sigma Chemical Co. (USA). Major apparatus were used were UV-Visible spectrophotometer(7305 JENWAY Spectrophotometer), cuvette, gas chromatography mass spectrometry (Agilent 7000C Triple Quadrupole GC/MS), liquid chromatography mass spectrometry (AB Sciex Instruments Linear Ion Trap Quadrupole LC/MS/MS), screw cap bottle, syringe filter, soxhlet apparatus, rotary evaporator, round-bottom flask, grinding machine. Biodiesel was purchased from Weschem Technologies Sdn. Bhd.

3.3 Extraction of Plants Materials

In order to extract the bioactive compound from plant material, method of best efficiency that yielded the highest extraction is by using Soxhlet extraction method (Kothari *et al.,* 2012). The polarity of compounds is taken into consideration by using two solvent having the opposite polarity such as methanol and hexane. Roselle seeds and Cashew nut shell samples were dried, grinded into fine powder and extraction was done by using Soxhlet method. 30 grams of powdered samples were extracted with 350 ml solvent of hexane and methanol for 8 hours at 70°C. The solvent was then evaporated by using rotary evaporator at 60°C. Yield of crude extract were calculated by using the equation (3.1):

$$\frac{\text{Extracted dried mass (g)}}{\text{Dried crude mass (g)}} \times 100\% \qquad \text{.....Equation (3.1)}$$

3.4 Antioxidants Activities Test

In this study three different types of antioxidant test were used for confirmation as depending for only one test is rather indistinct and this is aligned with the study of Siramon & Ohtani (2007) stated that the use of multiple method is needed as specificity and sensitivity between test might be different. The percentage of concentration that was required to scavenge the free radicals was calculated from 5 different concentrations of the fractions and the absorbance were taken by using UV-Visible spectrophotometer. Then the IC_{50} of each extract was calculated from the graph plotted. All data were statistically analyzed by using SPSS to determine whether there are significant differences between the samples that contribute to the oxidation activity. All experiments were performed in triplicate (n=3) and results were expressed as mean ± SEM. Statistical analysis was carried out with (SPSS package version 10.0) by using ANOVA followed by Tukey's post-hoc test ($P<0.05$).

3.4.1 DPPH free radical-scavenging activity

The DPPH free radical scavenging activity was determined according to the method of Mohd-Esa *et al.* (2010) with slight modification. 1ml freshly prepared 0.1M DPPH in methanol was added into each cuvette containing samples extract in the range of 2.92, 5.83, 8.74 and 11.66 and 14.58 mg/ml and tested after 5 minutes. On the other hand, a control will be prepared by adding 100 µl methanol in 1ml of DPPH solution. BHA and Trolox® with concentration starting from 20 mg/ml with 5 times dilution factor were measured as positive control. Antioxidant activity was calculated by using Equation (3.2):

$$\frac{\text{Absorbance of control - absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad \text{.....(Equation 3.2)}$$

3.4.2 β-Carotene bleaching assay

This method was prepared according to the method of Barros *et al.* (2008) with slight modification. 2 mg of β-Carotene was dissolved in 10 ml of chloroform and then 2 ml of this solution were pipetted into a 100 ml round-bottom flask. The chloroform was removed at 40°C by using rotary evaporator and 100 ml of methanol was added followed by 20 µl linoleic acid and 200 µl of Tween 20 emulsifier. Briefly each sample was diluted 10 times starting from 72.88 mg/ml until 5 consequence concentration. For every 100 µl samples, 1 ml of aliquots was used and was measured at 470 nm by using UV-visible spectrometer at 0 minute as initial reading. Trolox® and BHA undergo similar serial dilution but starting from 50 and 0.0004 mg/ml respectively as positive control. Then the samples were incubated at 50°C in water bath and final reading was taken at 120 minutes. A blank, devoid of β-Carotene, was prepared for background subtraction. Antioxidant activity was calculated by using Equation (3.3):

$$\frac{\text{Absorbance reading after 120 minutes}}{\text{Absorbance reading at 0 minute}} \times 100 \quad \text{.....(Equation 3.3)}$$

3.4.3 ABTS radical scavenging activity

The ABTS radical cation was produced using Miller *et al.* (1993) method with slight modification. A solution of ABTS (10 mg) and potassium peroxodisulfate (2.9 mg) was diluted with 0.01 M pH 7.4 phosphate buffer (10 mL). The mixture was protected from light and stored at room temperature for 12–16 h. Formation of ABTS was checked by its absorbance at 734 nm. The ABTS solution was diluted with methanol to an absorbance of 0.80 (±0.05) at 734 nm. For the assays, briefly, 2.92, 5.83, 8.74 and 11.66 and 14.58 mg/ml samples were mixed with ABTS solution of 1 ml. Reduction of absorbance was measured at 734 nm after 5 min. BHA with

concentration starting from 20 mg/ml with 5 times dilution factor was measured as positive control while Trolox® was measured by using 2, 0.4, 0.2, 0.04 and 0.02 mg/ml as positive control. Antioxidant activity was calculated by using Equation (3.2).

3.5 Phenolic Determination

All data were statistically analyzed by using SPSS software (version 10.0) to determine whether there are significant differences between the samples that contribute to the oxidation activity. All experiments were performed in triplicate (n=3) and results were expressed as mean ± SEM. Statistical analysis was carried out with ANOVA (SPSS version 10.0) followed by Tukey's post-hoc test ($P<0.05$).

3.5.1 Total Phenolic Content Determination

Predetermined concentrations (2.92, 5.83, 8.74 and 11.66 and 14.58 mg/ml) of each sample extracts were then determined according to the method of Mohd-Esa *et al.* (2010). Each sample were added with 1 ml of 0.5 M Folin–Ciocalteu reagent then shaken for 5 minutes. Then 1 ml of 75 g/L sodium bicarbonate was added and the mixture was shaken again for 30 seconds. After 5 minutes, the absorbance was read with UV/Vis spectrophotometer at 760 nm. The phenolic content were determined by plotting the galic acid calibration curve with 5 different concentrations (refer appendix A.1) starting from 2 mg with dilution factors of ten and expressed as milligrams of gallic acid equivalent (GAE) per gram of dried extract.

3.5.2 Total Flavonoid Content Determination

100 ml of 10% aluminum chloride with 1M of potassium acetate was prepared. Then 3.8 ml of methanol was added at room temperature for 40 minutes. 14.58 mg/ml of sample extract was mixed up with the above aliquot and analyzed using UV-visible spectrophotometer at 415 nm. The flavonoids were determined by plotting the quercetin calibration curve with 5 different concentrations starting from 2 mg with dilution factors of ten and expressed as milligrams of quercetin equivalent per gram of dried extract.

