EXTRACTION OF MIMOSINE FROM DRIED LEAVES AND DEFATTED SEED OF *Leucaena leucocephala* AND ITS BIODIESEL OXIDATIVE STABILITY

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EXTRACTION OF MIMOSINE FROM DRIED LEAVES AND DEFATTED SEED OF *Leucaena leucocephala* AND ITS BIODIESEL OXIDATIVE STABILITY

ABSTRACT

Leucaena leucocephala has long been recognized by human as one of the miracle tree with multifunctional benefits and uses. Due to its highly nutritious, palatable, widely available and drought tolerant characteristics, it has been regards as a good option for animal fodder. Animals feed with *Leucaena leucocephala* foliage had shown an increment in weight compared to other fodder choices. However, this tree contains a non-protein amino acid toxin known as mimosine. The detrimental effect of mimosine on animals such as alopecia, reduced in weight, and excessive saliva becomes a major drawback for farmers to incorporate *Leucaena leucocephala* in the animal's diet. Works have been done to remove mimosine from *Leucaena leucocephala* leaves and seeds such as drying, soaking and even cloning. Despite its anti-nutritional characteristic, mimosine possesses antioxidative behaviour which could be utilised as biodiesel additives. Therefore, this study is carried out to seek an effective method in removing mimosine from *Leucaena leucocephala* leaves and seeds as well as its potential antioxidative properties in biodiesel oxidative stability. The study was initiated by determining the mimosine yield from local *Leucaena leucocephala* leaves and seeds using two different extraction methods; HCl digestion and Soxhlet extraction (SXE) with water as extraction solvent. The result showed SXE yielded higher mimosine extraction followed by HCl digestion method with 21.633 µg and 9.764 µg in seeds while 10.261 µg and7.2 µg per 100g dry mass in leaves respectively. Next, SXE method was employed to remove mimosine from seed meals (defatted seeds) after oil extraction process to investigate whether leftover seed meals still contain mimosine before being given to the animals. The result depicted defatted seeds (DS) score lower mimosine

value with 8.150 µg compared to the undefatted seeds (UDS) which is 10.203 µg per 100g dry mass during first SXE cycle. However, the result was slightly reverse for mimosine content where the value was higher in DS compared to the UDS during the second cycle of SXE. The effect of heat also has been evaluated where UDS has higher mimosine concentration (24.5µg per 100g dry mass) compared to DS (19.417µg per 100g dry mass). Meanwhile, oxidative stability tests were conducted at a temperature of 110 °C and airflow of 10 L/h using a Rancimat machine with automatic induction period (IP) determination following the EN 14112 Method. The highest stabilization efficacy of mimosine was obtained at 10,000 ppm with IP 69.4 hours. For FRAP assay, UTS has higher FRAP value of 66.02 µmol compared to TS extracts with 55 µmol /Trolox equivalent (TE). Overall, SXE showed to be more effective in removing mimosine while heat plays a major role in reducing mimosine in seed meals. Whereas, antioxidative properties of mimosine showed a promising result to be used as a biodiesel additive.

Keywords: Mimosine, *Leucaena leucocephala*, Soxhlet, oxidative stability, antioxidant

PENGEKSTRAKAN MIMOSINE DARIPADA DAUN KERING DAN BIJI BENIH YANG DIBUANG LEMAK DARIPADA *Leucaena leucocephala* SERTA OKSIDATIF STABILITI BIODIESELNYA

ABSTRAK

Leucaena leucocephala telah lama diiktiraf sebagai salah satu daripada pokok luar biasa yang mempunyai pelbagai fungsi dan kegunaan. Ciri-ciri tumbuhan ini seperti tinggi khasiat, sedap, mudah didapati dan toleran terhadap kemarau telah menjadikan pokok ini pilihan yang baik untuk dijadikan sebagai makanan haiwan. Haiwan yang diberi makan dedaunan *Leucaena leucocephala* telah menunjukkan peningkatan dalam berat badan berbanding pilihan makanan yang lain. Walau bagaimanapun, pokok ini mengandungi toksin asid amino bukan protein yang dikenali sebagai mimosine. Kesan negatif daripada mimosine pada haiwan seperti keguguran bulu, pengurangan berat badan, dan air liur yang berlebihan menjadi kelemahan utama bagi petani untuk menggabungkan *Leucaena Leucocephala* dalam diet haiwan. Kaedah-kaedah untuk membuang mimosine dari daun dan biji benih *Leucaena leucocephala* seperti pengeringan, rendaman dan juga pengklonan yang telah dilakukan secara meluas. Namun begitu, walaupun mimosine mempunyai ciri anti-pemakanan, ia mempunyai ciri antioksida yang boleh digunakan sebagai bahan tambahan biodiesel. Oleh itu, kajian ini dijalankan untuk mendapatkan kaedah yang berkesan dalam mengeluarkan mimosine dari daun dan biji benih *Leucaena leucocephala* serta menganalisa potensi sifat-sifat antioksidanya dalam kestabilan oksidatif biodiesel. Kajian ini dimulakan dengan menentukan kandungan mimosine dalam daun dan biji benih *Leucaena leucocephala* tempatan dengan menggunakan dua kaedah pengekstrakan yang berbeza; penghadaman HCl dan pengekstraan Soxhlet (SXE) dengan air sebagai pelarut. Hasilnya menunjukkan SXE menghasilkan pengekstrakan mimosine yang lebih tinggi diikuti dengan kaedah penghadaman HCl dengan 21,633 μg

dan 9,764 µg dalam biji benih manakala 10,261 µg and7.2 µg per 100g jisim kering dalam daun. Seterusnya, kaedah SXE telah digunakan untuk mengeluarkan mimosine daripada makanan benih (benih dirawat) selepas proses pengekstrakan minyak untuk menyiasat sama ada makanan sisa biji benih masih mengandungi mimosine sebelum diberikan kepada haiwan. Hasilnya digambarkan benih yang dirawat (DS) memaparkan nilai mimosine lebih rendah dengan 8,150 µg berbanding benih yang tidak dirawat (UDS) iaitu 10,203 µg per 100g jisim kering semasa kitaran SXE yang pertama. Walau bagaimanapun, hasil untuk kandungan mimosine pada TS adalah lebih tinggi berbanding UDS semasa kitaran kedua SXE. Kesan haba juga dinilai di mana UDS mempunyai kepekatan mimosine yang lebih tinggi (24.5µg per 100g jisim kering) berbanding DS (19.417µg per 100g jisim kering). Sementara itu, ujian kestabilan oksidatif telah dijalankan pada suhu 110 °C dan aliran udara 10 L / h menggunakan mesin Rancimat dengan tempoh induksi automatik (IP) berpandukan Kaedah EN 14112. Penstabilan keberkesanan tertinggi mimosine telah diperolehi pada 10,000 ppm dengan IP 69.4 jam. Untuk FRAP assay, UTS mempunyai nilai FRAP lebih tinggi sebanyak 66,02 µmol berbanding DS dengan 55 µmol / Trolox setara (TE). Secara keseluruhan, SXE menunjukkan kaedah yang lebih berkesan dalam mengeluarkan mimosine manakala haba memainkan peranan besar dalam mengurangkan mimosine dalam makanan benih. Manakala, sifat-sifat antioksida daripada mimosine menunjukkan hasil yang memberangsangkan untuk digunakan sebagai biodiesel bahan tambahan.

Kata kunci: Mimosine, *Leucaena leucocephala*, Soxhlet, kestabilan oksidatif, antioksida

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LIST OF SYMBOLS AND ABBREVIATIONS

◦C	:	Degree celcius
µg	:	Microgram
ANOVA	:	Analysis of variance
APE	:	Allylic position equivalent
ASTM	:	American Society for Testing and Materials
BAPE	:	Bis-allylic position equivalent
BHA	:	Butylated hydroxyanisole
BHT	:	Butylated hydroxytoluene
C	:	Carbon
CN	:	Cetane number
CP	:	Cloud point
EN	:	European Standard
FA	:	Fatty acid
FFA	:	Free fatty acid
FAME	:	Fatty acid methyl ester
FRAP	:	Ferric reducing antioxidant power
g	:	Gram
H	:	Hydrogen
IV	:	Iodine value
MAE	:	Microwave-assisted extraction
MW	:	Molecular weight
OS	:	Oxidative stability
OSI	:	Oxidative stability index
PP	:	Pour point
ppm	:	Parts per million

PV	:	Peroxide value
RNS	:	Reactive nitrogen species
ROOH	:	Hydroperoxide
ROS	:	Reactive oxygen species
RSS	:	Reactive sulphur species
SD	:	Standard deviation
SEM	:	Standard error of mean
SXE	:	Soxhlet extraction
TAN	:	Total acid number
TE	:	Trolox equivalent
TBHQ	:	Tert-Butylhydroquinone
DS	:	Defatted seeds
UAE	:	Ultrasonic-assisted extraction
UDS	:	Undefatted seeds

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CHAPTER 1: INTRODUCTION

1.1 Background

Leucaena leucocephala (*petai belalang* in Malay) is commonly known as wild tamarind or coffee bush is a leguminous fast-growing tree that is native to southern tropical America, but now has been naturalised in most part of the subtropics and tropical regions around the world (Navie & Adkins, 2008). This tree has extensively introduced as agro forestry and forage legume due to its high protein crude, highly palatable, long-lived, and drought tolerant. Previous studies on different parts of *Leucaena leucocephala* have been assessed for food and non-food application ranging from wood production, phytoremediation and poultry feeds (Shelton & Dalzell, 2007; Feria et al., 2011; Jayanthy et al., 2014). Findings revealed that the oil content in *Leucaena leucocephala* seed is about 5 to 20%, high in vitamin E and vitamin C activities with about 4 to 5% presence of mimosine (Chowdhury et al., 1984; Chanwitheesuk et al., 2005; Nehdi et al., 2014). Recently, due to an increasing demand for petrol substitutes, the oil in *Leucaena leucocephala* seeds has been gaining attention to be utilized as a renewable biodiesel alternative (Khan & Ali, 2014).

Mimosine is a plant alkaloid and toxic in nature, which is found largely in *Leucaena* and *Mimosa* genera (Soedarjo & Borthakur, 1996). Due to its toxicity, animals fed with *Leucaena leucocephala* tend to have reduction in growth, alopecia, loss of hair and mortality which brings major drawback to farmers. The mimosine can be found in all parts of the tree, from seeds to shoots with varying amount from 3 to 10% (Lalitha et al., 1993). Currently, mimosine is now being perceived as an antioxidant in pharmaceutical treatment and lipid oxidation (Benjakul et al., 2013). Nevertheless, this value-added characteristic of mimosine has not been thoroughly studied especially for its application as a potential biodiesel antioxidant.

Different methods have been developed to remove mimosine from leaves of *Leucaena leucocephala* leaves and seeds including boiling, drying, soaking in water and HCl digestion (Chanchay & Poosaran, 2009; Adekojo et al., 2014). Meanwhile, other studies suggested incorporating mimosine-degrading bacteria strain in soil and introduction of transgenic Leucaena that possesses low mimosine content (Borthakur et al., 2003; Zayed et al., 2014). Extraction procedures have been widely used especially in extracting plant metabolites for industrial and medicinal purposes. However different extraction methods exhibit differences in compound yield and efficiency. Soxhlet extraction was commonly used for various compound extractions both in small scale laboratories and large scale industries with minimal expertise (Castro & Ayuso, 1998). Yet, there is no study has been conducted regarding removal of mimosine using Soxhlet extraction.

Therefore, this study was set out to assess the effect of extraction methods on the removal of mimosine from *Leucaena leucocephala* seed meals and antioxidative properties of mimosine on biodiesel quality.

1.2 Problem Statement

The global transportation sectors are now advancing towards utilising biofuels as an alternative to petrol-diesel because it is renewable and sustainable. The trend of biofuel liquid annual growth has been increasing about 15% since 2000 while biomass growth rate can only supply 2.3% globally (Kummamuru, 2017).

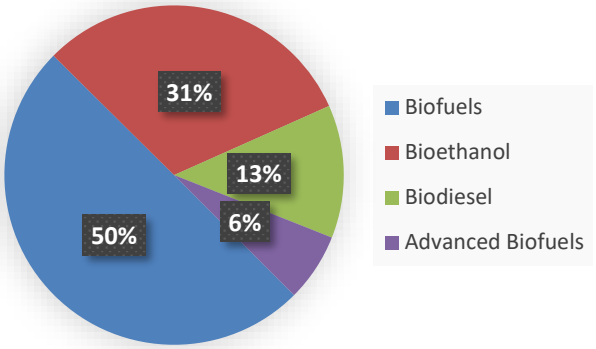

Figure 1.1: Fraction of renewable bioenergy in transportation sector. (Kummamuru, 2017)

The utilization of *Leucaena leucocephala* either as food consumption or bioenergy alternatives are limited with the presence of toxic non-protein free amino acid called mimosine; beta-(N-(3-hydroxy-4-oxypyridyl))(α)-aminopropionic acid. The content of mimosine is relatively high in seeds compare to leaves. The negative effects of mimosine could be seen in animals after prolong exposure in diet and/or increase in *Leucaena leucocephala* concentrate. Such undesired effect manifest is alopecia, excessive salivary, reduced weight gain and mortality (Meulen et al., 1979).

The feasibility of *Leucaena leucocephala* seed oil as biodiesel candidate has been identified previously. Khan &Ali, (2014) discovered that biodiesel from *Leucaena leucocephala* seed oil yielded 88% of biodiesel via microwave oven-assisted synthesis and exhibit a satisfactory score as biodiesel properties. Nevertheless, no known effect of mimosine existance in *Leucaena leucocephala* oil and its derived-biodiesel which requires further investigation.

Despite the extensive experimental works done to remove mimosine from raw *Leucaena leucocephala* seeds and leaves, it seems that there is no report on the content of mimosine in seeds after the extraction of oil (defatted seeds) which could be a new approach in utilizing this tree. Mimosine extraction methods mostly have been focusing on conventional techniques such as sun-drying and soaking in water.

However, there is no research made using Soxhlet extraction to reduce mimosine content in *Leucaena leucocephala* seeds and leaves.

1.3 Research Objectives

The research aim is to evaluate the feasibility of mimosine in *Leucaena leucocephala* seeds as antioxidant in biodiesel. In releasing this aim, three objectives were decided as below;

i. To extract and compare mimosine content in *Leucaena leucocephala* seeds and dried leaves using Soxhlet extraction (SXE) and HCl digestion.
ii. To investigate the mimosine content in *Leucaena leucocephala* defatted seed after oil extraction.
iii. To determine the effect of mimosine on biodiesel oxidative stability.

1.4 Scope of Work

This study will focus on finding efficient extraction methods to remove mimosine from *Leucaena leucocephala* dried seeds (undefatted seeds), defatted seeds and dried leaves, particularly using HCl digestion and Soxhlet (SXE) extraction. Then, the study will expose the seeds to two treatments (i) to investigate the effect of temperature on the mimosine concentration being extracted using aforementioned methods and (ii) to determine the effect of Soxhlet extraction cycles on the mimosine content in undefatted seeds (UDS) and defatted seeds (DS). The treatments were conducted in order to know the effect of external factors that may contribute to the decrease in mimosine concentration in the seeds. Finally, this study will look into the uses of mimosine in biodiesel aspect. Commercial biodiesel like rapeseed fatty acid methyl ester (FAME) will be added with mimosine in oxidative stability test to identify the suitability of mimosine to be used as biodiesel antioxidant.

1.5 Dissertation Outline

The dissertation is organized into five chapters: introduction, literature review, research methodology, results and discussion, and conclusion with future study recommendation.

The first chapter is a general introduction that gives a brief explanation regarding this study, which will emphasize on the recap of study literature, objectives, problem statement, and dissertation structure.

Next, chapter two focuses on a literature review of the past studies such as background and uses of *Leucena leucocephala*, effect of mimosine toxicity, different types of method employed for mimosine removal, biodiesel properties and antioxidant roles as biodiesel additive.

The third chapter will cover materials used and methodology for mimosine extraction using different techniques namely HCl digestion and Soxhlet extraction. This chapter will also discuss methods used for biodiesel oxidative stability test and mimosine antioxidative activity.

The fourth chapter will emphasize on the mimosine content in *Leucaena leucochepala* dried seeds, defatted seeds and dried leaves obtained from different techniques used and effect of temperature on it. This chapter will also discuss oxidation stability of biodiesel when added with mimosine.

Last but not least, the fifth chapter will summarize the findings of the study and a few recommendations for future research will be proposed in the chapter.

CHAPTER 2: LITERATURE REVIEW

2.1 *Leucaena leucocephala*

2.1.1 History and distribution

Leucaena has been identified to comprise of twenty-four species, six intraspecific taxa, two named hybrids and was put under Mimoseae of the subfamily Mimosoideae of the family Leguminosae. Most naturally occurring Leucaena, *Leucaena leucocephala* is a native species to southern Mexico and northern Central America. It also has been naturalized in Hawaii and spread through the old world tropics (Hughes & Harris, 1998). Over 400 years ago, the Spanish conquistadors recognized *Leucaena leucocephala* 's fodder value who then carried the foliage and seeds to the Philippines to feed their stock. This tree was later been introduced or widespread to Africa and Asia by the late of 19th century (Aganga & Tshwenyane, 2003).

2.1.2 Agronomic characteristic

Leucaena leucocephala is a thornless, woody, long-lived shrub with small to medium-sized tree that may grow in the range from 4 to 5 metre to 20 to 25 metre. It grows well in a long, warm, and wet growing season. Naturally, it is found mostly restricted elevation below 500 m with annual rainfall between 500 to 2000 mm (Pund et al., 2017). However, its growth rate is slower at higher altitudes. One of the *Leucaena leucocephala* unique properties is it has high tolerance to drought. The tree thrives on a wide range of soils but grows poorly on acidic latosis. Its deep-rooted system characteristic permits it to tolerate any types of soil, from heavy soils to porous coral thus, enabling it to produce a high-quality leaf during dry times (Aganga & Tshwenyane, 2003).

2.1.3 Chemical composition

Leucaena leucocephala leaves and its edible parts could make high protein feeds where it has been shown to contain 14 % to 34.4 % of crude protein in dry matter. The protein is made of high quality of nutrients and well balanced which is comparable to that of Alfafa (Table 2.1) (Meulen et al., 1979).

Table 2.1: Content of crude protein and mimosine in Leucaena leaves and seeds. (Meulen et al., 1979)

	% Crude protein	Mimosine
Leucena leaves	34.4	7.19
Leucaena seeds	31.0	12.13

Besides, *Leucaena leucocephala* also rich in vitamins and other minerals (Table 2.2). It provides a good source of b-carotene, vitamin K, calcium, phosphorus and other dietary minerals for poultry feeds.

Table 2.2: Amino acids composition of *Leucaena leucocephala.* (Meulen & Elharith, 1985).

Amino acid	Leucaena	Alfafa
Arginine	294	357
Cysteine	88	77
Histidine	125	139
Isoleucine	563	290
Leucine	469	494
Lysine	313	368
Methionine	100	96
Methionine + Cysteine	188	173
Phenylalanine	294	307
Threonine	231	290
Tyrosine	263	232

2.1.4 Uses of *Leucaena leucocephala*

One of the most widely use of *Leucaena leucocephala* is in agriculture is as an animal feedstock. The high crude protein contents in its leaves, palatable and drought tolerant characteristic highlights the importance of a continuous supply of high quality poultry feeds throughout the year.

Animal benefitted from the use of *Leucaena leucocephala's* inclusion in the diet shows a rapid weight gain which is needed to meet the global animal production's demand. In Australia, steers grazing brigalow pastures of buffel grass *(Cenchrus ciliaris)*, Rhodes grass *(Chloris gayana)* and green panic (*Panicum maximum)* showed weight gain only 140 to190 kg LW/yr, while leucaena-fed steers gain about 250 to 300 kg LW/yr (Shelton & Dalzell, 2007).

Soil could lose its nutrient especially mineral nitrogen (N) after decades of cropping or grazing. This requires added mineralising step or N fertilizers to the soil in order to achieve high animal production for exports. However, this step is just temporary and costly in a long term. Alternatively, farmers has been using vigorous forage legume like *Leucaena leucocephala* to to boost soil N levels by biological N fixation for a more sustainable and cost-effective method.

This tree also has been extensively introduced as an agro-forestry product and forage legume. The improvement of crop yield when intercropping *Leucaena leucocephala* with food crops has been widely documented. A study by Imogie et al., (2008) reported a noticeable increase in fresh fruit bunch production when intercropping *Leucaena leucocephala* with oil palm. Its deep root system and ability to fix nitrogen could aid in soil erosion, soil fertility and aeration, creating a healthy nitrogen cycle in crops. It also has use in bioremediation to treat industrial waste (Jayanthy et al., 2014). Unlike common ruminant diets that contribute to methane (CH_4) emission, research has shown that *Leucaena leucocephala* pastures were able to mitigate the greenhouse gas emissions by approximately 91,000 t carbon dioxide equivalent carbon ($CO_{2\text{-}e}$) annually (Tan et al., 2011). This anti-methanogenic characteristic is due to the presence of a plant secondary compound known as condensed tannins (CT).

CT has protein binding properties and could form CT-protein complexes that prevent the protein from being degraded into CH_4 in the rumen, thus enabling the protein to escape to the ruminant intestine (Soltan et al., 2013) .

The dynamic uses of *Leucaena leucocephala* have shifted the world's interest to explore its potential as a fuel crop as showed in Table 2.3. The composition of *Leucaena leucocephala* provides incentive for industries to utilize it, particularly in wood, pulp and paper production. Studies showed that *Leucaena leucocephala* liquor obtained by auto hydrolysis gives a potential energy yield. In wood production, *Leucaena leucocephala* has met the requirement set by the European Standard (EN) standard for general purpose wood, with a target density of 700 kg/m^3, making it a suitable candidate raw material in wood composite manufacture (Feria et al., 2011; Hilmi et al., 2012). It was suggested that the rapid growth and high dry matter production of *Leucaena leucocephala* serves as a potential biomass for generating electricity, based on the heating value and wood density as it requires about 1.5kg of dry wood to produce $1kwhr^{-1}$ (Rengsirikul et al., 2011). Likewise, it was found that *Leucaena leucocephala* feasibility for bio ethanol in the motor gasoline industry and its by-product electricity generation could meet 8% of state wide energy demand (Keffer et al., 2009).

Table 2.3 : Biomass utilisation from *Leucaena leucocephala* by different methods.

