Characterisation of Biodiesel from *Litsea glutinosa*

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***SUBMISSION APPROVED FOR EXAMINATION**

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REFERENCE DECLARATION

I, Miss. A.A. Perumal – 20607567 and Prof Bharti Odhav (full name of supervisor) do hereby declare that in respect of the following dissertation:

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AUTHOR'S DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of Prof Bharti Odhav

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Student's signature

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DEDICATION

DEDICATED TO MY PARENTS,

MR ANTHONY PERUMAL

AND

MRS JOYCE PERUMAL

THANKS FOR EVERYTHING; FOR YOUR GUIDANCE, SUPPORT AND SUPERVISION.

A Special thanks to my siblings

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†

Glória in excélsis Deo

et in terra pax homínibus bonae voluntátis.

Laudámus te,

benedícimus te,

adorámus te,

glorificámus te,

grátias ágimus tibi propter magnam glóriam tuam,

Dómine Deus, Rex cæléstis,

Deus Pater omnípotens.

Dómine Fili Unigénite, Iesu Christe,

Dómine Deus, Agnus Dei, Fílius Patris,

qui tollis peccáta mundi, miserére nobis;

qui tollis peccáta mundi, súscipe deprecatiónem nostram.

Qui sedes ad déxteram Patris, miserére nobis.

Quóniam tu solus Sanctus, tu solus Dóminus, tu solus Altíssimus,

Iesu Christe, cum Sancto Spíritu:

IN GLÓRIA DEI PATRIS. AMEN.

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LIST OF ABBREVIATIONS

2, 4-D	2,4-dichlorophenoxyacetic acid
ASTM	American Society for Testing and Materials
BAP	6-Benzylaminopurine
BP	Boiling Point
CN	Cetane number
DCM	Dichloromethane
EN	European Standards Organisations
FAME	Fatty Acid Methyl Esters
G	Grams
HCl	Hydrochloric acid
hr	Hour
IV	Iodine value
J. curcas	*Jatropha curcas*
K	Kelvin
KI	Potassium iodide
KOH	potassium hydroxide
L. glutinosa	*Litsea glutinosa*
Min	minutes
Ml	Milliliters
N	Normal
N/A	Not applicable
NaClO	Sodium Hypochloride
Na_2SO_4	Sodium Sulphate
NaOH	Sodium Hydroxide
NaOMe	Sodium Methioxide

No.	Number
No.2 Diesel fuel	Petroleum diesel
N. tabacum	*Nicotiana tabacum*
OD	Optical Density
ppm	Parts per million
Rpm	Revolutions per minute
SAPREF	South African Petroleum Refineries
Sec	Seconds
SMD	Sauter Mean Diameter
SN	Saponification number
Vs	versus

ABSTRACT

Global warming is a major concern to the world's population. It is caused by greenhouse gases that result from the burning of fossil fuel. The fossil fuel reserves are rapidly depleting as the needs and wants of man in the world increases. Biodiesel is one of the solutions proposed to remedy this environmental crisis facing the world today. The aim of this study was to characterise the biodiesel that can be produced from the oil of *Litsea glutinosa* by transesterification. Biodiesel can be used in a diesel engine without modification and be produced from many different natural renewable oil sources such as algae, plants and kitchen waste material. *Jatropha curcas* has been identified as a potential producer of oil for biodiesel. The biodiesel properties of *Jatropha curcas* meet the required American Society for Testing and Materials (ASTM) standards. The fruit of *Jatropha curcas* contains 40.0% lipids. The oil has a saponification number of 202.6 and an iodine value of 93.0. However *Jatropha curcas* cannot be grown in South Africa because it is a highly invasive plant. Cetane number is the most important parameter of biodiesel. The higher the cetane value, the better the quality of the biodiesel. Oil from *Jatropha curcas* has a cetane number of 57.1. An alternative is the oil from *Litsea glutinosa*, which is found as a naturalised free forest along the South African coastline, and is also found in many Asian countries. It has many medicinal properties, however, it is not edible and hence its use for biodiesel does not add to the debate of fuel versus food production. The cetane number of oil from *Litsea glutinosa* is 64.79, which is ideal for ignition, and the fruit with 61.29% lipids can yield valuable quantities of biodiesel. Thus, the aim of the research was to determine the potential of *Litsea glutinosa* as a source of biodiesel. Furthermore, to maintain a sustainable source, *Litsea glutinosa* was micropropagated, and transformation of *Litsea glutinosa* was attempted for hairy root cultures.

The Clevenger apparatus was used to extract fatty acids from dried crushed fruit of *Litsea glutinosa*. Fatty acids were converted to fatty acid methyl esters by transesterification. Transesterification was conducted in the presence of nitrogen and the reaction was catalysed with a mixture of methanol and sodium hydroxide (NaOH). The ratio 1 : 3 of oil to catalyst mixture was used for optimum transesterification to ensure a forward reaction

and it was transferred to a separating funnel to allow the glycerol and fatty acid methyl esters to separate. GC-MS was used to determine the fatty acids. The iodine number, saponification number, acid value, viscosity, kinematic viscosity, density, specific gravity, thermostability, distillation point and sulphur content were determined. The seeds of *Litsea glutinosa* were germinated and tissue culture callus was produced from the seeds and leaves. The leaves and stems were used to produce hairy root cultures by inoculating them with *Agrobacterium rhizogenes.*

Litsea glutinosa yielded 61% oil, which included 47 fatty acids in the fruit and 24 fatty acids in the seeds. The fatty acid profile of the oils indicated that the predominant fatty acids present were those that are essential for good quality biodiesel. The dominant fatty acids found in the fruit were 65.4% 9-octadecenoic acid and 13.6% hexadecanoic acid. The dominating fatty acids found in the seeds contained 36.3% 9-octadecenoic acid, 13.9%, hexadecanoic acid and 39.1%, dodecanoic acid. The iodine value was 6.3. The saponification value was 274. The acid value was 0.45 mg KOH. g^{-1}. The viscosity was 22.48 mm^2. s^{-1} and the kinematic viscosity was 23.84 mm^2. s^{-1}. The density was 942.69 kg. m^{-3} and the specific gravity was found to be 0.9 g. cm^{-3}. The distillation temperature ranged between 52.2°C to 610.2°C. The sulphur content was found to be 383 µg. ml^{-1}.