3.6 Analyses of Bioactive Compounds.

Samples that were extracted with hexane (nonpolar) were analyzed with GCMS while samples extracted with methanol (polar) were analyzed with LCMS.

3.6.1 Gas Chromatography Mass Spectrometry (GCMS)

Gas chromatography mass spectrophotometer analysis was done using Agilent 7000 GC-QQQ gas chromatography coupled to Ion trap mass spectrometer. The column that used was HP-5MS with 5% phenyl methyl silox. The oven temperature was programmed to have the initial temperature of 70°C for 0 minute and increase gradually with 10°C/min up to 300°C and was held for 6 minutes. Carrier gas used was Helium at 1 mL/min in constant flow mode with split less injection temperature of 250°C, auxiliary temperature of 280°C and threshold at 150.

3.6.2 Liquid Chromatography Mass Spectrometry (LCMS)

The sample were determined by LCMS/MS method using AB Sciex 3200Q Trap LCMS/MS with Perkin Elmer FX 15 uHPLC system. Agilent Zrbax C18, 150mm x 4.6mm x 5µm was used as column. Two types of buffer were used; Solvent A : water with 0.1% formic acid and 5mM ammonium formate and Solvent B is Acetonitrile with 0.1% formic acid and 5mM ammonium formate. For the MS settings, the voltage IS and source temperature was set at 5500V with temperature of 500°C each. The gradient run program was set at 10% B to 90% B from 0.01 minute to 8minutes. It was hold for 3 minutes and back to 10% B in 0.1min and re-equilibrated for 5 minutes. The sample was diluted in 2mL solvent and filtered with nylon 0.22µM.Analysis was carried out by injecting 20µL of diluted sample solution.

3.7 Oxidative Stability Test

The usage of natural plants extract with different extraction solvent were tested by using the Metrohm Rancimat (Metrohm 873) following the European Standard of EN 14214 and EN 14112. Synthetic antioxidants of BHA, 3-pentadecyl phenol (analytical grade) and Trolox® were also tested with same procedure as positive control. Each sample and positive control were tested with palm oil biodiesel (fatty acid methyl esters) from Weschem Technologies Sdn. Bhd. with 0.016, 0.033 and 0.066 weight/weight percentage (w/w %) at temperature of 110 °C with gas flow rate of 10 L/h.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

This chapter contains the experimental and discussion result which divided into 6 sections. The first section explains about percentage yield of extract and followed by the section about antioxidant activity of the 3 tests of DPPH, β-Carotene and ABTS. The third section discussed about phenolic determination and followed the by the correlation coefficient of DPPH and TPC. The fifth section discussed about the analyses done using GCMS and LCMS to identify the bioactive compounds of the extracts and the last section discussing the oxidative stability of biodiesel with addition of the extract as an antioxidant.

4.2 Determination of Oil Extracts Percentage

Extraction of plant material is commonly done by using organic solvent as the nature of antioxidants and that of plant materials becomes the two preliminary factors of determination (Pokorny & Korczak, 2011). The oil extract percentage of Cashew nut shell with methanol extract (CM) and hexane extract (CH) while Roselle seeds with methanol extract (RM) and hexane extract (RH) was tabulated in Table 4.1. In comparison both samples' extraction, Cashew nut shell regardless of extraction solvent show better yield with double the value of Roselle extract and appeared dark brown in color and correlated with the study of Akinhanmi *et al.* (2008) while Andrade *et al.* (2011) mentioned the oil as viscous dark liquid.

Table 4.1: Oil extract.

	CM	**RM**	**CH**	**RH**
Dried weight (g)	11.16	5.21	10.17	4.31
Percentage (%)	37.20	17.37	33.90	14.37

4.3 Antioxidant Activity Test

The use of single assay is insufficient to validate the antioxidant activity of the oil extract. Therefore, 3 assays were applied to further investigate antioxidant activity of extracts as Huang & Prior (2005) stated that using a single assay is rather unreliable and not easy to conclude as there is no consensus and vague of opinion to validate the reliability of which of the numerous methods to be used. Assays are complement with each other in order to give better result for antioxidant activity.

In this study 3 type of assays were used to analyze the samples for confirmation of result of both *Hibiscus sabdariffa* seed and *Anacardium occidentale* nut shell plant extracts. The extracts were compared according to amount of concentration needed to inhibit 50% of free radical activity (IC_{50}). BHA and Trolox® were used as positive control because BHA is a polar and most of the previous research used this synthetic compound thus it will ease in interpretation of data while Trolox®, which is analogous to vitamin C was used because according to Prior (2005), this compound typically used to calibrate reagents for both lipophilic and hydrophilic antioxidation test, thus it can express in both polarity.

4.3.1 DPPH Free Radical Scavenging Activity Assay

Statistical analyses showed that samples mean varies significantly with $F(3,8) = 145.295$, $p < 0.05$ and were presented in Figure 4.1. The results showed the order of DPPH radical scavenging activities of the extracts as Trolox® > BHA > CM > RM and their IC_{50} concentration of inhibition was tabulated in Table 4.2.

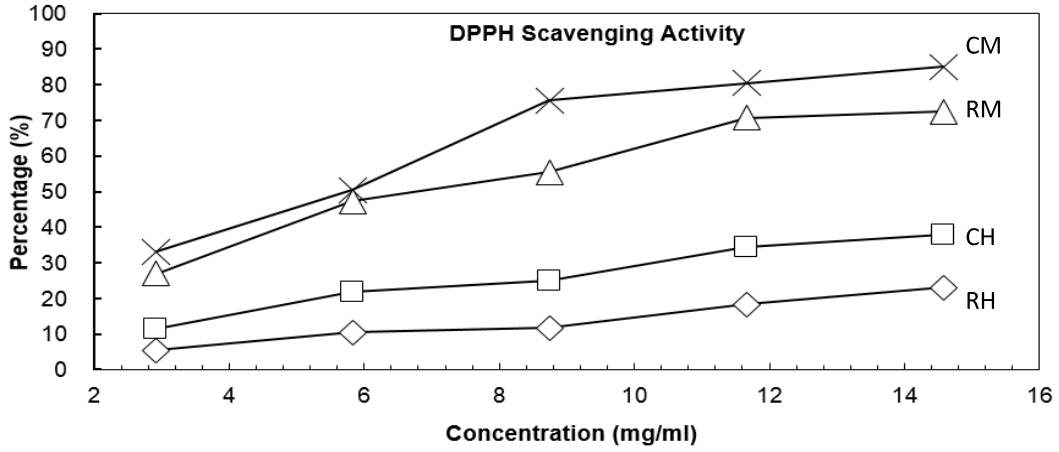


Figure 4.1 : DPPH scavenging activity of samples extract and positive controls. Data for Trolox® and BHA not shown on the graph.