Biofuel Types	Methods	References
Biomass based power generation	Gasification of Leucaena's wood	(Kalbande et al., 2010)
Bio ethanol	Fermentation of Leucaena's legumes	(Khan & Ali, 2014)
Biodiesel	Microwave assisted irradiation of Leucaena's legumes	(Khan & Ali, 2014)
Bio-oil	Pyrolysis of Leucaena's trunk	(Payormhorm et al., 2013)
Bio char	Pyrolysis of Leucaena's bark	(Anupam et al., 2015)

2.2 Mimosine Toxicity – A problem for L. *leucocephala* to be used as food

Despite of the nutritional attributes showed, *Leucaena leucocephala* is considered as an invasive weed and its benefits are limited with the presence of toxic non-protein free amino acid called mimosine; beta-(N-(3-hydroxy-4-oxypyridyl))(alpha)-aminopropionic acid. The content of mimosineis relatively high in seeds compare to leaves (Xuan et al., 2006) . Mimosine also known to be allelopathic, inhibiting the germination and growth of other horticultural and forestry species. The presence of mimosine varies among Leucaena species, growth rates, seasons and parts of the plant such that about 2.03 to 4.89% mimosine in the dry matter leaves, 0.68% in bark, 0.11% in xylem, 6 to12% in the growing tips, 3 to 5% in young pods, 3.9 to 5% in seeds and 2% in green stems (Xuan et al., 2006; Rengsirikul et al., 2011). Nevertheless, the content of mimosine in leaf decreases as the tree mature. Meanwhile, Adeneye, (1991) reported a noticeable absence of mimosine in green and brown seedcoats as well as empty brown pods, thus suggesting that supplement using empty green and brown pods without any further treatment are poised safe for ruminant consumption.

In ruminant, mimosine is degraded to its immediate secondary metabolite; 3-hydroxy-4-1(H)-pyridone (3,4-DHP). There is also certain endogenous plant enzyme presence in leaves and seed that capable of catalysing this conversion. The toxicity of *Leucaena leucocephala* is believed to be from mimosine and 3,4-DHP, which will be further degraded into its isomer 2,3- dihydroxypyridine (2,3-DHP) in the rumen. Yet, these converted intermediates do not detoxify the toxicity effect. Generally, ruminants (cattle, sheep, and goats) are better at tolerating *Leucaena leucocephala* than non-ruminant (horses, pigs and poultry) due to the presence of microflora in the rumen. Structurally, mimosine is known to be tyrosine analogue that supress tyrosinase and tyrosine decarboxylase.

It also recognised to be anti-peroxidase, inhibiting peroxidase and lactoperoxidase reaction that is, by interfering with the iodination of tyrosine, thus affecting the synthesis of thyroid hormones such as T_1, T_2, T_3 and T_4 (Halliday et al., 2013). Findings showed that circulating DHP in blood inhibit metal-chelating enzymes such that it forms complexes with Zn and Cu, or Fe leading to excretion and depletion of these metals.

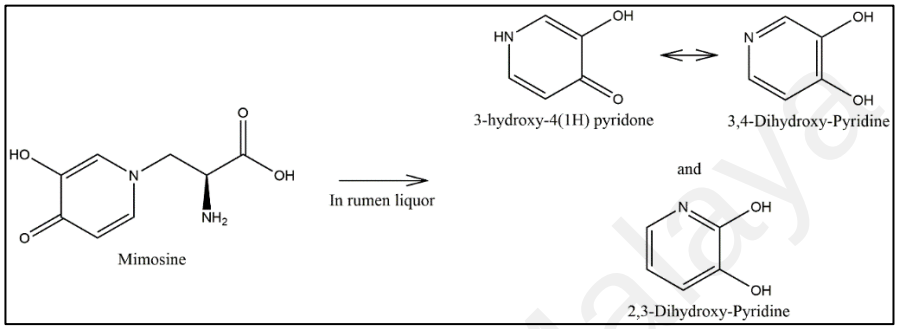

Figure 2.1: Degradation of Mimosine into its metabolites by ruminal microorganism.(Ramli et al., 2017)

Typical symptoms associated with mimosine and 3,4-DHP toxicity include alopecia, loss of appetite, growth retardation, excessive salivation in cattle and buffalo, reduced fertility, goitre and death. However, it was observed that only actively growing hair (proliferative phase) are affected rather than resting hair (keratinized phase)(Ghosh & Bandyopadhyay, 2007). The prevalence of toxicity was believed to take effect with respect of amount of *Leucaena leucocephala* and the duration of consumption where extended consumption causes decrease in liveweight instead of increasing it (Table 2.4). In contrast, there is no reduction in milk and meat yield when consuming *Leucaena leucocephala* leaf meal, reflects no mimosine and DHP toxicity possibly due to that barrier between the blood and udder, thus safe for human consumption (Gupta & Atreja, 1998).

Table 2.4: Symptoms of *Leucaena leucocephala* toxicity in animals.

Symptoms	Animals	References
Alopecia, loss of appetite, excessive salivation, poor breeding performance, thyroid hypertrophy, loss of body weight	Cattle, Buffalo, Sheep, Pig	(Jones et al., 1967; Hamilton et al., 1968; Adejumo & Ademosun, 1991; Laswai et al., 1997)
Depression of T_3 and T_4 level	Cattle, Buffalo	(Gupta, 1995; Ghosh et al., 2007)
Ulceration	Cattle, Sheep	(Pachauri & Pathak, 1989; Gupta, 1995)
Alopecia, infant mortality	Lemur	(Crawford et al., 2015)
Reversible paralysis	Rat	(El-Harith et al., 1979)
Alopecia, rapid weight reduction, serious liver and kidney degenerative	Rabbit	(Onwudike, 1995; Fayemi et al., 2011)
Reduced weight gains, egg mass and egg production	Chicken	(Abou-Elezz et al., 2011)

2.3 Mimosine removal techniques to be used as food

There are considerable methods have been proposed to reduce the toxicity of *Leucaena leucocephala* for ruminal feedstock. Factors such as leaf condition (fresh leaf and dry leaf) also plays role in determining mimosine degradation effectiveness. A study by Adekojo et al., (2014), revealed that different removal methods has significantly affect the mimosine content and its conversion. They found that the most effective approach employed for mimosine degradation was by soaking fresh leaves with water at room temperature for 36 hours, followed by soaking leaves at 60 ºC in hot water for 24 hours and fermentation for 5 days were the most effective at 40% inclusion in rabbit diet. The observed effect was pronounced as the temperature rises together with prolonged soaking however, the macerated leaves gave only slight increase in mimosine degradation. Chanchay &Poosaran, (2009) has affirm that about 94% reduction of mimosine content and virtually all tannins are reduced in the leaf meal could be obtained when employing the drying–soaking-drying techniques. It is speculated that instead of overexpression of mimosinase at high temperature, the study found that the release of mimosinase and other enzymes to catalyse mimosine was due to the breakdown of chlorophyll as the temperature rises in the intact leaves, though enzyme efficiency decreases slightly as a consequences of denaturation effect.

Table 2.5: Summary of mimosine extraction methods.

Extraction methods	Plant parts	Mimosine content	References
Hot water treatment at 60℃ Fresh water soaking Fermentation Air-dried treatment	Leaves	0.00mg/100g 0.14mg/100g 0.10mg/100g 0.26mg/100g	(Adekojo et al., 2014)
Sundried for 24h Sundried for 48h Sundried for 72h Hot water soaking at 100℃	Leaves	1.25mg/100g 1.22mg/100g 1.28mg/100g 1.07mg/100g	(Agbo et al., 2017)
Drying, soaking, drying for 24h Drying, soaking, drying for 48h Drying, soaking, drying for 72h Drying, autoclaving, drying for 24h Drying, autoclaving, drying for 48h Drying, autoclaving, drying for 72h Drying at 80℃ for 24h Drying at 80℃ for 48h Drying at 80℃ for 72h	Leaves	0.816 % 0.458 % 0.227 % 2.925% 2.847% 0.914% 1.644% 1.434% 1.251%	(Chanchay & Poosaran, 2009)
HCl digestion	Leaves	2.22g	(Matsumoto & Sherman, 1951)
Water kettle extraction	Leaves	Not specified	(Pund et al., 2017)

In nature, the utilization of mimosine was probably in the case of stress for energy source, where the nitrogen and carbon become scarce (Figure 2.2) (Negi et al., 2014).

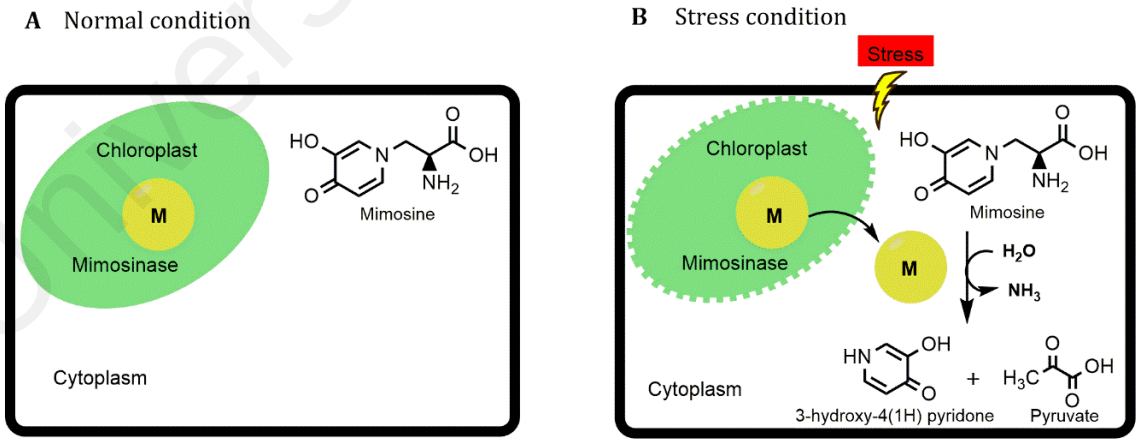

Figure 2.2: Illustration of Mimosine in cytoplasm and Mimosinase in chlorophyll of *Leucaena leucocephala* plant. A. Normal condition. B. Stress condition. (Ramli et al., 2017)

Inactivation of mimosine toxicity also could be seen through the adaptation of animal, which is considered geographical. Studies found that goat in Hawaii experience no adverse effect of mimosine toxicity, but not goat in Australia (Halliday et al., 2013).

Owing to this, attempts have been made to detect the microbes responsible for the mimosine-degrading characteristic, which is known to be *Synergistes jonesii; S. jonesii*. They found a high level of 2,3 - DHP content in the urine and faeces after inoculating *S.jonesii* in the ruminant and this microbial population tend to persist several months once the Leucaena diet stop. It is suggested that a modification in diet where rumen degradation of mimosine in sheep fed on lucerne-oat diet is more rapid than that sheep fed on lucerne hay only and the degradation process was achieved by bacteria-fraction rather than protozo-fraction (Tangendjaja et al., 1983).

In term of mechanism, it was postulated that the mechanism of desired result was due to the naturally occurring enzymatic reaction from the plant, a mimosinase that degrade mimosineinto 3-hydroxy-4-pyridone (3H4P), whereas others identified the mimosine-degrading enzyme in seedling extracts as a carbon-nitrogen (C-N) lyase that converted mimosine into 3,4-dihydroxypyridine (3,4DHP) and its by-products pyruvic acid, and ammonia (Figure 2.3) (Negi et al., 2014).

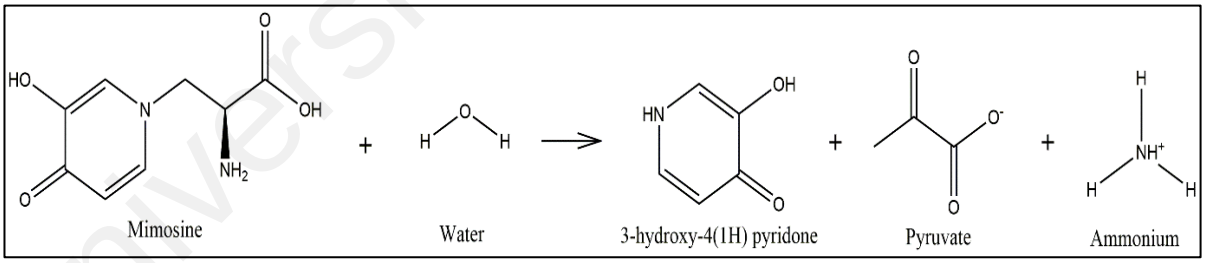

Figure 2.3: Illustration of balanced reaction of mimosine degradation catalysed by C-N lyase enzyme. (Ramli et al., 2017)

Physicochemical inactivation approaches such as supplement inclusion of ferric chloride in rabbit ration exhibit no mimosine excreted in faeces, while increase 3,4-DHP excretion in treated *Leucaena leucocephala* leaf meal indicating that mimosine forms chelate with Fe^{3+} ions, hence preventing the mimosine from being absorb in the intestine leading to substantial reduced toxicity symptoms (Gupta & Atreja, 1998).

Result from treating *Leucaena leucocephala* leaf with ferrous sulphate also shows a comparable fashion in growing pig ration with 20% inclusion of whole diet (Laswai et al., 1997). Similarly, chelation between mimosine and copper was observed when given at 10mg kg^{-1} and higher, together with iron at 8 g kg^{-1} in calves diet (Samanta et al., 1994). While others found incorporating iodine in goat diet increase the level of T_4 (tyroxine) significantly, pose a possibility to alleviate the toxicity effect on thyroid gland, although the result might be inconclusive due to short period of time (Rajendran et al., 2001).

On the other hand, researchers are now focusing on developing transgenic *Leucaena leucocephala* that has low mimosine content. A *Leucaena leucocephala* clone, was introduced by soaking the seedlings into ethyl methanesulphonate (EMS) at different concentrations prior to planting. The result showed 0.6% of EMS produced the lowest mimosine containing *Leucaena leucocephala* (87.5% reduction) however, it appears that there is slight decrease in nutritive values of cloned Leucaena (18.69%) though the value still exceeding that alfalfa (14.83%) crude protein (Zayed et al., 2014).

Borthakur et al., (2003) reported TAL1145, a *Leucaena leucocephala* nodulating *Rhizobium sp.* strain could degrade mimosine (Mid+) into 3-hydroxy-4-pyridone readily.

The corresponding enzyme with polypeptide of 45kDa; an aminotransferase encoded by *midD*gene provides TAL1145 strain a competitive advantage over other *Rhizobium*, *Sinorhizobium* and *Bradyrhizobium* spp. Another study by Pandey &Dwivedi, (2007) identified first bacterial strain found in soil independently from Leucaena tree was from *Pseudomonas* species; *P. putida* STM 905 that capable of degrading mimosine into carbon and nitrogen as energy source.

The genes responsible for the degradation activity possess a molecular mass of 70 kDa, and believe to be more efficient than that of *Rhizobium sp*. rhizosphere strain that capable of degrading mimosine.

2.4 Biodiesel

By 2030, the world will need more than 50% more energy than today with 45% of it producers come from China and India (Atabani et al., 2012). It is estimated that the world's total energy consumption will increase by 71% in 30 years' time between 2000 and 2030, and consequently, the carbon dioxide emission is expected to increase by up to 35% (Atabani et al., 2012).

Second largest energy consuming sector after the industrial sector, the transportation sector accounts for 30% of the world's total delivered energy, of which 80% is road transport. Most of the energy resources from fossil fuels are in a form of oil which constitutes 97.6% while the remaining are in a form of natural gas. The growth of transportation sector has been steadily increasing with more cars being produced, making the energy fossil fuel reservoir to exhaust.

Due to foreseeable problems such as depletion of fossil fuel and increased carbon emission, the world has shifting to renewable resources and more eco-friendly alternatives.

Biodiesel has been the worldwide focus as a renewable liquid fuel option due to its clean combustion and renewability (Dewulf et al., 2005; Knothe, 2008). It is defined by American Society for Testing and Materials (ASTM) as "a fuel comprised of monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats, designated B100" (Demirbas, 2009).

Some lucrative advantages of biodiesel is it is biodegradable and non-toxic, free of sulfur and aromatics contents, higher cetane number, higher combustion efficiency, producing lower exhaust emissions compared to conventional petroleum-derived diesel but capable to exert similar properties in terms of fuel efficiency (Anitescu & Bruno, 2012; Mofijur et al., 2012).The compatibility of biodiesel has been used in many applications including trucks and automobiles, farm vehicles, locomotives, aircraft , stationary power and heat generation.

2.4.1 Biodiesel feedstock

Globally, it has been identified that there are more than 350 oil-bearing crops which could be a potential source for biodiesel production. Table 2.5 shows the main widely-used feedstock of biodiesel (Atabani et al., 2012).

The wide range of feedstock provides accessibility in biodiesel production which represents one of the most significant factors of biodiesel production. Not only that, good biodiesel criteria should has a low production costs and large production scale. The availability of feedstock for producing biodiesel depends on the regional climate, geographical locations, local soil condition as well as agricultural practices (Khalid et al., 2015).

Table 2.6: Main feedstocks of biodiesel. (Atabani et al., 2012)

Group	Edible oils	Non-edible oils	Animal fats	Other sources
Name of feedstock	Soybeans (*Glycine max)* Rapeseed *(Brassica napus L.)* Safflower Rice bran oil (*Oryza sativum)* Barley Sesame Wheat Corn Coconut Canola Peanut Palm and palm kernel (Elaeis guineensis) Sunflower (Heliantus annuus)	*Jatropha curcas* Mahua (*Madhuca indica*) Pongamia *(Pongamia pinnata)* Camelina *(Camelina sativa*) Cotton seed (*Gossypium hirsutum)* Karanja or honge (*Pongamia pinnata)* Cumaru *Abutilon muticum* *Cynara cardunculus* Neem (*Azadirachta indica)* Jojoba (*Simmondsia chinensis)* Passion seed *(Passiflora edulis)* Moringa (*Moringa oleifera*) Tobacco seed Rubber seed tree *(Hevca brasiliensis)* Salmon oil Tall (*Carnegiea gigantea)* Coffee ground (*Coffea arabica)* Nagchampa (*Calophyllum inophyllum)* *Croton megalocarpus* *Pachira glabra* *Aleurites moluccana* *Terminalia belerica*	Beef tallow Poultry fat Fish oil Chicken fat	Algae (*Cyanobacteria*) Microalgae (*Chlorellavulgaris)* Tarpenes Poplar Switchgrass Miscanthus Fungi Latexes

2.4.2 Biodiesel production methods

There are four identifiable major possible ways where vegetable oil and/or animal fat can be converted to fuel for diesel engine: direct use or blending of oils, micro-emulsion, thermal cracking or pyrolysis and transesterification reaction. Among these methods, the most preferred process used is transesterification reaction (Gebremariam & Marchetti, 2017). This transesterification reaction enables the use of diverse feedstock types to produce a fuel that is highly resembled to conventional diesel in quality.

Through this method, oils and fats (triglycerides) reacts with alcohol with the presence of catalyst and are converted into their alkyl esters, mainly known as fatty acid methyl esters (FAMEs). Transesterification reaction can be catalyzed or non-catalyzed. The catalysis of transesterification is usually either chemically like base catalyzed transesterification and acid catalyzed transesterification, or using enzyme catalysts like lipase-catalyzed transesterification (Mishra & Goswami, 2017).

2.4.3 Properties and qualities of biodiesel

Standardization of fuel quality is important since biodiesel is produced from different plants of varying origins and qualities, it is necessary to install a in order to guarantee an engine performance without any difficulties (Meher et al., 2006). Austria was the first country in the world to define and approve the standards for rapeseed oil methyl esters as diesel fuel. Other countries such as in Germany, Italy, France, the Czech Republic and the United States also has set up a guideline for standards and the quality of biodiesel. Currently, the properties and qualities of biodiesel must adhere with the international biodiesel standard specifications mainly known as American Standards for Testing Materials (ASTM 6751-3) or the European Union (EN 14214) Standards (Atadashi et al., 2010).

The properties of biodiesel are characterized by physicochemical properties of the fuel. This includes; caloric value (MJ/kg), cetane number, density (kg/m^3), kinetic viscosity (mm^2/s), acid value (mg KOH/g-oil), cloud and pour points (°C), flash point (°C), copper corrosion, carbon residue, water content and sediment, distillation range, ash content (%), sulfur content, glycerine (% m/m), phosphorus (mg/kg) and oxidation stability as shown in Table 2.6. In brief, the physical and chemical fuel properties of biodiesel basically depend on the type of feedstock and their fatty acids composition.

Table 2.7: Biodiesel specifications according to EN 14 214 and ASTM D 6751 standards. (Monteiroa, 2008; Moser, 2009)

Properties	Test Method	Limits		Units
		Minimum	Maximum	
Iodine value	EN 14111	-	120	g I_2/100 g
Ester content	EN 14103	96.5	-	% (mol/mol)
Cetane number	EN ISO 5165 ASTM D 613	51 47	- -	
Density, 15 °C	EN ISO 3675, EN ISO 12185	860	900	kg m^{-3}
Kinetic viscosity, 40 °C	EN ISO 3104 ASTM D 445	3.5 1.9	5.0 6.0	mm^2 s^{-1} mm^2 s^{-1}
Acid value	EN 14104 ASTM D 664	- -	0.50 0.50	mg KOH g^{-1}
Cloud point	ASTM D 2500	Not specified		-
Oxidative stability, 110 °C	EN 14112	6	-	h
Flash point	EN ISO 3679 ASTM D 93	120 130	- -	°C °C
Free glycerine	EN 14105 ASTM D 6584	- -	0.020 0.020	% (mol/mol) % (mol/mol)
Total glycerine	EN 14105 ASTM D 6584	- -	0.25 0.240	% (mol/mol) % (mol/mol)
Sulphur content	EN ISO 20864, EN ISO 20884 ASTM D 5453	- -	10.0 0.05	mg kg^{-1} % (w/w)
Phosphorus content	EN 14107 ASTM D 4951	- -	10.0 0.001	mg kg^{-1} % (w/w)

2.4.3.1 Kinematic viscosity

Viscosity is one of the important measures in biodiesel quality as it indicates the ability of a material to flow (Hoekman et al., 2012). Consequently, the operation of the fuel injection equipment and spray automation could be affected by this biodiesel's flow behavior. Generally, higher viscosity leads to poorer fuel atomization because high viscosity can cause larger droplet sizes, poorer vaporization, increase exhaust smoke, narrower injection spray angle, and greater in-cylinder penetration of the fuel spray (Ejim et al., 2007; Alptekin & Canakci, 2008; Haşimoğlu et al., 2008).

Since biodiesel is synonym to have larger molecular mass and large chemical structure, the kinematic viscosity of biodiesel tends to be greater than diesel fossil fuels. There is high degree of correlation between biodiesel density and viscosity whereby high density leading to lower viscosity. Furthermore, researchers agreed that viscosity correlates more strongly with the degree of unsaturation of fatty acids, with higher unsaturation leads to lower viscosity, although coconut-derived FAME is an exception. In some cases, at low temperatures, a few biodiesel can become very viscous or even solidified which can compromise the mechanical integrity of the injection pump drive systems. The maximum allowable limit according to ASTM D445 ranges are (1.9 to 6.0 mm^2/s) and (3.5 to 5.0 mm^2/s) in EN ISO 3104 (Atabani et al., 2012).

2.4.3.2 Density

Density is weight per unit volume. Oils that are denser contain more energy (Atabani et al., 2012). Density is measured according to EN ISO 3675/12185 and ASTM D1298 where it should be tested at the temperature reference of 15 ºC or 20ºC (Torres-Jimenez et al., 2011).

In general, densities of biodiesel fuels are slightly higher than those of petroleum diesel, and increasing the B-level of biodiesel blends indirectly increases the blend's density. Previous studies showed that neem biodiesel has the highest density ranging from 912 kg/m^3 to 965 kg/m^3 while jojoba biodiesel has the lowest density ranging from 863 kg/m^3 to 866 kg/m^3. In contrast, diesel fuel has a density range of 816 to 840 kg/m^3 (Ashraful et al., 2014).

