KINETICS AND IMPROVEMENT OF BIODIESEL OXIDATIVE STABILITY BY A NATURAL ANTIOXIDANT FROM *Brucea javanica* SEEDS

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ABSTRACT

The study was conducted to determine antioxidant activity of *Brucea javanica* seed and test its potential to be used as natural antioxidant for biodiesel. 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing antioxidant Power (FRAP) and Metal Chelating Assay are the methodologies used to test antioxidant activity. The results indicated that higher flavonoids and phenolic contents could be detected in ethyl acetate and methanol extracts as compared to hexane extract. Ethyl acetate extract of *B.javanica* seed showed highest DPPH inhibition activity up to 90% with (IC_{50}= 31.2 μg/ml). On the other hand, methanol extract had a highest FRAP activity (0.180 ±0.03 mmol Fe^{2+} /g extract) with 71% inhibition. In the metal chelating assay, ethyl acetate extract indicated the highest chelating activity with 59% inhibition (IC_{50}=299μg/ml). In addition, the bioactive compounds were also analysed using LCMS and GCMS for confirmation. The chemical compounds found in *B. javanica* seed extract were brevifolin, ellagic acid, gallic acid, quinic acid, strictinin, O-methyl ellagic acid, protocatechuic acid and ellagic acid isomers from LCMS analyses. 35 chemical have been identified in GCMS analyses. In order to confirm anti-oxidation effect of *B. javanica* seed extract, rancimat and thermal oxidation tests were performed at 110°C and 80°C respectively. For rancimat test, Gallic acid showed the highest oxidative stability of biodiesel up to 69 hours induction period (IP). However, on the other end, ethyl acetate crude extract at 1000 ppm suggested better stability up to 6h in thermal oxidation test. While, kinetics evaluation showed a high degree of correlation coefficient (R^2 =0.9) confirming that the degradation of antioxidant in improving oxidative stability of biodiesel follows first order kinetics.

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ABSTRAK

Satu kajian penyelidikan telah dijalankan bagi menentukan aktiviti antioksida biji benih *Brucea javanica* dan potensinya sebagai antioksida semulajadi untuk biodiesel. Bagi mengukur aktiviti antioksida, kaedah-kaedah seperti 2, 2-diphenyl-1-picrylhydrazyl (DPPH), *Ferric Reducing Antioxidant Power* (FRAP) dan *Metal Chelating Assay* telah digunakan. Keputusan kajian menunjukkan bahawa kandungan flavonoid dan fenol dalam ekstrak etil asetat dan ekstrak metanol adalah lebih tinggi berbanding ekstrak heksana. Biji benih *B. javanica* dalam ekstrak etil asetat menunjukkan aktiviti perencatan DPPH pada kadar paling tinggi iaitu sebanyak 90% dengan (IC_{50}= 31.2 µg/ml). Manakala ekstrak metanol pula menunjukkan aktiviti FRAP paling tinggi (0.180 ± 0.03) dengan 71% kadar perencatan. Selanjutnya, bagi *metal chelating assay*, ekstrak etil asetat menunjukkan 59% kadar perencatan (IC_{50}= 299 µ g/ml). Selain itu, analisa kompaun bioaktif bagi pengesahan juga dilaksanakan dengan menggunakan LCMS dan GCMS. Hasil kompaun kimia yang didapati dalam ekstrak biji benih *B. javanica* melalui LCMS adalah brevifolin, asid ellargic, asid gallic, asid quinic, strictinin, asid O-methyl ellargic, asid protocatechuic dan isomer asid ellargic. Tambahan lagi, sebanyak 35 kompaun kimia telah diidentifikasi Melalui analisa GCMS. Bagi mengesahkan kesan antioksida daripada ekstrak biji benih *B. javanica*, ujian rancimat (110°C) dan pengoksidaan terma (80°C) dijalankan. Untuk ujian rancimat, asid gallic menunjukkan kestabilan oksidatif paling tinggi untuk biodiesel dengan 69 jam tempoh induksi. Walaubagaimanapun, ekstrak mentah etil asetat menunjukkan kestabilan yang lebih baik pada 1000 ppm iaitu sebanyak 6 jam tempoh induksi. Selanjutnya, penilaian kinetik menunjukkan kadar korelasi pekali yang tinggi (R^2 = 0.9), seterusnya mengesahkan bahawa degradasi antioksida dalam meningkatkan kestabilan oksida untuk biodiesel mengikuti kinetik tertib pertama.

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LIST OF SYMBOLS AND ABBREVIATIONS

TLC	Thin Layer Chromatography
DPPH	2, 2-diphenyl-1-picrylhydrazyl
LCMS	Liquid Chromatography Mass Spectrometry
GCMS	Gas Chromatography Mass Spectrometry
FRAP	Ferric Reducing Antioxidant Power assay
TPTZ	2, 4, 6-tripyridyl-striazine
G	Gram
M	Millilitre
mg/ml	Milligram/millilitre
ug/ml	Microgram/millitre
ul	Microliter
%	Percentage
OD	Optical density
Psi	per square inch
IC_{50}	Inhibition Coefficient at 50 %
IP	Induction Period
POME	Palm Oil Methyl Ester

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CHAPTER 1

INTRODUCTION

1.1. General Introduction

The biodiesel is considered as green and safe fuel for the environmental sustainability. There are various reasons to promote biodiesel as a green fuel such as renewable fuel, environmental concern, depleting reservoir of fuel and energy security. The petroleum based fuels are depleting fast and rise in price increase trend toward alternative sources. Some of the feedstocks to produce biodiesel are vegetable oils, animal fats and cooking oil. The biodiesel or fatty acid methyl esters (FAME) could be obtained from these sources by trans-esterification of oil/tri-glycerides using alcohol (Meher et al., 2006). The biodiesel is made up of long-chained FAME. United States and European countries are actively engaging the production of biodiesel from renewable resources in order to reduce their dependency on limited sources and to reduce air pollution in the environment. These countries are the major producer of sunflower, soybean and rapeseed oils (Jain and Sharma, 2010a).

The drawback of biodiesel is its vulnerablity towards oxidative degradation, which lead to fuel quality disruption. Therefore, the biodiesel oxidative stability is critically important as it determines resistance to chemical modifications caused by oxidative reaction. In addition to oxidation, polymers may form in the presence of unsaturated fatty acid which in turn will lead to higher molecular weight products that will increases its viscosity. The oxidative stability of biodiesel depends greatly on fatty acid compositions and degree of unsaturation. Saturated fatty acid methyl ester is more stable than those of unsaturated, while polyunsaturated fatty acid methl ester is at least two time more reactive to auto-oxidation than monounsaturated fatty acid methl ester (Neff et al., 1997). For the same number of double bonds per molecule, fatty acid

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methyl ester with longer chain or higher molecular weight would be less prone to auto-oxidation due to lower molar concentration of double bond (Knothe and Dunn, 2003). The oxidation of biodiesel could lead to many mechanical problems such as deposits formation, fuel system corrosion and filtering problem. These issues could be overcome by either adding antioxidants or altering the fatty acid chain via hydrogenation (Monyem and Gerpen, 2001).

Furthermore, this study will focus on utilization of bioactive compounds of *Brucea javanica* seed as potential natural antioxidant for biodiesel in order to boost up biodiesel oxidative stability.

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1.2. Research Background

Malaysia depends on its fossil fuel to complete its oil need. But the oil production has been fallen so fast. There is 13 % decline in oil production seen in Malaysia from 2006 to 2008. It is reported that these crude oil reservoir may deplet with in next 20 years (Oh et al., 2010). It will become a major problem as Malaysia petroleum consumption depends on two-third of its petrol and diesel reservoirs (Jayed et al., 2011). The oil production is very much important for every country in order to complete its oil requirements and energy. But fossil fuel reservoirs are declining very fast but energy need is increasing on daily basis. Biodiesel is the potential renewable fuel and environmental friendly. That is one of the reason that biodiesel is gaining so much interest and its production showed an increasing trend. However, biodiesel has low oxidative stability in comparsion with fossil fuel .

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1.3. Problem Statement

The biodiesel is an emerging source of renewable energy. It is basically produced from vegetable oil, fats and waste cooking oil. The biodiesel is contributing to the energy need of many countries blended with petroleum in different proportions such as B20. The biodiesel production is increasing on large scale and in last ten to fifteen years million tons has been produced. United states is the major producer of biodiesel which produced it from sunflower and soybean oil mostly.

However, the problem is with biodiesel is its low oxidative stability. It is produced from fats/oil which consist of unsaturated fatty acid that weakens the oxidative stability of biodiesel which in turn effect the storage of biodiesel. It oxidised very quickly when expose to air or water and if stored for long time. It is a mixture of methyl esters produced from vegetable oil, is more susceptible to oxidation than mineral diesel. The storage stability of methyl esters is found to be deteriorated during storage, and it is found the addition of antioxidants can ensure storage stability as they prolong the oxidative strength.

The synthetic and natural antioxidants were used previously which show good results (Mittelbach and Gangl, 2003). But the synthetic antioxidants such as Butylated hydroxyanisole (BHA) produces higher hydrocarbon emission and Butylated hydroxytoluene (BHT) produces higher nitrogen oxide (Fattah et al., 2014). Furthermore, this study will focus on utilization of bioactive compounds of *B. javanica* seed as potential natural antioxidant for biodiesel.

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1.4. Objectives of the Proposed Study

i. To test antioxidant activity of *Brucea javanica* seed.

ii. To identify bioactive compounds that are responsible in giving the antioxidation properties of *Brucea javanica* seed.

iii. To examine the optimum concentration of the antioxidants that will improve the oxidative stability of biodiesel.

iv. To study first order kinetics of the reaction of antioxidant with biodiesel in rancimat.

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1.5. Scope of Work

This study will focus on utilisation of bioactive compound of *B. javanica* seed as potential antioxidant for biodiesel. The biodiesel will be a part of renewable energy in future. Therefore, consideration should be given to the factors that can effect biodiesel oxidative stability which is also considered as disadvantage of it. The scope of this study is to find some ways or methods that can be used to prolong its oxidative stability. The synthetic antioxidant were used but these are toxic and non-renewale to meet the required amount needed to improve storage of biodiesel. The literature has emphasized the importance of natural antioxidant because of it non-toxicity and availability. This study will also evaluate the bioactive compound activity towards oxidation stability of biodiesel by using several designated test proposed. It should be noted that natural antioxidant from *B. javanica* seed has not beed used as potential antioxidants for oils/fats. It will create an alternative pathway if the proposed study showed high potential through series of positive results.

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1.6. Dissertation Outline

In this study, the natural antioxidant from *B. javanica* seeds were used as natural antioxidant to prolong oxidative stability of biodiesel. The experimently methodology and obtained results are presented comprehensively and significantly. There are five chapters in this dissertation which begin with,

Chapter 1, give comphrensive introduction the background, problems, purposes and aims of this study.

Chapter 2, which highlight some of the background of the study related to biodiesel, antioxidants and studied plant.

Chapter 3, describes methods and properties necessary for extraction continued with methods of testing antioxidant with biodiesel.

Chapter 4, this chapter discuss the result of the study together with reference of previous findings.

The summary of the result are presented in Chapter 5, together with recommandations for the future work.

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CHAPTER 2

LITERATURE REVIEW

2.1. Biodiesel

Petroleum products consumption is increasing and resources are limited. Since 1990, the prices of these products are rising very fast. The energy needed to power transportation sector and the demand for energy is increasing with time and there is a need to look for an alternative source of fuel which should be economical and viable (Srivastava and Prasad, 2000). The diminishing of petroleum based energy and increasing cost of it forced us to think for a alternative source of energy (Meher et al., 2006). On one hand petro-diesel is depleting and on the other hand it also creates air and water pollutions catalysing climates changes (Orecchini and Bocci, 2007). The biodiesel is thought to be the better option for the fulfilment of the need of energy of the world. The biodiesel is becoming more and more essential as fossil fuels are depleting with time (Basha *et al.*, 2009). Malaysia's oil producing capacity has dropped considerably up to 13 % from 2006 to 2008 and it is predicated that under surface crude oil in Malaysia could diminished in 20 year time (Oh et al., 2010). This will become a huge concern to the country as two-third of consumption in Malaysia is petro-diesel (Jayed et al., 2011). Although the fossil fuel resources are declining, but the demands for energy is increasing. Thus, an alternative energy is needed. Due to biodiesel potential as diesel substitute, the production of biodiesel has showed an increasing trend. However, the biodiesel tend to oxidise easily during storage when compared to the fossil fuel. Increasing environmental issues due to accumulation of wastes forced global measures to resolve these troubles. Various worldwide treaties, including European union (EU) regulations and commands have been taken to normalise emissions of greenhouse gases

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to the air in order to increase the use of natural energy sources, and to ensure effective management and utilization of waste of all forms. The atmospheric pollution causes by greenhouse gases, majorly contributed by road transportation. EU member states are supporting the production and use of biofuel in order to lessen the influence of these complications (Sendzikiene et al., 2005). The biodiesel has many advantages over petro-diesel when compared in term of performance as biodiesel such as lower exhaust emission, nontoxic, biodegradable essentially free of sulphur and renewable and hence considered as environmental friendly and sustainable energy (Knothe, 2010).

The biodiesel and diesel engine shares the same history and utilising of vegetable oils were studied at the time when diesel engine was invented. The word biodiesel was actually derived from the word "bio" which means life and the word "diesel" referred to Rudolf Diesel, a famous German inventor that invented diesel engine in 1893 (Jayed et al., 2011). Rudolf Diesel (1858-1913), the diesel engine inventor tested various oils for his engine such as cottonseed oil, palm oil, castor oil and soybean oil. The definition of biodiesel could be “it is a mono alkyl ester of long chain fatty acid”. In vegetable oil these esters may be prepared from triglycerides through transesterification with alcohols. The biodiesel could be miscible with petro-diesel so it could be effectively used as natural biodiesel (B100) or mixed with petrodiesel. The biodiesel is renewable fats and oils from mono alkyl ester usually methyl ester that was created by the transesterification reaction to create long chain fatty acid ester. As biodiesel is naturally produce by chemical reaction of vegetable oil or animal fat, carbon in the fats/oil normally originated from carbon dioxide. Thus, it adds less influence mainly in global warming compared to by product emitted from fossil fuels such as petrol (Gerpen, 2005). Different raw materials available for producing biodiesel are rapeseed oil, mahuva oil, linseed oil, soya bean oil, sunflower oil, beef tallow, lard, palm oil, cotton seed oil, jatropha oil, pongamia oil, olive oil, rice bran oil

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and guang-pi. The use of particular raw material depends upon the availability, price and policy (Sekhar et al., 2010).