From DPPH assay, CM showed the strongest inhibition capacity compared to the rest of the samples with IC_{50} of 5.489±0.31 mg/ml but lower than positive control. Although CM exhibit the best IC_{50} among all the samples but the inhibition value is less than the study of Andrade *et al.* (2011) which has the value of 0.12774 mg/ml in methanol. RH and CH did not achieve 50% of inhibition even at the highest sample concentration. However by using graph equation, both CH and RH estimation of IC_{50} were 19.412±0.09 and 33.205±0.67 respectively.

4.3.1.1 Comparison Polar and Nonpolar Extract in DPPH Assay

The lower inhibition percentage of CH and RH compared to CM and RM could be explained by the difference in polarity of the solvent used during assay testing. DPPH scavenging assay favored an extraction in polar solvent rather than nonpolar solvent. This is aligned with the study of Makari *et al.* (2008) when inhibition percentage of *Cordia wallichii* plants extract by using hexane is lower compared to methanol extract. In addition Ng *et al.* (2012) showed hexane displayed the least effective DPPH radical scavenging activity compared ethanol, methanol and acetone. This

study also stated that methanol with polar index of 5.1 showed the highest total phenolic content compared to the rest of the solvent. This polarity index of solvent must be taken into account for extraction of antioxidants and phenolic compound as some solvent is highly polar such as deionized water or nonpolar such as hexane. Thus solvent with intermediate polarity is preferable compared to either extremely polar or nonpolar (Pokarny & Korczak, 2001). The structural differences of bulk alcohols itself influences the kinetic reactions in scavenging assays such as in ABTS. The cluster structures of hepta-, hexa-, penta-, tetra- and trimeric have a different ability of proton transmission (Dawidowic & Olszowy, 2013). Although DPPH test is performed in polar medium of methanol, it did not obey the polar paradox theory because of the reason DPPH test medium is not an oil-in-water or water-in-oil environment but is rather as free antioxidants in polar medium. Thus it is best for polar antioxidants to move freely to scavenge the free radical DPPH rather than nonpolar antioxidants that will accumulate and hardly move around the assay environment.

4.3.2 β-Carotene Bleaching Assay

The antioxidant present presence of antioxidants preventing the free radicals which are form from linoleic acid that attack the highly unsaturated β-Carotene. Delaying the color change and decreasing of absorbance (Barros *et al.,* 2008). All samples' antioxidant activity were presented in Figure 4.2 and the samples mean are varies significantly with $F(2, 6) = 1942$, $p < 0.05$. The order of β-Carotene radical scavenging activities of the extracts as Trolox® > CH > RH. Their concentration of inhibition was tabulated in Table 4.2.

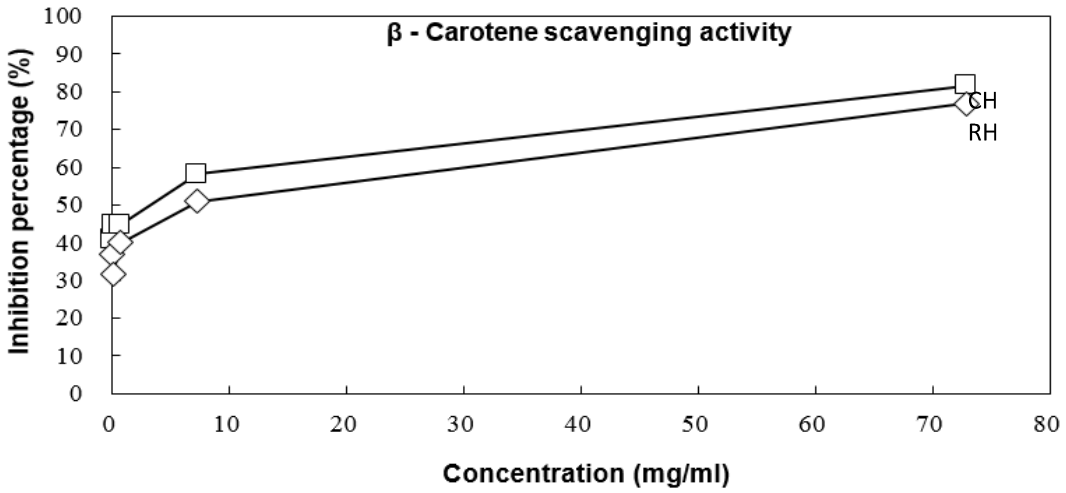


Figure 4.2 : β-Carotene scavenging activity of samples extract. Data for Trolox® not shown on the graph.

From the assay done, there is no consistency of linear correlation of antioxidant activity for polar extracted samples of CM and RM using 5 different concentrations. CH showed a better concentration in inhibiting half of the free radical with almost triple the value of RH.

4.3.2.1 Comparison of Using Polar and Nonpolar Extract in β-Carotene Assay

The inconsistency of absorbance reading of polar extracted compound such as CM, RM and positive control of BHA could be explained by polar paradox theory as it is earliest reported by Frankel (1996). This kind of nonpolar lipophilic antioxidants hindered the transport of free radicals across the droplet membrane as the nonpolar antioxidants forming the protective layer around the oil droplet. However in β-Carotene emulsion system, which is in the condition of oil-in-water or oil in polar medium causing nonpolar antioxidants is more effective than polar antioxidants which is more effective in bulk oil (Yim *et al.,* 2010).

This is supported by Brand-Williams *et al.* (1995), stated that lipophilic antioxidants are more active in polar mediums, the mechanism of polar paradox. According to Siramon & Ohtani (2007), polar compound such as ascorbic acid that tested with β-Carotene assay showed to be weak antioxidants but by using other method such as DPPH, this antioxidant proven to be a strong one. This revealed that β-Carotene assay is not suitable in determining polar antioxidants activity rather to nonpolar antioxidants. Thus polar extract showed a similar trend as polar paradox as none of the polar extracted samples showed a consistent correlation that polar extracted compound did in this assay. Although BHA is nonpolar lipophilic as stated by Yim *et al.* (2010), the dilution made for BHA is by using methanol which is a polar compound. Thus it is exhibit the same trend as the other polar extracted samples.