These characteristics indicate that *Litsea glutinosa* can be used as a source of biodiesel, because the properties meet the required ASTM standards. However, the production of biodiesel from *Litsea glutinosa* has not been commercialised because the production of fuel is dependent on the fruit of the plant, which is seasonal. To overcome this, a part of this study investigated micropragation of *Litsea glutinosa* and transformation of *Litsea glutinosa* by *Agrobacterium rhizogenes* into hairy roots and attempts where made to determine whether fatty acid could be produced by these techniques. Callus cultures were grown on MS media and McCowns woody plant media supplemented with 1 ml BAP and 1 ml 2,4-D per 1 L of media. Callus cultures were obtained in the light. However, *Litsea glutinosa* resisted transformation by *Agrobacterium rhizogenes*.

CHAPTER ONE: INTRODUCTION

The Industrial Development Corporation (IDC) of South Africa has made R5.1 billion available to develop liquid biofuels (Buthelezi, 2012). The money is to support the production of 300 million litres of greener fuels every year from 2016 onwards. The project will explore feedstock such as *Zea mays*, *Saccharum edule*, *Sorghum bicolor*, *Glycine max* and algae for fuel generation, which includes bioethanol and biodiesel. According to Buthelezi (2012), it has been estimated by the sugar companies that replacing only 10% of the country's liquid fuel supply with sugar-based bioethanol would create 110 000 jobs in the industry. Brazil has employed this method with great success (Buthelezi, 2012).

Currently, the dominant feedstock for biodiesel in Europe is rapeseed oil whereas *Glycine max* is predominantly used in the United States of America (Muldoon, 2006). The world demand for *Sorghum bicolour* has declined sufficiently enough for it to be included as a potential biofuel feedstock (Buthelezi, 2012). A problem with the production of greener fuels is that it leads to an increase of feedstock production and consequently leading to an increase in water consumption. There is great interest in developing algal lipids as biodiesel feedstocks. Research on algae as a source for fuel is currently being conducted by Sasol, Rhodes University and the Nelson Mandela Metropolitan University (Buthelezi, 2012). South Africa is endowed with many plants that can also be investigated for biodiesel production.

Biodiesel is defined by the ASTM as "a fuel comprised of monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats". It can be produced by transesterification of triglycerides. Animal fat (beef tallow) and spent cooking oil represent a significant market for biodiesel in many locations. Other vegetable oils of commercial interest as biodiesel feedstocks include *Camelina sativa*, *Brassica napus*, *Cocos nucifera*, *Zea mays*, *Jatropha curcas* and *Helianthus annuus*.

The compositional profile of the fatty acid methyl esters (FAME) provides insights into the similarities and differences amongst fuels from various sources. According to Singh and Singh (2010), common fatty acids found in biofuels are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and linolenic acid (18:3). The FAME profiles

determine the chemical properties of biodiesel (Hoekman *et al*., 2012). Standard specifications have been established for biodiesel by ASTM D6751 in U.S and EN 14214 by the European Standards Organisation (EU) (Jääskeläinen, 2009). **Table 1** shows the specifications for biodiesel. **Table 1** compares the requirements of biodiesel against petroleum diesel (No. 2 diesel). It can be in **Table 1** according to Singh and Singh (2010); that the specifications for biodiesel and petroleum diesel are different, even though they serve the same purpose. These specifications include sulphur content, kinematic viscosity, density, flash point, cetane number, cloud point (CP), pour point (PP) and water content, to name a few. The average chain length and average degree of unsaturation correlates to viscosity, specific gravity, iodine value, cloud point, pour point, and cold flow plug point (Hoekman *et al*., 2012). The cetane number is higher for FAMEs having a high saturate content such as those from *Cocos nucifera*, *Roystonea regia* and tallow and lower for FAMEs having high unsaturate content such as *Camelina sativa*, *Carthamus tinctorius*, *Glycine max* and *Helianthus annuus* (Sokoto *et al*., 2011). Similarly, iodine values are high for FAMEs with high unsaturated content, and low for FAMEs with high saturated content (Hoekman *et al*., 2012).

FAME chain length correlates with fuel properties such as cetane number and heating value (Bello *et al*., 2012). The average chain length metric does not distinguish between saturated and unsaturated FAME groups (Sokoto *et al*., 2011). A mixture of FAMEs can balance the characteristics of a biofuel. Low temperature operability is an important characteristic of biofuel (Hoekman *et al*., 2012); a poor cold flow temperature is an attribute of long-chain, saturated fatty acid esters dominating the biodiesel (Hoekman *et al*., 2012). The longer the carbon chain the poorer the low temperature operability (Hoekman *et al*., 2012). Maintaining proper engine temperature is important for efficient engine operation.

Oxidative stability is another important property with respect to performance of biodiesel (Hansen *et al*., 2011). Oxidative stability is related to the degree of unsaturation (Sokoto *et al*., 2011). The higher the unsaturation of biodiesel, the lower the stability of the biodiesel (Sokoto *et al*., 2011). Fuels from vegetable feedstock's, *Camelina sativa*, *Zea mays*, *Carthamus tinctorius*, *Glycine max* and *Helianthus annuus*, have over 50% poly-unsaturated fats. Biodiesel sourced from these would have poorer inherent oxidative stability compared to fuels from *Cocos nucifera*, *Jatropha curcas*, *Roystonea regia* and *Brassica napus*.

Changes in a single compositional feature such as chain length, chain branching and unsaturation produces both desirable and undesirable changes in FAME properties (Bello *et al*., 2012; Nwadike *et al*., 2013). Factors that favour good oxidative stability lead to poor low temperature operability (Hoekman *et al*., 2012). Due to the conflicting impacts of these FAME compositional features upon biofuel properties, it is not possible to define a specific FAME composition that is optimum for all biofuels (Sokoto *et al*., 2011).