Biodiesel is also considered as sustainable for the society and the environment because it produce less exhaust emissions. Many methods and procedures are established in order to met the standard specification of biodiesel such as European standard for biodiesel shown in Table 02. In transesterification, triglycerides are converted fatty acid methl ester and glycerol through consective reversible reactions. One molecule of triglycerides produces three ester molecules. There are many methods developed for the production of biodiesel such as acid-catalyzed method, enzymatic catalysis, alkali-catalyzed method and supercritical method (Fukuda et al., 2001). Butanol , propanol, ethanol and methanol are commonly used alcohols in biodiesel production (Ramadhas et al., 2005).

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Table 2.1: European standard for Biodiesel

Contractual Specifications (EN 14214 : 2003)		
Parameter	**Units**	**Limits**
Total Ester Content	%(m/m)	Min 96.5
Density @ 15°C	g/cm^3	Max 0.860 - 0.900
Viscosity @ 40°C	mm^2/s	3.50 - 5.00
Flash Point	°C	Min 120
Sulphur Content	mg/kg	Max 10.0
Carbon Residue (on 10% Distillation Residue)	% (m/m)	Max 0.30
Cetane Number	-	Min 51.0
Sulfated Ash Content	% (m/m)	Max 0.20
Moisture	Ppm	Max 500
Total Contamination	mg/kg	Max 24
Copper Strip Corrosion (3hrs @ 50°C)	Rating	Class 1
Oxidative Stability @ 110°C	Hours	Min 6.0
Acid Value	mg KOH/g	Max 0.50
Iodine Value	g iodine/100g	Max 120
Linolenic Acid Methyl Ester	% (m/m)	Max 12.0
Polyunsaturated (>=4 double bond) Methyl Ester	% (m/m)	Max 1.0
Methanol Content	% (m/m)	Max 0.20
Monoglyceride	% (m/m)	Max 0.80
Diglyceride	% (m/m)	Max 0.20
Triglyceride	% (m/m)	Max 0.20
Free Glycerol	% (m/m)	Max 0.02
Total Glycerol	% (m/m)	Max 0.25
Group I Metals (Na + K)	mg/kg	Max 5.0
Group II Metals (Ca + Mg)	mg/kg	Max 5.0
Phosphorus Content	mg/kg	Max 10.0

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2.2. Advantages and Disadvantages of Biodiesel

In broad-spectrum, biodiesel means a fuel derived from biological source as a substitute of the conventional energy sourcing. The advantages of biodiesel are;

i. Higher boiling point.

ii. Lower emission of toxic gases

iii. Biodiesel is an oxygenated fuel, so it contributes to a more complete fuel burn and a greatly improved emissions.

iv. Biodiesel can be used as blend with petroleum, there is no need of installing special equipment, a good substitute for petroleum, so there is no need to buy special vehicles or engines to run on biodiesel.

v. Less carbon monoxide.

vi. Less sulphur dioxide emiisions which help in reducing public health risk.

vii. It will reduce the country's dependence on imported oil and it is safe to handle, store, and transport (Sekhar et al., 2010).

Apart from being green, biodiesel oxidative stability is low, espically when it is produced from polyunsatrurated fatty acids. Storage time of biodiesel is reduced if it consists of unsaturated fatty acid and degraded easily by the attack of free radical which possess unpaired electron (Umamaheswari and Chatterjee, 2008). Other drawbacks could be its power, torque and fuel economy is less as compared to diesel due to its lower energy content. No_x emiision are higher in biodiesel. Since its cloud and pour point is around -10, it solidifies at that temperature during winter in European and American Countries (Sekhar et al., 2010). The oxidation of biodiesel could lead to many mechanical problems such as deposits formation, fuel system corrosion and filtering problem .These issues could be overcome by either adding antioxidants or altering the fatty acid chain via hydrogenation (Monyem and Gerpen, 2001). In addition to oxidation, polymers may form in the presence of unsaturated fatty acid which in turn will lead to higher molecular weight products that will increases its viscosity (Neff et al., 1997). The disadvantage of the above mentioned methods is that it utilizes a part of total energy developed in the engine and in few cases engine modification is required which is not at all desired.

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2.3. Biodiesel Degradation

The two central nemesis of biodiesel degradation are thermal stability and oxidative stability. The thermal stability is initiated by the exposure of high temperatures usually exceeding 250°C whereas the oxidative stability is affected by oxygen either in the gas form or that are been dissolved that come in connection with the fuel during a sufficiently extended period of time (Velasco et al., 2009). The oxidative stability also known as storage stability because of the reason of oxygen in air interaction with the fuel under storage conditions which likely to interfere with the fuel stability (Dunn, 2008).

Whenever, biodiesel is exposed to oxygen or air, there is a chance for hydrolysis to occur because it is an ester molecule. The flash point of biodiesel will be reduced if there is a presence of alcohol and total acid number will increase with alcohol presence. The above mentioned factors will make biodiesel unstable when stored for long duration and damage the chemistry of it. The oxidative strength of biodiesel is less than petro-diesel. Therefore, mixing it into petro-diesel will affect fuel strength considerably. The double bond in biodiesel is reason of its poor stability due to that gum formation occur (Dunn and Knothe, 2003).

The biodiesel degradation is caused by a free radical. A free radical is any atom or molecule having unpaired electrons. Free radicals are classified as primary oxygen derived free radical, superoxide anion (O2·), hydroxyl (OH·), hydroperoxyl (OOH·), peroxyl (ROO·) and alkoxyl (RO·) 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 together known as reactive oxygen species (ROS) (Umamaheswari and Chatterjee, 2008).

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The additional minor causes of biodiesel degradation are light, water and metal presence in the fuel that will speed up the oxidation process (Jain and Sharma, 2010b). The light exposure cause photo-oxidation mechanism, though it is unlikely to occur in biodiesel since it needs the exposure to ultraviolet and the occurrence of a photo-sensitizer. Similarly photo-oxidation and auto-oxidation usually take place in biodiesel (Knothe, 2007; Lapuerta et al., 2012). The oxidation strength of vegetable oil is more stable in comparison to animal oil even though a large amount of polyunsaturated fatty acid is present. This is due the lack of natural antioxidant of fatty acid methyl ester in animal chubby (Sendzikiene et al., 2005). These issues could be overcome by either adding antioxidants or altering the fatty acid chain via hydrogenation.

2.4. Antioxidants

Antioxidants are compounds which can slow down or inhibit oxidative stress of fatty acids by breaking oxidative chain of propagation (Velioglu, 1998). Plants consist of secondary metabolites which have the ability to inhibit oxidation process. It is suggested that secondary metabolites could be used as natural antioxidants to boost up oxidative stability (Kranl et al., 2005). Plants contain a large amount of natural antioxidant compounds, vitamins and carotenoids (Velioglu, 1998). A lot of experimental work had been carried out to check the amount of phenolic antioxidant compounds in plant extracts through usage of qualitative and quantitative determination (Nakatani, 2000). Plant crude extracts are rich in phenolic compounds which possess strong antioxidant activity that can inhibit the oxidative stress of lipids (Javanmardi et al., 2003). There are various methods available to test the antioxidants activities of the plants. The most commonly used assays are ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity, metal chelating activity, 2,2-dphenyl-1-

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picrylhydrazyl (DPPH) and trolox equivalent antioxidant capacity (TEAC) (Cao and Prior, 2001).

It is usually believe that antioxidant can play a vital role in combating oxidation of fats /oils and to reduce free radical in the oxidation process. Antioxidants are group of molecules that are capable of preventing and slowing down the oxidation of other molecule, antioxidant is also termed as radical scavengers. Antioxidant can also play major role in human health by preventing human body from free ROS species which are the cause of numerous diseases such as anaemia, asthma, arthritis, aging process and dementias (Borrelli and Izzo, 2000).

Antioxidant research grows very fast in previous era because of their potentials (Huang and Prior, 2005). According to *Webster's Third New International Dictionary*, the antioxidants could hinder reactions stimulated by oxygen or peroxides. Various of the antioxidant ha been used as additives in many products such as in gasoline to delay the expansion of rancidity, in fuel manufactured goods to retard the gum development and in rubber to slow down the aging process. In order for a compound to act as an antioxidant, it should has the capability to stabilizing the formed phenoxyl radical after reaction with lipid radicals and formed delocalized unpaired electrons. This action will let the molecule to act as hydrogen donor, singlet oxygen donor and reducing agents (Matthäus, 2002) .

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2.5. Mechanism of Antioxidants

The initial study on reaction mechanism of antioxidants was done by Bolland Ten (Bolland and Ten, 1947), where they stated a reaction chain process for free radical terminator, it contain a highly liable hydrogen which is quickly offered to peroxy

radical which later interfere with oxidation [(reaction (1) and (2)] (Yang, Hollebone, et al., 2013). The chain propagation reaction shown in (3) and (4), the reaction is exothermic in nature.

$$R^{\cdot} + O \longrightarrow ROO^{\cdot} \quad (1)$$

$$ROO + RH \longrightarrow R\text{-}OOH + R^{\cdot} \quad (2)$$

$$R^{\cdot} + R^{\cdot} \longrightarrow R + R \quad (3)$$

$$ROO^{\cdot} + R^{\cdot} \longrightarrow R\text{-}OO\text{-}R \quad (4)$$

Antioxidants uses various mechanisms to interrupt oxidation chain reaction such as scavenging activities, chelating metal ions, peroxides formation preventing, decreasing oxygen concentration, and inhibiting autoxidation chain reaction. Reactive oxygen species (ROS) are the free radicals that include and hydroperoxyl (HOO), hydroxyl radical (OH), peroxy radical (ROO) and super anions (O_{2}.-). Hydrocholorous acid (HOCl) and hydrogen peroxide (H_2O_2) are also member of ROS family but usually not considered as free radical as there electron pairing is complete. Reactive nitrogen species are also included in reactive oxygen species like peroxynitrate ($ONOO^-$), nitrogen dioxide (NO_2) and nitric oxide (NO) (Dusting and Triggle, 2005).

Phenolic antioxidants including Butylated hydroxytoulene (BHT), butylated hydroxyanisole(BHA), 2,5-di-tert-butylhydroquinone (DTBHQ), tert-butyl hydroquinone (TBHQ) and propyl gallate (PG) are considered as valuable antioxidants based on liability of hydrogen, flavonoids and amines are used as well in reaction. The antioxidant concentration effect on oxidation be determined by factors like prevailing conditions, structure of antioxidant and nature of sample being oxidised (Shahidi and Wanasundara, 1992).

The factors that can affect antioxidant activity are oxidation-reduction capability and rate constant and energy of activation (volatility, heat susceptibility and solubility) of antioxidants. Antioxidants that can inhibit or break free radical chain reaction are considered as potential antioxidants by donating hydrogen (H) to free radical composed during oxidative process and act as radicals themselves. These antioxidants contains phenolic and aromatic rings (Nawar, 1996)

2.6. Different Classifications of Antioxidants

Lee *et al.,* (2007), categorized antioxidants into two key classes of non-enzymatic and enzymatic antioxidants. The enzymatic antioxidants are those which are created endogenously while non-enzymatic are those which are created as exogenously. Antioxidants are divided into two groups known as primary and secondary antioxidant that vary in term of mechanism of action (Hue et al., 2012). Primary antioxidants stabilize the free radical by scavenging it and give a hydrogen atoms or electron and secondary antioxidants suppress the formation of free radicals and avoid the oxidative damage (Prior et al., 2005).

Antioxidants can be classified into two groups, the synthetic and the natural antioxidants. The synthetic antioxidants have been used for maintaining the oxidative stability of lipids/oils but it doesn't get so much importance because of its toxic and oncogenic nature as compared to natural antioxidants (Jeong et al., 2004). Antioxidants also can be classified into different groups on the basis of activity performed such as metal ion chelator, oxygen scavengers and free radical terminators (Shahidi and Wanasundara, 1992).

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2.7. Antioxidants and Biodiesel Oxidative Stability

Many scientists studied the effects of synthetic and natural antioxidants on biodiesel oxidative strength from the different sources. The caffeic acid (CA), ferulic acid and TBHQ were tested using the Rancimat test and other techniques in which it was found that CA meet European standard (EN 14214) specifications limit (Damasceno et al., 2013). BHA and TBHQ which are synthetic antioxidants were used to check their efficiency on soybean biodiesel oxidation, both shows higher potential to prevent the oxidation of biodiesel (Maia et al., 2011). The synergistic effect of BHA and BHT on rapeseed biodiesel had been studied at various concentrations and the result was promising at 400ppm (Sendzikiene et al., 2005). A study had also been done on jatropa biodiesel oxidation stability in which propyl gallate (PY) was used as antioxidant at various concentrations (200 ppm to 800 ppm) and induction period (IP) was retained for 6 hours up to six months (Jain and Sharma, 2013). PG, PY and BHA were used on methyl ester produced from Croton megalocarpus oil at different concentration and their effect was determined. PG and PY showed the higher potential as compare to BHA (Kivevele et al., 2011). Three different antioxidants BHA, TBHQ and PG were applied on linseed oil biodiesel, among them TBHQ was most effective antioxidant (Pantoja et al., 2013). Synthetic antioxidants such as BHa and BHT that are commercially available in industry have caused several problems. Synthetic antioxidant such as BHT yield greater nitrogen oxide and BHA produces higher hydrocarbon emission (Fattah et al., 2014).