4.3.3 ABTS Radical Scavenging Activity Assay

The ABTS radical scavenging activity of all samples with different extraction solvent were presented in Figure 4.3 and samples mean are varies significantly with $F(5,12) = 345.959$, $p < 0.05$ except for RM and RH. The order of ABTS radical scavenging activities of the extracts as Trolox® > BHA > CM and CH > RM and RH. Their concentration of inhibition was tabulated in Table 4.2.

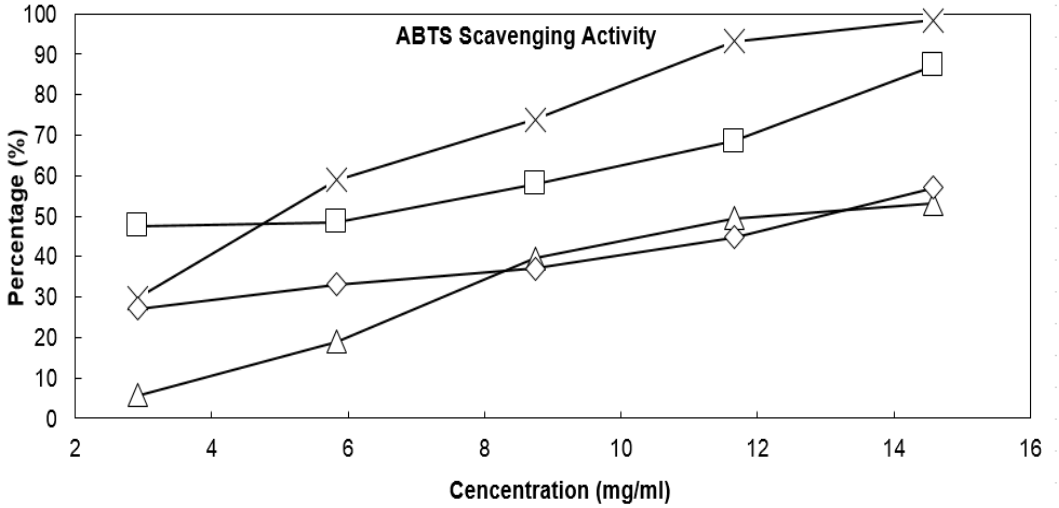


Figure 4.3 : ABTS scavenging activity of samples extract. Data for Trolox® and BHA not shown on the graph.

The IC_{50} of CM and RM means were significantly different. The IC_{50} for RM was 7.584±0.15 mg/ml and the concentration is far higher compare to the finding by Cissouma *et al.* (2013) with 1.68±0.01 mg/ml. This might due to the differences of method used such as the initial absorbance of control and less incubation time after the sample was mix with ABTS solution. In the study of Cissouma *et al.* (2013), the incubation time was 2 hours while in this study it was only 5 minutes. Longer incubation time might give enough time for the antioxidants in the sample to react completely thus give a better discoloration with lower absorbance reading. By this way smaller sample concentration is needed to achieve 50% of inhibition. RH exhibit weakest scavenging activity compared to CH and even with other samples tested.

4.3.3.1 Comparison of using Polar and Nonpolar Extract in ABTS Assay

In ABTS scavenging activity, it should be noted that the CH and RH sample did achieve 50% of inhibition but not in DPPH scavenging activity. This is due to the characteristic of ABTS that favored both polar and nonpolar extracts. ABTS is not affected by ionic strength with the ability to be soluble in both aqueous and organic solvents, thus this assay is favorable to determine both hydrophilic and lipophilic antioxidants (Prior *et al.,* 2005; Dawidowicz & Olszowy, 2013). This also supported by the study of Arnao *et al.* (2001), stated that ABTS assay can be used to evaluate the plants extracts of both organic or aqueous media. It is noteworthy to know that Trolox® is polar as according to Gutiérrez*et al.* (2001) but it performed well as antioxidant in both DPPH and β-Carotene assay which respectively favored polar and nonpolar samples. This suggests that Trolox® is a compound that have a characteristic of amphiphilic which can undergo reaction in both polar and nonpolar assays.

Table 4.2 : IC_{50} scavenging activity as in mg/ml.

	CM	RM	CH	RH	Trolox®	BHA
DPPH	5.48± 0.31	7.58± 0.15	IC_{50} not achieved	IC_{50} not achieved	3.84± 0.19	4.76± 0.25
β-Carotene	No correla-tion[1]	No correla-tion[1]	8.01± 0.24	21.12± 0.16	10.80± 0.03	No Correla-tion[1]
ABTS	5.20± 0.41	12.61± 0.11	5.23± 0.37	12.884± 0.28	0.78± 0.24	3.88± 0.32

[1]The IC_{50} could not be identified as there are no consistencies of correlation between each concentration.

In identifying the antioxidant activity of a sample, it is noteworthy to make sure that the reactions have completed before taking the absorbance as according to Brand-Williams *et al.* (1995), there are three type of kinetic behavior of the antioxidants namely rapid kinetic behavior (less than one minute), intermediate kinetic behavior (5 to 30 minutes) and slow kinetic behavior (1 to 6 hours) to reach steady state. Therefore analyzing data in certain interval is not accurate as some of the antioxidants is still under reaction. Thus it is recommended to test the antioxidants under steady state where the absorbance is constant or almost. In ABTS antioxidation assay, generation of ABTS cation need 16 to 17 hours of generation. Thus it is recommended by Cano *et al.* (2002), to use HPLC-ABTS test which only need 2 to 5 minutes to generate the ABTS cation by enzymatically method.

4.4 Phenol Determination.

A compound containing hydroxyl group attached to an aromatic ring is classified as phenol which is commonly named as hydroxybenzene. High association of intermolecular hydrogen bonding caused the phenol to increase its polarity resulting on higher melting and boiling points. There are two type of classification of this compound, one is according to common system and the other is using IUPAC system (Mukherji *et al.,* 2004).