Research conducted by Mohibbe *et al*. (2005) on 75 plant species showed that seed oils have 30% fatty acids in their seed / kernel. Fuel characteristics such as saponification number (SN), iodine value (IV) and cetane number (CN) of FAME of oils are empirically determined (Mohibbe *et al*., 2005). Fatty acid compositions IV and CN were used to predict the quality of various biodiesels. FAME from oils of 26 species including *Azadirachta indica*, *Calophyllum inophyllum*, *Jatropha curcas* and *Pongamia pinnata* were found to be the most suitable biodiesel sources since they meet the biodiesel specification of the US and European Standards Organisations (Hoekman *et al*., 2012). Mohibbe *et al*. (2005) pointed out that the FAME of another 11 species meet the specification of the biodiesel standard of U.S. These selected plants have great potential as sources of biodiesel. Amongst the 11 species was a woody plant named *Litsea glutinosa*. This plant has been declared an invasive weed and does not compete as a food crop. The oil from this woody plant is known to produce fatty acids for industrial use and feedstock for biodiesel. *Litsea glutinosa* is indigenous to India, Malaysia and the Philippines, and is naturalised along the coastline of South Africa (GRIN).

The aim of the research was to determine the characteristics of oil from *Litsea glutinosa* for the production of biodiesel by transesterification. This was achieved by the following objectives:

1. extraction and identification of fatty acids from the fruit of *Litsea glutinosa;*.
2. to determine the properties of the biodiesel produced from the oils of *Litsea glutinosa*;
3. to induce hairy roots in *Litsea glutinosa* using *Agrobacterium rhizogenes*;
4. to produce *in-vitro* callus cultures of *Litsea glutinosa* and
5. to determine whether biodiesel can be produced from hairy roots and callus culture if induction was successful and the characterisation of the biodiesel thereof.

The study was designed to determine the charateristics of oil extracted and transesterified from *Litsea glutinosa, as* outlined in **Figure 1**. The biodiesel was then investigated for its physical properties and compared to biodiesel derived from *Jatropha curcas,* and the ASTM standards for biodiesel. It also provides an overview of the efforts made to micropropagate *Litsea glutinosa* and to transform it for hairy roots.

A point of departure for this dissertation would be **Table 1**. **Table 1** provides a benchmark against which to check the characteristics of biodiesel from *Litsea glutinosa*. Frequent references are made to **Table 1** to determine whether *Litsea glutinosa* has potential as a feedstock for biodiesel. **Figure 1** provides an overview of the structure of this thesis.

Table 1: Biodiesel compared against No. 2 diesel (adapted from Singh and Singh 2010)

Fuel Properties	Biodiesel	No. 2 Diesel
Fuel Standard	**ASTM PS 121**	**ASTM D975**
Fuel Composition	C12-C22 FAME	C10-C12 HC
Lower Heating Value (Kj. L^{-1})	32.634	36.593
Kinermatic Viscosity, @ 40 ° C ($mm^2. s^{-1}$)	1.9 - 6.0	1.3 - 4.1
Specific Gravity kg. L^{-1} @ 15.55° C	0.88	0.85
Density, $kg.m^{-3}$ @15°C	0.0611	0.0590
Water, mg. kg^{-1} by wt	0.05% MAX	161
Carbon (wt %)	77	87
Hydrogen (wt %)	12	13
Oxygen, by dif. (wt %)	11	0
Sulfur (wt %)	0.0 - 0.0024	0.5 MAX
Boiling Point (°C)	182 - 338	188 - 343
Flash Point (°C)	100 - 170	60 - 80
Cloud Point (°C)	-3 to 12	-15 to 5
Pour Point (°C)	-15 to 10	-35 to -15
Cetane Number	48 to 65	40 to 55
Stoichiometric Air/Fuel Ratio ($w.w^{-1}$)	13.8	15
BOCLE Scuff (g)	> 7.000	3.600
HFRR (μm)	314	685
Fuel Standard	ASTM PS 121	ASTM D975
Fuel Composition	C12-C22 FAME	C10-C12 HC