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2.8. Structures of Natural Antioxidants

Natural antioxidants found in plants as secondary metabolites. Some of the most important natural antioxidants structures are shown below;

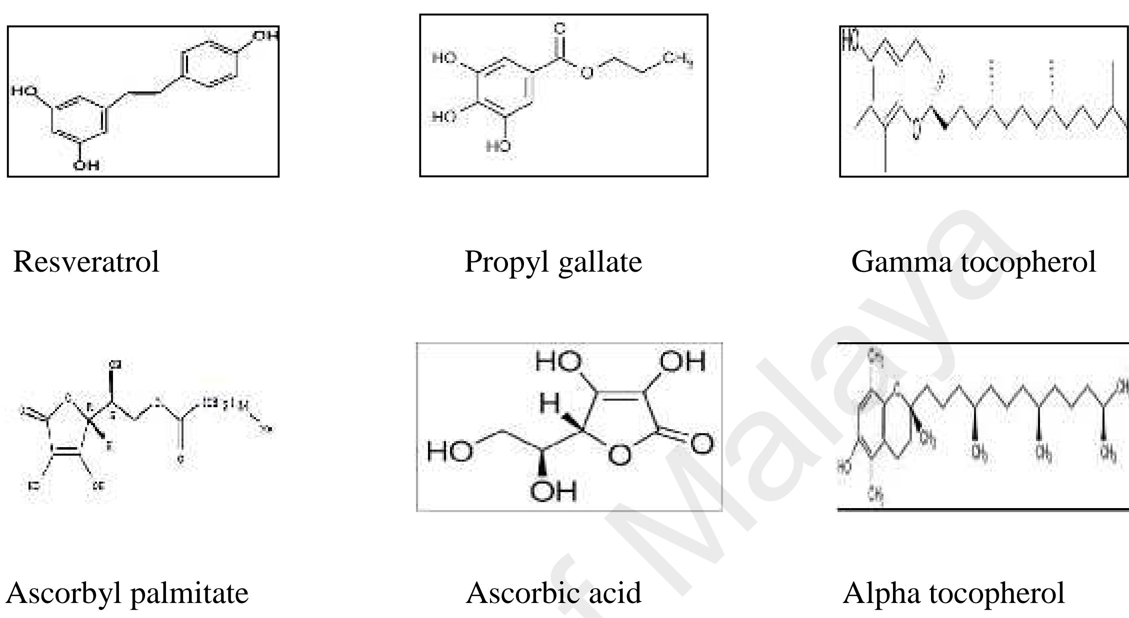

Figure 2.1: Natural antioxidants

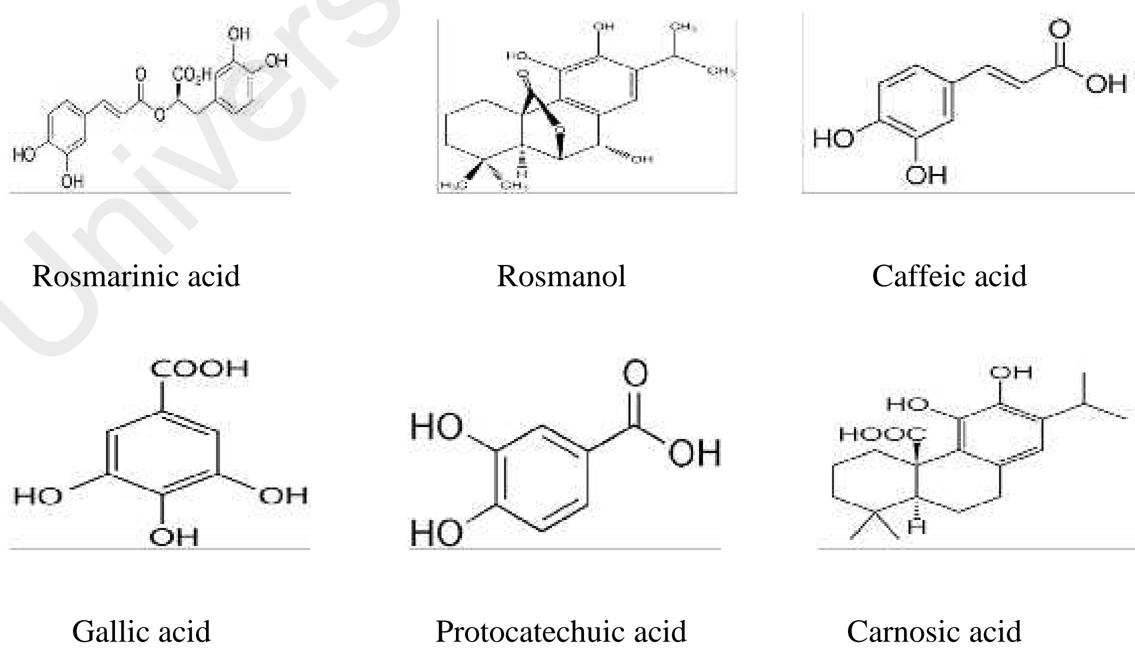

Figure 2.2: Phenolic antioxidant compounds found in plants

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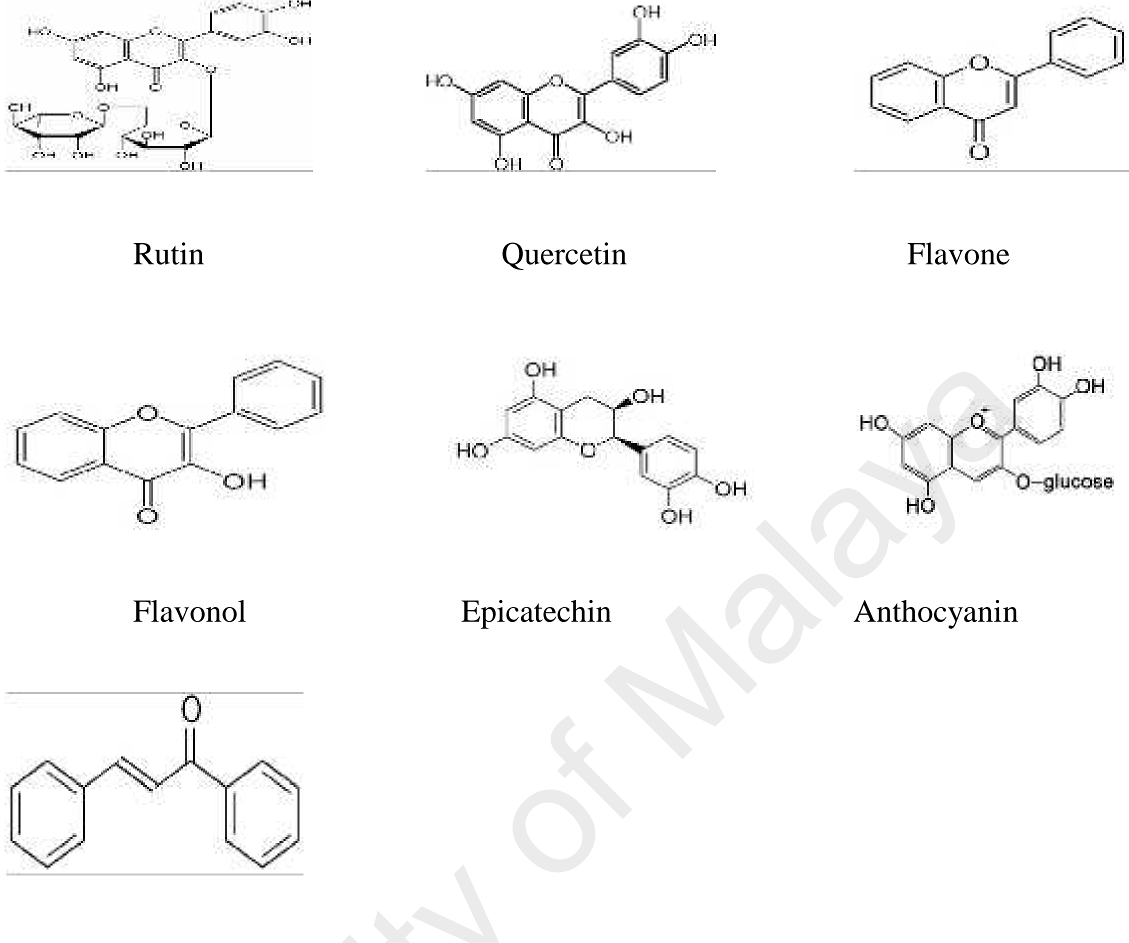
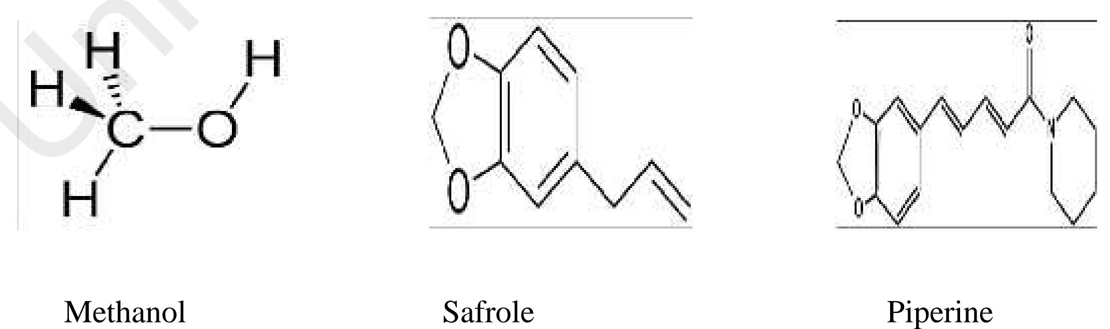


Figure 2.3: Flavonoid antioxidant compounds found in plant extracts



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Myristicin Eugenol

Figure 2.4: Volatile oils antioxidants in plant extracts

2.9. Antioxidant Assays

Though numerous *in vitro* techniques detection are available to permit quick screening and investigating antioxidant activity but their limits and advantages are still being debate and no agreement has been reached to set a standard including all the characteristic features of different classes of antioxidants because each method offer different idea and way of stating the result. Indirect *in vitro* method such as 2,2'-azino-bis, 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ferric reducing antioxidant power assay (FRAP) that include electron transfer reaction are simple to apply but have some limitations. For example, free radical scavenging capability of antioxidant compounds that are assessed by these indirect approaches are not essentially mirrored the real oxidative degradation even though in some amount the donation of hydrogen atoms or electrons compares with antioxidant activity (Tiveron et al., 2012).

2.10. Antioxidants in Plants

The plant extracts are extremely effectively and potential antioxidants due to their strong H-donating property. The plants mostly contains phenols (caffeic, rosmarinic acids and gallic acids), phenolic diterpenes (rosmanol, carnosol and carsonic

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acid), flavonoids (catechin, quercetin and kaempferol) and volatile oils, thus can be used as a potential antioxidants against oxidation. The lipids /fatty acids contain double bond polyunsaturated fatty acids chains and prone to oxidation whenever oxidation stress occur which in turn greatly affect the quality. Adding antioxidants to reduce the oxidation is the better option (Brewer, 2011). BHT, BHA and PY are synthetic antioxidants which can break oxidative stress chain and thus reduce oxidation process effectively. Some chelating agent can also avert oxidation such as ethylene diamine tetra acetic acid (EDTA). A huge quantity of antioxidant compounds are present in plant extracts, herbs and spices (Hinneburg et al., 2006).

2.11. Kinetic study

The biodiesel is produced from fats, its oxidation is same as lipid oxidation. A chemical reaction occur when oxidation of lipid takes place. Chemical reaction occurs when a molecule breakdown into several compounds. The rate at which the reaction take place is called as chemical kinetics. Reaction of reaction is describe by temperature, reactant and concentration. Lipid oxidation is the most important reaction, chain reaction that cause rancidity in oil and fats. The pH, temperature and reactant play important role in kinetics. Chemical kinetics deals with the rate at which chemical reactions take place. A chemical reaction occurs when sufficient energy is present in one or more molecules to produce rupture or formation of covalent bonds among atoms of these molecules when they are in proximity. Therefore, at the molecular level, chemical kinetics deals with the rate at which energy is brought to a molecule to react. The study of chemical kinetics is entirely based on the “law of mass action”, published in 1864 by Guldberg and Waage (Smith, 1981)

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Kinetics of lipid oxidation are often analysed when determining the shelf life of a product. Rate constants must be determined experimentally and depend upon parameters such as oxygen concentrations, surface area, and ionic strength. Oxidative reactions are fastest and thus, shelf life experiments are shortest at high temperatures, but using high temperatures runs the risk of changing factors such as oxygen solubility and partial pressure, and/or forming antioxidative side reaction products like those from Maillard browning or caramelization. Thus, it is strongly recommended that kinetic studies be conducted at multiple temperatures (Sullivan et al., 2011)

Lipid oxidation reaction kinetics are not simple since each step, initiation, propagation, and termination has its own rate constant (Labuza and Dugan, 1971). Even when considering the kinetics of hydro peroxide formation in commercial fish oil products found the data fit first-order kinetics only within certain temperature ranges and depending on the particular PUFA composition of the oil (Sullivan et al., 2011).

To simplify the complications of lipid oxidation kinetics, some scientists assume a linear approach when, in fact, the overall lipid oxidation reaction does not follow simple first order kinetics.

2.12. *Brucea javanica*

The plant of *B. javanica* was initially found in China and Vietnam. *B. javanica* plant belongs to family Simaroubaceae, genus *Brucea* and common name is *Brucea* fruit. The synonyms are *Rhu chinensis*, *B. sumatrana* and the common names include lada pahit, Chinese gall brucea, Chinese sumac, gallnut and ya tan tze (Chinese). This plant can preserve its evergreen property throughout the year, its seed is called as yadanzi , which was first cited in the herbal medicine encyclopaedia of China known as collections of *Materia Medica* issued in the 16th century (Wei et al., 2007).

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B. javanica plant can be describes as an evergreen shrub. The flower is polygamous, 0.5 cm, long, axillary panicles. The flowery portions rise in acro-petal succession in the perfectly tetramerous flower and axillary panicles. The floral parts arise in acropetal succession in the perfectly tetramerous flower. The pedicel and external surface of the sepals display unicellular, multicellular, and glandular bairs.

2.13. Chemical Compounds in *Brucea javanica*

The literature search revealed that a total of 72 compound have been isolated from this plant in which 52 are quassinoids (Fukamiya et al., 1992). The components inside fruit of *B. javanica* are oils (glycroltrileate, linoleic acid and oleic acid), quassinoids, bruceins (A and H), brustol and alkaloids. Some phytochemicals are identified in *B. javanica* such as quassinoids, triterpenoids, alkaloids, lignans, flavonoids, steroids and fatty acids has been identified (Kim et al., 2004; Murnigsih et al., 2005). The quercetine is a well known flavonoid was reported in fruit of *B. javanica* (Guru et al., 1983). There are 10 alkaloids in total are also have been isolated from *B. javanica* cell suspension and plant material (Su et al., 2002). The different part of *B. javanica* such as seed, fruits, leaves stems were reported to contain 6 type of steroidal compounds also called as steroids (Bawm et al., 2008). Seventeen phenyl propanoids were isolated from fruit of same plant (Cheng et al., 2011). The recent literature data and advancement showed that, to date 153 bioactive compounds have been stated from the aerial parts and seed of *B. javanica.* Two monoterpenes were isolated from the ripe fruits of *B. javanica* .One of them is monoterpenoid glycoside together with sequiterpenes were found in the seeds of *B. javanica (*Chen et al., 2009).