4.4.1 Total Phenolic Content (TPC) Determination

From physical observation, color of test aliquot will change from light yellow to blue or dark blue for an extract with higher phenolic content and this is corresponding to the study of Mandana *et al.* (2012). In total, CM exhibits the highest phenolic content with 3.776±0.13 mg/g while the lowest belong to RH with 1.043±0.21 mg/g. The concentration of RH is comparable with the study of Cissouma *et al.* (2013) showed that regardless the solvent of extraction, the phenolic content is in the range of 1.66 to 1.99 mg GAE/g. However for the RM, the phenolic content is far way better than the study of Cissouma *et al.* (2013) with 3.194±0.11. The data are tabulated in Table 4.3 and the result showed in the order of CM > RM and CH > RH respected to gallic acid concentration. Samples mean are varies significantly with $F(3.8) = 133.295$, $p < 0.05$ except for RM and RH. The use of Folin-Ciocalteau reagent was not specific for phenol only because other compound such as ascorbic acid can interfere with the reagent (Siramon & Ohtani, 2007). Thus, a confirmation test needs to be done. In this study profiling (in section 5) was done to observe the composition of the extracted compound.

4.4.1.1 Comparison of using Polar and Nonpolar Solvent in Phenol Determination

From the Table 4.3, polar extract showed a better phenolic content than nonpolar extract. This might due to the reason of the natural polarity of the phenol which is polar and favors an extraction in polar solvent such as methanol thus has better extraction than in hexane (Mandana *et al.,* 2012).

4.4.2 Total Flavonoid Content (TFC) Determination

Being as one of the most widespread plant secondary metabolite, flavonoids are common component in fruits and vegetables have shown free-radical scavenging activity and protection against oxidative stress (Victor *et al.,* 2012). The total flavonoid content for all four extracts are presented in Table 4.3 with the highest content exhibit by RM while the lowest is by RH at the value of 4.814±0.486 and 1.1438±0.33 mg/g respectively. Note that the flavonoid content RM is about 4 times higher than RH while the rest is in the range of 4 to 5 mg/g. The result showed in the order of RM and CM > CH > RH. From the statistical analysis, samples mean are varies significantly except for RM and CM with the value of $F(3, 8) = 275.940$, $p < 0.05$.

Table 4.3 : Total phenolic and flavonoid contents in mg/g at concentration of 14.57 mg/ml.

	CM	RM	CH	RH
TPC(mg/g GAE)	3.776±0.13	3.194±0.11	2.806±0.17	1.043±0.21
TFC(mg/g QAE)	4.538±0.34	4.814±0.48	4.147±0.47	1.143±0.33

4.5 Correlation Coefficient of DPPH and TPC

The IC_{50} of DPPH and TPC correlation was tabulated in Table 4.4. However despite of CH and RH did not achieve the 50% inhibition and not shown in the graph, both of this extracts still possessed a positive correlation with $r > 0.9$. Figure 4.4 showed the graph of DPPH percentage plotted against TPC concentration, both CM and RM produced a positive correlation with $r = 0.9743$ and 0.9853 accordingly.

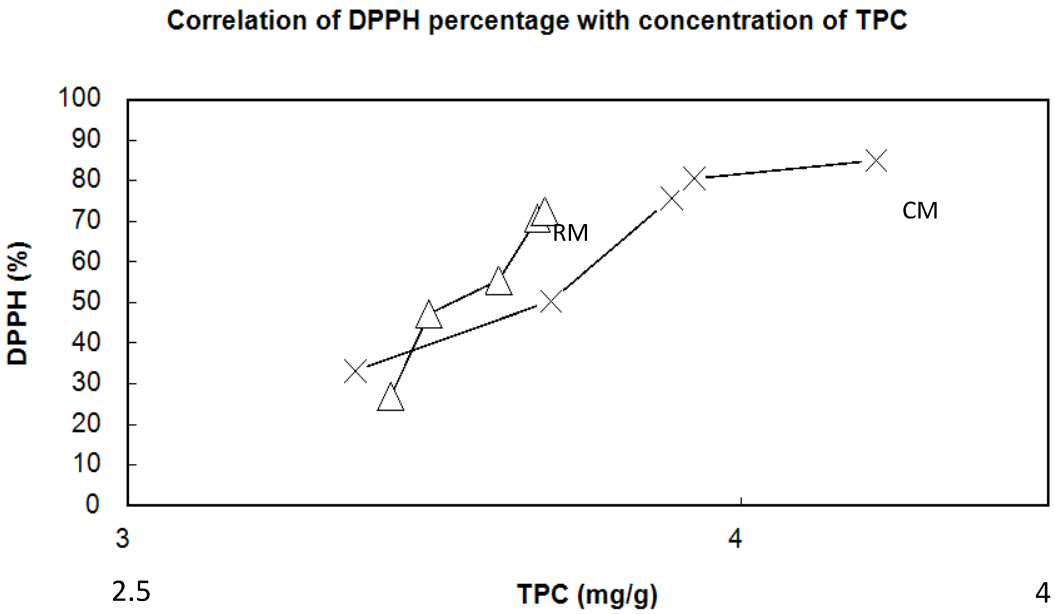


Figure 4.4 : Correlation of DPPH inhibition percentage with concentration of TPC.

Table 4.4 : IC_{50} of DPPH and TPC correlation.

	CM	RM	CH	RH
TPC(mg/g GAE)	3.106±0.11	3.052±0.09	IC_{50} not achieved	IC_{50} not achieved

The strong correlation of the antioxidant activity of DPPH and total phenolic content suggests that phenolic compounds were one of the contributor of the antioxidant activity and weak correlation showed that phenolic is not main contributor

of antioxidant activity as aligned with the study of Ogunlade *et al.* (2014) showing that poor correlation of the two factors did not contribute to any antioxidant activity. This also supported by the study of Dudonne *et al.* (2009) significant correlation found between DPPH and TPC method indicate that phenolic compound which are composed one or more aromatics ring that consist one or more aromatic groups in the extracts contribute significantly in the antioxidant capacity.

4.6 Analyses of Bioactive Compounds

Analyses of potential bioactive compound were done using Gas Chromatography Mass Spectrometry (GCMS) for nonpolar extract while Liquid Chromatography Mass Spectrometry (LCMS) for polar extract.

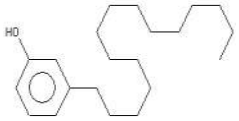
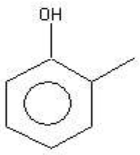
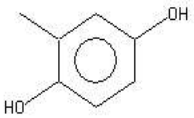
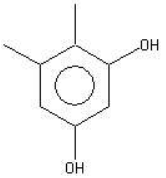
4.6.1 Gas Chromatography Mass Spectrometry (GCMS)

From the GCMS profiling, 4 compounds of phenols were identified from CH and none from RH. 2-methyl phenol was found with the highest abundancy while the lowest is 3-pentadecyl phenol (3DP). 2-methy phenol has the common name of *o*-cresol (Weber & Weber, 2010) with a methyl group attached at the *ortho*- position of the benzene ring (Mukherji *et al.,* 2012). This compound has the retention time in the range of 20.28 to 21.96 minutes. Other compounds that were identified in this extract were 2-methyl-1,4-benzenediol and 4,5-methyl-1,3-benzenediol, but there is not much information from previous study about these two compounds correlating to Cashew nut shell.