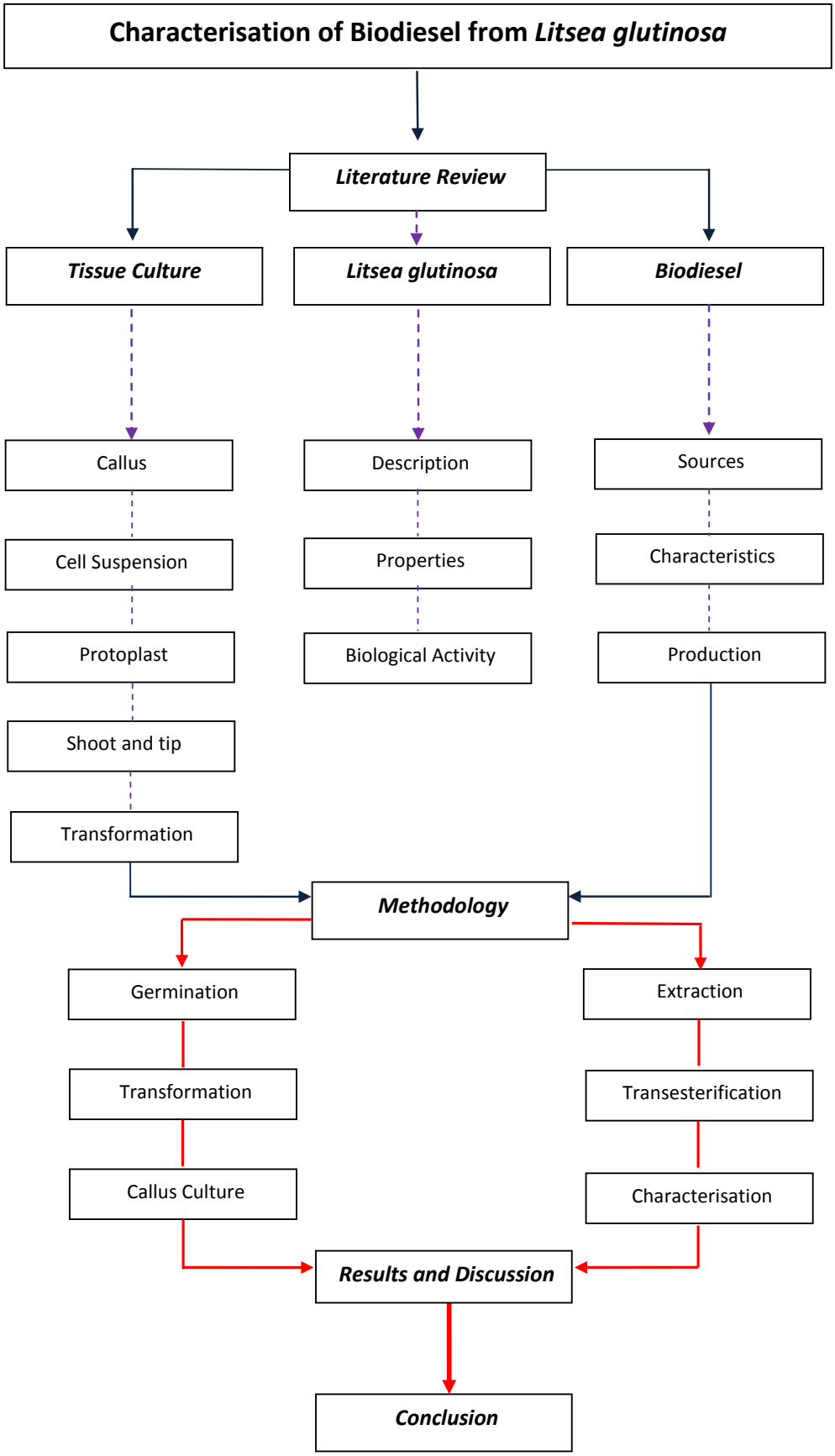

Figure 1: Overview of the thesis

CHAPTER TWO: LITERATURE REVIEW

2.1 *Litsea glutinosa*

The taxonomy of *Litsea glutinosa* indicates that the plant belongs to the C.B. Robinson Family: Lauraceae (Lemmens *et al*., 1995). It is referred to as *Litsea glutinosa* (Lour) C. B. Rob. *Litsea glutinosa* is found in India, Malaysia and the Philippines (Mishra *et al*., 2010). It has naturalised in South Africa. **Figure 2** illustrates the plant. The scientific synonyms are: *Litsea glutinosa* (Lour) C. B. Rob, *Litsea chinensis* Lamk, *Litsea geminata* Blume, *Litsea glabraria* A.L. Juss., *Litsea tetranthera* (Willd.) Pers. In South Africa and India this plant is known by its common name, Indian Laural (Hosmath, 2011). *Litsea glutinosa* is known in many countries by different names.

2.2 Description

Litsea glutinosa is a small tree reaching a height of 20 metres (Pradeepa *et al*., 2013). The leaves are elliptical to oblong-elliptical (9 to 20 cm long). They are alternate to opposite or sometimes subverticallated, penninerved, glabrous and aromatic (**Figure 2**). Flowers are small and yellow (**Figure 3**). The researcher noted that the fruits were round and 8 mm in diameter. Fruits turn from green to purple when ripe (**Figure 3**).

Figure 2: *Litsea glutinosa* (Perumal, 2010)

The fruit are a red drupe, globose-ovoid or subcylindrical and seated in a developed cup or disc. The seed contains 49% aromatic and tallow like oil (Chowdhury *et al*., 2008). The fatty acid laurostearin constitutes 85% of the oil and the rest is olein (Chowdhury *et al*., 2008).

2.2.1 Properties and uses

The roots, barks, leaves and seeds of *Litsea glutinosa* have many industrial and medicinal properties. Prusti *et al*. (2008) reported that the leaves have antispasmodic activity. They can be used as an emollient and have wound healing properties. The roots and leaves are used for healing of sprains and bruises in humans (Prusti *et al*., 2008). The bark is used to heal intestinal ailments (Mandal *et al*., 2000). Pounded seeds are used for boils and ground bark is used for wounds and bruises (Mandal *et al*., 2000; Devi and Meera, 2010). Devi and Meera (2010) has pointed out the following uses of *Litsea glutinosa*:

- The oil extracted from berries is used for rheumatism.
- The paste of the bark is used to heal wounds in India.
- It is used as an ingredient to cure intestinal parasitism in Mauritius.
- The Chinese use the oil from the seed to make soap.

Figure 3: Different stages of the fruit development (Perumal, 2010)

2.2.2 Biological activities

Mandal *et al*. (2000) showed that a methanolic extract of *Litsea glutinosa* inhibits Gram-positive and Gram-negative bacteria (Prusti *et al*., 2008). The highest activity was against *Staphylococcus aureus* and lowest activity was against *Pseudomonas aeruginosa* (Prusti *et al*., 2008). Hosamath (2011) found the following compounds in the bark of *Litsea glutinosa:* tannins, beta-sitosterols, boldine, norboldine, laurotetanine, n-methylactinodaphnine, quercetine, sebiferine, litsiferine, kaempferol-3-glucoside, amino acids, quercetine-3-rhamnoside, kaempferol-7-aminoglucoside, pelargonidine-5-glucoside, naringenin-7-monorhamnoside, monoquiterpenes, sesquiterpenes and beta-amirine acetate. The essential oils of *Litsea glutinosa* were found to reduce blood pressure in rats. The effect was attributed to the compound ligustilide in the essential oil of *Litsea glutinosa* (Prusti *et al*., 2008).

Litsea glutinosa is a woody plant with a high concentration of oils in the fruits and leaves. Chen *et al*. (2008) preferred *Litsea glutinosa* over other oil crops for two reasons: (i) The woody plant *Litsea glutinosa* does not compete as a food source; (ii) The oils from woody plants produce fatty acid for industrial usage and feedstock for biodiesel. The fatty acids found in the fruit of *Litsea glutinosa* makes this a reasonable choice as a feedstock for biodiesel. The fatty acids of *Litsea glutinosa* are shown in **Table 2** (Chowdhury *et al*., 2008). Prusti *et al*. (2008) found that the major constituent from the leaf was phytol, caryophyllene, thujopsene and β-myrcene. The major constituencies found in the fruit oil were lauric acid, a-cubebene and caryophyllene (Prusti *et al*., 2008).

Table 2: Compounds found in the leaves and ripe fruit of *Litsea glutinosa* (Chowdhury *et al.*, 2008)

Leaves	Fruit
α-muurolene	α-cubebene
β-myrcene	(E)-ocimene
α-phellandrene	3-octen-5-yne
β-pinene	2,7-dimethyl
caryophyllene	caryophyllene
cinnamyl acetate	cauric acid
cyclohexanol	n-hexadecanoic acid and
2-methylene-5-(1-methylethenyl)-Diepi-.α	cleic acid
cedrene epoxide	
limonene	
n-hexadecanoic acid	
phytol, tau.-cadinol	
thujopsene	
Z-ocimene	

2.3 Biodiesel

Biodiesel is defined by ASTM as a fuel comprised of monoalkyl esters of long-chain fatty acids derived from vegetable oils or animal fats, designated B100 (Hoekman *et al*., 2012). Biodiesel is derived from the transesterification of triglycerides. It is a suitable alternative to petroleum diesel (Su *et al*., 2009).