2.4.3.3 Flash point

Flash point refers to an ignition of fuel when exposed to a flame or a spark at certain temperature. It varies inversely with the fuel's volatility.

The flash point of biodiesel has been noted to be higher than the diesel fossil fuel, which means biodiesel is safe for transport, handling and storage purpose (Atadashi et al., 2010). Majority of biodiesel that were studied has a flash point more than 150ºC, while conventional diesel fuel has been showing a flash point of 55ºC to 66ºC (Atabani et al., 2012). According to Demirbas, (2009) the flash point values of fatty acid methyl esters (FAME) are significantly lower than those of vegetable oils whilst relationship between viscosity and flash point of FAME is considerably regular. In ASTM D93 and in EN ISO 3679, the limit of flash point ranges is 93ºC and 120ºC respectively (Atabani et al., 2012).

2.4.3.4 Cloud point (CP) and Pour point (PP)

It is noteworthy that the behavior of biodiesel at low temperature serves an important quality benchmark. This is because partial or full solidification of the fuel may cause blockage of the fuel lines and filters that leads to fuel starvation, problems of starting, driving and engine damage due to inadequate lubrication. (Atabani et al., 2012). The definition of cloud point (CP) is the temperature at which wax crystals first becomes visible when the fuel is cooled whereas pour point (PP) is the temperature at which the amount of wax agglomerates, sufficient to gel the fuel preventing the fuel's flow, thus it refers to the lowest temperature at which the fuel can flow (Monirul et al., 2015). Cloud and pour points are measured using ASTM D2500, EN ISO 23015 and D97 procedures. Generally, biodiesel has higher CP and PP compared to conventional diesel which can be a drawback compared to petrol-diesel. In brief, the longer the carbon chain, the higher the melting point, and poorer the low temperature performance of biodiesel. Briefly, CP is determined by the type and amount of saturation of fatty acid esters but does not account for unsaturated chains and other components which are observed to have little effect in pure biodiesel performance (Krishna et al., 2007).

2.4.3.5 Cetane number

The cetane number (CN) is the indication of ignition characteristics or ability of fuel to auto-ignite quickly after being injected. In general, the higher the cetane number (CN), the shorter the ignition delays (Lapuerta et al., 2008). Numerous researches agree that it is one of the most important parameters, which should be considered during the selection procedure of methyl esters for using as biodiesel (Atabani et al., 2012; Miraboutalebi et al., 2016; Mishra et al., 2016). Factors such as increasing chain length of fatty acids and increasing saturation are directly affecting cetane number (CN) value such as olive oil, palm oil and rapeseed oil that rich in saturated fatty acids (Karmakar et al., 2010).

Biodiesel has higher cetane number (CN) than conventional diesel fuel, thus results in higher combustion efficiency due to its higher oxygen content (Demirbas, 2005). The cetane number (CN) of diesel, specified by ASTM D613 is 47 min and EN ISO 5165 is 51.0 min. Since biodiesel is largely composed of long-chain hydrocarbon groups (with virtually no branching or aromatic structures) it is typically has a higher cetane number (CN) than petroleum diesel. In addition, increasing the B-level of biodiesel blends could therefore increase the cetane number (CN) of the blends making it more suitable for biodiesel application (Atabani et al., 2012).

2.4.3.6 Heating value

Heating value or heat of combustion is the amount of heating energy released by the combustion of a unit value of fuels. One of the most important determinants of heating value is the moisture content of the feedstock oil (Karmakar et al., 2010). Biodiesel possess higher oxygen content in its fatty acid chain compared to conventional fuel, thus lower mass energy value.

The heating value could determine the energy released after the biodiesel completely burnt, however it is not specified in the biodiesel standards ASTM D6751 and EN 14214 but is prescribed in EN 14213 (biodiesel for heating purpose) with a minimum of 35 MJ/kg (Rashid et al., 2009).

2.4.3.7 Lubrication properties

Lubricity in fuel is considered to be critical in protecting fuel injection system. Lubricity refers to the reduction of friction between solid surfaces in relative motion. Normally, biodiesel's good lubricity can be attributed to the ester group within the FAME molecules, but a higher degree of lubricity is can be seen due to the trace impurities in the biodiesel (Atabani et al., 2012). In particular, free fatty acids and free and monoacylglycerols which is contaminants produced from biodiesel production are responsible for the lubricity of low-level blends of biodiesel (Knothe & Steidley, 2005). It has been noted that purification of biodiesel by means of distillation reduces its lubricity because these impurities are removed. Xue et al., (2011) shows that high lubricity of biodiesel might result in the reduced friction loss and thus improve the brake effective power.

2.4.3.8 Oxidative stability

Another critical characteristic in biodiesel makings is oxidative stability of the fuel. It refers to a reaction between unsaturated fatty acid chains and the double bond in the parent molecule with oxygen upon exposure to the air (Atadashi et al., 2010).

It is observed that the chemical composition of biodiesel fuels makes it more susceptible to oxidative degradation than fossil diesel fuel. In general, higher unsaturation leads to poorer stability.

As studies done by Bouaid et al., (2007) conclude that the amount of highly unsaturated fatty compound (double bonds) and their position (allylic and bis-allylic) plays role in the rate of oxidation process, with bis-allylic has more pronounce effect of instability compared to that of allylic position (Figure 2.4). In term of mechanism, oxidative degradation processes are initiated at the allylic and bis-allylic position of fatty acid chain by the extraction of a hydrogen atom from a carbon (C) atom adjacent to a double bond (Arisoy, 2008; Refaat, 2009). Following the removal of this hydrogen, rapid reaction with molecular oxygen leads to formation of allylic compounds such as hydroperoxides, aldehydes, alcohols, and carboxylic acids. It is also found that linoleic acid and linolenic acid has higher oxidation instability which related to their methylene-interrupted double bonds and fatty acids with conjugated double bonds respectively. Hence, due to this reason the European biodiesel standard (EN 14214) includes a separate specification for linolenic acid methyl ester, which contains two bis-allylic groups. (Hoekman et al., 2012).

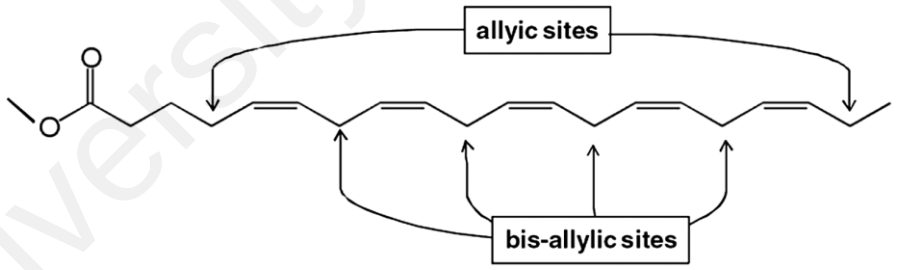

Figure 2.4: Locations of the allylic sites and the bis-allylic sites in the hydrocarbon chain. (Gopinath et al., 2014)

The Rancimat method (EN ISO 14112) is listed as the oxidative stability specification in ASTM D6751 and EN 14214. A minimum IP (110ºC) of 3 hours is required for ASTM D6751, whereas a more stringent limit of 6 hours or greater is specified in EN 14214 (Atabani et al., 2012).

2.5 Factors affecting biodiesel oxidation stability

Generally, oxidation stability of biodiesel FAME is characterized by Rancimat induction period (RIP) according to test method EN 14112 that could be affected by various factors including fatty acid (FA) composition, the saturation degree of FAME, configuration of double bonds, the molecular weight and the relative proportions of different type of FA present. Other than that, the amount of impurities presents in the biodiesel such as metals, free fatty acids, additives and antioxidants also exert effects on oxidative stability of biodiesel. Moreover, prior exposure of FAME sample to pro-oxidizing conditions such as air, heat, and light has been found to accelerate this oxidation processes.

2.5.1 Fatty acid composition

The fatty acid (FA) composition of different oils and fats can vary considerably among oil feedstocks. Many of the oils and fats listed have been investigated its potential for the use as biodiesel. The composition of fatty acid (FA) of FAME is a major factor in influencing oxidation.

There are four feedstocks that dominate world-wide biodiesel production which is known as soybean, rapeseed, palm and sunflower (Atabani et al., 2012). The fatty acid chains of these feedstocks contain primarily 16 or 18 carbon atoms with zero to three double bonds. For example, 18 carbon atoms with respective number of double bonds are primarily known as oleic (18:1), linoleic (18:2), and linolenic (18:3). The relative oxidation rates for these C18 esters are linolenic = linoleic ≫ oleic (Karavalakis & Stournas, 2010).

In addition, di- and tri- unsaturated fatty acids (FA) contain the most reactive bis-allylic sites for initiating the autoxidation chain reaction.

In other report, oxidation stability was shown to correlate not with the total number of double bonds, but with the total number of bis-allylic sites (McCormick et al., 2007). Polyunsaturated linoleic and linolenic acids are usually known to be high in vegetable oils, hence it tends to give methyl ester (FAME) poor oxidation stability (Ramos et al., 2009).

2.5.2 Position of the double bond

Another study tested on 14 fatty acids and esters of several high purity, mono-ene methyl ester of the same carbon length, where their oxidation stability (OSI values at 70ºC and 90ºC) were compared and showed that oxidation stability varies according to the position of the double bond. The oxidation stability of carbon chain 18:1 methyl esters reduced and then increased, as the double bond site changed from the 6th (D6) carbon position to D9, and to D11 respectively (Knothe & Dunn, 2003).

2.5.3 Molecular weight

The molecular weight (MW) of the alkyl-ester chains of biodiesel could affects the concentration or density of unsaturation although, oxidation stability commonly depends more on the nature of the double bonds in a molecule rather than on the MW (Pullen & Saeed, 2012). Researcher hypothesized the FAME samples of precisely equivalent mass; using two samples. First is a sample of pure mono-unsaturated with shorter-chain oleic acid methyl ester and another sample is a pure monounsaturated with longer chain erucic acid methyl esters. They elucidate that the oleic acid sample (i) will contain a greater number of molecules, hence possess a greater density of unsaturation compared to the (ii) sample. Similarly, the type of alcohol such as methanol or higher alcohols that is used to make biodiesel during transesterification process can affect oxidation stability by relatively altering the MW of the product alkyl-ester (Pullen & Saeed, 2012).

Using oxidation stability test (OSI), Knothe &Dunn, (2003) reported a higher molecular mass of esters such as oleic acid (methyl, ethyl, propyl and butyl oleate) did exhibit greater stability, though this did not follow a clear trend. The authors stated that OSI value however, could not compare samples of different MW although they have the same number of double bonds per molecule. Theoretically, if the two samples have a constant number of double bonds but increasing MW value, the OSI will increase as the corresponding 'molar' concentration of double bonds decreases. In contrast, when the two samples have a constant number of double bonds but decreasing MW value, the OSI will therefore decreasing as the 'molar' concentration of double bonds increasing. To address the problem, they proposed two approaches which is to compare OSI value from pure compound with different MW but consist the same number of double bonds and another test was to vary the 5g weight of pure sample since the varying weight infer varying 'molar' concentration of double bonds instead of the number of double bonds in the molecule.

The result of oxidation stability of higher MW compounds, such as neat methyl 11-eicosenoate (C20:1) and methyl erucate (C22:1) were compared to those of lower MW, such as methyl oleate (C18:1) showed longer OSI value, proving the proposed theory that OSI value is dependent on MW value and 'molar' concentration.

2.5.4 Proportions of different FAME

Researchers have been considering another approach to increased oxidative stability of biodiesel that is to blend a mixture of fatty acid methyl esters. Pullen &Saeed, (2012) has been proposed that a when higher MW compound increases, the oxidation stability of the mixture should increase too since the concentration or amount of double bonds in a given mass of sample is reduced such as a mixture of pure methyl oleate mixed (C18:1) with pure methyl 13-docosenoate (C22:1).

In general, increased oxidation stability of some biodiesel FAME may be attributed to the proportions of higher MW ester compounds.

A study by Park et al., (2008) examined the effects of blending different biodiesels on the oxidation stability and low temperature properties or CP-PP of the aggregate fuel blends. They found out that blending more saturated and more stable biodiesel like palm FAME with more unsaturated and more unstable biodiesel like rapeseed FAME was demonstrated as a method which simultaneously improving oxidation stability of the more unstable FAME, whilst at the same time capable of improving the cold flow properties of the more saturated type of FAME. In that study, 21 different blends of palm, rapeseed and soybean biodiesels were compared.

The fatty acid (FA) compositions of the individual biodiesel samples for palm, rapeseed and soybean biodiesels that were blended together showed the 'linoleic + linolenic' acid contents to be 11.24%, 29.30% and 60.04% respectively. In addition, the total content of unsaturated fatty acid (FA) for palm, rapeseed and soybean biodiesels were 54.26%, 92.88% and 83.16% respectively. While, the order of oxidative stability was palm (11 h) > rapeseed (6.94 h) > soybean (3.87 h).

In terms of cold flow ability, the order was palm (+10 ºC) > soybean (-3 ºC) > rapeseed (-20 ºC) respectively. In brief, the blend combinations of the three biodiesels showed an inversely proportional correlation between oxidative stability (h) and 'linoleic + linolenic' content. Moreover Park et al., (2008) also revealed that it had shown a similar benefit in terms of cold flow ability. This blending technique has served a new method that enables the commercialization of feedstocks for biodiesel that otherwise would be impossible.

For example the poor cold flow properties of biodiesel derived from palm oil is no longer a major problem to be used especially in colder climates like the UK which otherwise could solidified and clog the engine (Pullen & Saeed, 2012).

Meanwhile, Hoekman et al., (2012) compared FA compositional profiles of TAG fractions found in algal lipids from variety of algal strains which mostly have been studied as potential biodiesel feedstocks. They compare the algal FA profiles with a well-known vegetable oils/ animal fat and found some similarities in the fatty acid (FA) especially C16 and C18 components. However, the content of C16 and C18 present in algal species were not as dominant as in most vegetable oils as they found that algal FA profiles were broader, containing lighter species (C12 to C15) and heavier species (C20–C22).

For example, highly unsaturated species including FAs with 3 to 6 double bonds were found rampant in many algal species, typically Eicosapentaenoic acid (20:5) and also lower levels of Docosahexaenoic acid (22:6). The impact from these highly unsaturated species would cause implication in biodiesel properties such as the density, heating value, IV, CN and oxidation stability.

Another study done by Bucy et al., (2012) found the high content of long chain polyunsaturated fatty acids (LC-PUFA) of methyl ester which was derived from algae could be potential candidate for large scale cultivation in biodiesel production. However, these constituents could become problematic in terms of oxidation stability and cetane number (CN). Therefore, they suggested the removal of 50 to 80% of the LC-PUFA from the algal oil investigated was necessary for meeting existing specifications on oxidation stability.

2.5.5 Presence of impurities

Generally, feedstock origin and prior processing of biodiesel will account for the amount of each impurity present in biodiesel. Impurities that are known to affect oxidation stability of FAME includes metals, free fatty acids, contaminant peroxides, fuel additives which may be acidic, and antioxidants including those naturally present as well as additives.

Previous study has emphasized that for commercial biodiesel samples containing various impurities, the correlation of oxidation stability with the number of bis-allylic sites may be skewed or overshadowed by these factors (Pullen & Saeed, 2012).

2.5.6 Metals

A number of authors who has conducted biodiesel oxidation studies have confirmed the catalyzing effect of metals on oxidation (Knothe & Dunn, 2003; Jain & Sharma, 2011; Yang et al., 2013). Metals such as copper (Cu), iron (Fe), nickel (Ni), and brass are likely to increase oxidizability of fatty acid chains. The worst offender was copper where as little as 70 ppm of Cu in rapeseed oil can greatly increase oxidation process.

Similarly, a more pronounced effect of iron has been shown to be a potent hydroperoxide decomposer at higher temperature. In addition, it has been reported iron also capable of increasing acidity of biodiesel more than copper.

Knothe &Dunn, (2003) examined the oxidation stability of methyl oleate in the presence of Cu, Fe and Ni where Cu showed the strongest catalyzing effect. In other work, peroxide value (PV) of biodiesel samples was shown to increase more rapidly in Cu containers compared to steel types. Despite the detrimental effect of metal on oxidation, however the influence of increasing bis-allylic carbons was found to exert greater magnitude than the effect of metals on oxidation process.

Hence reduction of highly unsaturated components will likely enhance oxidation stability more than preventing exposure to metals. McCormick et al., (2007) analysed the metal contents of 27 commercially available US biodiesels, reporting on Cu, Fe and Zn properties. They found out no correlation was observed between metal content of individual Cu, Fe, Zn and the sum of all three metals with either insolubles (ASTM D 2274) or with oxidation stability test.

However, samples with individual metal concentrations or total metal concentration more than 6 ppm showed a very short IP as well as higher total insolubles 4mg/100 ml. These particular results infer that poor stability linked to metal contamination may not be a great issue for commercial biodiesel.

Another study done by Shiotani &Goto, (2007) evaluated different metals that are used in automotive fuel and its effects on the oxidation stability of PME. Metals including zinc, tin copper, iron, aluminum, and various aluminum alloys shown to decrease oxidation stability of palm methyl ester (PME) over several weeks storage The order of metal effect from strongest to weakest trend was copper, tin, iron, zinc, aluminum, with the strongest effect from copper reducing induction period of PME from 13 hours down to almost 0 hour. Various alloys of aluminum had a stronger effect relative to pure aluminum. They also tested terne sheet of steel influences towards oxidative stability. Results exhibit PME sample in closed terne sheet of steel cup accelerates oxidation compare to open air terne sheet of steel cup. The closed terne sheet of steel cup is thought to be caused by the impurities being unable to evaporated thus enhanced corrosion of the closed sample.

This work highlights the importance of fuel system material compatibility with type of biodiesel used, as certain metals will accelerate oxidative degradation of the fuel.

The composition PME was also monitored as oxidation proceeded showing that unsaturated components were consumed by the oxidation process, where the concentration of linoleate and oleate decreased while the level of saturated palmitate that has not been consumed by oxidation showed a relatively increase in concentration.

2.5.7 Free fatty acids

Several papers have affirmed that free fatty acids have been shown to have a significant effect on biodiesel oxidizability by the action from carboxylic acids.

Free carboxylic acids were found to be far more oxidatively unstable than their corresponding methyl esters as it accelerated the rate of decomposition of hydroperoxides (Knothe & Dunn, 2003).

2.5.8 Antioxidants

The effects of various antioxidants on biodiesel oxidation stability have been investigated extensively in the literature (Mittelbach & Schober, 2003; Karavalakis & Stournas, 2010; Prabu et al., 2017). One of the solutions to overcome oxidative instability is by addition of antioxidant as it could significantly delay the biodiesel degradation process by inhibiting the formation of free radicals or by interrupting propagation of the free radical. In summary, synthetic types such as tertiary butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) are generally more effective than natural types (tocopherols) so are generally preferred commercially. In fact, many commercial additive formulations contain two or more antioxidants (Dunn, 2008) . It is observed that effective antioxidant concentrations appear to be between 200 to1000 ppm, depending on the substrate and the type of stability test used to evaluate additive performance (Pullen & Saeed, 2012).

Antioxidant process can be classified by one or more of several mechanisms such as (1) scavenging species that initiate peroxidation, (2) chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, (3) quenching O_2- preventing formation of peroxides, (4) breaking the autoxidative chain reaction, and/or (5) reducing localized O_2 concentrations (Brewer, 2011).

Generally, the most effective antioxidants are in categories that interrupt the free radical chain reaction. They usually contain aromatic or phenolic rings, these antioxidants donate hydrogen to the free radicals that formed during oxidation, and becoming a radical themselves.

However, these radical intermediates behavior are stabilized by the resonance delocalization of the electron within the aromatic ring and formation of quinone structures. In addition, many of the phenolics lack positions available for molecular oxygen attack giving it an advantage (Brewer, 2011).

Karavalakis &Stournas, (2010) has tested five different antioxidants in pure biodiesel and biodiesel blend, where they have found that the efficiency of the antioxidants used at a loading of 1000 mg/kg was in the order of PG > pyrogallol (PA) > TBHQ > BHT > BHA. This efficiency of antioxidant depends on several factors such as the phenol groups occupying 1 and 2 or 1 and 4 positions in an aromatic ring, as well as the volume and electronic characteristics of the ring substituent present. In general, the hydroxyl group provides proton that inhibit the formation of free radicals or interrupt the propagation of free radical and, thus, delay the rate of oxidation. Furthermore, based on the result, the effectiveness of TBHQ, PG, and PA can be explained on the basis of their molecular structure. These additives possess two OH groups attached to the aromatic ring, as compared to BHT and BHA that possess only one OH group attached to the aromatic ring.

Thus, according to the electro-negativities, TBHQ, PG, and PA offer more sites for the formation of the complex between the free radical and antioxidant radical for the stabilization of the ester chain. However, they also found that the effect of antioxidant TBHQ was reversed when added to biodiesel blend where prooxidant activity was observed. On the contrary, both BHT and BHA were found to be very effective when used as additives in hydrocarbon fuel mixtures with biodiesel. Whilst, PG and PA were among the most effective antioxidants screened in both pure and blended biodiesel.

On the other hand, Tang et al., (2008) studied the effectiveness of eight antioxidants: alphatocopherol (α-T), BHA, BHT, TBHQ, 2, 5-di-tert-butyl-hydroquinone (DTBHQ), PG, PA and a commercial antioxidant, Ionol BF in short- and long-term storage.

In the study, PG,and PY were found to be the most effective antioxidants at 250 ppm and TBHQ at 500 ppm, while DTBHQ, BHT, and BHA increased the oxidative stability > 6 hours at 1000 ppm. Meanwhile, the naturally occurring antioxidant, α-T, was found not very effective antioxidant. The study indicated that the synthetic antioxidants were more effective in increasing the oxidative stability of biodiesel, and biodiesel from different feedstocks showed different oxidative stability improvement.

Schober &Mittelbach, (2004) showed that an effective antioxidant such as TBHQ can improve biodiesel oxidative stability by a factor of around 2 or more at only 1000 ppm dose. Conversely, they also stated that over-dosing can reduce oxidative stability, while extravagant doses can deleteriously affect other fuel properties. At a lower 250 ppm dose up to 1000 ppm, the result showed to have no significant negative effects on other biodiesel properties as defined by EN 14214 for several common antioxidants, with the exception of acid value (mg KOH/g) which increased slightly. It was thus recommended to use antioxidants at the lowest concentrations possible.

Evaporation of certain antioxidants during Rancimat testing might be an issue that could influence the results. Studies by Bondioli et al., (2003) has showed that TBHQ scores the most effective synthetic antioxidant where initial dose of 400 ppm in un-distilled rapeseed methyl ester (RME) achieved oxidative stability of 32.77 hours from 36 hours within12 months of storage, compared to un-distilled rapeseed methyl ester (RME) without any antioxidant which achieved just 6.83 hours from 9.20 hours.

This indicates that as antioxidants are consumed, their effectiveness reduces and oxidation stability decreases although, biodiesel with antioxidant still exhibiting higher oxidative stability.