The modern analytical methods such as infrared spectroscopy, nuclear magnetica resolution, ultra violet spectroscopy and mass spectroscopy and high-

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performance liquid chromatography was helpful in advance research on the study of these compounds. The recent compounds isolated from seed are olein, glucosides, anthraquinone, oleic acid, linoleic acid, tetracyclic triterpene quassinoids and sesquiterpenes (Chen, Bai, et al., 2011; Liu et al., 2011). A total of nine are triterpenoids (Kitagawa et al., 1994; Liu et al., 2009; Pan et al., 2009), five of them are pregnane glycosides (Chen et al., 2011), two of them are sesquiterpenes (Chen et al., 2009), two are canthin- 6-one alkaloids, one monoterpenol (Chen et al., 2009). An extensive study has been done on *B. javanica* plant and some amazing secondary metabolites are isolated such as alkaloids, lignans, quassinoids, triterpenoids and flavonoids. Previous research findings suggested that *B. javanica* plant showed that a total of seventy four quassinoids compound are isolated, thirty three of them are glycosides (Sakaki et al., 1986; Kim et al., 2004). *B. javanica* also contain phenyl propaniods, seventeen of them are reported to be isolated from fruits or seed of this plant including three coumarinolignoids (Luyengi et al., 1996; Dong et al., 2013) .

2.14. Ethanomedicinal Uses of *Brucea javanica*

B. javanica has been used as ethanomedicine for long to treat diseases such as its antipyretic and detoxifying activities . It was also extensively used for the treatment of different medical difficulties such as potential antimalarial drug, used to cure inflammation, used for viral infection, the treatment of lung and prostate cancer and to treat gastrointestinal cancer. This plant is also used in traditional Chinese medicine and by people in Malaya peninsula for curing diabetes mellitus (Noor et al., 2009). Quassinoids are shown to have cytotoxic activities in various cancer cell lines (Ohnishi et al., 1995). The water extract of *B. javanica* shows great cytotoxicity and by induceing the growth of the breast cancer, hepatocellular carcinoma, lung cancer and oesophageal

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carcinoma cell lines (Lau et al., 2005). Xuan (1994) demonstrated the growth inhibitory activity of the emulsion of *B. javanica* to human squamous cell carcinoma cells. The *B. javanica* plant is also a useful anticancer drug.

The emulsions of *B. javanica* have been reported to show great synergistic effect with radio therapy treatment in brain metastasis in patient with the cancer of lungs (Wang, 1992). Some of the compound present in *B. javanica* shown to be an effective agent in treating inflammatory diseases (Yang et al., 2013). In tradition medicine, the fruit of *B. javanica* plant is also used to combat diseases such as fever, bleeding, killing of parasites, to cure food poisoning and sometime use to treat the pain of lower backbone. *B. javanica* seed and fruits are used in folk medicine in malaria, amoebic dysentery and inflammation (Subeki et al., 2007). *B. javanica* oil emulsion displayed noticeable *in vitro* inhibitory effects on human papilloma virus type 16(HPV16) infested cells. Its underlying mechanisms might be possibly associated with down-regulating expressions of 16 HPV16 E6 and E7 oncogenes (Hu et al., 2013).

The literature shows that this plant species is widely studied because of its various biological activities and it also contains a lot numbers of chemical compounds especially in its seed. *In vitro and in vivo* studied has been carried out to identify the effectiveness of *B. javanica*. The advanced and recent literature of *B. javanica* shows some wonderful knowledge and information about efficacy and active compounds present in it. The bisoprolol identified from *B. javanica* is usually used to lower blood pressure as it contains adrengenic receptors.

The water and hexane extract of *B. javanica* was to posses anti-hypersensitive agent and this might be due to the presence of alkaloid and flavonoid in *B. javanica* seed (Anna et al., 2012).

Tetracyclic triterpene quassinoids is the active ingredient in *B. javanica* and have the ability to induce apoptosis and reducing cell multiplication by inhibiting the

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expression of the *Bc.l 2* gene (Lau et al., 2008; Lou *et a*l., 2010). Immune system can also be enhanced by *B. javanica* (Yang et al., 2010). The literature review suggested that this plant possess antitumor activity and recent studies explained its mechanism of action (Chen et al., 2009), that drug resistance can be reversed in tumors cells through altering P-glucoprotein on the cell membrane by *B. javanica.* It is also reported that very interesting and recent information on activity of topoisomerase II enzyme. Topoisomerase activity can be inhibited by *B. javanica* which affect DNA synthesis and lead to cell cycle arrest in hepatoma cells.

Some recent studies explains the mechanism that in human acute myeloid leukemia cell lines, strong evidence are provided that apoptosis of cell through activation of caspase 8 and modulation of apoptosis- related protein can be induced by using oil from *B. javanica* (Zhang et al., 2011). Usually the treatment of cancer patients is done with chemotherapeutic drugs which are at a risk of immunocompromised because of that drug, so if the drugs are utilised in mixture with *B. javanica* then the protection and potential can be improved and which results in an improved immune quality and function of life expectancy in patient with malignant cancer as proliferating level of T-lymphocytes and NK cells (Hu et al., 2011).

Quassinoids and bruceantin was discovered and reported to have anti-leukemic activity in *B. antidysenterica* with in 1973. Quassinoisds are isolated as minor component from *B. javanica* and most studied quassinoid was bruceantin and its showes some great cytotoxicity activity against myeloma cell lines (Kupchan *et al*., 1973). It is reported that application of intravenous *B. javanica* oil extraction could reduce intracranial hypertension caused by brain metastasis from lung cancer and reduce pain caused by bone metastasis (Lu et al., 1994).

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B. javanica oil (BJO) unveiled a potential of killing many types of cancer cells from breast cancer cells to bladder cancer cells (Jiang et al., 2009). The anticancer activity of BJO might be attributed to following properties, induction of apoptosis (Wang et al., 2003; Zhang, et al., 2011) disrupted cellular energy metabolism, depression of expression of VEGF (vascular endothelial growth factor), up regulation of caspase-3 and caspase-9, inhibition of NF-$_k$B and Cox-2 (Lou et al., 2010), disruption of cell cycle. China has already commercialised the injection of oral and intravenous. BJO is not only used for different types of malignant cancer but also used for tumour metastasis (Zhang et al., 2011). Some promising result was obtained this plant was used as synergetic drug such as radiotherapy and chemotherapy combination. These synergetic effects improve patient life and reduce side effects (Nie et al., 2012). *B. javanica* oil shows a lot of potential as anticancer agent but the mechanism of action is very poorly understood yet, that may be due to its multifarious nature and some unknown compounds in *B. javanica* oil.

The extract from seed and aerial parts of *B. javanica* with different solvents showed biological activities especially the quassinoids was one of the breakthrough (Zhao et al., 2014). *B. javanica* plant demonstrated a range of activities from anti-malarial to anti-tumour. In recent years, Scientist has increasingly strengthened their focus on cancer proliferation process. *B. javanica* had been progressively attracting the interest of pharmacologists as well because of its stability, bioavailability and water miscibility make this plant as strong and potential treatment of tumours.

Nevertheless, on that point is a lot of mechanism unknown and not explored yet. Therefore, the unravelling of these complications and using potentials of this medicinal plant are probably will be the burning topics in the upcoming. Some most important concepts which are yet not understood. There is a requirement for advance investigation of the vigorous elements of *B. javanica* and the stimulated mono-mer mechanism.

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Secondly, it is important to isolate and refine the dynamic constituents and the monomer. Thirdly, study must be carried on the dispersal and metabolic rate of the dynamic constituents in living bodies. Last but not the least there is a need to develop novel nano-particulate formulations for clinical trials. Through additional analysis, *B. javanica* is one of the most potent and effecient herbal and traditional China medicine used to cures patients wich shows significant anti-tumor activity, may possibly be more commonly used in the clinic and helpful to human (Chen et al., 2013).

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CHAPTER 3

MATERIALS AND METHODS

3.1. Introduction

B. javanica seed was bought from a local supplier from Sungai Buloh and Negeri Sembilan, Malaysia. The seed was totally dried in 40 ^{o}C in order to avoid fungus growth. After dry, the seed was kept 8 ^{o}C so that it cannot be effected by any microorganism. The plant materials collected were then extracted with different solvent polarity. Then, the extracted sample were tested with three different tests of antioxidant acivities, phenolic determination, profiling of extracted compounds, thermal stability, oxidative stability and kinetics of biodiesel added the extracted sample.

All laboratory grade of organic solvent, heating element and glasswares for standard biodiesel was used. LCMS and GCMS was used in profiling of compounds. DPPH, FRAP and Metal chelating activity assay was used to test antioxidant activities. Total Phenolic Content and Total Flavonoid Content were used to determine phenolic compounds and thermal and oxidative stability tests were used to test antioxidant for biodiesel. All tests were separately done. In doing experiment test done in triplicates to get accurate reading while avoiding machinery errors. Summary of methodology is shown in Flowchart in Figure 3.1.

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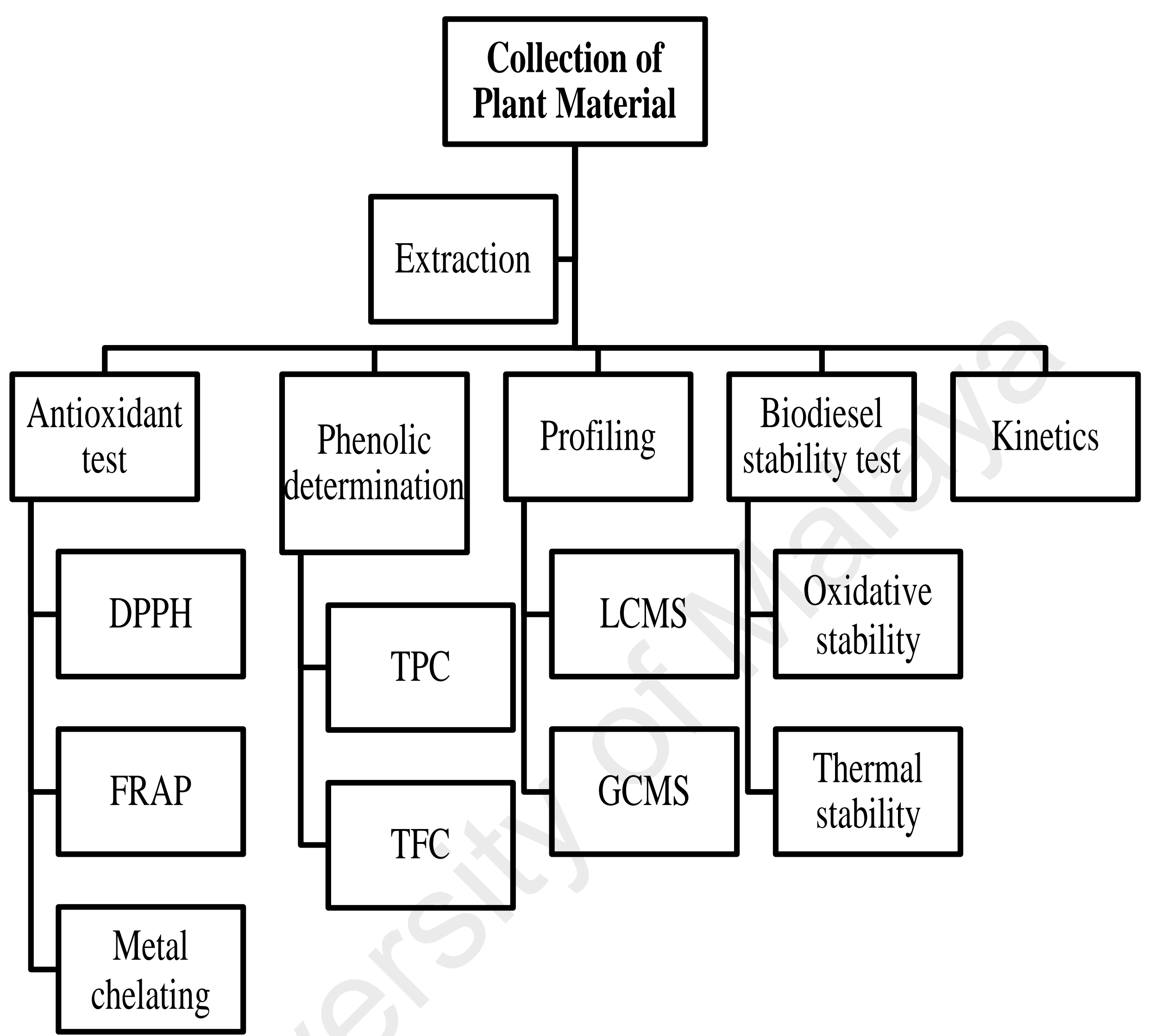

Figure 3.1: Overall Flow of Methodology

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3.2. Chemicals and Reagents

Palm oil methyl esters, butylated hydroxyanisole (BHA), ferrozine, sodiumnitroferricyanide (III) dehydrate, sodium acetate trihydrate, 2,2 -diphenyl-1-picrylhydrazyl (DPPH), gallic acid monohydrate, 2, 4, 6-tripyridyl-s-triazine (TPTZ) , Folin–Ciocalteu reagent and sodium phosphate mono were purchased from Sigma Chemical Co. (USA). Acetic acid glacial, ascorbic acid, ferric sulfate, ferric chloride hexahydrate, sodium chloride, aluminum chloride, potassium acetate, quercetin, ethylenediaminetetra acetic acid disodium dehydrate and sodium bicarbonate were purchased from Merck Chemical Co. (Malaysia). Ethyl acetate, methanol and hexane purchased from Systerm. High Performance Liquid Chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Malaysia). All the chemicals used were of analytical grade and were used without further refinements.

3.3. Sample Collection

Brucea javanica plant is available in the forest of Malaysia. *B. javanica* seed (10 kg) were purchased from Sungai Buloh and Negeri Sembilan, Malaysia between October 2013 and November 2013. Only mature *B. javanica* seed with adequate tenderness were being taken. The color of mature seed was dark brown that could be well distinguished from an immature one that was green. The seed should be free of any contaminants such as fungus. Plant has been identified by plant taxonomist Prof Dr. Ong Hean Chooi from Institute of Biological Science (ISB), Faculty of Science, University Malaya. The seeds were completely dry at 40 ^{o}C and stored at 8 ^{0}C in capped bottles prior to analysis.

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3.4. Preparation of Extracts

The *B. javanica* seed was washed carefully in order to eliminate impurities and lessen the number of microbes such as fungs that sometime grows on the coating of seed. The seed was air dried under indirect sunlight until it's totally dry. *B. javanica* seed was grinded into powder form, weighed and data was recorded. The dried powder was stored at 8 ºC for further processing.