Biodiesel is produced from fatty acids which can be obtained from a variety of plants and animal sources (Fangrui and Milford, 1999). Biodiesel from plant material is composed of organic carbon as a result of its photosynthetic origin. It is sustainable, renewable, and C-neutral (Chanakya *et al*., 2012) and therefore the emission of pollutants such as greenhouse gas is reduced (Fangrui and Milford, 1999). **Table 3** shows common fatty acids found in biodiesel. Diesel engines can be powered by biodiesel without modification, since diesel engines were originally designed to run on biodiesel (Meher *et al*., 2006).

Table 3: Chemical structure of common fatty acids (Singh and Singh, 2010)

Name of fatty acid	Chemical name of fatty acids	Structure (xx:y)	Formula
lauric	dodecanoic	12:0	$C_{12}H_{24}O_2$
myristic	tetradecanoic	14:0	$C_{14}H_{28}O_2$
palmitic	hexadecanoic	16:0	$C_{16}H_{32}O_2$
stearic	octadecanoic	18:0	$C_{18}H_{34}O_2$
oleic *cis-9-*	octadecanoic	18:1	$C_{18}H_{32}O_2$
linoeleic *cis-9, cis-12-*	octadecadienoic	18:2	$C_{18}H_{30}O_2$
linolenic	*cis-9,cis-12,cis-15-* octadecadienoic	18:3	$C_{18}H_{30}O_2$
arachidic	eicosanoic	20:0	$C_{20}H_{40}O_2$
behenic	docosanoic	22:0	$C_{22}H_{44}O_2$
erucle	*cis-13*-docosenoic	22:1	$C_{32}H_{42}O_2$
lignoceric	tetracosanoic	24:0	$C_{24}H_{48}O_2$

Although biodiesel was originally designed for diesel engines, biodiesel has disadvantages (Singh and Singh, 2010). The disadvantages of biodiesel are that it has high viscosity, low volatility and poor cold flow property (Singh and Singh, 2010). Biodiesel can be blended with petroleum diesel, to overcome the disadvantages of biodiesel (Demirba, 2002; Meher *et al.*, 2006, Nwadike *et al*., 2013). The advantages of biodiesel are that it is renewable, non-toxic and produces reduced toxic emissions due to a low sulphur and aromatic content (Wang *et al*., 2000).

2.3.1 Sources of biodiesel

Table 4 shows the common sources of biodiesel are vegetable oils, non-edible oils and animal fats (Singh and Singh, 2010). Waste vegetable oils and non-edible crude vegetable oils are cheaper biodiesel sources than edible vegetable oils as there is no demand for them as a consumable item. Common plant sources are: *Glycine max*, *Helianthus annuus*, *Roystonea regia*, *Brassica napus* and *Jatropha curcas* (*J. curcas*) (Bezergianni *et al*., 2010). Most of these plants are edible and there is a concern regarding competition between food or fuel

production (Karmakar *et al*., 2010). Unfortunately biodiesel from animal fats are not given equal attention as plant souces (Fangrui and Milford, 1999). Biodiesel from animal fats contain a higher concentration of saturated fatty acids (Fangrui and Milford, 1999). The biodiesel source is determined by the country in which it is produced, since the climate dictates the availability of the source (Knothe, 2005).

A summary of biodiesel properties from vegetable oils, non-edible oils, animal fats and other sources is presented in **Table 5** (Karmakar *et al*., 2010). Kinematic viscosity is one of the properties of biodiesel. The ASTM D6751 kinematic viscosity requirement of biodiesel is 1.9 to 6.0 $mm^2. s^{-1}$ (**Table 8**). *Glycine max*, *Jatropha curcas*, tallow and yellow grease have kinematic viscosities of 22.87 $mm^2. s^{-1}$, 33.90 $mm^2. s^{-1}$, 45.34 $mm^2. s^{-1}$ and 132.10 $mm^2. s^{-1}$ respectively (**Table 5**). The kinematic viscosity requirement for biodiesel is 1.9 to 6.0 $mm^2. s^{-1}$ (**Table 1**). Yellow grease had the highest kinematic viscosity followed by tallow, *Jatropha curcas* and *Glycine max*. None of these oils meet the ASTM requirements for kinematic viscosity when compared to the limits in **Table 8**. All the kinematic viscosity values were high. However, this can be corrected with blending the oil, with oil from *Olea europaea*, *Anacardium occidentale* or petroleum diesel (Nwadike *et al*., 2013).

The saponification value of *Glycine max*, *Jatropha curcas*, tallow and yellow grease are 195.3, 200.8, 198.0 and 198.4 respectively (**Table 5**). *Jatropha curcas* had the highest saponification value followed by yellow grease, tallow and *Glycine max*. There is no ASTM requirement for this value since it is used as an indicator to determine the free fatty acid composition. The free fatty acid composition determines the efficiency of transesterification and pre-treatment required before transesterification (Meher *et al*., 2006; Sharma *et al*., 2008). *Jatropha curcas* would require the most pre-treatment to obtain maximum yield of FAME compared to tallow which has the lowest saponification value. However *Jatropha curcas* has the second lowest kinematic viscosity compared to the other three sources above. India (Gmünder *et al*., 2012) and Zimbawe (Karavina *et al*., 2011) are using oil from *Jatropha curcas* to produce biodiesel and yet *Jatropha curcas* needs major pretreatment before transesterification. Although *Jatropha curcas* has a kinematic viscosity that can be corrected by blending yet the saponification value suggests that it is not a suitable feedstock for transesterification. Factors such as oil characteristics, availability and financial viability must also be taken into account when selecting a feedstock for biodiesel.