Typically, natural antioxidants are consumed during frying of vegetable oil where unsaturated fatty acids starting to polymerized, causing lipid antioxidant to perform poorly leading to poor oxidation stability of biodiesel made from used-frying oils (Aladedunye, 2014). To overcome this problem, it was suggested to use added antioxidant. A report by Xin et al., (2008) investigated the kinetics of safflower biodiesel oxidation, stabilized by the addition of PG antioxidant with varied doses from 0 to 5000 ppm with different temperature from 100ºC to 120ºC using Rancimat test. They showed that oxidative stability of the various dosed samples increases with respect of antioxidant dose. However, at a particular antioxidant concentration, the oxidative stability was shown to fall dramatically as Rancimat test temperature increasing. Next, they also determine the composition and the tocopherol content, a natural antioxidant present in biodiesel by high-performance liquid chromatography (HPLC) method, which revealed highly unsaturated FAME content and relatively low tocopherol content. This explains the consequently very low (0.86 h) oxidative stability of safflower biodiesel.

Similarly, since free fatty acids (FFAs) could be attractive biodiesel alternatives without competing with edible-oil derived biodiesel, Chen &Luo, (2011) investigated the oxidation stability FFAs biodiesel, which were added with various antioxidant additives; one natural and 10 synthetic antioxidants at different concentrations between 100 and 1000 ppm.

They discovered the oxidative stability of un-dosed FFA derived biodiesel was initially very low with only 0.20 hours, while dosed FFA by the addition of antioxidants exhibit satisfactory increment of oxidative stability with some of which were more effective than others.

They conclude that the order of antioxidant effectiveness with respect to the oxidation stability of the FFA-based biodiesel was PY > Ethanox (4760E) > PG > Ethanox (4740) > PDA ~ BHA > BHT > MBMTBP ~ TBHQ > DTBHQ > α-Tocopherol. Overall, the study recommended commercial antoxidant; Ethanox 4760E (250 to 1000 ppm) has showed better solubility compared to PY and PG. However, PY and PG also found to slightly increase the acid value of biodiesel. The effectiveness of an antioxidant added to biodiesel was attributed to a variety of factors including chemical structures, the fatty acid profile of FAMEs, the amount of natural antioxidants present, and the storage conditions. Generally, oxidative stability increased with antioxidant concentration, and decreased with higher Rancimat test temperature.

Meanwhile, studies by Obadiah et al., (2012) examines the effect of antioxidant on changes of acid value and viscosity in Pongamia biodiesel using five different antioxidants which are BHT, BHA, PY, Gallic acid (GA), and TBHQ. In the studies, kinematic viscosity (KV) and acid value (AV) of samples were monitored accordingly.

It was observed that the KV and AV of Pongamia biodiesel rose dramatically and addition of antioxidants proved to retard oxidation process effectively at 3000 ppm concentration. Initial oxidative stability of the Pongamia biodiesel was 0.33 hours but performed best when PY is used which increased the oxidative stability to 34 hours at 3000 ppm. In contrast, GA performed worst as it did not increase the oxidative stability of biodiesel, with score above 0.88 hours at 3000 ppm. The other antioxidants performed similarly, increasing RIP to approximately 5 to 6 h at 3000 ppm. In conclusion, storage stability study results showed that for all samples used, KV and AV significantly increased with storage time. It was also noted that samples loaded with more antioxidant showed relatively smaller increases in KV and AV, hence higher concentration of antioxidant suppressed oxidation progression better. Results indicated that PY and TBHQ better suppressed oxidation progression, compared to the other antioxidants.

Kivevele et al., (2011) studied the effects of synthetic antioxidants; PY, PG and BHA on the oxidation stability of methyl ester biodiesel produced from non-edible *Croton Megalocarpus* oil (COME). The COME has high linoleic acid profile (72%), hence low oxidative stability is expected. Three antioxidants were tested; dosed at 200, 500 and 1000 ppm, respectively in separate tests. The results showed antioxidant efficacy was in the order PY >PG > BHA. The efficacy of PY and PG were more effective than BHA is due presence of three −OH groups in their aromatic rings while BHA has only one −OH group. They also tested the thermal stability of the COME. The thermal stability of could be regard as the temperature where the oxidation starting to take place. The un-dosed biodiesel recorded an onset temperature of 211ºC. When PY antioxidant is added, the onset temperature observed was 218.53ºC at 500 ppm and has decreases to 217.07ºC at 1000ppm. They concluded the effect of antioxidant on the onset temperature however was not clearly established.

2.5.9 Mass and viscosity of the sample

A downward trend in oxidation stability was observed with increasing sample mass. This has been shown in studies conducted by Knothe &Dunn, (2003) using methyl oleate and triolein with varying weight from 2 to 8 g. It was observed that as number of double bonds present in the sample increasing, it indirectly increasing the mass of sample that contains a greater number of allylic positions available to react with oxygen. Thus, they suggested a careful weighing of samples for accelerated oxidation tests is important. In the same study, the effect of sample viscosity on oxidative stability using Oil Stability Index (OSI) method was examined.

The data found that a more viscous TAG of oleic acid (triolein) was shown to exhibit greater oxidative stability than methyl oleate. It was explained that viscosity can affect a few factors such as: the mass-transfer of oxidation products to the air–oil interface, how fast bubbles traverse the sample, the size of bubbles, and the rate at which oxygen from the bubbles dissolves into the sample. Hence more viscous samples may yield higher oxidative stability. Generally, biodiesel viscosity is increased as a consequence of incomplete transesterification, where unreacted monodi- and tri-acylglycerides remain in the fuel as impurities.

2.5.10 Effects of temperature on oxidation stability

Dunn, (2005) examined the effects of temperature on the oil stability index (OSI) of soybean oil fatty acid methyl esters (SME) and used cooking oil fatty acid methyl esters (UCOME), which were compared to pure methyl oleate (MO). Increased temperature resulted in accelerated oxidation reaction and decreased OSI value. The iodine value (IV) of SME and MO were found to be consistent with OSI although, iodine value (IV) is only measure a total double bonds present in biodiesel. At constant temperature, SME yielded lower OSI than either UCOME or MO.

This was due to correlation of OSI with values of bis-allylic position equivalents (BAPE) and allylic position equivalents (APE) of double bonds in respective FAME. It was observed that SME had much higher C18:2 and C18:3 contents (53.5 and 6.5 wt %) than either UCOME (24.4 and 2.0 wt %) or MO, showing higher BAPE value in SME which is prone to oxidation; hence explaining the lower OSI of SME. UCOME had a higher BAPE than MO. However, the result also showed that MO had a much higher APE value than UCOME. In addition, bis-allylic positions are about 2.5 times more reactive than allylic positions therefore activity including OSI value of MO might be expected similar to that of UCOME (Pullen & Saeed, 2012).

However, Dunn did not observe this trend in this experiment as he found UCOME yielded significantly higher OSI than MO despite having comparable APE values. The likely explanation presumably to be the possible presence of antioxidants in the UCOME, compared to zero present of antioxidant in the pure MO.

Meanwhile, studies by Frankel, (2012) explained the kinetics of antioxidant. It was observed that effect of antioxidants is to increase the activation energy of oxidation. This means antioxidant lower the rates of oxidation by increasing the overall activation energy which is contradict to the metal effects. The Arrhenius plot of log induction period versus reciprocal of temperature exhibit the effectiveness of antioxidant increases as the temperature decreases. As the temperature increases, antioxidant efficacy decreases and at higher temperature, the effect of antioxidants could vanish and may even act as pro-oxidant. This infers that biodiesel FAME that is protected with added antioxidants, will inevitably lose that protection at sufficiently high temperature.

2.5.11 Processing of biodiesel and storage conditions

Another factor that contributes to decreasing in oxidation stability is exposure to pro-oxidizing conditions. These conditions are the effect that occurs during manufacturing, handling and storage phase. Pro-oxidizing parameters which have been investigated including the exposure to air, heat, light as well as influences by the nature of the storage container like metal, plastic, and glass. The oxidation process that is catalyzed by exposure to light is negligible during manufacturing and transport of biodiesel fuel provided that as the exposure of biodiesel to strong light sources is minimal (Pullen & Saeed, 2012). Prior studies has affirm these general observations, two identical biodiesel samples can be kept under different conditions; one exposed to more oxidizing conditions (higher temperature, oxygen, light); the other kept refrigerated in an air-sealed container in darkness (Shiotani & Goto, 2007). After prolonged storage, the refrigerated sample shall exhibit improved oxidative stability. Relatively increased levels of oxidation products would be present in the unrefrigerated sample, and its physical properties would be more substantially altered.

To see the effect of biodiesel processing, Bondioli et al., (2003) explored its impact on oxidative stability that is subjected to commercial storage conditions over one year. Eleven biodiesel samples derived from various feedstocks and manufacturing processes (distilled and non-distilled) were prepared, some with antioxidant additives, and each biodiesel was stored in a 200 liter drum.

They then recorded the fuel properties of each sample and were periodically analysed, whilst ambient storage temperatures were monitored. One drum was stored outdoors and shaken occasionally, to promote biodiesel/ air mixing which is also as imitation to the real situation. They found that only this sample recorded increased in acidity and polymer levels.

Meanwhile, the rest of samples showed no significant changes in these and several other fuel properties. However, all samples showed clear increases in PV levels peaked and dropped in certain cases, indicating hydroperoxide degradation with probable formation of secondary oxidation products. Meanwhile, kinematic viscosity (KV) of all samples recorded slight increase in value. Distilled samples showed comparatively lower KV probably because of the near complete removal of non-methyl ester materials such as glycerides. On the other hand, the outdoor agitated drum exhibited the most dramatic decline in oxidative stability, compared to other samples. Results of this study revealed that poor oxidation stability can be influenced by incorrect storage conditions, contact with air and agitation. For tocopherol content, the study finds little variation in most samples with notable exception for the outdoor stored sample which showed a dramatic drop.

Ambient storage temperatures were monitored and were found not to have a large influence on FAME quality at temperatures below 30ºC. However, proper long-term study of storage stability sensitivity to temperature variation would require control of sample temperature.

It was noted that storage temperature strongly affects the trends seen in oxidation parameters. Elevated temperatures have been shown to decrease oxidative stability, but increase the PV, total acid number (TAN), viscosity, and polymer levels. At ambient or colder temperatures, oxidative stability decreases more slowly but causes biodiesel to form wax crystals, thus clogging the line and fuel systems. Whilst PV, TAN, viscosity, and polymer levels either plateau or increase only modestly (Pullen & Saeed, 2012; Zuleta et al., 2012).

Biodiesel oxidative degradation under four different storage conditions were investigated by Leung et al., (2006). Experimental results suggested that high temperature with air exposure greatly increased biodiesel degradation rate, whilst high temperature or air exposure alone had little effect. Moreover, the biodiesel purity was found to likely impact biodiesel degradation directly. This implies more effective biodiesel storage can be achieved by filling the biodiesel in opaque storage containers and properly sealing them in order to minimize air contact with the fuel, also storing containers in cool and ideally dark environments. The effect of water contamination in biodiesel leading to hydrolytic degradation of esters to alcohol and free acids was found to be small compared with the degradation effects of air and temperature.

Bouaid et al., (2007) studied the storage stability of biodiesel derived from vegetable and used frying oils over a period of 30 months. Methyl esters of high oleic sunflower oil, high and low erucic content *Brassica carinata* (Ethiopian mustard) oil, and used frying oil were stored at room temperature in air-sealed, glass containers that were clear and coloured; varying the exposure of contents to light. Results showed that AV, PV, viscosity and insolubles increased while IV decreased with ageing during storage. Samples exposed to daylight tended to degrade at a higher rate, indicated by PV and AV values. It was observed that increased moisture content within the sunflower oil esters appeared to promote degradation.

2.6 Antioxidant

Biodiesel or known as fatty acid methyl ester (FAME) usually derived from lipids of various resources such as vegetables-edible oil, non- edible oil, animal fats, cooking oil and others.

This feedstock will undergo processes that commonly used involving transesterification where triglyceride molecules of the oil react with alcohol, mainly methanol with the aid of catalyst to form fatty acid methyl ester (FAME) and glycerol (byproduct). The fatty acid profile provide useful information such as chain length and level of unsaturation of FAME produced which corresponds to the type of oil used. The fatty acid profile also plays an important role in biodiesel's physicochemical properties, which in turn affecting the quality and performance of biodiesel.

Fuel instability refers to the susceptibility of fuel to degradation processes by alteration of fatty acid composition that forms undesirable species. Although biodiesel is thermodynamically stable, the exposure to the oxygen at ambient air stimulates its instability which referred to as oxidative instability. The term 'oxidation stability' (OS) is a general term, which differs from 'storage stability' and 'thermal stability', as the conditions for oxidative degradation may occur dependent on certain factors such as during extended storage period, transportation and biodiesel's end use (Rizwanul Fattah et al., 2014).

Other instabilities of the fuel could occur if the fuel is exposed to air and/or light, experience higher temperature and factors that causing sediment formation in the long-term storage.

Free radical species refers to any atoms, molecules or ions that contain unpaired electrons which are highly unstable and active towards reaction with other molecules. Basically, free radical derived from three main elements; oxygen, sulfur and nitrogen thus creating reactive oxygen species (ROS), reactive sulfur species (RSS) and reactive nitrogen species (RNS). Among ROS includes hydroperoxyl radical (HO_2•), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl), peroxynitrite (ONOO-) and superoxide anion (O_2-•).

Whilst, RNS derive from NO by reacting with O_2 to form ONOO and RSS are easily formed by the reaction of ROS with thiols (Lü et al., 2010).

Some biodiesel unavoidably has higher unsaturated fatty acids compositions such as plant-derived biodiesel that tends to have polyunsaturated fatty acid chain. Therefore, one of the ways in improving biodiesel stability is by blending of two feedstocks of different levels of inherent oxidative stability. Reported examples of this include blending high stability palm FAME with poorer stability jatropha FAME and soy FAME with palm FAME ratio should be equal or more than 50% of the soy FAME (Moser, 2008; Sarin et al., 2009). Not only that, another practical and effective method for improving biodiesel stability is by utilizing antioxidant additives.

2.6.1 Antioxidant: Mode of action

There are two types of autoxidation process take place mainly know as primary oxidation and secondary oxidation. In primary oxidation, three phases of radical reactions can be distinguished as: initiation, propagation and termination (Figure 2.5).

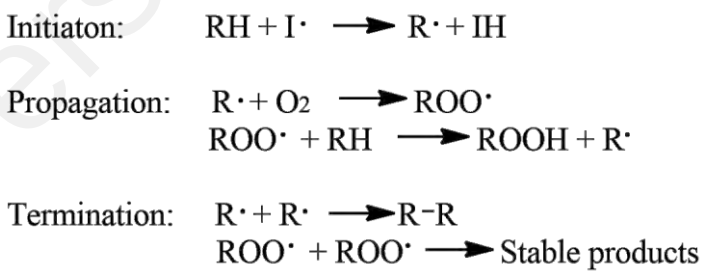

Figure 2.5: Stages of autoxidation process. Kivevele et al., (2011)

In the initiation phase, the hydrogen is eliminated from the carbon atom of unsaturated fatty acid to form a carbon free radical (R•) by free radicals (I•) and other reactive species like metals. When being exposed to air or oxygen, subsequent reaction occur between carbon free radical and diatomic oxygen to forms a peroxy free radical (ROO•).

Peroxy free radical then swiftly reacts to eliminate hydrogen from another carbon atom forming another carbon free radical and ROOH. The ROOH level remains low until certain period of time, often called as induction period (IP). Upon exposure to oxygen and temperature, this ROOH level will build up as a consequence of oxidation and can directly or indirectly change the properties of biodiesel. Lastly, in termination step the two free radicals are reacting and form a stable product. If there is insufficient level of oxygen in the surrounding, the formation of ROOH will either slowed down or eventually stops. A type of antioxidant known as free radical terminators are considered primary antioxidants, which react with high-energy lipid radicals and convert them into more stable products. Whilst secondary oxidation produce esters, aldehydes, acids, and ketones (McCormick et al., 2007) and contributes to the change in physical properties such as acid number, oxidation stability, and viscosity of the fuel (Bondioli et al., 2003).

In secondary antioxidants category, the antioxidant work by blocking the rate of chain initiation by decomposing the hydroperoxides. Researcher has postulated antioxidant reaction (1) and (2) as the mechanisms of action of free radical terminators. The free radical terminators contain hydrogen, which is rapidly donated to peroxyl radical that will interfere with lipid oxidation process (reaction (1) and (2)). The latter reactions (3) and (4) compete with the chain propagation reaction (Figure. 2.6).

$$ROO^{\cdot} + AH \rightarrow ROOH + A^{\cdot} \quad (1)$$

$$RO^{\cdot} + AH \rightarrow ROH + A^{\cdot} \quad (2)$$

$$ROO^{\cdot} + A^{\cdot} \rightarrow ROOA \quad (3)$$

$$RO^{\cdot} + A^{\cdot} \rightarrow ROA \quad (4)$$

Figure 2.6: Action of antioxidant during oxidation process. (Rizwanul Fattah et al., 2014)

These reactions are exothermic in nature. As the bond dissociation energy of AH and RH increases, the activation energy of these reaction increase. Therefore, as the bond strength of AH decreases, the efficiency of antioxidant increases.

2.6.2 Types of antioxidant

2.6.2.1 Natural antioxidants

Antioxidant system is divided into two major groups known as enzymatic antioxidants and non-enzymatic oxidants (Figure 2.7). In enzymatic antioxidants family, they are divided into primary and secondary enzymatic defenses.

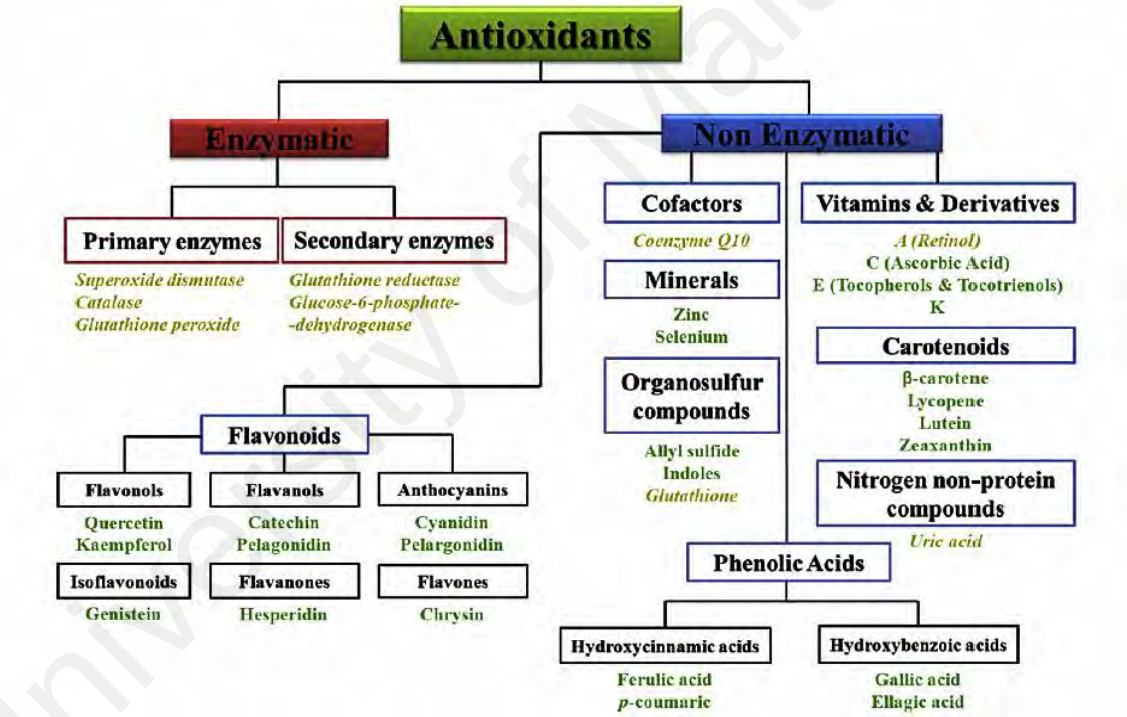

Figure 2.7: Categories of antioxidants.(Carocho & Ferreira, 2013)

Regarding to the primary defense, it is composed of three important enzymes that prevent the formation or to neutralize free radicals. Firstly, a glutathione peroxidase, which responsible for forming selenoles (Se-OH) by donating two electrons to reduce peroxides and also eliminates peroxides as potential substrate for the Fenton reaction.

There are two forms of this enzyme known as selenium-dependent and selenium-independent.

Secondly, a catalase enzyme that converts hydrogen peroxide into water and molecular oxygen. It has one of the biggest turnover rates known to man, by allowing just one molecule of catalase which is able to convert 6 billion molecules of hydrogen peroxide. Finally, a superoxide dismutase, one of the most effective intracellular enzymatic antioxidant that converts superoxide anions into hydrogen peroxide and dioxygen (Rahman, 2007).

The secondary enzymatic defense includes glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase works by reducing glutathione antioxidant from its oxidized state into its reduced form, thus recycling it to continue neutralizing more free radicals. Meanwhile, glucose-6-phosphate regenerates nicotinamide adenine dinucleotide phosphate (NADPH), a coenzyme that commonly used in anabolic reactions thus creating a reducing environment. These two enzymes however do not neutralize free radicals directly, but plays roles in cascade other endogenous antioxidants into action (Rahman, 2007).

Subsequently, there are quite a number of non-enzymatic endogenous antioxidants, namely vitamins A, enzyme cofactors (Q10), nitrogen compounds (uric acid), and peptides (glutathione). Vitamin A or retinol is a carotenoid that produced in the liver and could be a result from the breakdown of b-carotene. Vitamin A is known to have beneficial impact on the skin, eyes and internal organs.

Researchers had found what confers the antioxidant activity of vitamin A is the ability to combine with peroxyl radicals before they propagate peroxidation to lipids (Carocho & Ferreira, 2013).

Coenzyme Q10 also known as ubiquinone is present in all cells and membranes where it plays an important role in the respiratory chain and in other cellular metabolism. A specific reduced form of Coenzyme Q10 ($CoQH_2$) acts by preventing the formation of lipid peroxyl radicals (LOO•), although it has been reported that this coenzyme can neutralize these radicals even after their formation. Another important function of this enzyme is the ability to regenerate vitamin E; some authors describe this process to be more likely than regeneration of vitamin E through ascorbate (vitamin C) (Turunen et al., 2004).

Next, uric acid is the end product of purine nucleotide metabolism in humans and during evolution its concentrations have been rising. About 90% of uric acid is reabsorbed by the body after undergoing kidney filtration, indicating that it has important functions within the body. Uric acid also have biological significance as an antioxidant where it acts as a powerful scavenger of peroxyl radicals (RO_2•), hydroxyl radicals (•OH) and reactive oxygen species (Ridi & Tallima 2017). In fact, uric acid is known to play roles in tissue healing and prevent the overproduction of oxo-hem oxidants that result from the reaction of hemoglobin with peroxides (Carocho & Ferreira, 2013).

Glutathione is an endogenous tripeptide which protects the cells against free radicals either by donating a hydrogen atom or an electron. It plays important role in biological system including as an antioxidant defense, detoxification of electrophilic xenobiotics, modulation of redox regulated signal transduction, storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotide synthesis, regulation of immune responses, and regulation of leukotriene and prostaglandin metabolism.