Extraction was performed with three different solvents. A total of 3 kg of powder was extracted with 5ml 99 % methanol for a week. The extraction was done by using Soxhlet apparatus and the mixture was filtered with Whatman filter papers and the extraction was dried using rotary vacuum evaporator. A total of 50g of grinded seed was extracted with ethyl acetate in Soxhlet extraction for 8 to 10 hours. After extraction the solution was filtered with whatman filters. The remaining solvent was evaporated using rotarty vaccum evaporater. A total of 50g of grinded seed was extracted with hexane in Soxhlet extraction for 8 to 10 hours. After extraction the solution was filtered with whatman filters. The remaining solvent was evaporated using rotarty vaccum evaporater. The concentrated fraction was stored at 4 ºC for future uses. These fractions were later tested for antioxidant activity.

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3.5. Thin Layer Chromatographic (TLC) Separation of Chemical Compounds

The plates used for TLC are commercially available as either silica gel or alumina adsorbents on either a plastic or aluminium foil support. The plates are supplied in a 20 x 20 cm size. For organic chemistry, the plates are usually silica gel supported on a plastic support. The size of the plate does not have much to do with the resolution of the compounds, but does affect the time required for analysis. For rapid analysis, a

plate cut to 6.5 cm high by 2.5-5 cm wide will provide good results in a minimum amount of time. A plate higher than 6.5 cm requires a more elaborate set-up than the one described below for comparable results. It is possible to get 4 – 6 separate spots on a 2.5 cm wide plate, so don't cut more plate than is required. Using a pencil, carefully draw the spotting points on the plate about 1 cm from the bottom. Carefully draw a line 50 mm from your spotting points. This will be the solvent front when you remove the plate from the development tank.

Thin layer chromatography (TLC) plates had been used for separating chemical compounds of *B. javanica* seed. Thin layer chromatography (TLC) plates were cut in rectangular shape with dimension estimated 8 cm x 2 cm. A straight line has been taken out on both ends of paper of TLC about 2 cm from end point.

An aliquot of *B. javanica* seed solution from each extract were directly put as a spot onto TLC plate. TLC were established in a pre-saturated solvent flask with chloroform-methanol (85:15) and chloroform-methanol (90:10) as developed solvent till the solvent front touched 1 cm from the upper parts of the plates. The plates then were taken out from the vessel and permitted to dehydrate for 5-10 minutes, followed by spraying reagents vanillin, dragendorff and folin colcateu reagent for visualisation. Each plates were then monitored under UV light at 254 nanometer (nm). Bands appeared on TLC were then isolated kept in centrifuge tubes and stored at room temperature for further antioxidant tests.

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3.6. Detection and Identification of Chemical Compounds by LCMS and GCMSAnalysis.

3.6.1. Sample Preparation

Ethyl acetate, methanol and hexane extracts of *B. javanica* seed have been further examined in order to distinguish and find out their chemical compounds by using Liquid chromatography Mass spectrometry (LCMS) and Gas Chromatography Mass Spectrometry (GCMS) analysis. A total of 10mg of *B. javanica* seed were extracted using ethyl acetate, methanol and hexane, respectively.

3.6.2. Liquid Chromatography Mass Spectrometry (LCMS) Analysis

LCMS analysis was carried out on Agilent 5973 MSD with Triple-Axis HED-EM detector (Agilent USA) with an inert ion source programmable up to 350 degree. Meanwhile, the data acquisition and processing were performed using Agilent MSD Chemstation. All parameters were as follows; nitrogen was used as ion source gas, curtain gas at 15 psi and collision gas at 10 psi.

3.6.3. Gas Chromatography Mass Spectrometry (GCMS) Analysis

GCMS analysis was done using Trace GC 2000 gas chromatograph coupled to a Polaris-Q Ion trap mass spectrometer (Thermo Finnigan, Austin, TX, USA). The column that used was Zebron ZB-5ms (Phenomenex, Torrance, CA, USA) fused silica capillary column (30m long x 0.25mm I.D. x 0.25 film thickness). The oven temperature was programmed to have the initial temperature of 40 °C. It was held for 5 min before being increased gradually every 10°C for 2-10 min up to 280 °C. Carrier gas

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used was Helium at 1 mL/min in constant flow mode with injection temperature of 200 °C and auxiliary temperature of 250 °C.

3.7. Phenolic Determination

3.7.1. Total phenolic content determination

The total phenolic content was measured using the Folin Ciocalteu method described by (Singleton and Rossi, 1965) with some modifications. A total of 100 µl samples extracts were added with 1 ml of 0.5 M Folin–Ciocalteu reagent then shaken for 5 minutes. 1 ml of 75 g/L sodium bicarbonate was added and the mixture was shaken again for 30 sec. After incubation of 2 h in the darkness at room temperature and 96 multiwell microplates was used to measure the absorbance at 765 nm. Gallic acid was applied as standard and the result was determined using standard curve of gallic acid. The result was expressed as gallic acid equivalents (GAE) mg/g of dry material.

3.7.2. Total flavonoid content determination

Total flavonoid content determination was evaluated according to method (Kim, Chun et al., 2003) with some modifications. A total of 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 mins. 20 µl of sample extract was mixed up with the above aliquot and analysed using 96-multi well microplate at 510 nm. The total flavonoids contents were determined by plotting the quercetin calibration curve with 5 different concentrations and expressed as milligrams of quercetin equivalents per gram of dried extract.

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3.8. Antioxidant Activity of *B. javanica* seed extracts

3.8.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical-Scavenging Activity

The free radial scavenging activity of extracts was measured in terms of hydrogen donating ability using DPPH radical as described by Müller (2010) with a little adjustmen. Briefly, a total of 40 μL of sample extracts of different concentrations (2 mg/mL) were mixed with 200 μL of 50 μMDPPH solution in ethanol. The mixture was immediately shaken and incubated for 15 min in the dark at room temperature. The decrease in absorbance was measured at 517 nm with a microplate reader. Ascorbic acid (5–80 μg/mL) was used as a standard and the control was ethanol. The percentage of inhibition activity of the extracts was calculated according to the following equation:

Percentage Inhibition= Acontrol - Asample or standard/ Acontrol × 100.

Where,

$A_{control}$ = absorbance value of control

A_{sample} = absorbance value of sample

The concentration of extracts required to scavenge 50 % of DPPH radical was estimated from the graph plotted against the percentage inhibition and compared with the standard. All the tests were performed in triplicate, and the results were expressed as μg/mL.

3.8.2. Ferric Reducing Antioxidant Power (FRAP)

The activity of FRAP assay was determined as stated by Müller (2010) with some modifications. 20 μl of extracts in methanol were mixed with 200 μL of FRAP assay reagent which was prepared on daily base (5 mL 10 mMTPTZ in 40 mMHCl, 5

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mL of 20 mM FeCl3, and 50 mL of 0.3 M acetate buffer (pH 4) in micro-well plate reader. The incubation period was 10 min, the formation of the TPTZ-Fe2+ complex in the presence of antioxidant compounds in the extract was measured at 595 nm with a microplate reader. The blank was methanol. Standard curve calibration was plotted using ferrous sulphate ($FeSO_4$) as standard. The linear regression was used to evaluate FRAP value. The absorbance at 595 nm was measured and the results were stated as mmol $Fe^{2+/}$g of dry extract from tri-plicated test.

3.8.3. Metal Chelating Activity

The procedure used to determine metal chelating activity was ion chelating activity of ferrous by looking at Fe^{+} ferrozine complex based on the method as described by (Welch, 1990). Extracts with various concentration were mixed with dH_2O (120 μL) and $Fecl_2$ (2 mM) 10 μL in 96-wellmicroplate. Ferrozine (5 mM, 20 μL) was added to the mixture to initiate the reaction. The incubation period was 20 min and the measurement of absorbance at 562 nm with standard EDTA Na_2 (5 to 80 μg/mL) as metal chelator. Ethanol (100 μL) was used as a control. Ferrozine was not added into the blank (20 μL dH_2O). The following formula was used to express result:

$$\% \text{ age Inhibition} = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100$$

Where,

$Abs_{control}$ = absorbance reading of control in Spectophotometer

Abs_{sample} = absorbance reading of sample in Spectophotometer

Percentage Inhibition graph plotted and used to measure Fe^{2+} ion chelation (IC_{50}) of plant extracts. All the tests were performed in tri-plicate, and the results were stated as μg/mL.

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3.9. Thermal Stability of Biodiesel using DPPH assay

3.9.1. Biodiesel samples for thermally induced oxidation at 80 ºC.

Two g of palm oil methyl esters (POME) or biodiesels were placed singly in a 10mL caped test tube. 1000-5000 ppm of *B. javanica* seed extract (methanol and n-hexane extracts) were added respectively for each test tube and oxidized thermally at 80 ºC for 8 h. All samples were prepared in triplicate and analysed using DPPH method. Antioxidant possesses some what differing solubilities and effectiveness. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Liu and Yao, 2007). The reduction capability of DPPH is determined by the decrease in its absorbance at 517 nm induced by antioxidants.

3.9.2. DPPH measurement

DPPH method was ued described by Lee (2007). Two millilitres of 0.01 mM DPPH in methanol were mixed with 2 g of oil sample in caped test tube and after 30 min standing in dark, the absorbance of the sample mixture was measured at 517 nm using 96 microplates reader. BHA used as standard.

3.10. Oxidative Stability Test

The oxidation stability of biodiesel with natural antioxidant extracted from *B. javanica* was tested using the EN14112 in Rancimat equipment model 743 (Metrohm, Herisau, Switzerland), which was operated under the following conditions, air flow rate, 10 L/h, a total of 3 g biodiesel sample was placed at a heating block with temperature set from 100 to 120 ºC, the vapors discharged to a flask containing 0.06 L distilled water and the conductivity change will be recorded by a computer

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simultaneously. The induction periods of biodiesel samples with antioxidant concentrations from 0 to 10,000 ppm was determined.

3.11. Kinetics

To calculate kinetics the rate of consumption of antioxidant was considered on the basis of the kinetics of the first order reaction for which the rate equation is,

$$dc/ dt = - kc \quad (1)$$

Where

c is the concentration of samples, *t* is the oxidation time tested by Rancimat method, while *k* is the reaction constant of extract consumption. Integration of (1) within the concentration range from C_0 to C_{cr} and time limits $t = 0$ to $t = ti$ results in the dependence as following equation:

$$\text{In } C_0 = k(ti - tio)\} + \text{In}C_{cr} \quad (2)$$

This shows a linear relation between initial concentration of antioxidant C_0 and induction period ti Shows a dependence of the induction period of biodiesel on the Napierian (natural) logarithm (ln) of *B. javanica* extract of different concentration (2000-10,000 ppm) at 110 °C. Straight lines will be determined to fit the data in order to adopt the first order rate equation.

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3.11.1. Integrated Rate Laws

First-order

$$\nu = k[A] \Rightarrow \frac{d[A]}{dt} = -k[A]$$

$$\frac{d[A]}{dt} = -k[A] \Rightarrow \frac{d[A]}{[A]} = -k\,dt \Rightarrow \int_{[A]_0}^{[A]} \frac{d[A]}{[A]} = -\int_0^t k\,dt \Rightarrow \ln\frac{[A]}{[A]_0} = -k\,t$$

$$\Rightarrow [A] = [A]_0\, e^{-kt}$$

Note that plot of ln [A] vs. t is linear.

First-order half-life

$$\ln\frac{[A]}{[A]_0} = -k\,t \Rightarrow \ln\frac{\frac{1}{2}[A]_0}{[A]_0} = -k\,t_{\frac{1}{2}} \Rightarrow \ln\frac{1}{2} = -k\,t_{\frac{1}{2}}$$

$$-\ln 2 = -k\,t_{\frac{1}{2}} \Rightarrow t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

3.12. Statistical Analysis

All the data were statistically analyzed 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) using ANOVA test ($P<0.05$)

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CHAPTER 4

RESULTS AND DISCUSSION

4.1. Introduction

This chapter contain the result and discussion part which is divided into 6 sections. The first section explain about percentage yield of extract followed by second section which discuss about TLC, LCMS and GCMS section. The third section discussing about TFC and TPC determination. The fourth section discusing about antioxidant activity assays. The fifth section discussing about thermal and oxidative stability tests of biodiesel with natural antioxidants and last section discussing about kinetics of antioxidant in rancimat test.

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4.2. Determination of yield percentage

The extraction of plant materials are commonly done using organic solvent. Antioxidant depends on extraction method as well as the nature of antioxidants and that of plant materials becomes the two preliminary factors of determination.

The percentage yields of *B. javanica* seed with methanol extract (BM), ethyl acetate extract (BE) and hexane extract (BH) is listed in Table 4.1.

Table 4.1. Percentage yield of the ethyl acetate (BE), methanol (BM) and hexane (BH) extracts of *B. javanica* seed .

BM	BE	BH
39.20mg	32.90mg	15.40mg

4.3. Analyses of Bioactive Compounds

4.3.1. Thin Layer Chromatography (TLC) analysis of *B. javanica* seed extraction

The thin layer chromatography was used to screen *B. javanica* seed extracts for the presence of various classes of secondary metabolites. The phytochemical profile of the methanol extract of *B. javanica* showed that terpenoid, phenolic and flavonoids were present (Table 4.2 - 4.4). Methanol extract (Table 4.2) showed the presence of seven bands with three bands were conforming to phenols, one terpenoids and one alkaloid. Ethyl acetate extract presented in Table 4.3 indicates that a total of seven bands in which three corresponding to phenols, one terpenoid and one flavonoid. The hexane extract shows the lowest number of four bands with one terpenoid and one flavonoid. The chemical compounds reported in *B. javanica* include alkaloids, terpenoids, lignans (Karin et al., 1990) and quassinoids (Kim et al., 2003).

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However, the current screening reported that phenolic, flavonoid and terpenoids compounds presence in ethyl acetate, methanol and hexane seed extract of *B. javanica*. The absence of some of phytochemical compound in TLC may be due to complexity of major compound like starch, protein and fats which are usually difficult to separate during TLC (Ismail et al., 2010). Basically TLC screening indicated the presence of antioxidant compounds in extracts of *B. javanica* in 3 solvents.