Table 4: Different biodiesel sources adapted from Singh and Singh, (2010)

Vegetable oils	Non-edible oils	Animal Fats	Other Sources
Glycine max	*Prunus dulcis*	Lard	Bacteria
Brassica napus	*Abutilon muticum*	Tallow	Algae
Carthamus tinctorius	*Carapa megistocarpa*	Poultry Fat	Fungi
Hordeum vulgare	*Attalea speciosa*	Fish oil	Cooking Oil (Yellow Grease)
Cocos nucifera	*Brassica carinata*		Microalgae (*Chlorella vulgaris*)
Arachis hypogaea	*Camelina sativa*		
Avena sativa	*Dipteryx odorata*		
Oryza sativa	*Cynara cardunculus*		
Sorghum bicolor	*Jatropha curcas*		
Sesamum indicum	*Jatropha nana*		
	Simmondsia chinensis		
	Pongamia glabra		
	Laurus nobilis		
	Lesquerella fendleri		
	Madhuca longifolia		
	Roystonea regia		
	Nicotiana Tabacum		
	Oryza sativa		

Table 5: Properties of common feed stocks adapted from Karmakar *et al*., 2010

Oils/fats	Density	Kinematic viscosity (40°C) $mm^2.s^{-1}$	Cetane No.	Flash point	Saponification value	Iodine value
canola	911.5	34.72	37.6	246	189.80	-
soybean	931.8	28.87	37.9	254	195.30	128-143
sunflower	961.1	35.84	37.1	274	193.14	125-140
palm	918.0	44.79	42.0	267	208.63	48-58
peanut	902.6	39.60	41.8	271	191.50	84-100
corn	909.5	30.75	37.6	277	183.06	103-128
rice bran	918.5	36.68	-	-	201.27	90-108
sesame	913.3	36.00	41.8	260	196.50	103-116
cottonseed	914.8	33.50	-	234	198.50	103-115
jatropha	940	33.90	-	225	200.80	82-98
neem	918.5	50.30	-	-	290.66	65-80
karanja	936.5	43.61	-	-	188.50	81-90
mahua	960	24.50	-	232	190.5	58-70
linseed	923.6	35.75	34.6	241	187.63	-
coconut	918	37.26	-	-	267.56	7.5-10.5
castor	955	251.20	42.3	-	191.08	83-86
tobacco	917.5	27.70	-	-	191.50	125-154
beef tallow	-	45.34	-	-	198	-
yellow grease	-	132.10	-	-	198.36	-

2.3.2 Characteristics of biodiesel

Literature review has highlighted the following biodiesel properties: acid number, viscosity, cloud point, pour point, methanol content, flash point, water and sediment, visual properties, cetane number, iodine value, saponification number, glycerol and fatty acids. American Society for Testing and Materials (ASTM) determine parameters that define the biofuel and recommended testing methods which are listed in **Table 8**.

The ASTM was established in 1898, by a group of engineers and scientists who gathered to discuss and address the constant rail breaks in the burgeoning railroad industry in the United States of America. The solution to this problem was the standardisation of the steel used in the railroad industry, to prevent further breakage and for public safety. New industrial, governmental and environmental products were being created and developed. This required new standards to protect the public and ensure standard and cost effective products. The ASTM became international and created the ASTM manual of standards, which are regularly updated. They are now known as ASTM International today. It is made up of 30,000 members representing a variety of sectors: producers, users, consumers, government and academia, from over 120 countries. ASTM International standards are easily accessible to the public through the internet.

Biodiesel specifications are country specific. However, the U.S. (ASTM D6751-08) and Europe (EN 14214) have developed standards and specifications that are used as guidelines worldwide. The European Standards Organisations (EN) is composed of three organisations. The three organisations are Committee for European Standardisation (CEN), European Committee for Electrotechnical Standardisation (CENELEC) and European Telecommunications Standards Institute (ETSI). The EN was created as an international non-profit association in Brussels on 30 October 1975. They facilitate the European industry by setting standards that comply with international requirements and thereby aiding in global trading.

2.3.2.1 Iodine value

Iodine value is used to determine the concentration of unsaturated fatty acids present in the biofuel (Hoekman *et al*., 2012). IV is inversely proportional to CN (Van Gerpen, 1996). An increase of CN results in a decrease of IV (Gopinath *et al*., 2009). A high iodine number indicates a high concentration of unsaturated fatty acids (Akbar *et al*., 2009). A low iodine number indicates a high concentration of saturation. The iodine value (IV) for biodiesel set by the European Standards Organisations is 120 g. l_2. 100 g^{-1} max (Hoekman *et al*., 2012).

2.3.2.2 Saponification number

Saponification is a side reaction that occurs during transesterification with an alkali catalyst (Atadashi *et al*., 2012). Soap is the unwanted by-product of transeserification (Atadashi *et al*., 2012). It occurs when the oil has a high free fatty acid concentration (Jansri and Prateepchaikul, 2011). NaOH forms sodium methoxide when dissolved in methanol and produces water. The water and free fatty acids reacts with Na^+ and K^+ salts to form sodium or potassium soaps (Sharma *et al*., 2008). The soap may accumulate with the catalyst in the glycerol phase or occasionally in the biodiesel (Sharma *et al*., 2008). Animal fats and waste oil produce more soap than crude vegetable oil. The presence of soap deposits in biodiesel will result in poor ignition, filter clogging, fuel pump problems and microbial growth causing corrosion. There is no ASTM requirement for saponification number.

2.3.2.3 Acid value

The acid number / value is an indication of all acidic components which indicates the level of free fatty acids (FFA) present in biodiesel, including process acids and the by-products of degradation (Prajapat and Jani, 2013). The acid number is related to the fuels long-term qualities (stability and corrosiveness) (Singh *et al*., 2012). The lower the acid number the higher the quality of the biodiesel. A high acid number reduces control over the process, such as methanol carry over during transesterification. These residues affect the engine because they dissolve rubber seals and hoses in the engine, due to their solvency effects (Singh *et al*., 2012). The ASTM standard for fatty acid number for biofuels is a maximum of 0.8 mg. $KOH.g^{-1}$ (**Table 8**).