Despite its remarkable efficiency, the endogenous antioxidant system does not suffice, and humans depend on various types of antioxidants that are present in the diet to maintain free radical concentrations at low levels (Pietta, 2000).

Vitamins C and E are generic names for ascorbic acid and tocopherols. Ascorbic acid consist of two compounds with antioxidant activity namely L-ascorbic acid and L-dehydroascorbic acid are probably the most potent hydrophilic antioxidant in protection against disease. Both types of ascorbic acids are readily absorbed through the gastrointestinal tract and can be interchanged enzymatically in vivo. Ascorbic acid is effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitrogen oxide (Barros et al., 2011).

Vitamin E is composed of eight isoforms, with four tocopherols (α-tocopherol, b-tocopherol, c-tocopherol and d-tocopherol) and four tocotrienols (α-tocotrienol, b-tocotrienol, c-tocotrienol and d-tocotrienol). However, only a-tocopherol is being the most potent isoform that fulfill human requirement in biological systems due to hepatic discrimination favoring a-tocopherol and preference to metabolizes non α-tocopherol forms (Traber & Atkinson, 2007). Structurally, the chroman head group accountable for the antioxidant activity to tocopherols, but the phytyl tail has no influence.

Vitamin E works by halting lipid peroxidation through donating its phenolic hydrogen to the peroxyl radicals to form tocopheroxyl radicals. Although they are being radicals, they are unreactive and unable to continue the oxidative chain reaction. Not only that, Vitamin E is the only major lipid-soluble, chain breaking antioxidant found in plasma, red cells and tissues, allowing it to protect the integrity of lipid structures, mainly membranes (Burton & Traber, 1990).

These two vitamins also display a synergistic behavior with the regeneration of vitamin E through vitamin C from the tocopheroxyl radical to an intermediate form, therefore reinstating its antioxidant potential (Carocho & Ferreira, 2013).

Vitamin K belongs to a fat-soluble compounds group. It is essential for posttranslational conversion of protein-bound glutamates into carboxyglutamates in various target proteins. The 1,4-naphthoquinone structure of these vitamins confers the antioxidant protective effect. There are two natural isoforms of this vitamin, known as K1 and K2 (Carocho & Ferreira, 2013).

Meanwhile, flavonoids are an antioxidant group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones.

All these sub-groups of compounds share the same diphenylpropane (C6C3C6) skeleton and commonly found in plants. Flavanones and flavones are usually found in the same fruits and are connected by specific enzymes, while flavonols and flavones do not share this and are rarely found together. In addition, anthocyanins are derived from flavanones which has undergone a series of transformations, also absent in flavanone-rich plants. The antioxidant properties of flavonoids are mainly by the phenolic hydroxyl groups attached to ring structures and they can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators. They also activate antioxidant enzymes, reduce a-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress, and increase levels of uric acid and enhance the antioxidant properties of low molecular antioxidants. However, flavonoids could also act as prooxidant depending on the cellular environment such as contribution of flavonoids as prooxidant in DNA breakage thus suggesting their mutagenicity activities (Procházková et al., 2011; Carocho & Ferreira, 2013).

Hydroxycinnamic and hydroxybenzoic acids are two members under phenolic acids group. They are ubiquitous to plant material and sometimes present as esters and glycosides. They have antioxidant activity as chelators and free radical scavengers with special impact over hydroxyl and peroxyl radicals, and superoxide anions. One of the most studied and promising compounds in the hydroxybenzoic group is gallic acid which is also the precursor of many tannins, while cinnamic acid is the precursor of all the hydroxycinnamic acids (Krimmel et al., 2010; Terpinc et al., 2011).

Another non-enzymatic antioxidant, carotenoids are natural pigments that are synthesized by plants and microorganisms but not by animals. They can be separated into two vast groups: (1) the carotenoid hydrocarbons known as the carotenes which contain specific end groups like lycopene and b-carotene that acts as a vitamin A precursor; and (2) the oxygenated carotenoids known as xanthophyls, like violaxanthin, zeaxanthin and lutein (Mezzomo & Ferreira, 2016). The mechanism of antioxidant property of carotenoids is due to singlet oxygen quenching which resulted in excited carotenoids that dissipate the newly acquired energy through a series of rotational and vibrational interactions with the solvent, thus returning to the unexcited state and allowing them to quench more radical species. This can occur while the carotenoids have conjugated double bonds within. The only free radicals that completely destroy these pigments are peroxyl radicals.

At high concentration, it was observed that formation of carotenoid cation radicals may results in prooxidant effects (Paiva & Russell, 1999).

Lastly, minerals that only found in trace quantities in animals and are made up of small proportion of dietary antioxidants, but play important roles in their metabolism. The most important minerals that have antioxidative activity are selenium and zinc.

Selenium can be found in both organic such as selenocysteine and selenomethionine and inorganic form such as selenite and selenite in the human body. It does not act directly on free radicals but act as a component in most antioxidant enzymes such as metalloenzymes, glutathione peroxidase, thioredoxin reductase and selenoproteins that would otherwise have no effect without it (Tinggi, 2008).

Zinc is a mineral that is essential for various pathways in metabolism. Just like selenium, it does not directly attack free radicals, instead it acts as co-factor for important enzymatic activities in the prevention of free radicals formation. Zinc has been shown to give protection of sulfhydryl against oxidation. It also acts as inhibitor of NADPH oxidases, a prooxidant which catalyze the production of the singlet oxygen radical from oxygen by using NADPH as an electron donor (Marreiro et al., 2017).

It is present in superoxide dismutase, an important antioxidant enzyme that converts the singlet oxygen radical into hydrogen peroxide. Zinc induces the production of metallothionein that is a scavenger of the hydroxyl radical. Finally zinc also competes with copper for binding to the cell wall, thus decreasing the production of hydroxyl radicals (Carocho & Ferreira, 2013).

2.6.2.2 Synthetic antioxidants

In food industry, a standard antioxidant activity measurement system to compare with natural antioxidants and to be incorporated into food was developed. Synthetic antioxidants were believed to be more efficient compared to natural antioxidant where these pure compounds are added to food so it can withstand various treatments and conditions as well as to prolong shelf life. Almost all processed foods have synthetic antioxidants incorporated, which are reported to be safe, although some studies indicate otherwise.

Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the most widely used chemical antioxidants as they possess almost 90% DPPH inhibition.

Further, tert-Butylhydroquinone (TBHQ) could stabilize and preserves the freshness, nutritive value, flavour and color of animal food products (Carocho & Ferreira, 2013). While, octyl gallate is considered safe to use as a food additive because it is hydrolysed into gallic acid and octanol after consumption. It also found in many plants and do not pose a threat to human health (Joung et al., 2004). Lastly, Nordihydroguaiaretic acid (NDGA), a potent scavenger of ROS has shown to exert apoptosis in tumor cell and cytoprotective effect in non-tumor cell. Despite being a food antioxidant, NDGA is also known to cause renal cystic disease in rodents (Hernández-Damián et al., 2014).

2.6.3 Plant extracts as biodiesel additives

Plant extracts that rich in phenolic compound can be a good antioxidant in oils. The use of natural antioxidants obtained from plants in biofuel sector has been a long-standing interest due to a health safety reason. There are many plant-based phenolic compounds that been mass-produced for commercial uses such as carotenoids, lycopenes, gallic acids, caffeic acids, zeaxanthin, sinapic acids, vanillin, resveratrol, ferulic acids, sesamol and eugenol (Varatharajan & Pushparani, 2018).

Recently, the effect of ginger extracts as biodiesel additive has been investigated (Devi et al., 2017). The oxidative stability of ginger extract in pongamia biodiesel showed a robust increased in induction period (IP) from 4.03 h to 23.99 h in pure pongamia biodiesel and pongamia biodiesel with ginger extract (2000 ppm) respectively. Meanwhile, another natural antioxidant sage and thyme extracts were investigated and showed to increase the rapeseed biodiesel induction period (IP) to 2 and 1.7 times respectively (Kreivaitis et al., 2013).

Studies involving rosemary, senna, chamomile, fennel and coriander extracts has been conducted by Cordeiro et al., (2013), found that natural antioxidant rosemary extracts in soybean oil gave IP value of 1.7 times higher than IP value of synthetic antioxidant BHT.

2.6.4 Antioxidative activity in Mimosine from medicinal perspective

Mimosine has phenolic, amine, and amino carboxylic groups which is responsible for its antioxidative role. It is commonly used as a cell synchronization agent due to its ability to inhibits mammalian DNA replication, arresting cells in the late G_1 or early S phase of the cell cycle (Kubota et al., 2014).

In addition, previous studies showed that mimosine possesses anti-proliferative effects and induces apoptosis in human pancreatic, lung cancer xenografts in vivo and on various cancer cell lines including human U-937, human HL60 and Chinese hamster fibroblasts (PJ. Mosca et al., 1992; Renò et al., 1999; Zalatnai A & J., 2003; M; Hallak et al., 2007).

According to Benjakul et al., (2013) mimosine could be potential antioxidant in pharmaceutical treatment and lipid oxidation. This has been demonstrated thoroughly by Pluchino et al., (2015) where mimosine could prevent cells from carcinogen-induced elevation of ROS and oxidative damage of chromosomal DNA, contributing to the suppression of breast cell carcinogenesis progression induced by cumulative carcinogen exposures.

Other studies also been reported that mimosine exhibit antioxidant and free radical scavenger activity both in cellular environment and seedlings (Sreekala et al., 1999; Lalitha & Kulothungan, 2007).

2.6.5 Mimosine as cell cycle blocking inhibitor

Investigation on mimosine as cell cycle blocking has been widely documented. There are two proposed modes of action of mimosine on the cell cycle known as (i) blocking entry into S phase (late G1 phase), and (ii) suppressing elongation of DNA replication (S phase).

In mammalian cells, mimosine has been suggested to arrest the cell cycle in the late G1 phase, whilst in insect cells, similar effect were observed but at lower mimosine concentration compared to mammalian cells (Szüts & Krude, 2004; Fallon, 2015). The effect of mimosine on late G1 phase was significant, hence it has been utilized as synchronization agent in an attempt to stop or to clarify the biochemical events that trigger the initiation of S phase (Ouhibi et al., 1994; Kulp & Vulliet, 1996).

For example, in a study comparing the effect of mimosine on different DNA synthesis in specialized systems such as cellular, mitochondrial, and viral SV40 chromosomes, they found that mimosine inhibited DNA replication plus prevent the elongation of nascent DNA chains in S phase. However, mimosine showed no inhibition of DNA replication in lysates of mammalian cells supplied with exogenous deoxyribonucleotide triphosphate (dNTP) precursors and had no effect on the initiation or elongation of DNA replication in extracts of intact Xenopus eggs or Xenopus eggs containing high levels of deoxyribonucleotide triphosphates (dNTPs). The author concluded that mimosine block the cell cycle at the elongation level of nascent DNA chains by causing the decline in intracellular pools of deoxyribonucleotide (Gilbert et al., 1995).

Kubota et al., (2014) investigate a novel mimosine mechanism in the mimosine-induced G1 checkpoint. Mimosine arrested the cell cycle at the G1–S boundary upon ataxia telangiectasia mutated (ATM) activation.

Cells treated with mimosine showed an increment of protein levels Hif-1α, a marker of hypoxic stress, indicating that mimosine induced hypoxia in cells which were mediated by reactive oxygen species (ROS). This in turn upregulate ATM phosphorylation, thus blocking cells from entering S phase in response to reactive oxygen species (ROS), eventually prevents induction of DNA damage from replication fork stalling.

Mimosine as a good iron chelator seem to exert effect in the event of cell cycle arrest. For example, mimosine successfully inhibit cell cycle progression in human breast cancer cells, and reduced DNA synthesis by more than 90% of the control within 4 hours of treatment at concentration of 400 μM. The inhibition was regard as the effect of iron chelation of mimosine since it acts in similar manner to desferrioxamine (DFO), a well-characterized iron chelator (Kulp & Vulliet, 1996).

In other works, HeLa cells that have been treated with mimsoine exhibit dramatically low level of p170, a putative subunits of eukaryotic initiation factors (eIFs) that catalyzed the initiation of mRNAs translation. Over expression of this p170 has been associated with several human cancers such as breast and lung cancers, where it is low in G1 phase and high in S phase of the cell cycle. Mimosine also has been found to inhibit p170 synthesis by the action of iron-chelating activity.

Owing to this, several mechanisms involving iron chelation has been proposed such as the inhibition of ribonucleotide reductase via chelation of iron in the R2 subunit, causing the deprivation of deoxynucleotide (dGTP and dATP) pools (Dai et al., 1994) , preventing synthesis of histone H1 kinase, a crucial regulator of cell cycle progression into replication phase (Feldman and Schönthal, 1994) or prevention of hypusine generation in translation factor eIF-5A, which is associated with cellular proliferation (Hanauske et al., 1995) and DNA damage (Szüts & Krude, 2004).

2.6.6 Mimosine as anti-cancer and apoptosis inducer

There are several studies on mimosine that has been shown to have antitumor effect and anticancer activities both in vitro and in vivo (Chang et al., 1999). A study by Chang et al., (2000) tested on three lung cancer cell lines which were incubated with mimosine for 48 h at different concentrations revealed that mimosine inhibited 40 to 60% growth of these cell lines at 400 μM of mimosine in a dose-dependent manner. This interference is mediated by multiple mechanisms. Moreover, they also found that mimosine was capable to exert significant anticancer activity in nude mice *in vivo*. This inhibition of cancer activity was resulted from downregulation of cyclin D1 expression and induction of cyclin-dependent kinase inhibitor p21WAF1 expression in mimosine-treated lung cancer cells, which is similar in *in vitro* experiments.

The antiproliferative activity of mimosine was also demonstrated in porcine granulose cells and in prostate carcinoma cells (Vacková et al., 2003). In prostate carcinoma cells experiment, researchers found the incubation of carcinoma cells in mimosine has elevated the numbers of G0/G1 cells which in turn inhibit PC-3 cell proliferation. They also found that mimosine could modulate the expression of B-cell translocation gene 2(Btg2) and N-myc downstream regulated gene 1 (Ndrg1) genes through different mechanisms. Mimosine showed to stabilizes HIF-1 protein and enhances expression of B-cell translocation gene 2(Btg2) and N-myc downstream regulated gene 1(Ndrg1) at transcriptional level, which attenuates cell proliferation (Chung et al., 2012).

An additional effect of mimosine is the induction of apoptosis that has been reported such as HL60 human promyelocytic leukemia cells (Renò et al., 1999), human U-937 leukemic cells (Hallak et al., 2008), xenotransplanted human pancreatic cancer cells (Zalatnai A & J., 2003) and rat ovarian cancer cells (Restivo et al., 2005).

The apoptotic event was elucidated to be dependent on cell type, mimosine concentration, and time of treatment. For example, mimosine induced apotosis was prominent after 18 hours incubation with 0.3 and 1mM mimosine for U-937 cells, and in HL60 tumor cells at 10 μM and reached a maximum at 200 to 400 μM. Meanwhile, the percentage of apoptotic C6 glioma cells was 12.6% after 24 hours at 250 μM (Renò et al., 1999; Qiao et al., 2011).

The new biochemical pathways underlying mimosine's apoptotic effect have been suggested. Mimosine-mediated apoptosis via mitochondrial activation took place when there was depolarization of the membrane potential or through ROS production associated with depletion in glutathione levels (Hallak et al., 2008).

This induction by mimosine was by promoting ROS formation in mitochondria and the release of cytochrome c from mitochondria, followed by caspase 3 activation causing the phosphorylation level of c- Jun-N-terminal protein kinase and p38, which are downstream effectors of ROS accumulation. Because of the apoptotic effect on rat C6 glioma cells, a new role of mimosine has emerged as a promising agent for the treatment of malignant gliomas (Qiao et al., 2011).

2.6.7 Mimosine as anti-inflammation and anti-fibrosis

Research showed that mimosine is capable of inhibiting molecular targets involved in inflammation. In mice muscle tissue infected with the *Trichinella spiralis* tumor, mimosine strongly inhibited the production of several cytokines such as necrosis factor-alpha (TNFα) and interleukin-6 (IL-6) that are produced by macrophage but not effective in inhibiting interleukin-4 (IL-4) that is produced by T-cells (Frydas et al., 2002). Similarly in the study of chronic inflammation of minced glanuloma, it was also shown to have an anti-inflammatory effect that exhibit inhibition on TNFα and IL-6 generation, hence the reduced in size of the minced granulomas (Frydas et al., 2003).

Chemokines plays role in cell trafficking regulatory, categorized into two main functions as homeostatic chemokines and inflammatory chemokines (Deshmane et al., 2009). Other report has suggested that mimosine was found to have an inhibitory effect on MCP-1 chemokines by completely abolish their transcription and translation, while partially inhibit MIP-2 transcription and translation in cell infected with *T.spiralis* (Conti et al., 2002).

It was believed that mimosine act on enzyme serine hydromethyltransferase (L-serine: tetrahydrofolate 5, 10-serine-hydroxymethyl-trasferase, (SHMT) to down regulate chemokine, which may lead to arrest of cell proliferation in S phase of the cell cycle, hence consequently can inhibit chemokine production.The antifibrotic effect of mimosine is because of the inhibition of propyl-4-hydroxylase, which catalyzes the hydroxylation of collagen pro-α chains for the deposition of cardiac collagen (Nguyen & Tawata, 2016).

2.6.8 Mimosine as anti-microbial and anti-viral

The efficacy of mimosine as an antimicrobial agent has been reported. A research from Zinkernagel et al., (2008) shows the mode of action of mimosine could result in the production of an effective antibacterial formulation. A bacterial infection on mice skins were studied and found that mimosine treated skin was able to contain the spreading human pathogen *Staphylococcus aureus* by activation of hypoxia-inducible factor -1α (HIF-1 alpha) thus, regulates the innate immune functions of phagocytes. The results exhibited at 100 mol/L concentration, HIF-1α agonist mimosine elevated the capacity of human phagocytes and whole blood to kill the leading pathogen *S. aureus* by 2-fold and reduced the lesion size in a murine model of *S. aureus* skin infection (Zinkernagel et al., 2008). Meanwhile, another study revealed that mimosine inhibitory effect is more efficient as antifungal compared to antibacterial (Anitha et al., 2005).

On the other hand, mimosine was shown to play role in inhibition of various viruses replication of such as the adenovirus, adeno-associated virus, parvovirus H-1, SV40, and herpes simplex virus type 1 (HSV1) (Dai et al., 1994). Neuraminidase (NA) is one of two enzymes present in the influenza virus which is involved in the release of virus progeny and the spread of infection to new cells by removing sialic acid from the virus and cellular glycoproteins (Moscona, 2005).

It has been a target for designing anti-influenza drugs, similar to zanamivir and oseltamivir that target viral neuraminidase (NA) and are currently being marketed for the treatment of influenza viruses A and B (Zhang et al., 2006).

With the molecular structure similar to tyrosine inhibitor, the effect of mimosine against the influenza virus was recently reported. Mimosine displayed a competitive neuraminidase inhibitor (IC50 = 9.8 μM), which is the same mode of inhibition against tyrosinase. It is a slow and time-dependent inhibitor of neuraminidase, which is more like the drug Tamiflu (Upadhyay et al., 2011).

2.7 Extraction of Plant Methodologies

2.7.1 Background

Various plant extraction methods are widely used especially in medicinal plant to extract bioactive compounds for research. The study of plant extracts usually starts with the pre-extraction and the extraction procedures, which is an important step in the processing of bioactive constituents from plant materials. Conventional methods such as maceration and Soxhlet extraction are commonly used at laboratory setting or at Small Manufacturing Enterprise (SME) level.

A more advance procedures have been developed in the processing of plants' extract such as microwave-assisted (MAE), ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE), in which these advances are aimed to increase yield at lower cost (Azwanida, 2015).

2.7.2 Pre-extraction

In most experimental design cases, dried sample is preferred considering the time needed for usage is longer. According to Sulaiman et al., (2011) the limit of time interval between harvest and experimental work should be maximum period of 3 hours in order to maintain freshness of samples because fresh samples are fragile and tend to deteriorate faster than dried samples.

Comparison between fresh and dried *Moringa oleifera* leaves showed no significant effect in total phenolics using decoction method but there was significantly higher total flavonoids content in dried sample (Vongsak et al., 2013).

Another factor to consider in pre-extraction is surface contact. Surface contact between samples and extraction solvents is most efficient when the particle size is reduced. One of the ways is by grinding which resulted in coarse smaller samples. While, powdered samples have a more homogenized and smaller particle leading to better surface contact with extraction solvents. This particular pre-preparation is important for efficient extraction to occur, the solvent must make contact with the target analytes and particle size smaller than 0.5 mm is ideal for efficient extraction (Azwanida, 2015).

2.7.3 Extraction methods

2.7.3.1 Maceration

Maceration involved soaking plant materials (coarse or powdered) in a container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation. This process is intended to soften and break the plant's cell wall to release the soluble phytochemicals. After 3 days, the mixture is pressed or strained by filtration.

In this conventional method, heat is transferred through convection and conduction and the choice of solvents will determine the type of compound extracted from the samples (Azwanida, 2015) .

This technique is known to be the easiest and simple homemade method which is generally used in small scale productions.

However, organic waste becomes an issue as a large volume of solvents is normally involved and proper management of the waste is needed. Influence in temperature changes and choice of solvents enhance the extraction process could reduce the volume needed for extraction in maceration provided the variable do not cause any effects. A research done by Ong et al., (2010) found that boiling *Centella asiatica* at 90°C showed to increase in phenolics content and antioxidant activities, but it has been jeopardized the pH of the extracts with increase in extraction time.

2.7.3.2 Soxhlet extraction

In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is placed in the thimble of the Soxhlet apparatus (Figure 2.8). Extraction solvents is heated at certain temperature in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drip back.

When the liquid content reaches the siphon arm, the liquid contents emptied into the bottom flask again and the process is repeated until desired period of time reached. Usually, solvent is choosing based on the polarity of the sample of interest. The polarity, from least polar to most polar, of a few common solvents is as follows:

Hexane < Chloroform < Ethylacetate < Acetone < Methanol < Water

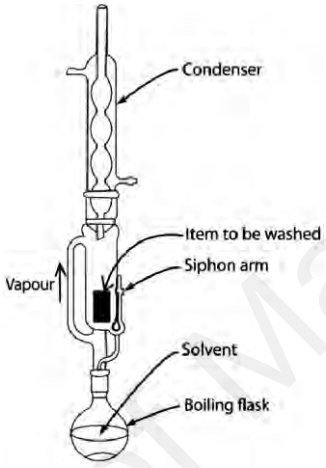

Figure 2.8: Schematic diagram of Soxhlet extraction apparatus.(Cumpson & Sano, 2012)

This method requires a smaller quantity of solvent compared to maceration (Azwanida, 2015). Besides, Soxhlet extraction (SXE) is capable of displacing solvent-sample by repeatedly bringing fresh solvent into contact with the solid matrix in equilibrium. It also could maintain a relatively high extraction temperature with heat from the distillation flask continuously, and no filtration requirement is needed after leaching/extraction. Economically, it has wide industrial applications, better reproducibility and efficiency, and less extract manipulation However, the Soxhlet extraction (SXE) comes with disadvantage such as exposure to hazardous and flammable liquid organic solvents, with potential toxic emissions during extraction is inevitable. Furthermore, solvents used in the extraction system need to be of high-purity that might add to cost and consume long time to extract compounds.