Table 4.2. Thin layer chromatography profile of methanol extract of *B. javanica* seed

Label compound	R_f value	Visible Light	Ultra violet (UV) Light	Reagents			Remarks
				Dragendr off's	Folin-Ciocalteu's	Vanillin	
A1	0.031	-	Purple (+)	-		Greenish (+)	Phenol
A2	0.113	-	Pale (+)	-	-	-	-
A3	0.265	-	Pale purple (+)	-	-	-	-
A4	0.443	-	Purple (+)	-	-	Greenish (++)	phenol
A5	0.506	-	Purple (+)	-	-	Greenish (+++)	phenol
A6	0.582	-	Pale purple(+)	-	-	Blue (+)	terpeniod
A7	0.645	-	Pale purple(+)	-	-	Red(+)	flavonoid
A8	0.715	-	Pale purple(+)	-	-	-	-
A9	0.917	-	Palepurple(+)	-	-	Greenish (+)	phenol

Indication for intensity of colour: (+++) =**Strong,** (++) = **Medium,** (+) = **weak,**

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Table 4.3. Thin layer chromatography profile of ethyl acetate extract of *B. javanica* seed

Label compound	R_f value	Visible Light	Ultra violet (UV) Light	Reagents			Remark
				Dragendroff's	Folin-Ciocalteu's	Vanillin	
A1	0.086	-	Purple (+)	-		Greenish (+)	Phenol
A2	0.258	-	Pale (++)	-	-	Blue (+)	terpeniod
A3	0.396	-	Pale purple (-)	-	-	-	-
A4	0.482	-	Purple (-)	-	-	-	-
A5	0.68	-	Purple (-)	-	-	Greenish (+++)	phenol
A6	0.793	-	Pale purple(+)	-	-	Red(+)	flavonoid
A7	0.93	-	Pale purple(+)	-	-	Greenish (++)	phenol

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Table 4.4. Thin layer chromatography profile of n-hexane extract of *B. javanica* seed

Label compound	R_f value	Visible Light	Ultra violet (UV) Light	Reagents			Remarks
				Dragendroff's	Folin-Ciocalteu's	Vanillin	
A1	0.085	-	Pale Purple (-)	-		-	-
A2	0.189	-	Pale purple (++)	-	-	Blue (+)	terpeniod
A3	0.413	-	Pale purple (+)	-	-	Red(+)	flavonoid
A4	0.913	-	Purple (-)	-	-	-	-

Indication for intensity of colour: (+++) =Strong, (++) = Medium, (+) = weak,

4.3.2. Liquid Chromatography Mass Spectrometry (LCMS)

The liquid chromatography mass spectrometry investigation has been applied on methanol and ethyl acetate extract of *B. javanica* seed. The LCMS investigation has been conducted to identify and confirm the presence of chemical compounds such as antioxidant compounds in *B. javanica* seed. Therefore, preparative chromatography technique was applied. A C_{18} solid phase extraction column cartridge was used in the analysis of LCMS. This cartridge was highly recommended because its ability to remove high polar compound such as sugars and proteins, which can give false peak to chromatogram. Figure 4.1 illustrates LCMS profile of methanol extract of *B.javanica* seed.

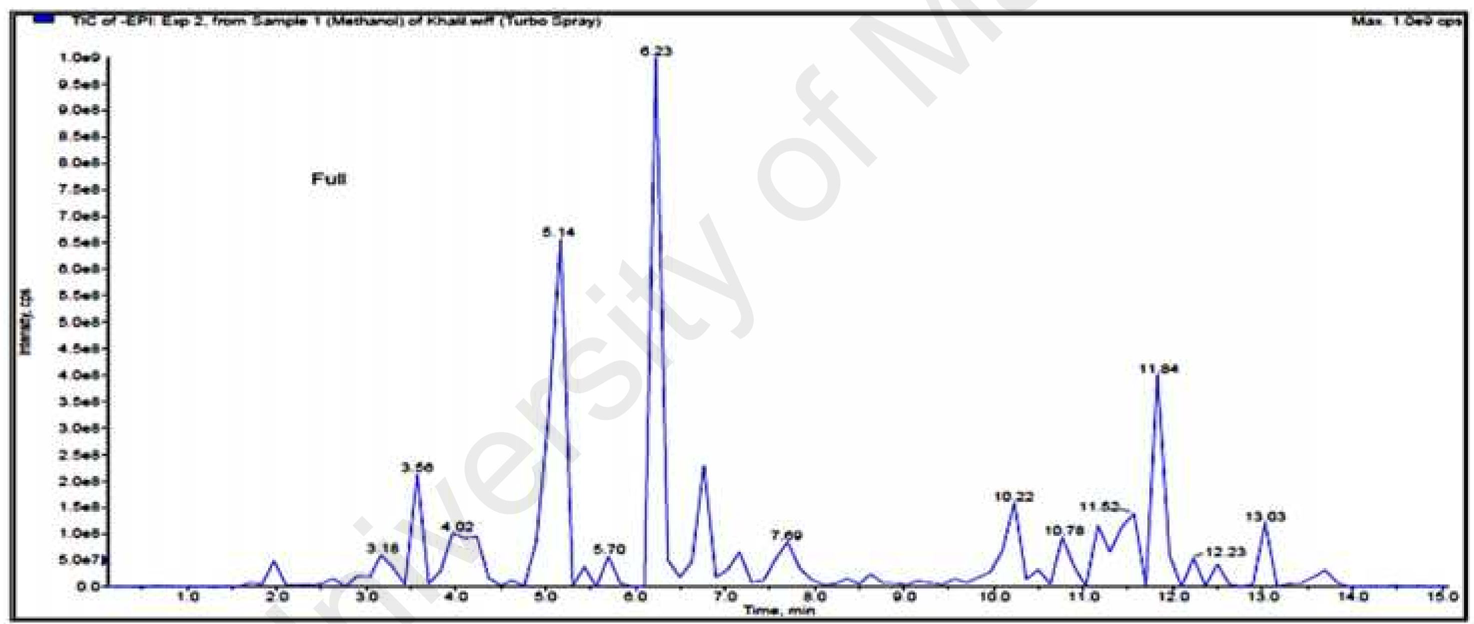

Figure 4.1. LCMS profile of methanol extract of *B. javanica* seed

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According to LCMS profile of methanol extract the identified compounds were brevifolin, ellagic acid, gallic acid, quinic acid and strictinin There were lot of unknown peaks also found in LCMS profile of methanol extract when crosschecked with library no identification was found. All the compounds with their peak no, retension time, molar mass and structures are tabulated in Table 4.2.

Table 4.5. Chemical compounds identified in LCMS analysis of methanol and ethyl acetae extract of *B. javanica* seed

Peak	Retention Time	Compound	Molar Mass	Structure
1	1.97	Quinic acid	192.17 g/mol.	
2	3.02	Gallic Acid	170.12 g/mol	
3	3.82	Protocatechuic acid	154.12 g/mol	
4	4.62	Ellagic acid	302.197 g/mol	
5	4.63	Brevifolin	196.20 g/mol	
6	5.40	Ellagic acid Isomer	------------	--------------
7	7.54	Di-o-methyl ellagic acid	462.36 g/mol	

4.3.3. Gas Chromatography Mass Spectrometry (GCMS)

Gas Chromatography Mass Spectrometry analysis was carried out in order to identify compounds that are possibly present inside *B. javanica* seed extracts. Hence, the identified compounds might be responsible for the antioxidant activity such as phenols and flavonoids. Phytochemical profile was determined using mass analysis through direct infusion technique in hexane extract of *B. javanica* seed. Chemical compounds investigated and detected in n-hexane extract along with the corresponding values for comparison are listed in Figure 4.2 and Table 4.3.

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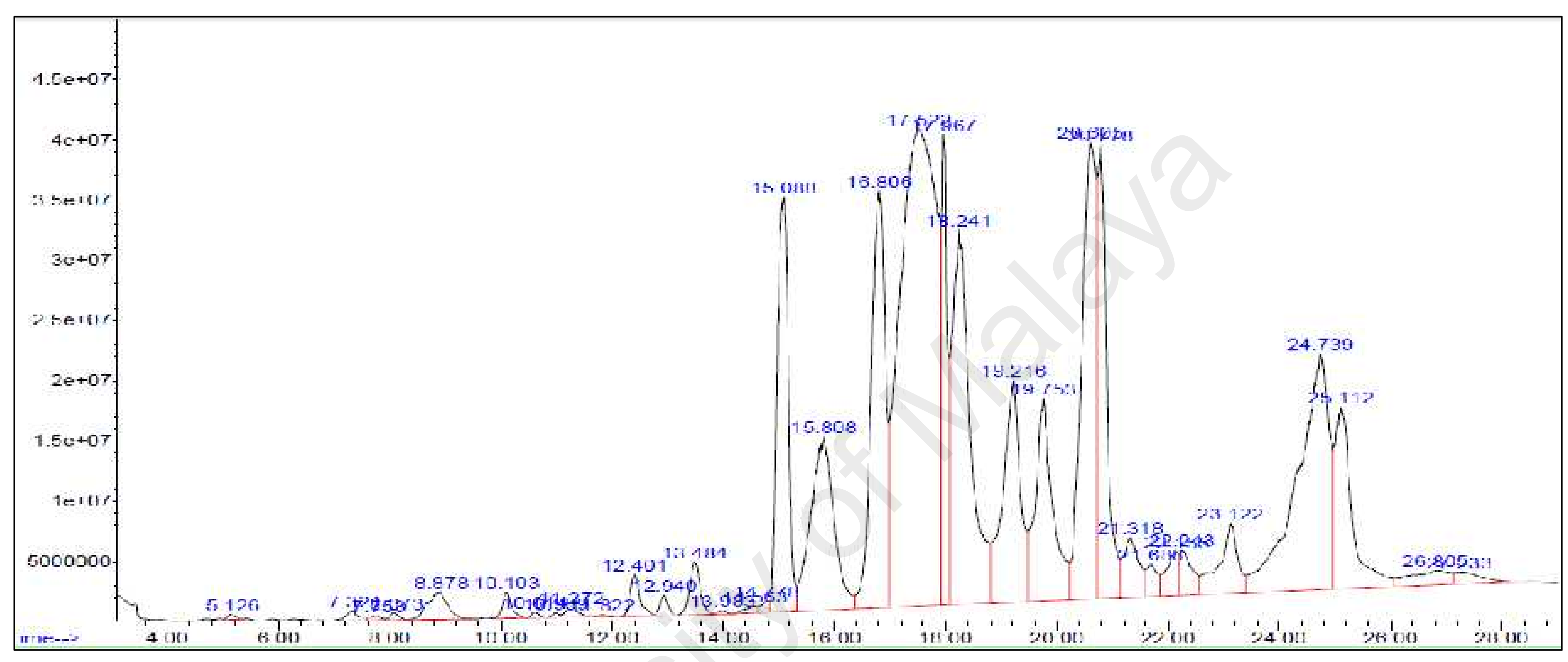

Figure 4.2. Gas Chromatography Mass Spectrometry profile of hexane extract of *B. javanica* seed

The total numbers of peaks were 187 out of which only 35 compound were detected by reference library and other all were unknown compounds. The further research can be done to identify those unknown compounds. Hexadecanoica cis-methyl ester, n-hexadecanoic acid, 9-octadecanoic acid methyl ester identified in GCMS analysis are oil in nature which suggest that *B. javanica* seed can also be a feedstock for biodiesel production.

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Table 4.6. Chemical compounds identified from GCMS analysis of hexane extract of *B .javanica* seed

Peak No	Retention Time	%Area	Chemical Compound	Reference
1	5.1239	0.0521	2-Decene, 6-methyl	27063
2	7.3211	0.1454	2-Decenal, (E)	26669
3	7.7503	0.0588	2,4-Decadienal, (E,E)	25129
4	8.0707	0.0775	2,4-Decadienal, (E,E)	25126
5	8.8775	0.7042	n-Decanoic acid	39473
6	10.102	0.3125	Cyclopentane, decyl	69587
7	10.6113	0.0564	Dodecanoic acid, methyl ester	72688
8	10.9889	0.039	Cyclohexene, 1-nonyl	67950
9	11.2693	0.1815	Cyclohexadecane	81247
10	11.8243	0.0204	11-Hexadecyn-1-ol	92516
11	12.4022	0.5184	8-Heptadecene	92565
12	12.9401	0.2045	Tridecanoicacid,12-methyl ester	95899
13	13.4837	0.7112	3-Octadecene, (E)	104188
14	13.9815	0.0318	Naphthalene	16914
15	14.5651	0.1061	1-Nonadecene	115904
16	14.7769	0.1189	cis-9-Hexadecenal	92517
17	15.0858	6.0428	Hexadecanoic acid, methyl ester	119407
18	15.8068	5.2165	n-Hexadecanoic acid	107549
19	16.8082	7.6304	9-Octadecenoic acid, methyl ester, (E)	141310
20	17.5234	22.5493	Oleic Acid	129337
21	17.9697	3.5004	Octadecanoic acid	131262
22	18.2386	8.5806	9-Eicosyne	126189
23	19.2171	4.9914	10-Heneicosene (c,t)	139793
24	19.755	4.6028	9,17-Octadecadienal, (Z)	114272
25	20.6075	8.2593	1,13-Tetradecadiene	56485
26	20.7792	5.6151	14-Tricosenyl formate	194460
27	21.317	1.179	9,12-Octadecadienoic acid (Z,Z)	127648
28	21.689	0.456	cis-Vaccenic acid	129339
29	22.1525	0.6666	Z,E-2,13-Octadecadien-1-ol	115874
30	22.244	0.7581	Triacontyl acetate	232691
31	23.1195	1.6968	Docosane	153223
32	24.7388	9.6426	Heneicosane	141424
33	25.1107	4.1019	Stigmastan-3,5-diene	210541
34	26.8044	0.7648	Erucic acid	175491
35	27.2336	0.4068	9,12-Octadecadienoic acid	127647

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4.4. Total Phenolic and Flavonoid Content Determination

4.4.1. Total Phenolic Content Determination

The method which is considered as a reliable for the determination of total phenolic content is Folin-Ciocalteu method. This method is adopted in majority of published work for the determination of antioxidants (Spigno et al., 2007). The Folin-Ciocalteu is a reagent which work by oxidizing phenolates to a complex of molybdenum-tungsten blue (Singleton and Rossi, 1965). The extractable phenolic compound content in *B. javanica* seed was determined by using linear gallic acid standard curve (R^2=0.966) (Figure 4.16).

The total phenolic content amount in *B. javanica* seed extracts is listed in Table 4.6. The extractable phenolic compound content in *B. javanica* seed extract was determined by using linear gallic acid standard curve (R^2=0.966). The total phenolic content amount in *B. javanica* seed extracts are mentioned in Table 4.6.