2.3.2.4 Viscosity

Viscosity is the resistance of a fluid to flow (Nwadike *et al*., 2013). Fuels must have suitable flow characteristics to ensure that an adequate supply reaches injectors in an engine at different operating temperatures (Nwadike *et al*., 2013). One method to remedy high viscosity is by cracking (Zarei *et al*., 2012). Cracking is a distillation method where hydrocarbons are separated. The ASTM D6751 viscosity requirement value range from 1.9 to

6.0 $mm^2.s^{-1}$ (**Table 8**). The viscosity of biodiesel is higher than No. 2 diesel since it has a higher molecular mass and chemical structure. The viscosity at 311 K ranges from 2.8 to 3.5 $mm^2.s^{-1}$ for biodiesel and 2.7 $mm^2.s^{-1}$ for No. 2 diesel (Demirba, 2002). Biodiesel is more viscous than No. 2 diesel fuel. Most diesel engines were designed to use high lubricating and high sulphur content fuel. Small amounts of sulphur for lubricating purposes are allowed in fuels. The high viscosity property of biodiesels assists as a lubricant in diesel engines (Meher *et al*., 2006). Yuan *et al*. (2009) stated that the viscosity is dependent on the FAME composition of the oil. The molecular weight of triglyceride molecules are between 800 and 900 $g.mol^{-1}$. This is nearly four times larger than diesel fuel molecules (Ali and Hanna, 1994). Transesterification reduces the molecular weight of the triglyceride by one-third and the viscosity by an eighth. However this increases the volatility of biofuel (Singh and Singh, 2010).

2.3.2.5 Cetane number

The cetane number (CN) is a significant property which specify the ignition quality of a fuel for use in a diesel engine (Bello *et al*., 2012). It is the ability of fuel to ignite quickly after being injected (Prajapat and Jani 2013). A higher CN value indicates better ignition quality of the fuel (Oliveira and Da Silva, 2013). The CN of esters from vegetable oils (biodiesel) is higher than No. 2 diesel fuel (Demirba, 2002). The CN relates to the readiness of the fuel to self-ignite when exposed to the high temperatures and pressure in the diesel engine combustion chamber (Meher *et al*., 2006). The number is also indicative of the relative fuel stability (Knothe, 2005). US biodiesel standards have specified the upper limit of CN to be 65 (**Table 1**). The European Standards Organisations have set the biodiesel standards for CN at 51 (Knothe, 2005).

Determination of cetane number

The CN equation in the standard ASTM D976, using the boiling point and density for the calculation of cetane index of petroleum products, is inaccurate for vegetable oils and fatty acid esters (Gopinath *et al*., 2009). Software has been designed to determine the cetane number of FAMEs. A number of methods have been developed to predict cetane number.

However, many of the methods are inaccurate. Therefore libraries of fatty acid methyl ester cetane values are being developed to help predict the cetane value of biodiesel based on their fatty acid methyl ester composition (Gopinath *et al*., 2009). A system called Artificial Neural Networks (ANNs) to predict the cetane number of individual components is currently being developed (Gopinath *et al*., 2009).

Methods to improve cetane number

The cetane number depends on the nature of the crude oil as well as the refinery blending strategies (Bello *et al*., 2012). Hydrotreating the molecules in biodiesel increases cetane number while reducing aromatics (Santana *et al*., 2006). Combining hydrotreatment and selective ring opening (SRO) process is a solution to improve the CN (Santana *et al*., 2006).

Cetane boosters improves cold-start performance, reduces combustion noise and may reduce particulate matter emissions (Santana *et al*., 2006). These cetane boosters are generally organic nitrates. The disadvantage of cetane boosters are: an increase in fuel flammability, decreases in storage stability and ultrafine particle emissions from the biodiesel, which are highly hazardous. Santana *et al*. (2006) believes that cetane boosters are not an economically viable solution to increase CN.

Bodiesel can be blended with fule obtained via the Fischer-Tropsch method (Santana *et al*., 2006). This blend of biofuel will result in increases cetane number and reduces concentrations of aromatics, sulphur and other impurities (Santana *et al*., 2006).

2.3.2.6 Cloud point

Cloud point is the lowest operational temperature of biodiesel. When the temperature is too low wax crystals begin to form (Knothe, 2005; Imahara *et al*., 2006). Wax crystals clog the engine. Biodiesel is more reactive to oxygen and has a higher cloud point temperature than No. 2 diesel. The cloud point temperature of biodiesel is -3°C to 12°C, and it is -15°C to 5°C for No.2 diesel (**Table 1**).

2.3.2.7 Pour point

Pour point is the lowest temperature at which the fuel ceases to flow (Imahara *et al*., 2006; Meher *et al*., 2006). The pour point temperature is lower for No. 2 diesel fuel than biodiesel. Pour point temperature can be controlled with additives and blends (Knothe, 2005; Imahara *et al*., 2006). Biodiesel has a higher pour point temperature than No. 2 diesel fuel. The pour point temperature of biodiesel is -15 °C to 10 °C and -35 °C to -15 °C for No. 2 diesel (**Table 1**).

2.3.2.8 Methanol concentration

Exess methanol results in the deterioration of rubber seals and gaskets (Meher *et al*., 2006). Methanol is essential in the transesterification process, because the reaction is conducted in excess alcohol (Meher *et al*., 2006). It is considered when determining flash point (Section 2.3.2.9). Methanol is hygroscopic and therefore plays a role in the water content of the biodiesel (Hayyan *et al*., 2010). The excess alcohol used during the production reaction can be removed and re-used (Elam, 2008). Thus the level of methanol is set by the ASTM for biodiesel to be 0.2% by mass (**Appendix 3**).

2.3.2.9 Flash point

The flash point of biodiesel is the temperature at which vapours above the fuel become flammable (Prajapat and Jani, 2013). The flash point of vegetable oils are above that of diesel fuel, showing the non-volatile nature of vegetable oils (Meher *et al*., 2006). Vegetable oils are not directly volatile but have to be cracked (distillation into a series of hydrocarbons) or converted by transesterification into more volatile methyl ester. The flash point temperature of biodiesel is 100°C to 170°C and 60°C to 80°C for No. 2 diesel (**Table 1**).

2.3.2.10 Water sediment

The water content in biodiesel contributes to the quality and performance of the biodiesel (Prajapat and Jani, 2013). The presence of water shows the water wash or removal process was not performed correctly or indicates improper storage. Water in biodiesel causes poor ignition, filter clogging and fuel pump problems. Furthermore, it may allow for microbial growth or corrosion (Prajapat and Jani, 2013). Water and sediment should be a maximum of 0.05% by volume to comply with ASTM requirements (**Table 8**).

2.3.2.11 Visual standard

Fuel should look clear and bright and free of visible particles. There is no ASTM standard requirement for visual standard.