2.7.3.3 Microwave assisted extraction (MAE)

One of non-conventional method is microwave assisted extraction (MAE) where the process acceleration and high extraction yield are the result combination of two transport phenomena which are heat and mass gradients that is working in the same direction (Chemat et al., 2009).

On the other hand, in conventional extractions (maceration and SXE) the mass transfer occurs from inside to the outside of the sample, although the heat transfer actually occurs from the outside into the inside of sample. Microwaves have electromagnectic radiation that usually occurs at frequencies between 300 MHz to 300 GHz, with wavelengths between 1 cm and 1 m (Saxena & Chandra, 2011). These electromagnetic waves consist of both an electrical field and a magnetic field. These are described as two perpendicular fields. The first mechanism of microwaves was to heat up objects that can absorb a part of the electromagnetic energy and convert it into heat. Microwave radiation interacts with dipoles of polar and polarizable materials such as. solvents and sample causes heating near the surface of the materials and heat is transferred by conduction. Dipole rotation of the molecules induced by microwave electromagnetic disrupts hydrogen bonding; enhancing the migration of dissolved ions and promotes solvent penetration into the matrix (Kaufmann & Christen, 2002). In non-polar solvents, poor heating occurs as the energy is transferred by dielectric absorption. MAE can be considered as selective methods that favour polar molecules and solvents with high dielectric constant.

This technique can reduce extraction time and solvent volume as compared to conventional method (maceration and SXE) considerably. Besides, there is also an improved extraction yields of samples and reproducibility were observed in MAE method but with caution of using proper conditions to avoid thermal degradation

(Kaufmann & Christen, 2002). It is notable that MAE is comparable to other modern extraction techniques such as supercritical fluid extraction (SFE) due to its simplicity and low cost. However, compared to SFE, an additional filtration or centrifugation is necessary to remove the solid residue during MAE.

Moreover, it was observed that the advantages of MAE is limited particularly in phenolic acids (gallic acid and ellagic acid), quacertin, isoflavin and trans-resveratrol because these molecules were stable under microwave heating conditions up to 100°C for 20 minutes.

Additional cycles of MAE resulted in drastic decrease in the yield of phenolics and flavanones, mainly caused by the oxidation of compounds (Trusheva et al., 2007). However, tannins and anthocyanins may not be suitable for MAE as they were potentially subjected to degradation at high temperature. Furthermore, the efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar, or when they are volatile (Azwanida, 2015).

2.7.3.4 Ultrasound-assisted extraction (UAE) or sonication extraction

Ultrasound-assisted extraction (UAE) involves the use of ultrasound ranging from 20 kHz to 2000 kHz (Azwanida, 2015). The mechanic effect of cavitation from the ultrasound increases the surface contact between solvents and samples as well as permeability of cell walls. The effect of implosion of cavitation bubbles on the material's surface could results in micro-jetting which generates several effects such as surface peeling, erosion and particle breakdown. Several studies reported that there was micro fractures appeared on the surface of materials after being exposed to ultrasound wave, which in turn making the material to become porous and readily dissolve in the solvent, thus boosting yield with shorter time.

In general, physical and chemical properties of the materials subjected to ultrasound are altered and the plant cell wall was disrupted; facilitating the release of compounds and enhancing mass transport of the solvents into the plant cells (Dhanani et al., 2017). This technique is simple and relatively low cost technology that can be used in both small and larger scale of phytochemical extraction.

Ultrasound-assisted extraction poses an inexpensive, simple and efficient alternative technique. The benefits of ultrasound-assisted extraction (UAE) is mainly due to reduction in extraction time and solvent consumption.

Furthermore, the ultrasound-assisted extraction (UAE), like Soxhlet extraction (SXE), can be used with any types of solvent for extracting a wide variety of natural compounds. However, use of ultrasound energy more than 20 kHz may have an effect on the active phytochemicals through the formation of free radicals (Azwanida, 2015).

CHAPTER 3: METHODOLOGY

3.1 Materials and Method

Collection and Preparation of Sample

Samples of fresh leaves and seeds of *Leucaena leucocephala* were collected from surrounding area of Glami Lemi Biotechnology Resesarch Centre, Jelebu, Negeri Sembilan (Figure 3.1). Only matured leaves and seeds were chosen from the tree as mimosine content in both parts of the tree are low compared to young growing leaves and seeds (Figure 3.2, Figure 3.3).

Figure 3.1: *Leucaena leucocephala* tree at the site of collection.

Figure 3.2: Matured *Leucaena leucocephala* brown seeds.

Figure 3.3: Matured *Leucaena leucocephala* leaves.

The leaves and seeds were let dried at room temperature for two days to remove the moisture. After two days, the dried leaves and seeds were stored in a glass bottle in a dark cupboard for further use. Next, the dried leaves and seeds were ground daily prior to use using laboratory blender until fine sized powder was achieved (Figure 3.4, Figure 3.5). The grinding process was carried out to speeds up the rate of reaction by increasing the surface area of the samples and to help extracting out the desired compound effectively. The ground dried seeds were then divided into two groups which is the normal dried seeds (undefatted seeds) and another group where the oil being removed from the seeds (defatted seeds) (Figure 3.6). The defatted seeds are used as the subject of research in order to recycle the seed wastes after the oil extraction process for biodiesel production. Lastly, the dried leaves, undefatted seeds (UDS) and defatted seeds (DS) will further be treated by using two different extraction methods before quantifying its mimosine concentration.

Figure 3.4: Powdered dried *Leucaena leucocephala* brown seeds.

Figure 3.5: Powdered dried *Leucaena leucocephala* leaves.

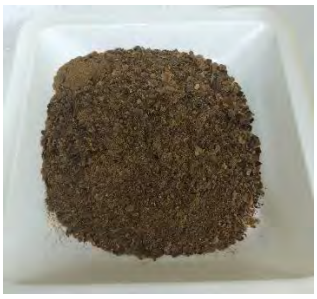

Figure 3.6: Powdered *Leucaena leucocephala* defatted seeds.

The proximate composition for *Leucaena leucocephala* leaves and seeds has been studied by other researchers and shown in table 3.1 as references before beginning the experiment.

Table 3.1: The proximate composition for *Leucaena leucocephala* leaves and seeds.

Treatment	*Leucaena leucocephala* leaves, g/100g	*Leucaena leucocephala* seeds, g/kg
Kcal	1166.84 455.29	2573.26 ± 4.24
Crude protein	11.82 ± 2.68	311.00 ± 3.61
Crude fiber	4.71 ± 3.29	132.00 ± 2.0
Ash	4.6 ± 1.98	45.00 ± 5.0
Nitrogen free extract	6.75 ± 5.59	404.00 ± 2.0
Moisture	66.10 ± 11.60	–
Phosphorus	10.02 ± 0.01	3.40 ± 0.001
Calcium	61.96 ± 1.77	3.70 ± 0.1
References	(Agbo et al., 2017)	(Ahmed & Abdelati, 2009)

Pure L-Mimosine from Koa hoale seeds (purity ≥ 98%) and 2,4,6-Tri(2-pyridyl)-s-triazine, TPTZ reagent (purity ≥ 98%) was purchased from Sigma-Aldrich Pty Ltd, St Louis, MO, USA. The hydrocloric acid of 37% concentration, n-Hexane 99% and Iron (III) chloride hexahydrate (FeCl3) was purchased from Friendemann Schmidt Chemicals, Parkwood,,WA, USA. The Trolox solution was purchased from Abcam, Cambridge, MA, USA. The equipment used were Laboratory blender 7011HS, Waring Commercial, USA, Top loading Balance TX3202L, Shimadzu, Kyoto Japan, IKA® C-MAG HS 10 IKAMAG hot plate, Syringe Filter with Sartorius 0.2µm for purification of activated charcoal and Jenway 7305 Series Spectrophotometer for determination of mimosine absorbance.

3.2 Extraction method for Mimosine content in leaves and seeds:

3.2.1 HCl digestion

HCl digestion method was conducted using method from Matsumoto &Sherman, (1951) with slight modification. A total of 40 g of dried *Leucaena leucocephala* leaves was weighed using TX3202L Top loading Balance, Shimadzu, Kyoto Japan and later was transferred into 800 mL beaker and a total volume of 400 mL of 0.1N HCl was added to the mixture for digestion process to take place.

Then, the mixture was transferred into a 250 mL Schott Duran® storage bottle and was allowed to stand still until most of the solids get settled to the bottom.

3.2.2 Soxhlet Extraction

Soxhlet extraction (SXE) method was conducted using method from Ilham et al., (2015). A total of 40 g of dried *Leucaena leucocephala* leaves was weighed using TX3202L Top loading Balance, Shimadzu, Kyoto Japan and placed in a Cellulose Thimble 43mmx123mm, Favorit, Malaysia. A total volume of 400 mL distilled water, which act as the solvent was placed in a round bottom flask and the soxhlet extractor apparatus was setup. The extraction cycles were ensured to be constant before it was let to run for 8 hours at 70 to 100 °C. The extract along with the solid was transferred into a 250 mL Schott Duran® storage bottle and was allowed to stand still until most of the solids get settled to the bottom. The experimental procedure was repeated using the dried *Leucaena leucocephala* seeds.

3.3 Extraction method for Mimosine content in seeds after removal of oil (defatted seeds)

Firstly, the dried seeds of *Leucaena leucocephala* was undergo Soxhlet extraction (SXE) for oil removal using hexane. About 40 g of dried *Leucaena leucocephala* seeds was weighed using TX3202L Top loading Balance, Shimadzu, Kyoto Japan and placed in a Cellulose Thimble 43mmx123mm, Favorit, Malaysia. A total volume of 400 mL hexane acting as a solvent was placed in a round bottom flask and the Soxhlet extractor apparatus was setup. The extraction cycles were ensured to be constant before it was let to run for 8 hours at 70°C. Then, the extracted solution is subjected to evaporate via Rotatory evaporator until the oil is obtained.

Meanwhile, the leftover seeds meal will then further utilize to extract mimosine using the same Soxhlet extraction (SXE) method with distilled water as a solvent and the obtained extracts will be used to determine the mimosine content in the seed meal. The extract along with the solid was transferred into 250 mL Schott Duran® storage bottle and was allowed to stand still until most of the solids get settled to the bottom.

3.4 Treatment of the seeds

3.4.1 Soxhlet extraction cycle

Once the Soxhlet extraction of the undefatted seeds (UDS) and defatted seeds (DS) were complete, the same powdered seeds were subject to another Soxhlet extraction (second cycle). This is to determine the effect of second cycle of Soxhlet extraction on the mimosine concentration in both type of seeds.

3.4.2 Heat

A total of 40 g of dried *Leucaena leucocephala* undefatted seeds (UDS) and defatted seeds (DS) was weighed using TX3202L Top loading Balance, Shimadzu, Kyoto Japan and later was transferred into 800 mL beaker. A total volume of 400 mL of 0.1N HCl was added and the mixture was boiled at the temperature of 85 °C on an IKA® C-MAG HS 10 IKAMAG hot plate for an hour. The mixture was transferred into a 250 mL Schott Duran® storage bottle and was allowed to stand still until most of the solids get settled to the bottom. This is to determine the effect of heat on the mimosine concentration in both type of seeds.

3.5 Preparation of Mimosine calibration curve

Exactly 0.025 g of L-Mimosine powder (Sigma-Aldrich, St Louis, MO, USA) from Koa hoale seeds was dissolved in a 50 mL conical flask with 25 mL 0.1N HCl. The 0.1N HCl stock solution was prepared beforehand by diluting 9.9 ml of 37% concentrated HCl into 990.1 ml of distilled water to make 1 litre of solution. Aliquot of

sample were transferred into 6 different conical flask and each were added with 10 mL of 0.1N HCl and 4 mL of 0.5% $FeCl_3$ in 0.1N HCl. The final volumes of 6 different aliquots were raised until 100 mL with distilled water. The absorbance of each diluted aliquots were read by using Jenway 7305 Series Spectrophotometer and a graph of concentration versus absorbance was plotted. The concentration of mimosine from the reaction mixture was expressed as μg/mL. For sample determination, 15 samples from each treatment; undefatted seeds (UDS) and defatted seeds (DS) were used and each absorbance was recorded.

3.6 Antioxidant test with FRAP assay

There are several techniques used to test antioxidative activity such as 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP). Ferric reducing antioxidant power (FRAP) assay is used due to the ability of phenolics to reduce yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants (Benzie et al. 1999). Its advantages over other methods such as simple, rapid performance, showed high reproducibility and highest correlation between ascorbic acid and total phenolics making it suitable for testing plant extracts (Moharram & Youssef, 2014). The total phenolic content (TPC) of *Leucaena leucocephala* seeds extract was investigate and evaluated by other authors, using different extraction solvents and during germination phase (Benjakul et al., 2014; Suryanti et al., 2016). The TPC varies widely proportionate to its antioxidant activity, thus suffice to show the presence of mimosine can indicate such antioxidant activity.

FRAP assay method was derived from Benjakul et al., (2014) with a slight modification. A stock solution is prepared which consists of 300mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40mM HCl and 20mM $FeCl_3.6H_2O$. The working solution or FRAP solution consists of 25 ml acetate buffer, 2.5 ml TPTZ solution and 2.5 ml $FeCl_3.6H_2O$ and the mixture is let to sit at 37ºC in water bath for 30 min. About 150µl of sample is added to 2850µl of FRAP solution and let to sit in a dark room for 30min. The coloured product was then measured with spectrophotometer at 593 nm. Blank solution consists of all solution except for $FeCl_3$ that has been replaced with water. Trolox will be used as a standard with range of 50 to 600 µmol concentrations and was expressed as µmol Trolox equivalents (TE)/100 g extract.

Calculated FRAP value of samples are as follows, Habu &Ibeh, (2015);

$$\text{FRAP value Sample (µM)} = \frac{\text{Change in absorbance of sample from 0 to 30 minute}}{\text{Change in absorbance of standard from 0 to 30 minute}} \times \text{FRAP value Standard (1000µM)}$$

3.7 Testing oxidative stability of biodiesel with Rancimat

Oxidation stability of biodiesel samples with varying dosage of antioxidants was studied using Rancimat instrument Metrohm 873 (Metrohm, USA). Biodiesel samples which are made from rapeseed fatty acid methyl ester (FAME) produced from Faculty of Engineering, University of Malaya, Malaysia will be added with mimosine of different concentrations (ppm) to acts as an antioxidant additive, then samples were kept at a constant temperature of 110 °C and aired at a flow rate of 10 l/h bubbles through each sample. Each measuring vessel contains 60 ml of distilled water. Results will be displayed in graph.

The effectiveness of all antioxidants was expressed as a stabilization factor,

$$F = IP_x/IP_0$$

where IP_X is the induction period in the presence of the antioxidant, and IP_0 is the induction period in the absence of the additive.

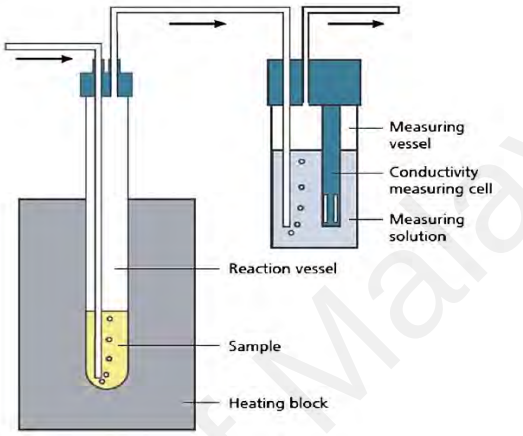

Figure 3.7: Diagram of Rancimat method. (Kivevele et al., 2011)

3.8 Statistical Analysis

All the 15 experimental runs for each extraction results were analysed by using Microsoft excel software due to minimal number of samples for analysis. The results are shown as the mean ± SD and all experiments were performed in triplicates. Statistical Package for the Social Sciences (SPSS) 16.0 software was used for further statistical analysis which include paired t-test to compare the amount of mimosine after a series of Soxhlet extraction (SXE) cycle and two-way ANOVA was used to compare the mean values in all four variables. This was to determine their significance. The level of statistical significance was set at 5% Post-hoc analysis and 95% confidence level.

Mimosine Quantification


A


B

Sample collection: A. Matured leaves. **B.** Matured seeds of *Leucaena leucocephala.*
Matured seeds conditions:
1) Normal
2) Oil removed prior to experiment (defatted)


A


B


C

Drying process: Room temperature for 2 days. Followed by grinding process prior to experiment. **A.** Powdered dried seeds. **B.** Powdered dried leaves. **C.** Powdered defatted seeds


A


B

Extraction process: **A.** Soxhlet extraction (SXE). **B.** HCl digestion.

TREATMENTS OF SEEDS

Second cycle of SXE

Heat

Quantification of Mimosine: Spectrophotometer 535nm

Mimosine Antioxidative Activity

Mimosine from Koa Hoale, Sigma Aldrich

Oxidative stability test: Rancimat using rapeseed biodiesel.

FRAP assay: Spectrophotometer 593nm

Figure 3.8: Experimental methodology flow chart

CHAPTER 4: RESULTS AND DISCUSSION

4.1 The presence of mimosine in *Leucaena leucocephala* leaves and seeds using Soxhlet extraction and HCl digestion.

This part of study will discuss the results regarding the presence of mimosine in leaves and seeds of *Leucaena leucocephala* using two extraction methods; which are (1) HCl digestion and (2) Soxhlet extraction (SXE) (Table 4.1). The total content of mimosine was determined using L-mimosine standard curve ($y = 0.008x - 0.015$), $R^2 = 0.991$.

Table 4.1: Average mimosine content in dried leaves and matured seeds of *Leucaena leucocephala.*

Average mimosine content in dried leaves (µg mimosine/100 g sample)		**Average mimosine content in matured seeds (µg mimosine/100 g sample)**	
Soxhlet	**HCl**	**Soxhlet**	**HCl**
10.261 ± 0.199	7.200 ± 0.095	21.633 ± 0.255	9.764 ± 0.155

Table 4.1 shows different extraction methods for mimosine removal using Soxhlet extraction (SXE) and HCl digestion. The table has demonstrated that the average mimosine concentration in matured seeds is higher than average mimosine concentration in the dried leaves. The highest mimosine removal was observed in Soxhlet extraction (SXE) of matured seeds (21.633 µg) and dried leaves (10.261 µg) followed by HCl digestion method with 9.764 µg in seeds and 7.2 µg per 100g dry mass in leaves respectively. For 40g of sample used, the mimosine value successfully extracted from dried leaves was 4.104 µg and 2.88 µg for SXE and HCl digestion respectively, while 8.653 µg and 3.906 µg for SXE and HCl digestion in matured seeds respectively.

This amount of mimosine in dried leaves and matured seeds were aligned with the result obtained from previous studies which confirmed that seed has higher mimosine content compared to the leaves (Kamada et al., 1997; Xuan et al., 2006; Ismail. et al., 2017).

Mimosine content varies within different parts of *Leucaena leucocephala* with young leaves contains (2.66%), flower (1.17%), xylem (0.11%), bark (0.68%) and matured seeds (2.38%) of mimosine (Xuan et al., 2006). From the table 4.1, it was observed that mimosine content is slightly higher than the result found by (Xuan et al., 2006) with mimosine value from seeds (1.79 mg/L) and mature leaves (11.05mg/L) was probably due to different extraction method employed. According to Adeneye, (1991) the highest mimosine concentration was found in yellow cotyledon possibly due to moisture stress faced during growing phase and seasonal changes. A similar report by Masafu &Linington, (2009) also revealed that mimosine content in *Leucaena leucocephala* during summer, with drying method prior was higher compared to winter season, concluding that mimosine concentration was significantly affected by dehydration, followed by the time of defoliation and season. This phenomenon can be explained by analyzing the rainfall distribution at the time when the seeds and leaves of *Leucaena leucocephala* were collected. The average rainfall recorded in June 2016 (142mm) showed a 37% decline compared to May 2016 (224mm) which had exerted some stress to the tree, thus inadvertently releasing more mimosine in order to survive. Irrigation and Drainage Department, (2016).

4.2 Mimosine content in seed meals of *Leucaena leucocephala* after oil removal

This part also will discuss the effect of different cycles of Soxhlet (SXE) in Table 4.2 and the effect of heat on the mimosine content in *Leucaena leucocephala*'s undefatted and defatted seeds (Table 4.3).

Table 4.2: Average mimosine content in seeds with different treatments.

Soxhlet Cycle	Average mimosine content in Undefatted seeds (µg mimosine/100 g sample)	Average mimosine content in Defatted seeds (µg mimosine/100 g sample)
First	10.203 ± 0.114	8.150 ± 0.059
Second	5.331 ± 0.094	6.719 ± 0.126

Table 4.2 shows the presence of mimosine in untreated and defatted seeds after removal of oil using Soxhlet (SXE) with water. In order to know whether mimosine could be fully removed from the *Leucaena leucocephala* seeds after the oil extraction, the second Soxhlet (SXE) was employed using the same seeds. From the result shown, defatted seeds score lower mimosine value with 8.150 µg compared to the undefatted seeds which is 10.203 µg per 100g dry mass. However, the result during the second cycle of Soxhlet extraction (SXE) was slightly reverse for mimosine content where the value was lowest in undefatted seeds compared to the defatted seeds.

Result showed the treatments to extract mimosine is twice higher in the first cycle of Soxhlet extraction (SXE) (mean= 10.20, SD= 0.21) than second cycle of Soxhlet extraction (SXE) (mean= 5.33, SD= 0.13). Data from paired t-test has found this difference to be significant, $t(14) = 75.11$, $p < 0.05$. This suggests that the interaction between first cycle and second cycle of the treatment has significant correlation on the content of mimosine being extracted from the seeds.

Initially high mimosine content after first Soxhlet extraction (SXE) in undefatted seeds (UDS) compared to defatted seeds (DS) may indicate that there was some typically occurrence of mimosinase degradation in the undefatted seeds (UDS) due to exposure of heat. Also, it is noticeable that defatted seeds (DS) have lower mimosine from the beginning of the first cycle which explains the effect of heat on the mimosine and almost similar amount of mimosine to undefatted seeds (UDS) in the second cycle of Soxhlet extraction (SXE).

After the second cycle of Soxhlet extraction (SXE), there was still a considerable amount of mimosine present, which is unable to be fully extracted out from the seed meals. This is consistent with the finding of Wee &Wang, (1987) that there was about 30% or more of the mimosine remain unchanged after soaking at 60°C for 20 minutes. This outcome was presumably attributed to mimosine forming complexes with other compound thus making it inaccessible for degradation.

4.3 Effect of Heat on Mimosine content in *Leucaena leucocephala* seeds.

The histogram illustrated the average mimosine concentration in undefatted seeds (UDS) and defatted seeds (DS) following heating and in normal condition (Figure 4.1).