Table 4.7. Total phenolic contents in *B. javanica* seed extracts

Seed Extract	Ethyl acetate	Methanol	Hexane
TPC(mg/g GAE)	98.5 ±0.15^{a}	85.3 ±0.6^{b}	36.9 ±0.8^{c}

Mean ± SEM (n=3). Mean with different lower cases (a, b, c) in the same rows are significantly different at $P < 0.05$ using ANOVA.

Among the three extracts of *B. javanica* seed, ethyl acetate extract exhibits the highest content of phenols of 98.5 ±0.15^{a} mg GAE/g of dry weight. The data implies that ethyl acetate extract is the more extractable solvent for phenolic content. The total phenolic content extracted from methanol extract was 85.3 ±0.6^{b} mg GAE/g of dry weight. Among the three extracts, the hexane extract has the smallest total phenolic content at 36.9 ±0.8^{c} mg GAE/g of dry weight. The data suggests that the amount of

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total phenolic content varies among the three extracts. The deviation may be expected due to the different phenols present in the extracts. This can be supported by the previous finding which stated that different solvents extract different phenolic compounds and shows different response in the Folin-Ciocalteu method (Heinonen et al., 1998). According to the data obtained, the proposed study recommend that the higher total phenolic content amount mighyt be due to the presence of phenolic compounds such as (gallic acid, ellagic acid, quinic acid and brevifolin) as shown LCMS profile. The findings in agreement with some previous studies which reported that gallic acid as strong natural antioxidant in plants (Badhani et al., 2015).

4.4.2. Total Flavonoid Content Determination

The most commonly distributed antioxidant groups found in plants are flavonoids that are characterized by a ring structure known as benzo-y-pyrone, which is distributed in most fruits and vegetables (Yumrutas and Saygideger, 2010). Flavonoids contain an aromatic ring which allow them to accept and donate electrons from free radicals (Kanner, 1994). Flavonoids have been proven to protect lipids against oxidation (Cao *et al.*, 1997). In the current study, the total flavonoids content in *B. javanica* seed extracts were measured using linear quercetin standard curve (R^2=0.996) (Figure 4.4).

The flavonoid content found were small in amount compared to the total phenolic content. The total flavonoid content is presented in Table 4.7. The total flavonoids amount varies among the extracts.

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Table 4.8. Total flavonoid in *B. javanica* seed extracts

Seed Extract	Ethyl acetate	Methanol	Hexane
TFC(mg/g QAE)	20.0. ±0.4^{a}	12.9 ±0.3^{b}	10.0 ±0.4^{c}

Mean ± SEM (n=3). Mean with different lower cases (a, b, c) in the same rows are significantly different at $P < 0.05$ using ANOVA

Data in Table 4.4 for total flavonoids content suggests that ethyl acetate extract exhibits the highest total flavonoid content at 20.0 ±0.4^{a} mg QAE/g of dry weight and followed by methanol extract with 12.9 ± 0.3^{b} mg QAE/g of dry weight. The hexane extract has the lowest amount of total flavonoid content at 10.0 ±0.4^{c} mg QAE/g of dry weight. Polar solvent showed more flavonoid content than non-polar (hexane) which agreed with the previous finding that polyphenolic compounds were best soluble in polar organic solvent (Wang *et al*., 2009). Season, extraction and location could also be the reason of variations. *B. javanica* is a monoecious or dioecious shrub or small tree that can grow up to 10 m tall with soft-haired twigs and leaves. It prefers open localities such as light secondary forests and thickets, forest edges, ridges, and even occurring in sunny places on sandy dunes and on limestone. It grows under both per-humid and seasonal conditions from sea level up to 900 m altitude. This plant is grown in the environmental condtion of China, India, Sri lanka, Malaysia and down to northern Australia.

Previous finding suggest that that fractions of *B. javanica* seed contained various amounts of TFC, TPC, TET, and phenol and greatly influenced their antioxidant properties. Most polyphenolic compounds were extracted with ethyl acetate and exhibited the highest activities to DPPH, FRAP, and metal chelating suggesting that

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polyphenol is the main contributor for antioxidant activities of the extracts (Ablat et al., 2014).

4.5. Antioxidant Activity Test

To test antioxidant activity of plant and oil extract , three test are usually proposed as single test is not sufficient to validate the antioxidant activity. Therefore, three assays were applied to further invstigate antioxidant activity of extract as it is stated that using a single assay is either unreliable and not easy to conclude as there is no consensus vague of opnion to validate the realiability of which of the numerous methods to be used (Prior, 2005).

In this study three types of assays were used to analysed the sample for confirming of result of *B. javanica* seed extracts. The extract were compared according to IC_{50} value, amount of concentration needed to inhibit 50 % of free radical activity. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay, Ferric Reducing Antioxidant Activity Assay (FRAP) and Metal chelating assay were used to test antioxidant activity of seed extracts.

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4.5.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

The free radial scavenging activity of extracts was measured in terms of hydrogen donating ability using the DPPH radical as described by the method (M rghita et al., 2009) with some modifications. The DPPH is a free stable radical mostly recommended to check the potential of extracts to perform scavenging radical activity or hydrogen donor and to assess antioxidant activity of lipid matrix (Loizzo et al., 2012) .

Figure 4.3, which illustrates the DPPH inhibition percentage of seed extracts at different concentration.

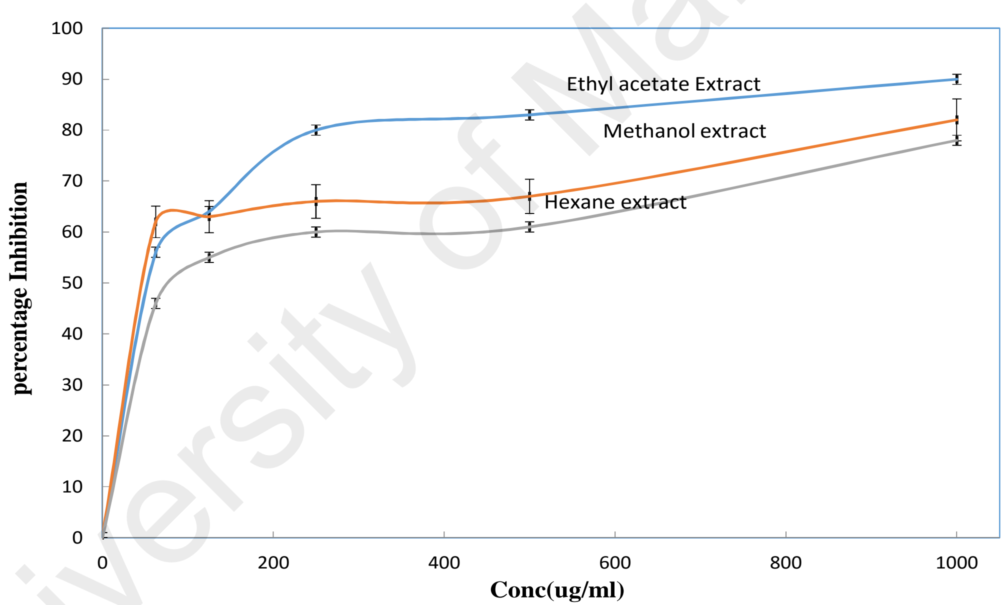

Figure 4.3. DPPH inhibition by ethyl acetate, methanol and hexane extract of *B. javanica* seed

Ethyl acetate extract of *B. javanica* seed shows the highest inhibition of 90 % at the concentration of 1000 μl with IC_{50}= 31.2 μg/ml followed by methanol extract with 82 % inhibition IC_{50}=184 μg/ml using concentrations from 200 μl to 1000 μl of seed extract (62.5,125,250,500 and 1000μg/ml) and the lowest inhibition is shown by n-hexane extract up to 78 % DPPH inhibition with IC_{50}= 319 μg/ml using different

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concentrations of seed extract. Free radical scavenging activity can be arranged as methanol extract with highest inhibition was followed by ethyl acetate extract and lowest inhibition by n-hexane extract.

These findings are similar in some ways with previous findings which suggested that change in extract polarity alter extract efficacy to extract specific group of antioxidant compounds (Zhou et al., 2004).

Table 4.9. *B. javanica* seed extract Inhibition Concentration

Seed extract	IC_{50} (μg/ml)
Ethyl acetate extract	31.2 ± 0.1 μg/ml
Methanol extract	184 ± 0.02 μg/ml
Hexane extract	319 ± 0.3 μg/ml

IC_{50} is the concentration required for 50 % DPPH inhibition

Free radical scavenging activity of *B. javanica* seed extract including the isolated compounds are in agree with analysis of TLC which already had indicated the presence of phytochemical compounds such as flavoniods, phenols and terpenoids. Polyphenol have a strong potential for biological activities such as antioxidant, anti inflammatory and antimicrobial activities (Kamatou *et al*., 2010; Šamec *et al*., 2010). Both antioxidant acitivity and polyphenols have a strong redox property which plays a vital role in neutrilizing the free radicals (Osawa, 1994). Remarkably, the radical scavenging activity of *B. javanica* seed extracts might be due to the presence of the phenolic compound such as gallic acid, ellagic acid, strictnin, protocatechuic acid, brevifolin and quinic acid which had been identified by LCMS analysis.

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4.5.2. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP activity of extracts were measured as previously described method by (Müller, et al., 2010) with some modifications. The efficacy of *B. javanica* seed extract to reduce ferric (III) to ferrous (II) was evaluated and compared with ferric sulphate which is considered as one the the strongest reducing agents, Ferric sulphate was used as a standard and shown in the Table 4.6.

The summary of the FRAP assay is shown in Table 4.9. The result suggests that methanol extract of seed of *B. javanica* seed showed the highest FRAP activity (0.180 ±0.03 mmol Fe^{2+} /g extract) with inhibition percentage of 71 % followed by ethyl acetate extract (0.163 ±0.02 mmol Fe^{2+} /g extract) with percentage inhibition 59 and lowest percentage inhibition was n-hexane with (0.15 ±0.04 mmol Fe^{2+} /g extract) with 48 % inhibition. The result is expressed as mill mole iron per gram dry extract.

FRAP activity assay in argreement with finding that *B. javanica* seed extracts fractions contains much amount of total phenolic content (TPC), total flavonoid content (TFC) and phenols, which greatly enhance antioxidant activity and showed highest activity of ferric reducing antioxidant power assay FRAP activity assay expressing that phenolic compounds are the major contributors in antioxidant activity

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Table 4.10. Ferric Reducing Antioxidant Power assay of *B. javanica* seed extract

Seed Extracts	FRAP (mmol Fe2+/g extract)
Methanol	0.180 ±0.03
Ethyl acetate	0.163 ± 0.02
Hexane	0.15 ± 0.04

4.5.3. Metal chelating Activity Assay

The metal chelating activity of methanol, ethyl acetate and hexane extract of *B. javanica* seed were evaluated against Fe^{2+} to estimate the potential antioxidant activity. The summary of metal chelating antioxidant activity is presented in Figure 4.4.

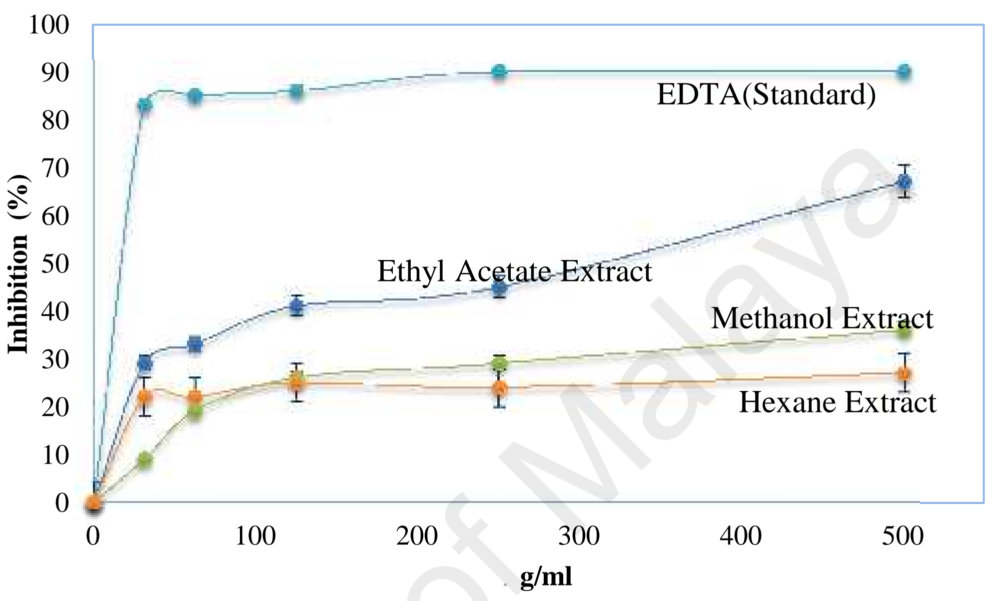

Figure 4.4. Metal chelating activity of Ethyl acetate methanol and hexane extract of *B. javanica* seed

The result indicates that the ethyl acetate extract of seed shows the highest inhibition up to 59 % with IC_{50}=299 μg/ml followed by methanol extract with 37 % inhibition with IC_{50}=656 μg/ml and the lowest activity is shown by hexane extract with 27 % inhibition IC_{50}= 1100 μg/ml at different concentrations of seed extract. The free radical scavenging activity can be arranged like methanol followed by ethyl acetate and lowest meatl chelating activity was shown by hexane.

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Table 4.11. ***B. javanica*** **seed extract Inhibition Concentration**

Seed extract	**IC_{50} (μg/ml)**
Ethyl acetate extract	299.40 ± 0.01μg/ml
Methanol extract	656.34 ± 0.2 μg/ml
Hexane extract	1100.20 ±0.1 μg/ml

IC_{50} is the concentration required for 50 % metal chelating activity

This suggests that phenolic compounds from *B. javanica* ethyl acetate seed extracts play major role in metal chelating activity. Though, there are differenent reports in literature reagarding polyphenol metal chelating activities (Wang, et al., 2009). In contrast, methanol and hexane extract shows less inhibition which agrees to some author who reported that metal chelating play a vital role in overall antioxidant activities of polyphenol (Rice-Evans et al., 1997), which corresponds to our finding of low metal chelating activity of methanol and hexane extracts of *B. javanica* seed . In the same way, the comparison of polyphenol and EDTA to chelate iron was reported and suggested that EDTA is far more stronger to chelate iron compared with phenolic compounds (Andjelkovi et al., 2006). Some proteins and peptides have the ability to chelates metal ions has been also reported (Saiga et al., 2003).