3-pentadecyl phenol which fall under cardanol group is present in spongy mesocarp of the cashew nut shell with recognized international name of alkyl phenolic oil (Attanasi *et al.,* 2009; Andrade *et al.,* 2011). The alkyl side chain (R) of cardanol which are the constituents of cashew nut shell liquid may be saturated, triolefinic, diolefinic and monolefinic (Attanasi *et al.,* 2009). In this study, 3DP showed 10.39% area percentage and it is not aligned with the study of Rodrigues *et al.* (2006), where roasting-extracted Cashew nut showed 60-65% while for solvent-extracted was only 10%. The lower percentage in this study might be due to the different in temperature and time applied in Soxhlet extraction, compound to the roasting method from the previous study. This is supported by the study of Andrade *et al.* (2011) stating that CNSL obtained by roasting at prolong high temperature of 195°C contained mainly

cardanol and cardol. However, a study by Trevisan *et al.* (2006) showed that cardanol is not as major compound in CNSL which is heated in the oven at 175°C for only 45 minutes. High amount of cardanol is related to temperature because anacardic acid presence in the Cashew nut shell is converted into cardanol through decarboxylation at high temperature (Paramashivappa *et al.,* 2001). This is supported by Rodrigues *et al.* (2006) stating that the anacardic acid is thermally unstable and easily decarboxylated into cardanol. The high temperature will accelerate a reaction and produce unwanted side chain (Kumoro *et al.,* 2009). Thus, long extraction time and the use of high temperature need to be considered to accelerate the transformation of anacardic acid to cardanol. For Roselle seed, the compound identified are *n*-hexadecanoic acid and 9, 12-octadecadienoic acid with sum area percentage of 25.32 and 34.01 respectively. Table 4.5 showed the structure of compound identified through GCMS with their respective retention times.

Table 4.5 : GCMS analysis of Cashew nut shell extracted with hexane (CH).

Retention Time	Area (%)	Name of Compound	Structure
16.83	2.59	3-pentadecyl phenol	
18.70	7.80		
20.28	28.87	2-methyl phenol	
21.96	8.6		
22.94	18.39	2-methyl-1,4-benzenediol	
23.15	8.76	4,5-methyl-1,3-benzenediol	

4.6.2 Liquid Chromatography Mass Spectrometry (LCMS)

From the analysis using LCMS, Cashew nut shell showed higher amount of phenolic compound compared to Roselle seed. Figure 4.5 showed a full chromatogram of cashew nut shell extract. All the compounds identified were polar which poses at least one hydroxyl group connected to the aromatic benzene ring.

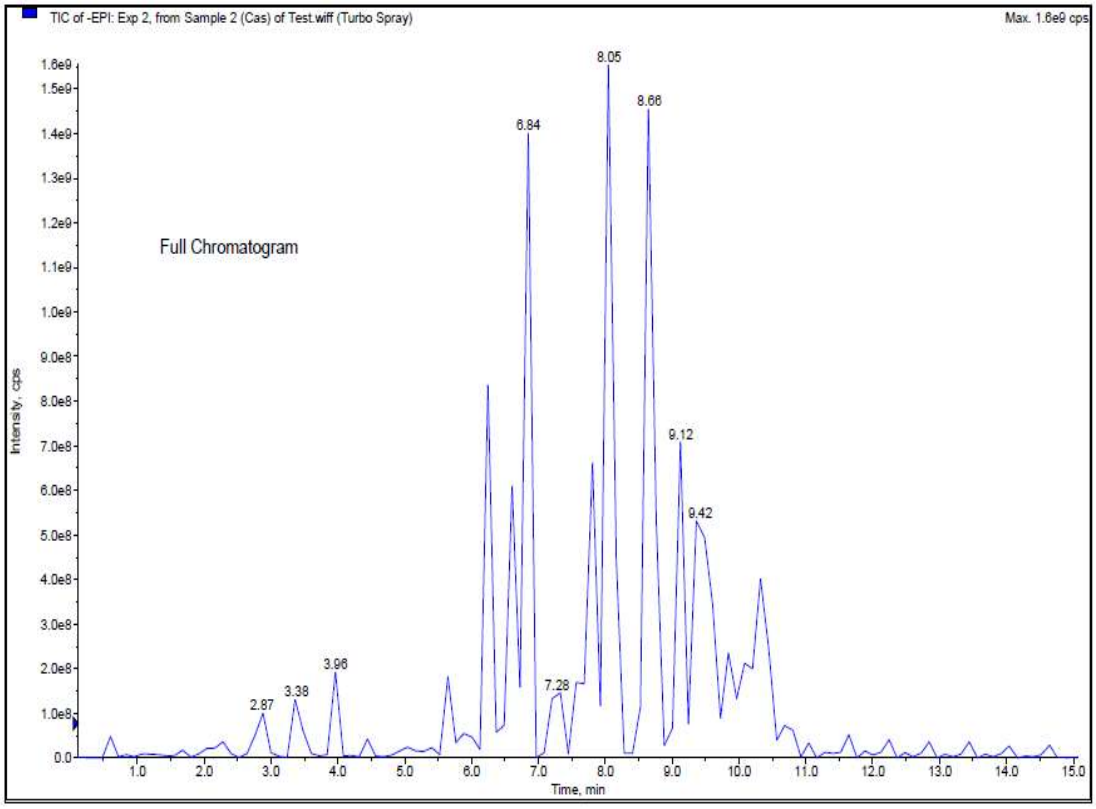


Figure 4.5 : All LCMS peaks chromatogram of Cashew nut shell extracted with methanol

Table 4.6 : LCMS analysis of Cashew nut shell extracted with methanol (CM).