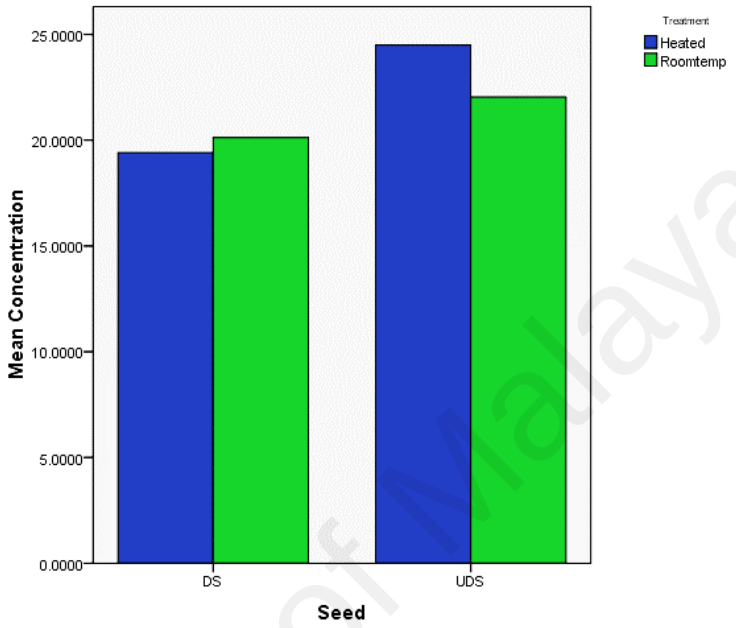

Figure 4.1: Mean concentration of mimosine in seeds with different treatment (heat).

Generally, undefatted seeds (UDS) have higher mimosine concentration compared to defatted seeds (DS). It was observed that defatted seeds (DS) with heat has the lowest mimosine content with 19.775µg per 100g dry mass while undefatted seeds (UDS) with heat has the highest mimosine content at 24.5µg per 100g dry mass (Table 4.3).

Table 4.3: Average mimosine content of seeds with different traement (heat).

Treatment	Average mimosine content in Undefatted seeds (µg mimosine/100 g sample)	Average mimosine content in Defatted seeds (µg mimosine/100 g sample)
Room temperature	21.633 ± 0.255	20.061 ± 0.242
Heat	24.269 ± 0.254	19.775 ± 0.136

Based on the result, there was significant correlation of treating the seeds by boiling method on the concentration of mimosine extracted $F(1,56) = 163.507$, $p < 0.005$ with R^2 value of 0.964. The estimated marginal means (Appendix) indicate that there is interaction between the types of seeds with regards of boiling. This result is supported by the findings of Adekojo et al., (2014) who found values of mimosine was significantly ($p<0.05$) higher in air-dried *Leucaena leucocephala* leaf and lowest in 60ºC hot water processed *Leucaena leucocephala* leaf. The effect of different processing methods towards reduction or elimination of anti-nutritional elements in plants also in line with the findings of Fayemi et al., (2011) that tannin and mimsoine were lowest in hot water treatment compared to sun dried *Leucaena leucocephala*.

However, the slight decrease in mimosine content observed in defatted seeds (DS) may be due to the overexposure to high temperature during initial Soxhlet extraction (SXE) with hexane in order to remove oil from the seeds, causing a considerable amount of mimosine might be lost before water extraction being used. This is supported by findings from Lowry et al., (1983) that revealed mimosine breakdown started at 55ºC and the rate will be increased as the temperature reached 70ºC. However, there was no degradation or inactivation of mimosinase after 70ºC due to prolong heating process thus, decreasing the efficiency to convert mimosine into DHP. Furthermore, at higher temperature, mimosine remains largely unaffected.

4.4 Biodiesel stability test

Part two will explain the findings of antioxidative activity on mimosine. This will include the result of using Rancimat test for biodiesel's additive efficiency. Figure 4.2 shows Rancimat oxidative stability test of rapeseed fatty acids methyl ester (FAME) with commercial mimosine from Koa Hoale. The commercial mimosine concentration was started at 2000 ppm, 4000 ppm, 6000 ppm, 8000 ppm and 10,000 ppm in 7.5 mg of rapeseed FAME for each concentration.

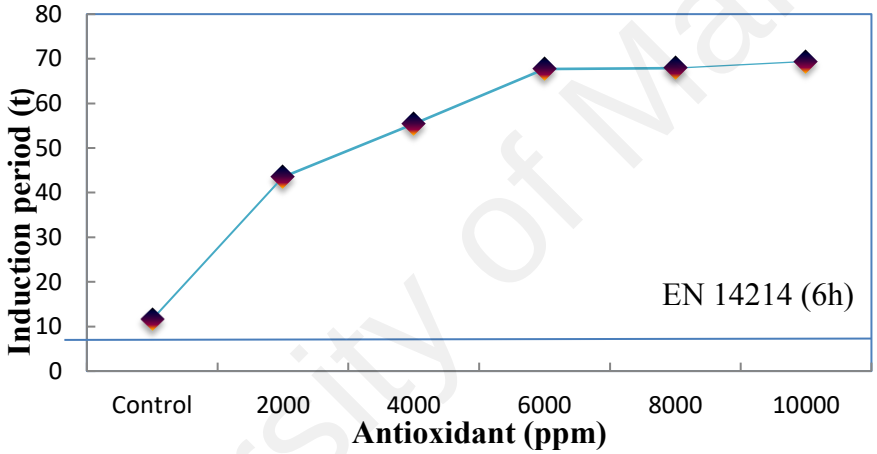

Figure 4.2: Graph of oxidative stability of rapeseed FAME with mimosine.

Table 4.4 shows induction period and stabilization factor of rapeseed FAME with mimosine. The highest stabilization efficacy of mimosine was obtained at 10,000 ppm with 5.98 and the lowest value at 2000 ppm with 3.75. It was observed that the efficacy of mimosine oxidative activity increased in respect of concentration (ppm) until it reaches maximum induction period at 69.4 hours.

Table 4.4: Induction period (IP) with stabilization factors (F) of rapessed FAME with mimosine.

Antioxidant (ppm)	IP, h	F
0 (Control)	11.6	
2000	43.5	3.75
4000	55.4	4.775862
6000	67.7	5.836207
8000	67.9	5.853448
10000	69.4	5.982759

Maximum IP at 69.4 hours when mimosine is added surpasses the minimum requirement of EN14214 of 6 hours shows that mimosine possess an effective stabilization of biodiesel characteristic where it is able to delay biodiesel oxidation process for a long duration in period of storage.

This may be ascribed to the structure of mimosine that contain double bonds at carbonyl, hydroxyl and amines groups which responsible as hydrogen or electron donor to free radicals. Functional groups at different position such that ortho-, meta-, and para-positions of the benzene ring gave different effect of antioxidative activity of phenolic antioxidants including BHA, BHT, DTBHQ, PG, PY, and TBHQ. The active hydroxyl group in the antioxidants can provide protons (H) that combine with oxidized free radicals at termination reaction, thus decelerating the oxidation rate and break the cycle of generating new radicals (Pereira et al., 2009).

Another successful additive characteristic exert by mimosine is it has high thermal stability (227 to 228 °C), thus making it a good candidate for natural antioxidant. The increment in the antioxidant concentration resulted in the increase of available hydrogen atoms that could be having reaction with the ROO′ during the oxidation reaction.

Hence, this reaction could prevent the formation of acids, esters, aldehydes, and ketones plus extending the oxidation stability of the fuel (Zuleta et al., 2012).

4.5 FRAP assay of Mimosine

The FRAP test was used to measure the total antioxidant capacity which is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous colored form in the presence of antioxidants, and is regarded as accurate indicators of total antioxidant power, since total reducing power is defined as the sum of the reducing powers of the individual compounds contained in a particular sample (Tezcan et al., 2011). The efficacy of mimosine extract to reduce ferric (III) to ferrous (II) was evaluated and compared with Trolox standard curve ($y = 0.001x - 0.017$), $R^2 = 0.972$, which is considered as one of the strongest reducing agents was shown in the graph (Figure 4.3).

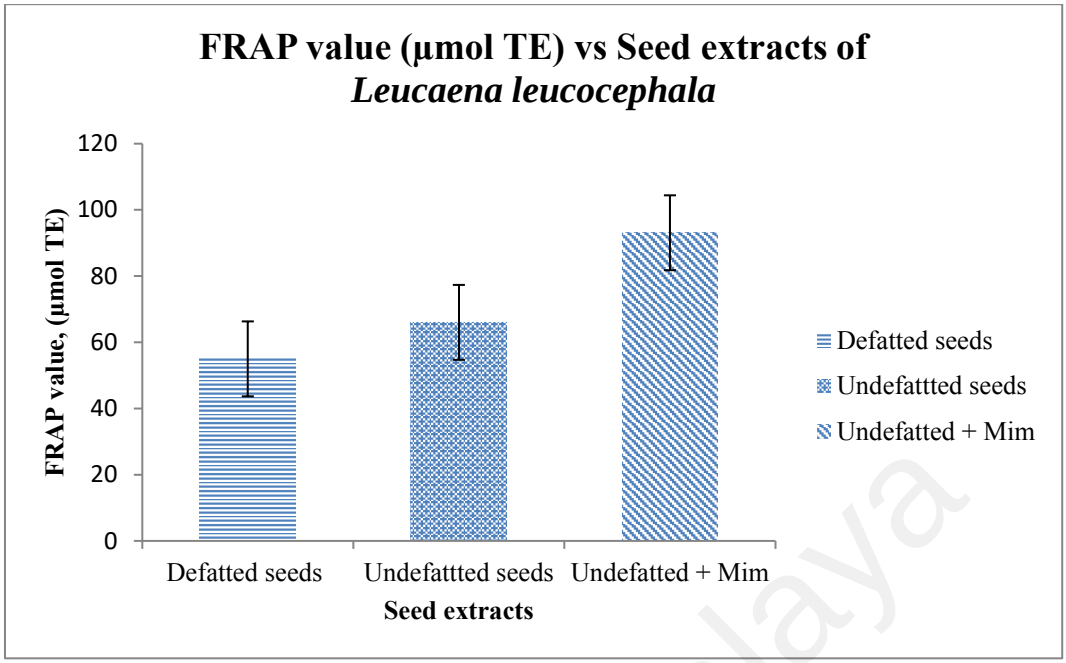

Figure 4.3: FRAP assay of mimosine content in seeds exposed to different treatments.

From figure 4.3, undefatted seeds (UDS) has higher FRAP value of 66.02 µmol compared to defatted seeds (DS) extracts with 55 µmol Trolox equivalent (TE). Whilst positive control mimosine showed FRAP value of 93.09 µmol Trolox equivalent (TE). This indicates that all extracts are able to reduce ferric (III) to ferrous (II) by the reaction of electron donating antioxidant. The lower FRAP value in defatted seeds (DS) exhibit that high temperature has negatively affect the antioxidative ability of mimosine. This finding has been supported by the study of Benjakul et al., (2014) where they have found that *Leucaena leucocephala* extracts treated with hot water has lower antioxidative activity compared to extracts with water and all ethanolic solvent (1.2 to 2.0% concentration), signifying that the heat from hot water might cause the destruction of compounds with FRAP to some degree. Thus, extraction solvent and extraction temperature had the profound impact on FRAP value of resulting extracts. The same author also evaluates the effect of FRAP incubation timing, reveals that extracts with 40 minutes showed higher antioxidative activity compared to extracts incubated for 10 minutes and 20 minutes.

This means the incubation time should be sufficiently long enough to allow complete reaction for all the reducing compounds present in the extracts, depicting true antioxidant capacities (Burawat et al., 2016). Meanwhile, the antioxidative effect of mimosine and its possible benefit in pharmaceutical have been discovered to originate from the -OH and -O of the pyridine rings, whereas its herbicidal and antifungal properties were relies on the α-amino and carboxyl groups (Xuan et al., 2016).

The possible structure of the Fe (III)-Mimosine chelation complex has also been proposed by Soedarjo &Borthakur, (1998) at low and high pH. The α-ketohydroxy metal binding site present in aromatic and heteroaromatic rings such in mimosine was identified as an original, specific and has strong affinity of chelating site for iron, hence forming stable iron complexes that is important in biological properties (Kontoghiorghe et al., 2015).

CHAPTER 5: CONCLUSION AND RECOMMENDATION

Research Conclusion

Removal of mimosine from *Leucaena leucocephala* leaves and seeds has long been researched especially in agricultural sector. Several methods have been put forward with their effectiveness in extracting mimosine such as drying, soaking, and boiling. More recent advancement has been explored at molecular level such as developing transgenic Leucaena with low mimosine content. Here, the present study was aiming to compare the presence of mimosine and their effectiveness in *Leucaena leucocephala* leaves and seeds using Soxhlet extraction and HCl digestion.

In conventional feedstock usage, raw *Leucaena leucocephala* leaves and seeds were being fed to the animal without removing the oil. A potential use of *Leucaena leucocephala* as biodiesel candidate has been explored where the oil-containing seeds showed a good biodiesel property that satisfies ASTM standard, thus provides us a new approach to fully utilize this tree. The second objective is therefore aimed to investigate the mimosine content in the seed meal after extraction of oil. The effect of heat treatment also was carried out to see whether heat has negative effect the mimosine concentration in the defatted seeds.

Since mimosine bears the antioxidative characteristic which profusely being studied in medicinal and pharmaceutical areas, its uses in biodiesel is still unknown. For this reason, the third objective in this study is to investigate the effect of mimosine on biodiesel oxidation stability. The result indicates that the use of mimosine as biodiesel additive exhibit a promising tool in enhancing biodiesel shelf-life.

In brief, as depicted in Table 5.1, the presence of mimosine in local *Leucaena leucocephala* leaves and seeds is slightly high, which implying the collection should be made during high rainfall season to minimize the spontaneously high mimosine content in *Leucaena leucocephala* tree. Furthermore, the use of correct extraction method such as Soxhlet extrcation (SXE) could help to extract toxic mimosine effectively and safely for food consumption as it showed similar result with the previous studies. Next, the mimosine content in seed meals derived from seeds undergone oil extraction (defatted seeds) is significantly affected by the Soxhlet extraction (SXE) cycle and heat exposed. Finally, mimosine shows a good trend as antioxidant which potentially could be used as a new biodiesel additive. Hence, the study concluded that the full utilization of *Leucaena leucocephala* seeds, leaves and undefatted seeds as animal feedstocks and bioenergy additives is possible and achievable.

Table 5.1: Summary of study findings

No.	Objectives	Methods	Findings
1.	To compare the presence of mimosine in *Leucaena leucocephala* leaves and seeds using Soxhlet extraction and HCl digestion.	HCl digestion Soxhlet extraction	i. The highest mimosine removal was observed in Soxhlet extraction (SXE) of seeds and leaves followed by HCl digestion method with 21.633 µg and 9.764 µg in seeds while 10.261 µg and7.2 µg per 100g dry mass in leaves respectively. ii. It was identified that June 2016 recorded a 37% decline in average rainfall compared to May 2016 from 224mm to 142mm rainfall, which had exert some stress to the tree, thus inadvertently releasing more mimosine in order to survive.
2.	To investigate the mimosine content in seed meal after extraction.	Soxhlet extraction	i. In order to know whether mimosine could be fully removed from the *Leucaena leucocephala* seeds after the oil extraction, the second Soxhlet (SXE) was employed using the same seeds. From the result shown, defatted seeds score lower mimosine value with 8.150 µg compared to the undefatted seeds which is 10.203 µg per 100g dry mass. However, the result was slightly reverse for mimosine content where the value was lowest in undefatted seeds compared to the defatted seeds during the second cycle of Soxhlet extraction (SXE). ii. Effect of heat: Undefatted seeds (UDS) have higher mimosine concentration compared to defatted seeds (DS). It was observed that defatted seeds (DS) with heat has the lowest mimosine content with 19.417µg per 100g dry mass while undefatted seeds (UDS) with heat has the highest mimosine with 24.5µg per 100g dry mass.
3.	To investigate the effect of mimosine on biodiesel oxidation stability.	Rancimat test (EN14214) FRAP Assay	i. Rancimat test: The highest stabilization efficacy of mimosine was obtained at 10,000 ppm with 5.98 and the lowest value at 2000 ppm with 3.75. It was observed that the efficacy of mimosine oxidative activity increased in respect of concentration (ppm) until it reaches maximum induction period at 69.4 hours. ii. FRAP assay: Undefatted seeds (UDS) has higher FRAP value of 66.02 µmol compared to defatted seeds (DS) extracts with 55 µmol Trolox equivalent (TE). Whilst positive control mimosine showed FRAP value of 93.09 µmol Trolox equivalent (TE). This indicate that all extracts are able to reduce ferric (III) to ferrous (II) by the reaction of electron donating antioxidant.

Future Research Recommendations

The following topics should be considered in order to improve the quantity and quality in *Leucaena Leucocephala* leaves, seed and seeds meals in future research's objectives:

In this study, mimosine extraction using HCl digestion and Soxhlet extraction was compared. Both extraction methods however could be categorized as conventional methods in compounds extraction. Due to technology enhancement, further experiments using other types of modern extractions including microwave-assisted extraction, ultrasonic-assisted extraction, and super-critical fluid extraction should be conducted in order to have a more robust comparison. It is also suggested the seed meals that underwent full removal of mimosine should be dedicated for compound analysis for the evaluation of a food grade standard. This is to ensure a safer quality guaranteed for animal consumption. Lastly, to ensure a good shelf life for biodiesel with mimosine additive, it is recommended to carry out the antioxidant components analysis including full range of biodiesel properties parameters that follows European (EN) standard. This is to ensure the biodiesel quality is suitable for industrial applications.

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LIST OF PUBLICATION AND PAPER PRESENTED

Publications

1. Ramli, N., Jamaludin, A. A., & Ilham, Z. (2017). Mimosine toxicity in Leucaena biomass: A hurdle impeding maximum use for bioproducts and bioenergy. *International Journal of Environmental Sciences & Natural Resources, 6*(4), 1-5

2. Ilham, Z., Hamidon, H., Rosji, N., Ramli, N., & Osman, N. (2015). Extraction and quantification of toxic compound mimosine from *Leucaena Leucocephala* leaves. *Procedia Chemistry* 16, 164 – 170.

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Extraction and Quantification of Toxic Compound Mimosine from *Leucaena leucocephala* Leaves

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Abstract

The existence of mimosine in *Leucaena leucocephala* prevents the utilization of its biomass residues (after energy conversion) as animal feed. In this study, mimosine quantification in *Leucaena leucocephala* was carried out by using rapid colorimetric method. In addition, two different extraction methods which are soxhlet extraction with either distilled water or ethyl acetate as extraction solvent and digestion method were used to compare its efficiency in extracting the mimosine from *Leucaena leucocephala* leaves. The samples from both extraction methods were then clarified by boiling it with 30 mg activated carbon and filtered. The absorbance of diluted aliquot of 1-2-3-4-5 mL was then obtained from spectrophotometer at the wavelength of 535 nm. The concentration of mimosine from soxhlet extraction with distilled water was found to be higher compared to the digestion method but in contrast with ethyl acetate solvent extraction.

Peer-review under responsibility of Research Center for Chemistry, Indonesian Institute of Sciences

Keywords: Mimosine, Digestion, Soxhlet, *Leucaena leucocephala*

1. Introduction

Leucaena leucocephala was first recognized in Central America and the Yucatan Peninsula of Mexico by Spanish adventurer who carried Leucaena feed and seed on their galleons to the Philippines to feed their stock. It has spread to most countries of the tropical world since then where it was used as shade plants for their crops. The genus Leucaena has over 50 names ascribe to it such as while leadtree in English, *subabul* in India, *lamtoro gung* in

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Mimosine Toxicity in Leucaena Biomass: A Hurdle Impeding Maximum use for Bioproducts and Bioenergy

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Abstract

Leucaena biomass could serve as a new commodity for bio energy production. It is well established as a feedstock for animal husbandry, due to its nutrient content and lower cost. However, the existence of mimosine caused loss of hair among ruminants. This paper provides a short review of current uses of Leucaena, from agriculture to bio energy. In the next section, the techniques to remove Leucaena toxicity caused by the toxic non-protein amino acid mimosine will be discussed. A common mimosine inactivation technique adopted for animal rations is dosage-dependent on the Leucaena portion and inclusion of iodine, iron, copper or sulphate in the diet. However, this technique is inefficient. Other approaches such as enzymes from ruminal microbes or soil bacteria and low-mimosine Leucaena clones have become new strategies to overcome this problem, although the toxicity is reduced it still can be seen. Future research is needed to if possible fully eliminate the toxicity and realize the goal of Leucaena utilization to produce both bio products and bio energy.

Keywords: Mimosine; Toxicity; Bio Energy; *Leucaena Leucocephala* and Biomass

Introduction

Leucaena leucocephala is a leguminous fast growing tree native to Mexico and Central America, but now found in most of the sub tropic and tropical regions around the world. L. leucocephala belongs to the *Fabaceae* family, with about 22 species in the Leucaena genus worldwide including two named acid-tolerant hybrids (*L. leucocephala Bahru and L. leucocephala Rendang*) [1]. It is known as *Subabul* in India, Ipil-ipil in the Philippines, Yin hue in China and *Petai Belalang* in Malaysia. In this review, *L. leucocephala* will be referred to using its general name, Leucaena. Due to factors such as increasing market demand for beef products, escalating land prices for agricultural activities and high cost of protein concentrates, there is urgency to find alternative animal feed stocks. Feedstock shortages faced by developing countries has driven adoption of Leucaena for its nutritive value, low cost and sustainability. This tree has been extensively introduced as an agro-forestry product and forage legume for its high crude protein, also for being highly palatable, long-lived, and drought tolerant. The improvement of crop yield when intercropping Leucaena with food crops has been widely documented. A study by Imogie reported a noticeable increase in fresh fruit bunch production when intercropping Leucaena with oil palm [2]. Its deep root system and ability to fix nitrogen could aid in soil erosion, soil fertility and aeration, creating a healthy nitrogen cycle in crops. It also has use in bioremediation to treat industrial waste [3]. Numerous papers show cattle fed with Leucaena show a gain in live weight more than with other forage legumes, from 50 kg/ha/yr to 200 kg/ha/yr [4]. Similarly, it is observed that Leucaena intake as a supplement significantly improves the rumen microbial population, improves N-retention and microbial supply, thus contributing to an efficient digestion process. However, inclusion of Leucaena in the diet has been shown to lower the weight of catfish and Nile tilapia when exceeding 20% and 50% of the diet, respectively [5,6].

Current Uses

Table 1: Biomass harvest from Leucaena by different methods.