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4.6. Thermal and Oxidative Stability Test

The biodiesel oxidised quickly when exposed to oxygen, air and high temperature (Knothe, 2007). In order to test thermal stability of biodiesel, the effect of *B. javanica* crude seed extracts was tested on the oxidative degradation of palm oil methyl ester at 80 ºC with a duration of 8 h. To test oxidative stability of biodiesel ramcinat test was carried out .

4.6.1. Thermal Stability Test

In order to confirm anti-oxidation effect of *B. javanica* seed extracts on oxidative stability of palm oil methyl ester (POME), the thermal oxidation test was performed. The DPPH is a free stable radical scavenger usually used to evaluate antioxidant propertiey of crude plant extracts. Resistance to oxidative changes in oils can also be assessed using a DPPH assay. This method is based on a single electron transfer mechanism and measures the ability of the antioxidants in oil to reduce a stable DPPH radical (Espín *et al.*, 2000). The DPPH assay has been shown to be a good predictor of the oxidative stability of oils as determined using thermal oxidations of oil (Lee *et al*., 2007). Palm oil methyl ester were tested for the antioxidant activity during thermal oxidation at 80 ºC. The addition of ethyl acetae (E.A), hexane (H.E) and methanol crude extracts (M.E) of *B. javanica* seed at different concentration from 1000 ppm to 5000 ppm were tested to see the optimum concentration needed for biodiesel is shown in Figure 4.5.

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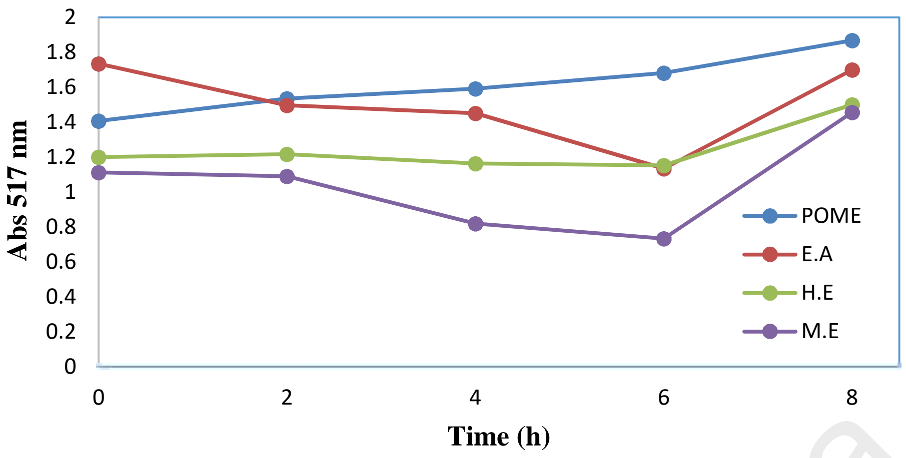

Figure 4.5. Effect of *B. javanica* seed extracts on oxidative degradation of Palm Oil Methyl Ester (POME).

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For the thermal oxidation of palm oil methyl ester (POME), a gradual increase in DPPH absorbance from 0 hour to 8 hours indicated the lack of oxidative stability. The hexane extract on the other hand is a showing stable or constant pattern over a duration of 0 hour to 8 hours and little increase in absorbance is indicating less scavenging activity compared to methanol extract where there is decrease in absorbance from 2 hours to 6 hours, which mean that the extract is protecting the biodiesel from degradation and as well as prolong its stability. BHA was used as standard and gradual decrease in absorbance showed that BHA protects biodiesel from thermal oxidation. It is observed that methanol extract was protecting biodiesel from degradation and can be used as a potential antioxidants to increase biodiesel oxidative stability. The results agreed with previous stated finding that jatropha plant methanolic crude extract show a stability upto 6 h when added to biodiesel (Diwani *et al*., 2009). After 6 h, a sudden increase in absorbance of DPPH is indicating that the antioxidants presents in extracts were already be used up and biodiesel start degrading and oxidising.

4.6.2. Oxidative Stability Test

The oxidation stability of biodiesel can be prolonged by adding antioxidant (Luo et al., 2012)Dorman *et al*., 2000; Trichopoulou *et al.,* 2000; Luo *et al*., 2012;). Plants are considered as strong natural antioxidant because of the presence of phenolic, flavonoids and anthocyanin compounds (Zhang and Wang, 2002). Most of the phenolic compounds have a great efficiency to trap oxygen reactive species like hydroxyl radical and superoxide radical which in turn inhibit oxidation of lipids/oils. The phenolic compound possess redox properties and considered as strong anti-radical agents (Rao et al., 2010)

Oxidative stability test was done using Rancimat machine following an European standard EN 14214 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 ester using accelerated oxidation test (Prankl and Schindlbauer, 1998). The most effective method reported for measuring oxidative stability of biodiesel, oils and vegetables oil is the Rancimat test (Metrohm, Switzerland) (Méndez et al., 1996; Mateos et al., 2006; Farhoosh et al., 2008;). The induction period of sample and positive control is shown in Figure 4.6. Various phenolic and flavonoid antioxidant compounds were identified in *B. javanica* seed extracts ranging from gallic acid, *O*-methyl ellagic acid, protocatechuic acid, brevifolin, ellagic acid isomers, ellagic acid to quinic acid in LCMS and GCMS profiling of ethyl acetate and methanol extracts. In order to confirm antioxidation effect of phenolic compounds of *B. javanica* seed, gallic acid was identified and detected in both extracts of *B. javanica* seed. Gallic acid was used as natural antioxidant for biodiesel to test its oxidative stability in the Rancimat machine with different concentrations from 2000 to 10,000 ppm Figure 4.6.

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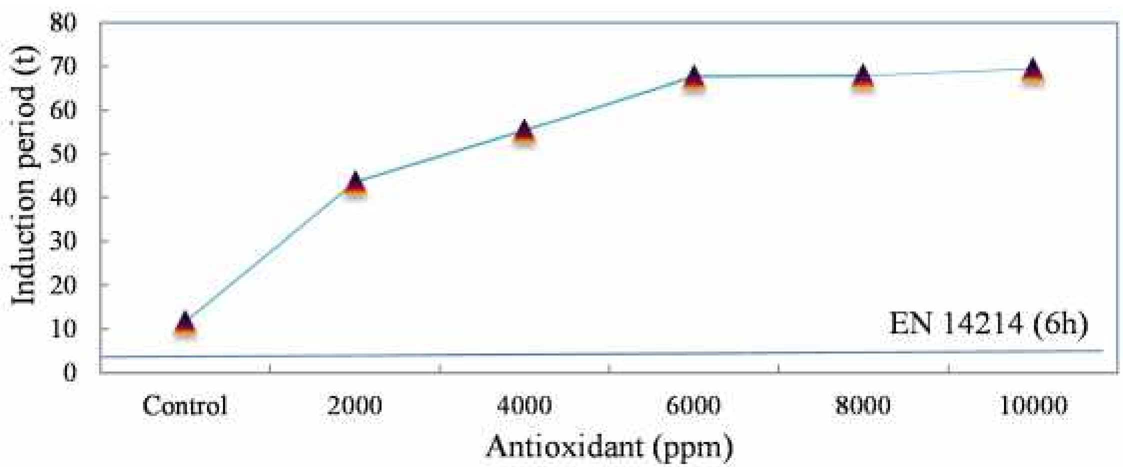

Figure .4.6. Influence of natural antioxidant (gallic acid) on oxidative stability of palm oil methyl ester. Data for BHA not shown in graph.

The result was significant and promising. It was observed that a significant increase in the induction period with gallic acid as antioxidant and which in turn prolong the oxidative stability of biodiesel. The induction period (IP) of Sample I was 43 h with 2000 ppm gallic acid concentration followed by Sample II (IP :55 h, 4000 ppm) followed by Sample III (IP: 67 h, 6000 ppm) which is followed by Sample IV (IP: 67 h, 8000 ppm) and maximum peak was obtained with Sample V (IP : 69 h, 10,000 ppm). BHA was used as standard with induction period 49 h.

On the basis of these findings it is suggested that gallic acid could be listed as strongest and potential natural antioxidant present in *B. javanica* seed. Which in agree with the finding that *B. javanica* is also rich in gallic acid (Samanidou et al., 2012). The result was also in agreement with the finding that gallic acid protects lipids /oil against oxidation in the same way increase oxidation stability of lipids (Abdelwahed et al., 2007). These finding could be relate in some way with the previous study which states that gallic acid as strong natural antioxidant in plants (Badhani, et al., 2015). The result suggests that the phytochemicals compounds present in *B. javanica* seed can be used as strongest antioxidants which could prolong the oxidation stability of biodiesel up to weeks depending on concentration.

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4.7. First order Kinetics of Antioxidant Consumption

Antioxidants play a vital role in determining the length of induction period of the biodiesel. The kinetics was calculated as the rate of consumption of antioxidant with respect to time were considered on the basis of the kinetics of the first order reaction mechanism. Figure 4.24 shows a linear relation between the concentration of antioxidant C_o and the induction period (t). The plot of lnC_o (natural log of concentration) versus induction period (time) are shown in Figure 4.7.

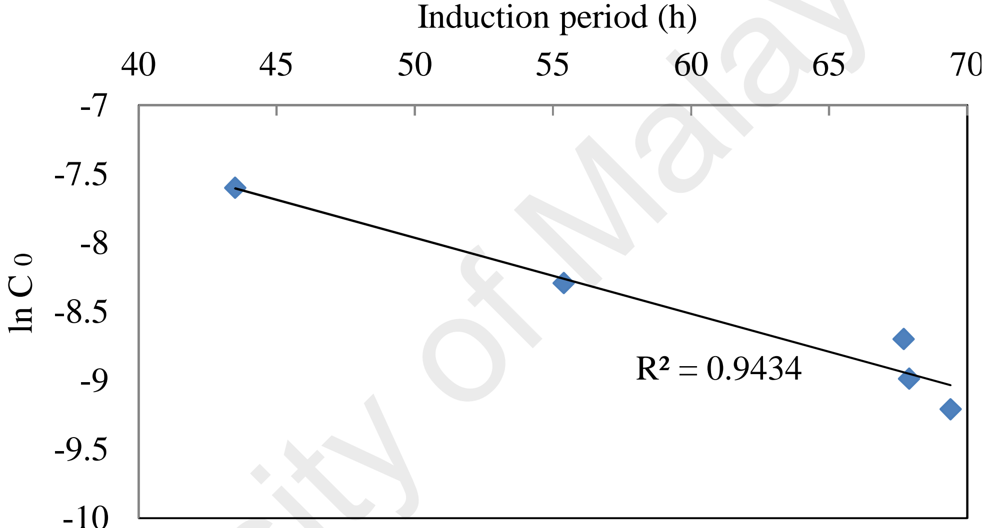

Figure 4.7. Dependence of induction period of palm oil biodiesel on logarithm (ln) of gallic acid

The first order kinetics rate was calculated from the slope of the straight line . The straight line were determine to fit the data in order to adopt first order rate equation. The straight line shows a high degree of correlation coefficient > 0.9 confirming that the degradation of antioxidant used for improving the oxidative stability of biodiesel follows first order kinetics in the rancimat machine at 110 ^{0}C.

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CHAPTER 5

CONCLUSION AND RECOMMENDATION

The aim of the study was to evaluate the oxidative stability of biodiesel with natural antioxidants from *B. javanica* seed. The antioxidant activity of *B. javanica* seed extracts was studied by applying different *in vitro* bioassays antioxidant assay such as Metal Chelating Assay, 2,2diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and Ferric Reducing Antioxidant power (FRAP). The *B. javanica* seed extracts contain many potential compounds such as polyphenols, alkaloids, terpenoids and flavonoids. LCMS and GCMS analysis revealed that *B. javanica* seed extracts contains potential antioxidant compounds such as brevifolin, ellagic acid, gallic acid, quinic acid, strictinin, *O*-methyl ellagic acid, protocatechuic acid and ellagic acid isomers. In total phenolic content determination, among the three extracts. In ferric reducing antioxidant power assay (FRAP), the methanol extract of *B. javanica* seed shows the highest FRAP activity. Based on in-vitro antioxidant activity assays, *B. javanica* seed extracts with three different solvents revealed varying degree of antioxidant activity assay. It is concluded that the type of solvent, temperature and methods of extractions are the fundamental aspects need to be considered in performing extraction because all these factors have a significant influence in the other tests.

The variation in degree of antioxidant activity may be due to the polarity of solvent that extracts different compound based on polarity. The oxidative stability test done showed that polar extract exhibit better induction period than non-polar such as gallic acid, ellagic acid and quinic acid. The ethyl acetate crude extract shows the highest protecting activity of protecting palm oil methy ester from degradation upto six hours. The data obtained in this study is noteworthy was further studied in the rancimat system for measuring the oxidation stability. The gallic acid shows the highest

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oxidative stability up to 69 hours induction periods. The first order kinetics rate was calculated from slope of the straight line. The straight line was determined to fit the data in order to adopt first order rate equation. The straight line shows a high degree of correlation coefficient > 0.9 confirming that the degradation of antioxidant used for improving the oxidative stability of biodiesel follows first order kinetics in rancimat machine at 110 ^{0}C. Thus, the result suggests that *B. javanica* seed can be used as a source of natural antioxidant and results also suggests that the plant selected for this study had a great antioxidant potential and properties that was sufficient to upgrade the oxidative stability of biodiesel.

For future work it is recommended to fractionate and isolate ethyl acetate and methanol extract into several partition by using other solvent with different polarity. Thus, by testing content of extract with different polarity such as acetone, choloroform and water, it will enable close identification of compounds responsible for antioxidant activity rather than a combination of miscellaneous compound acting together.

It is also recommended to do a pre-analysis test using HPLC to analyse an individual compound and do a downstream test with antioxidant assay. The reason of this is to identify which compound is responsible for the antioxidation activity as the extract is a mixture of compound. This could also help in determing 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. It is also found that, for GCMS test out of 187 peaks only 35 peak were known compounds when crosschecked with reference library. Thus, further test should be done by purification and separation the compound such as NMR. *B. javanica* seed consist of 65 % of oil that could be used as a feedstock for biodiesel production in future.

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Furthermore, in order to understand the advance investigation of the vigorous elements of *B. javanica* and the stimulated monomer mechanism is necessary. Secondly, it is important to isolate and refine the dynamic constituents and the monomer because the constituents might be a break through in ethanomedicine and natural antioxidant history.

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