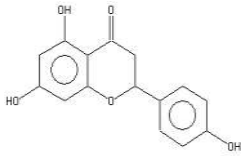
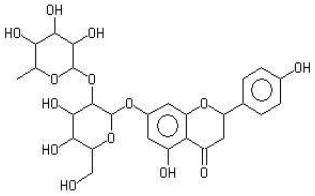
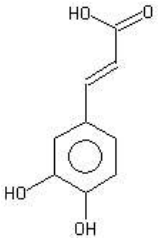
Retention Time	Name of Compound	Structure
2.87	Naringenin	
3.38 3.96	Naringin	
6.84	Caffeic acid	

Table 4.6 showed the structure of compound identified through LCMS with their respective retention times. From the previous study, naringenin and caffeic acid were highly present in cashew leaf and nut (Nugroho *et al.,* 2006). Caffeic acid is found in many plants (LeBlanc *et al.,* 2012) and the most concentrated phenolic compound identified in cashew apple juice (Marc *et al.,* 2012). On the other hand, naringenin belongs to the class of flavonoids namely flavanone. This compound is derived from the hydrolysis of other flavanone such as naringin and highly soluble in organic solvent (Wilcox *et al.*, 1999). However there is not much information relating these compound to cashew nut shell liquid and further identification by comparison with authentic standard is needed to be done in future work.

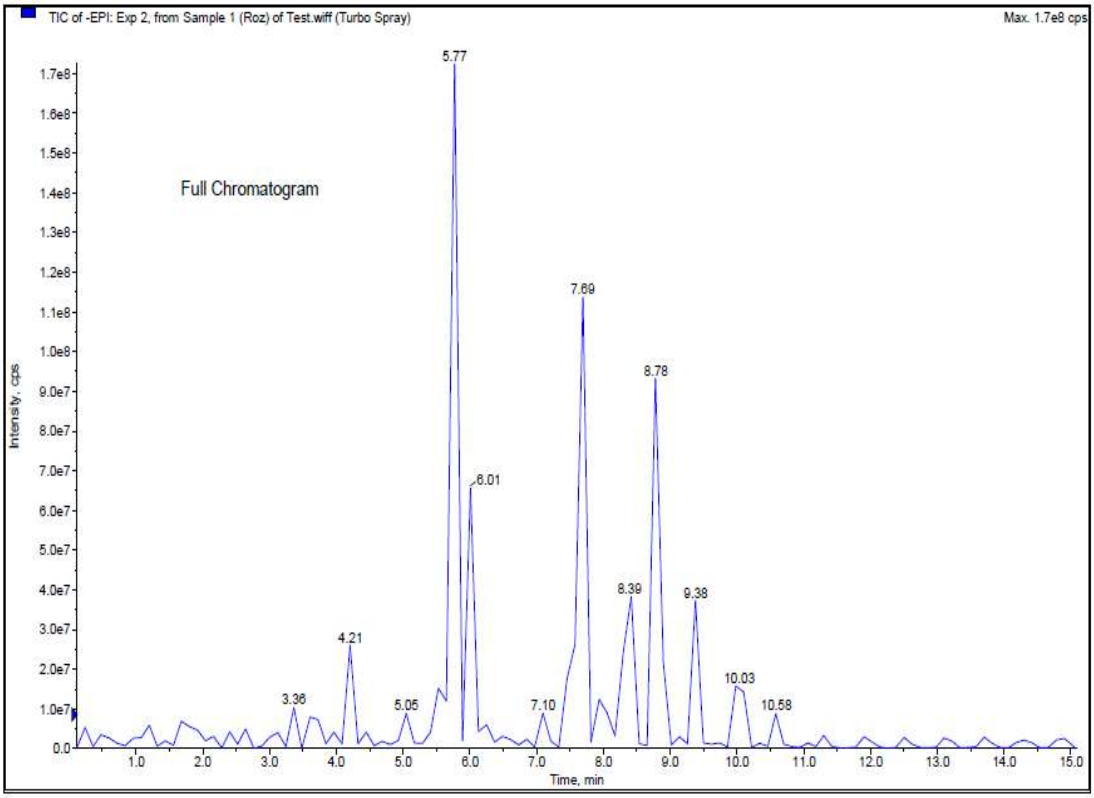
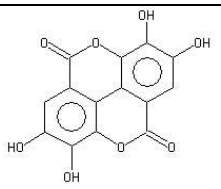


Figure 4.6 : All LCMS peaks chromatogram of Roselle seed extracted with methanol.

Figure 4.6 showed a full chromatogram of Roselle seed extract with one identified compound listed in Table 4.7. Ellagic acid is a flavonoid compound which can be found in the leaf of a Roselle plant (Da-Costa-Rocha *et al.,* 2014; Kang *et al.,* 2006).

Table 4.7 : LCMS analysis of Roselle's seed extracted with methanol (RM).

Retention Time	Name of Compound	Structure
4.21	Ellagic acid	

By correlating LCMS analysis with total flavonoid content of both methanol plant extract, RM showed a better TFC content than CM with 4.814±0.48 and 4.538±0.34 mg/g QAE respectively although it has only one flavonoid compound been identified. On the other hand, by comparing analysis using different solvent polarity, polar extraction solvent such as methanol showed better identification of compound compared to nonpolar solvent such as hexane.

4.7 Oxidative Stability Test

Oxidative stability test was done using Rancimat machine following a European standard EN14214 for biodiesel which set the induction period for 6 hours as the lower limit and EN 14112 standard method to determine the oxidation stability for fatty acid methyl esters using accelerated oxidation test. The induction period of samples and positive control were shown in Figure 4.7.

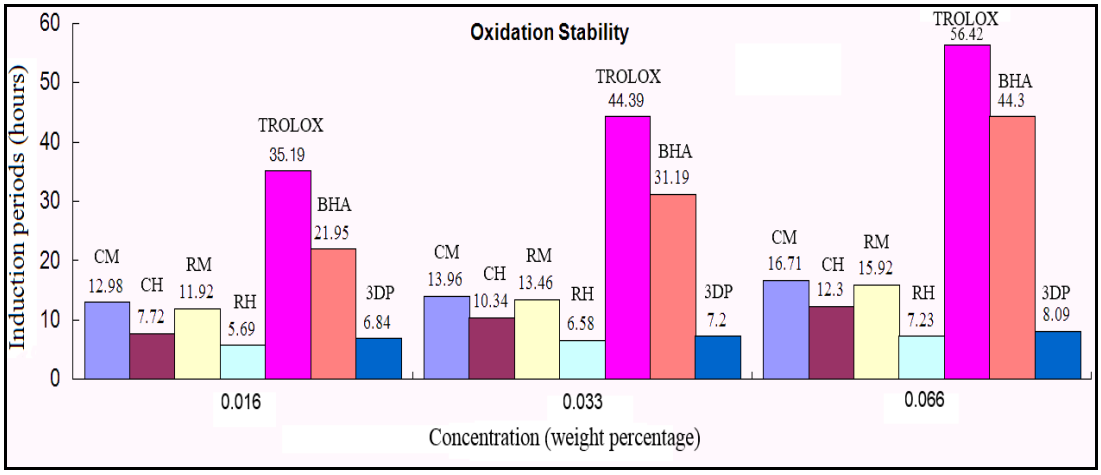


Figure 4.7 : Induction periods of samples and positive controls.