Biofuel Types	Methods	References
Biomass based power generation	Gasification of Leucaena's wood	[13]
Bio ethanol	Fermentation of Leucaena's legumes	[14]
Biodiesel	Microwave assisted irradiation of Leucaena's legumes	[14]
Bio-oil	Pyrolysis of Leucaena's trunk	[15]
Bio char	Pyrolysis of Leucaena's bark	[16]

APPENDIX A

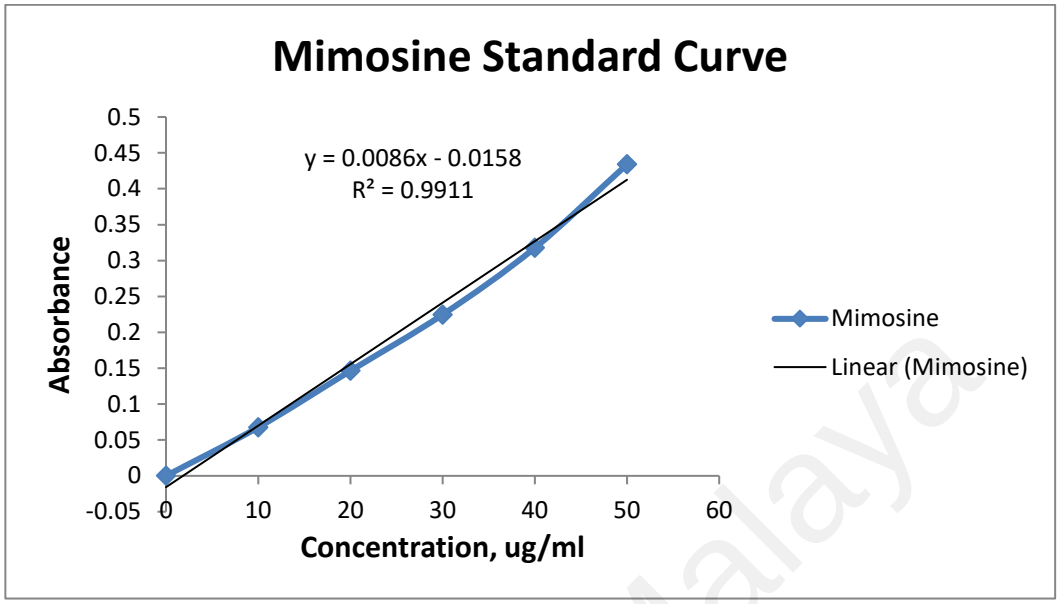

Figure A1: Graph of Mimosine standard curve.

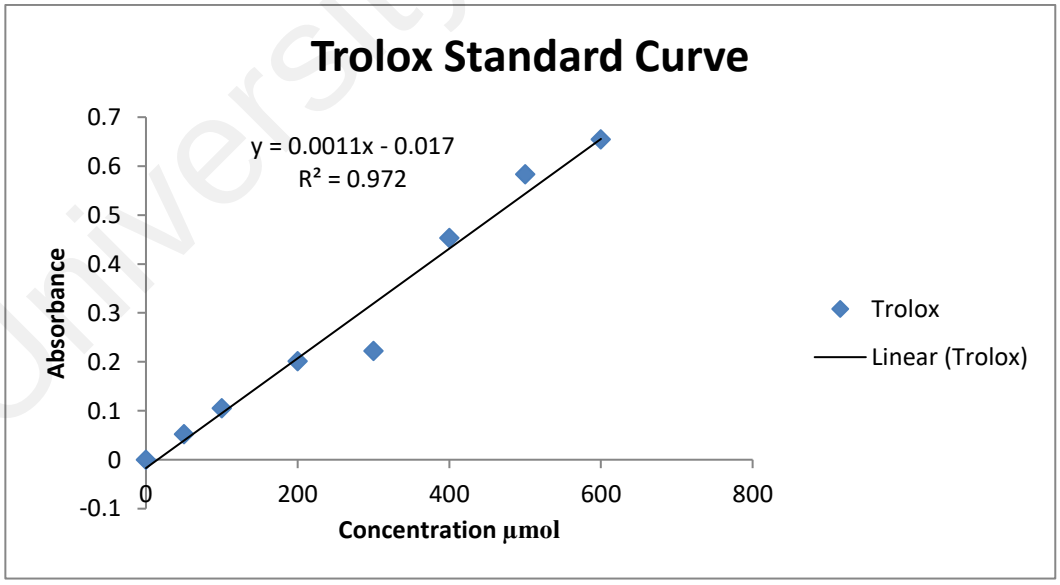

Figure A2: Graph of Trolox standard curve.

APPENDIX B

Table B1: Mimosine content in Undefatted Seeds (UDS) at room temperature using Soxhlet extraction (SXE).

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.161333	22.04167	0.886825	0.512009	0.786458
2	0.161333	22.04167	0.886825	0.512009	0.786458
3	0.158	21.625	0.433013	0.25	0.1875
4	0.156	21.375	0.125	0.072169	0.015625
5	0.155	21.25	0	0	0
6	0.155667	21.33333	0.190941	0.11024	0.036458
7	0.157	21.5	0.330719	0.190941	0.109375
8	0.153	21	0.330719	0.190941	0.109375
9	0.167667	22.83333	0.260208	0.150231	0.067708
10	0.154	21.125	0.125	0.072169	0.015625
11	0.154667	21.20833	0.360844	0.208333	0.130208
12	0.168333	22.91667	0.360844	0.208333	0.130208
13	0.157667	21.58333	0.288675	0.166667	0.083333
14	0.154333	21.16667	0.190941	0.11024	0.036458
15	0.157	21.5	0.125	0.072169	0.015625

Table B2: Mimosine content in Undefatted Seeds (UDS) at room temperature using HCl digestion.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.065333333	10.0416667	0.07216878	0.04166667	0.00520833
2	0.053333333	8.54166667	0.19094065	0.11023964	0.03645833
3	0.052	8.375	0	0	0
4	0.066666667	10.2083333	0.07216878	0.04166667	0.00520833
5	0.069333333	10.5416667	0.28867513	0.16666667	0.08333333
6	0.062	9.625	0.125	0.07216878	0.015625
7	0.065333333	10.0416667	0.07216878	0.04166667	0.00520833
8	0.062666667	9.70833333	0.26020825	0.1502313	0.06770833
9	0.065333333	10.0416667	0.07216878	0.04166667	0.00520833
10	0.062	9.625	0.33071891	0.19094065	0.109375
11	0.065666667	10.0833333	0.14433757	0.08333333	0.02083333
12	0.065666667	10.0833333	0.07216878	0.04166667	0.00520833
13	0.063666667	9.83333333	0.14433757	0.08333333	0.02083333
14	0.062	9.625	0.33071891	0.19094065	0.109375
15	0.065666667	10.0833333	0.14433757	0.08333333	0.02083333

Table B3: Mimosine content in leaves using Soxhlet extraction (SXE).

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.067667	10.33333	0.753464	0.435013	0.567708
2	0.065	10	0.125	0.072169	0.015625
3	0.068	10.375	0.330719	0.190941	0.109375
4	0.065333	10.04167	0.072169	0.041667	0.005208
5	0.065667	10.08333	0.072169	0.041667	0.005208
6	0.069333	10.54167	0.381881	0.220479	0.145833
7	0.064	9.875	0.125	0.072169	0.015625
8	0.062	9.625	0	0	0
9	0.065333	10.04167	0.072169	0.041667	0.005208
10	0.07	10.625	0	0	0
11	0.071333	10.79167	0.144338	0.083333	0.020833
12	0.07	10.625	0	0	0
13	0.069333	10.54167	0.314576	0.181621	0.098958
14	0.067	10.25	0.125	0.072169	0.015625
15	0.066333	10.16667	0.144338	0.083333	0.020833

Table B4: Mimosine content in leaves using HCl digestion.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.042666667	7.20833333	0.07216878	0.04166667	0.00520833
2	0.049333333	8.04166667	0.07216878	0.04166667	0.00520833
3	0.041333333	7.04166667	0.07216878	0.04166667	0.00520833
4	0.042666667	7.20833333	0.07216878	0.04166667	0.00520833
5	0.044666667	7.45833333	0.07216878	0.04166667	0.00520833
6	0.041666667	7.08333333	0.07216878	0.04166667	0.00520833
7	0.041333333	7.04166667	0.19094065	0.11023964	0.03645833
8	0.041	7	0	0	0
9	0.043333333	7.29166667	0.07216878	0.04166667	0.00520833
10	0.042666667	7.20833333	0.07216878	0.04166667	0.00520833
11	0.041	7	0.125	0.07216878	0.015625
12	0.041333333	7.04166667	0.14433757	0.08333333	0.02083333
13	0.041	7	0	0	0
14	0.042666667	7.20833333	0.31457643	0.18162079	0.09895833
15	0.042333333	7.16666667	0.07216878	0.04166667	0.00520833

Table B5: Mimosine content in Defatted Seeds (DS) at room temperature.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.146	20.125	0.125	0.072169	0.015625
2	0.144667	19.95833	0.144338	0.083333	0.020833
3	0.146333	20.16667	0.401819	0.23199	0.161458
4	0.146	20.125	0.125	0.072169	0.015625
5	0.145333	20.04167	0.072169	0.041667	0.005208
6	0.145333	20.04167	0.072169	0.041667	0.005208
7	0.148667	20.45833	0.144338	0.083333	0.020833
8	0.146333	20.16667	0.401819	0.23199	0.161458
9	0.144333	19.91667	0.190941	0.11024	0.036458
10	0.146667	20.20833	1.040833	0.600925	1.083333
11	0.146	20.125	0.125	0.072169	0.015625
12	0.145333	20.04167	0.190941	0.11024	0.036458
13	0.144	19.875	0.330719	0.190941	0.109375
14	0.145333	20.04167	0.260208	0.150231	0.067708
15	0.142	19.625	0.330719	0.190941	0.109375

Table B6: Mimosine content in Undefatted Seeds (UDS) with heat.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.181333	24.54167	0.072169	0.041667	0.005208
2	0.179	24.25	0.25	0.144338	0.0625
3	0.18	24.375	0.125	0.072169	0.015625
4	0.178667	24.20833	0.190941	0.11024	0.036458
5	0.181333	24.54167	0.072169	0.041667	0.005208
6	0.180667	24.45833	0.288675	0.166667	0.083333
7	0.174333	23.66667	1.120361	0.646841	1.255208
8	0.179	24.25	0.216506	0.125	0.046875
9	0.181	24.5	0.125	0.072169	0.015625
10	0.178333	24.16667	0.260208	0.150231	0.067708
11	0.180667	24.45833	0.144338	0.083333	0.020833
12	0.178667	24.20833	0.144338	0.083333	0.020833
13	0.177333	24.04167	0.314576	0.181621	0.098958
14	0.178333	24.16667	0.401819	0.23199	0.161458
15	0.178667	24.20833	0.288675	0.166667	0.083333

Table B7: Mimosine content in Defatted Seeds (DS) with heat.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.140333	19.41667	0.072169	0.041667	0.005208
2	0.139333	19.29167	0.072169	0.041667	0.005208
3	0.140333	19.41667	0.072169	0.041667	0.005208
4	0.144	19.875	0.125	0.072169	0.015625
5	0.146333	20.16667	0.072169	0.041667	0.005208
6	0.145	20	0.216506	0.125	0.046875
7	0.143667	19.83333	0.190941	0.11024	0.036458
8	0.145667	20.08333	0.190941	0.11024	0.036458
9	0.143333	19.79167	0.505181	0.291667	0.255208
10	0.145	20	0	0	0
11	0.141667	19.58333	0.260208	0.150231	0.067708
12	0.141333	19.54167	0.072169	0.041667	0.005208
13	0.143	19.75	0	0	0
14	0.146	20.125	0.330719	0.190941	0.109375
15	0.143	19.75	0.216506	0.125	0.046875

APPENDIX C

Table C1: Mimosine content in Undefatted Seeds (UDS) first cycle of Soxhlet extraction.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.068333	10.41667	0.260208	0.150231	0.067708
2	0.066333	10.16667	0.288675	0.166667	0.083333
3	0.065333	10.04167	0.072169	0.041667	0.005208
4	0.069667	10.58333	0.072169	0.041667	0.005208
5	0.064333	9.916667	0.072169	0.041667	0.005208
6	0.066667	10.20833	0.072169	0.041667	0.005208
7	0.066	10.125	0.216506	0.125	0.046875
8	0.067	10.25	0.330719	0.190941	0.109375
9	0.065667	10.08333	0.072169	0.041667	0.005208
10	0.066667	10.20833	0.360844	0.208333	0.130208
11	0.068	10.375	0.330719	0.190941	0.109375
12	0.066333	10.16667	0.288675	0.166667	0.083333
13	0.069333	10.54167	0.072169	0.041667	0.005208
14	0.064667	9.958333	0.144338	0.083333	0.020833
15	0.065	10	0.125	0.072169	0.015625

Table C2 : Mimosine content in Undefatted Seeds (UDS) second cycle of Soxhlet extraction.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.028333	5.416667	0.072169	0.041667	0.005208333
2	0.028333	5.416667	0.072169	0.041667	0.005208333
3	0.025667	5.083333	0.288675	0.166667	0.083333333
4	0.028	5.375	0.125	0.072169	0.015625
5	0.028667	5.458333	0.072169	0.041667	0.005208333
6	0.025333	5.041667	0.072169	0.041667	0.005208333
7	0.028333	5.416667	0.072169	0.041667	0.005208333
8	0.027667	5.333333	0.072169	0.041667	0.005208333
9	0.027333	5.291667	0.190941	0.11024	0.036458333
10	0.028	5.375	0	0	0
11	0.027	5.25	0	0	0
12	0.028667	5.458333	0.072169	0.041667	0.005208333
13	0.027	5.25	0.330719	0.190941	0.109375
14	0.027667	5.333333	0.072169	0.041667	0.005208333
15	0.028667	5.458333	0.072169	0.041667	0.005208333

Table C3: Mimosine content in Defatted Seeds (DS) first cycle of Soxhlet extraction.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.050667	8.208333	0.072169	0.041667	0.005208333
2	0.049667	8.083333	0.260208	0.150231	0.067708333
3	0.054	8.625	0.125	0.072169	0.015625
4	0.049333	8.041667	0.190941	0.11024	0.036458333
5	0.048333	7.916667	0.072169	0.041667	0.005208333
6	0.049333	8.041667	0.144338	0.083333	0.020833333
7	0.049667	8.083333	0.072169	0.041667	0.005208333
8	0.050333	8.166667	0.072169	0.041667	0.005208333
9	0.051333	8.291667	0.072169	0.041667	0.005208333
10	0.051667	8.333333	0.072169	0.041667	0.005208333
11	0.049667	8.083333	0.072169	0.041667	0.005208333
12	0.049333	8.041667	0.190941	0.11024	0.036458333
13	0.049333	8.041667	0.072169	0.041667	0.005208333
14	0.050333	8.166667	0.144338	0.083333	0.020833333
15	0.05	8.125	0.125	0.072169	0.015625

Table C4: Mimosine content in Defatted Seeds (DS) second cycle of Soxhlet extraction.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.037667	6.583333	0.401819	0.23199	0.161458
2	0.037	6.5	0.330719	0.190941	0.109375
3	0.039667	6.833333	0.072169	0.041667	0.005208
4	0.04	6.875	0	0	0
5	0.038333	6.666667	0.072169	0.041667	0.005208
6	0.038333	6.666667	0.190941	0.11024	0.036458
7	0.038667	6.708333	0.072169	0.041667	0.005208
8	0.039	6.75	0.125	0.072169	0.015625
9	0.039667	6.833333	0.072169	0.041667	0.005208
10	0.040333	6.916667	0.072169	0.041667	0.005208
11	0.039667	6.833333	0.144338	0.083333	0.020833
12	0.038333	6.666667	0.072169	0.041667	0.005208
13	0.039333	6.791667	0.144338	0.083333	0.020833
14	0.036333	6.416667	0.401819	0.23199	0.161458
15	0.039	6.75	0.125	0.072169	0.015625

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Firstcycle - Secondcycle	4.872222E0	.2512206	.0648649	4.7331009	5.0113435	75.113	14	.000

Figure C1: Paired-t tests of Between-Subjects Effects

APPENDIX D

Effect of heat on mimosine content.

Table D1: Mimosine content in Defatted Seeds (DS) at room temperature.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.050333	8.166667	0.688446	0.397475	0.473958
2	0.039333	6.791667	0.072169	0.041667	0.005208
3	0.041333	7.041667	0.288675	0.166667	0.083333
4	0.051	8.25	0.125	0.072169	0.015625
5	0.051333	8.291667	0.072169	0.041667	0.005208
6	0.051667	8.333333	0.190941	0.11024	0.036458
7	0.051333	8.291667	0.288675	0.166667	0.083333
8	0.051	8.25	0.125	0.072169	0.015625
9	0.048667	7.958333	0.520416	0.300463	0.270833
10	0.050333	8.166667	0.190941	0.11024	0.036458
11	0.051	8.25	0.330719	0.190941	0.109375
12	0.05	8.125	0.125	0.072169	0.015625
13	0.051667	8.333333	0.072169	0.041667	0.005208
14	0.052	8.375	0.125	0.072169	0.015625
15	0.05	8.125	0	0	0

Table D2: Mimosine content in Defatted Seeds (DS) with heat.

Sample	Average Absorbance	Average Concentration	Std	SEM	Variance
1	0.047	7.75	0.433013	0.25	0.1875
2	0.037	6.5	0	0	0
3	0.039333	6.791667	0.072169	0.041667	0.005208
4	0.041333	7.041667	0.288675	0.166667	0.083333
5	0.041333	7.041667	0.190941	0.11024	0.036458
6	0.040667	6.958333	0.260208	0.150231	0.067708
7	0.042333	7.166667	0.381881	0.220479	0.145833
8	0.042	7.125	0.25	0.144338	0.0625
9	0.037333	6.541667	0.832291	0.480523	0.692708
10	0.043333	7.291667	0.144338	0.083333	0.020833
11	0.043333	7.291667	0.190941	0.11024	0.036458
12	0.043333	7.291667	0.144338	0.083333	0.020833
13	0.042	7.125	0.216506	0.125	0.046875
14	0.037	6.5	0.544862	0.314576	0.296875
15	0.045	7.5	0.125	0.072169	0.015625

Table D3: Tests of Between-Subjects Effects

Dependent Variable:Concentration

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	190.749 [a]	3	63.583	502.006	.000
Intercept	27566.839	1	27566.839	2.176E5	.000
Group	138.017	1	138.017	1.090E3	.000
Treatment	20.709	1	20.709	163.507	.000
Group * Treatment	32.023	1	32.023	252.829	.000
Error	7.093	56	.127		

a. R Squared = .964 (Adjusted R Squared = .962)

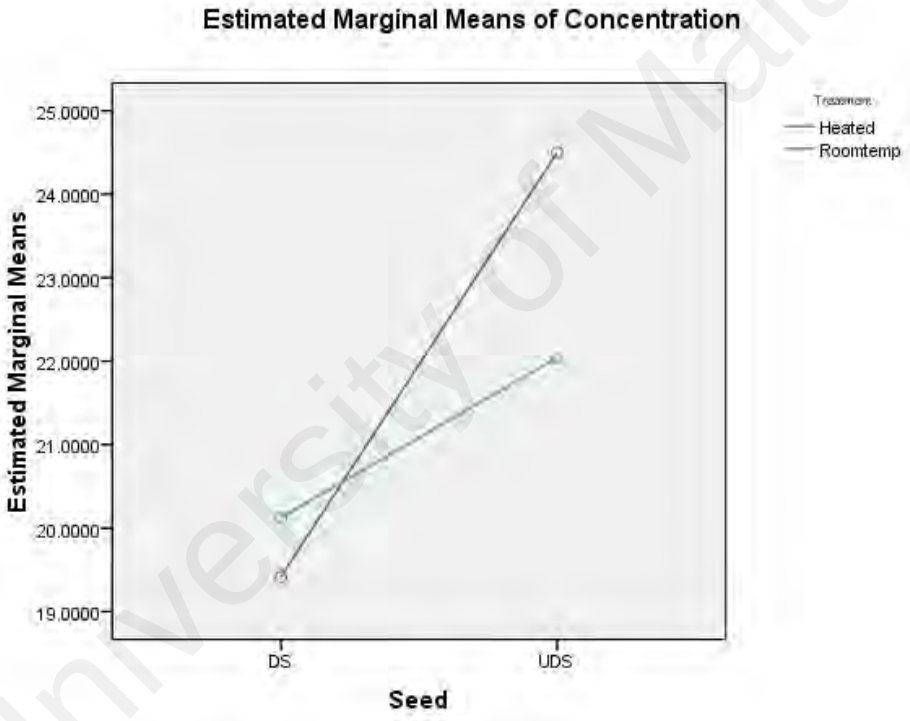

Figure D1: Graph of estimated marginal means of mimosine concentration in UDS and DS under heat treatment.

APPENDIX E

Table E1: FRAP absorbance with heat treatment.

Sample	Average Absorbance	Average Concentration	Variance	Std	SEM
1	0.037666667	54.66666667	1.333333333	1.154701	0.666667
2	0.038333333	55.33333333	0.333333333	0.57735	0.333333
3	0.038	55	3	1.732051	1
4	0.039666667	56.66666667	0.333333333	0.57735	0.333333
5	0.038	55	0	0	0
6	0.037666667	54.66666667	0.333333333	0.57735	0.333333
7	0.039	56	1	1	0.57735
8	0.038333333	55.33333333	2.333333333	1.527525	0.881917
9	0.037666667	54.66666667	0.333333333	0.57735	0.333333
10	0.037666667	54.66666667	1.333333333	1.154701	0.666667
11	0.037666667	54.66666667	0.333333333	0.57735	0.333333
12	0.037	54	1	1	0.57735
13	0.038333333	55.33333333	4.333333333	2.081666	1.20185
14	0.037666667	54.66666667	2.333333333	1.527525	0.881917
15	0.037333333	54.33333333	1.333333333	1.154701	0.666667

Table E2: FRAP absorbance at room temperature**.**

Sample	Average Absorbance	Average Concentration	Variance	Std	SEM
1	0.050333333	67.33333333	0.333333333	0.57735	0.333333
2	0.048333333	65.33333333	1.333333333	1.154701	0.666667
3	0.049666667	66.66666667	1.333333333	1.154701	0.666667
4	0.046666667	63.66666667	0.333333333	0.57735	0.333333
5	0.047333333	64.33333333	0.333333333	0.57735	0.333333
6	0.049	66	1	1	0.57735
7	0.051333333	68.33333333	2.333333333	1.527525	0.881917
8	0.046333333	63.33333333	1.333333333	1.154701	0.666667
9	0.049666667	66.66666667	1.333333333	1.154701	0.666667
10	0.049666667	66.66666667	0.333333333	0.57735	0.333333
11	0.048666667	65.66666667	0.333333333	0.57735	0.333333
12	0.047333333	64.33333333	0.333333333	0.57735	0.333333
13	0.049666667	66.66666667	4.333333333	2.081666	1.20185
14	0.050333333	67.33333333	0.333333333	0.57735	0.333333
15	0.051	68	3	1.732051	1

Table E3: FRAP absorbance with Mimosine addition.

Sample	Average Absorbance	Average Concentration	Variance	Std	SEM
1	0.075666667	92.66666667	1.333333333	1.154701	0.666667
2	0.078333333	95.33333333	0.333333333	0.57735	0.333333
3	0.068	85	7	2.645751	1.527525
4	0.078666667	95.66666667	0.333333333	0.57735	0.333333
5	0.079	96	0	0	0
6	0.074333333	91.33333333	0.333333333	0.57735	0.333333
7	0.074666667	91.66666667	0.333333333	0.57735	0.333333
8	0.076333333	93.33333333	0.333333333	0.57735	0.333333
9	0.076666667	93.66666667	1.333333333	1.154701	0.666667
10	0.076333333	93.33333333	0.333333333	0.57735	0.333333
11	0.078333333	95.33333333	0.333333333	0.57735	0.333333
12	0.075333333	92.33333333	0.333333333	0.57735	0.333333
13	0.076666667	93.66666667	0.333333333	0.57735	0.333333
14	0.077	94	1	1	0.57735
15	0.076	93	3	1.732051	1