Biodiesel without the addition of antioxidant served as a control for this test and has an induction period of 7.27 hours which is higher than biodiesel with the addition of 3-pentadecyl phenol (3DP) as antioxidant. From the previous studies, the hydroxyl group presence on aromatic ring of a cardanol such as 3DP and its side chain could be utilized for various chemical transformations. The reactive free hydroxyl group could be attached to a variety of functional group through series of reaction such as nucleophilic aromatic or aromatic substitution and condensation reaction (Lochab *et al.,* 2013). The study of (Souza *et al.,* 2012) stated in synthesizing porphyrins, intermediate compounds will be attacked by nucleophile cardanols and polymerize into a new compound. These suggest that 3-pentadecyl phenol which is a cardanol became a nucleophilic aromatic them self and attacked the unsaturated chain of the biodiesel thus brought down the biodiesel shelf life. Other reason is because of

the presence of high degree of unsaturated *meta*-substituent attached to the aromatic ring of 3DP and supported by the study of Rodrigues *et al.* (2006) saying that *meta*-alkylphenols attached to the benzene ring is vary in degree of unsaturation. Free radical tend to attack the unsaturated alkyl chain and undergone propagation reaction to form another chain of free radical. This is the same case for RH which showed lower induction period than control due to the fact that the extract has a high content of unsaturated fatty acid namely 9, 12-octadecdienoic acid rather than phenolic compound. The unsaturated bond is prone to oxidation which leads to lower induction period compared to control. CH which is nonpolar, showed an increasing trend of induction period. This proved that nonpolar extract can be used as an antioxidant addictive in biodiesel. The phenolic compounds identified by GCMS all contain at least a hydroxyl group attached to benzene ring.

However the availability of compound cannot guarantee a higher induction period. For example in CH, even though there are 4 types of phenolic compound identified, the induction period is lower compared to RM which has only one type of phenolic compound, ellagic acid. This is due to the consistent argument that all the compound in these plant extract did not act alone to combat the free radical as they are not high in purity, it is rather a combination of several compounds of antioxidant that work together in one mechanism to fight the free radicals. The antioxidant activity of a compound plays an important role and the quality is more important rather than quantity. According to Wang *et al.* (2014), less polar compound with hydroxyl group such as BHT showed better induction period compared to a strong polar compound that consist of carboxyl group such as citric acid. However in this study, Trolox®, a synthetic antioxidant analogue to vitamin C (Cano *et al.,* 2002) which contain of both hydroxyl and carboxyl group showed the best induction period among all.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

The type of solvent, temperature and method of extraction are the fundamental aspects need to be considered in performing extraction because all these factors have a significant influence in the other tests. Choosing a solvent for extraction is strongly dependent on the target of interest. In an antioxidation test such as ABTS radical scavenging activity, CH and RH extracts did achieve IC_{50} of inhibition rather than in DPPH radical scavenging activity. On the other hand, CM and CH need the lowest concentration to achieve IC_{50} while RM and RH need almost 3 times the values of CM or RM. The polarity of the solvent affects the antioxidant assay in determining the antioxidant activity of the extracts. For positive control standard, it must have an ability to fit numerous tests as different antioxidation test have different mechanism of actions. Trolox® which contain two functional groups of carboxyl and hydroxyl performed best in all test compared to BHA which has only one functional group. Numerous compound embed in a plant have different scale of polarity. So no ordinary solvent can act universally in extraction. Thus, a practice of using several different solvent for extraction is practical for this study because some compound such as flavonoid is better extracted using polar compound rather than nonpolar. This proved when Roselle seed that was extracted with polar solvent (RM) showed a presence of a phenolic compound rather than in nonpolar extraction solvent (RH). Thus it can be concluded that different polarity of solvent does have significant effect in extraction.

The oxidative stability test done showed that polar extract exhibit better induction period compared to nonpolar solvent. Even though CH contains different types of compound identified than CM, the induction period is far better in CM. The

polarity is the factor contributing to this affect. Favored by biodiesel is the key to this peculiar. Other argument might be because of the individual compound itself having better ability than the other compound. Ellagic acid, the single compound found in RM has almost the same induction period as CM which contains four compounds. By excluding the effect of polarity as both extracts are polar (methanol), it can be concluded that ellagic acid has a higher antioxidant capacity in biodiesel. In doing extraction, temperature is important in retaining compound in their natural state as some compounds might degraded by temperature and undergo minor changes in their side chain. In analysis done in Cashew nut shell, anacardic acid is not present even though there are studies showing that this compound is one of the main compounds in Cashew nut shell oil. The fact that anacardic acid is unstable and easily decarboxylated into cardanol become the reason to support this. Thus, it is recommended to use low temperature extraction methods with shorter extraction time as high temperature might break or alter the structure of a compound and cause compound to be fragmented into smaller nonfunctional structure. For future works it is recommended to separate the pure methanol extract into several partition by using other solvent with different polarity such as hexane and chloroform. Thus, by testing the content of extract in the partitioned solvent, it will enable close identification of the compounds responsible for antioxidant activity rather than a combination of miscellaneous compounds that acting together.

It is also recommended to purify the extract before analyzing the compound. The reason of doing this is to identify which compound is responsible for the antioxidant activity as the crude extracts is not pure. This could also help in determining which compound is contribute in combating the free radical and which is one promoting it. Thus further test should be done by purification and separation the

compound individually and testing it with antioxidation and oxidative test. Furthermore machine selection is very important according to the aim of the study because different specification of machine has different ability especially to detect compound. For example triple quodrupole (MS/MS) is better compared to single quadrupole (MS) because it is more reliable to detect analytes using multiple reaction monitoring (MRM) than selected ion monitoring (SIM) thus triple quadrupole (MS/MS) is highly selective. A further confirmation is also recommended by doing comparison of the compound been identified with authentic compound using the same method of profiling and an estimation of concentration of the sample can be made. Lastly in performing oxidative stability test, it is best to make an extrapolation graph by using at least 5 different concentrations of antioxidants so that the data can be further manipulated for further use such as finding an optimum of antioxidant concentration.

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LIST OF PAPER PRESENTED

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APPENDIX

A.1 Gallic acid standard curve