2.3.2.12 Total glycerol

Glycerol content is important since it is a by-product of transesterification (Oliveira and Da Silva, 2013). It is more polar than the FAME and has a higher density. Poor separation is caused by high free glycerol content (Hayyan *et al*., 2010). If the catalyst concentration is low or the reaction is not complete the glyceride content in the fuel would be high. High glycerol is problematic for consumers since it may separate out in storage, and cause filter clogging and fuel pump problems (Meher *et al*., 2006). It can cause dirty injectors or the formation of carbon deposits on mechanical parts in the car (nozzles, pistons, and valves), causing poor combustion conditions. The maximum allowed level of free glycerol is 0.240% by mass (**Table 8**).

2.3.2.13 Fatty acids

The FAME composition is used to determine the quality of the biodiesel to meet the specification of biodiesel standards set by the US and European Standards Organisations. Saponification number, iodine value and cetane number depends on the fatty acid

composition (Bello *et al*., 2012; Nwadike *et al*., 2013). Iodine value is the degree of unsaturation (Lapuerta *et al*., 2008). Increase in CN causes IV to decrease (Van Gerpen, 1996). This indicates that the degree of unsaturation decreases when CN is high (Bamgboye and Hansen, 2008).

Saturated fatty acids have no double bonds. Hydrogen atoms are removed when double bonds are formed. Saturated fatty acids contain many hydrogen to carbon bonds and saturated with hydrogen atoms. Unsaturated fatty acids have a lower energy output than saturated fatty acids. This is a result of the number of hydrogen present in the fatty acid chain. Unsaturated fatty acids are not as stable as saturated fatty acids since the oil goes rancid faster.

2.4 Production of Biodiesel

Biodiesel is derived from animal fats or any vegetable oils (Hayyan *et al*., 2010). The major problem associated with the use of pure vegetable oil as a basis for biodiesel, for diesel engines is the effect of the high viscosity of biodiesel on compression ignition (Demirba, 2002). Vegetable oil cannot be used directly in a diesel engine due to the high viscosity (Bamgboye and Hansen, 2008) and low volatility (Lapuerta *et al*., 2008). The techniques of pyrolysis, micro-emulsion, dilution and transesterification can be used to resolve the problems of high viscosity (Demirba, 2002; Meher *et al.*, 2006).

2.4.1 Pyrolysis

Pyrolysis is a process where organic matter is decomposed in the absence of oxygen using heat and a catalyst (Fangrui and Milford, 1999; Demirba, 2003; Atadashi *et al*., 2012). This process is simple and pollution free, but requires substantial energy input which creates pollutants in the first place.

2.4.2 Micro-emulsion

Micro-emulsion is a process that requires methanol and 2-octanol (Fangrui and Milford, 1999). However cetane improvers are required to ensure that the product meets the biofuel standards (Demirba, 2003; Atadashi *et al*., 2012).

2.4.3 Dilution

Dilution is a process where biodiesel is blended with petroleum diesel (Fangrui and Milford, 1999; Atadashi *et al*., 2012). A ratio from 10% to 50% of petroleum diesel can be used. The engine performance depends on the percentage blend.

2.4.4 Transesterification

Transesterification is taking a triglyceride molecule or a complex fatty acid and neutralising the free fatty acids by removing the glycerol and creating an alcohol ester (Demirba, 2002; Atadashi *et al*., 2012). It is a chemical process where fatty acid methyl esters and glycerol are produced from fatty acid oil in the presence of alcohol and a catalyst (Fangrui and Milford, 1999). Fatty acid methyl esters (which is the chemical name for biodiesel) (Van Gerpen, 1996) and glycerol is a byproduct of the reaction (Tan *et al*., 2012). Glycerol can be sold, since it is used in the soap and cosmetic industry (Tan *et al*., 2012). Details of transesterification are discussed below.

2.4.4.1 Conversion during transesterification

Three consecutive and reversible reactions take place (**Figure 4**) during transesterification (**Figure 5**). First the triglycerides are converted to diglycerides. The diglycerides are then converted to monoglycerides. Finally the monoglycerides are converted to glycerol which yields one methyl ester molecule per mole of glycerol per reaction step. These reactions are shown in **Figure 4**. Vegetable oils are a source of fatty acids or free fatty acids. **Table 3** lists the common fatty acids found in vegetable oils. Fatty acid characteristics are dependent on

the carbon chain lengths, and the number and situation of unsaturated or saturated bonds. Saturated fatty acids contain a single bond between carbons. Mono-unsaturated fatty acids contain one double bond in their carbon chain. Polyunsaturated fatty acids contain two or more double bonds. Lipids are composed of one mole of glycerol and three moles of fatty acids and oxygen (Maher and Bressler, 2007). Fats are composed of 90% – 98% triglycerides. Three identical fatty acids combine to form a triglyceride. Triglycerides break down to form mono-saturated fatty acids and polysaturated fatty acids. Triglycerides are lipids, which are non-polar compounds. Mono-saturated fatty acids have one double bond and one hydrogen atom missing whereas polysaturated fatty acids have more than one double bond and one hydrogen atom missing. Completely saturated triglycerides causes excessive carbon deposits in engines.

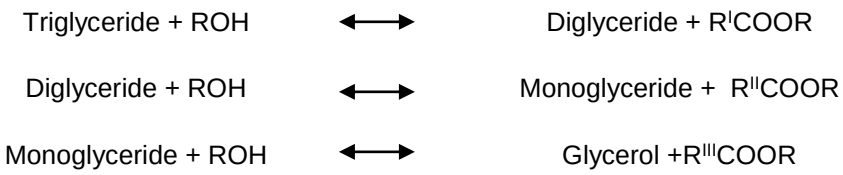

Figure 4 : Three consecutive and reversible reactions of transesterification (Luković *et al*., 2011)

2.4.4.2 Transesterification Chemical Reaction

An alkaline or acid catalyst can be used for transesterification of biodiesel. The catalyst used determines yield of biodiesel (Mathiyazhagan and Ganapathi, 2011). An alkaline catalyst is favoured over an acid catalyst for the transesterification reaction. Alkaline catalyst is 4000 times faster than an acid catalyst. Common alkaline catalysts used successfully in the transesterification reaction are sodium hydroxide and potassium hydroxide (Meher *et al*., 2006).

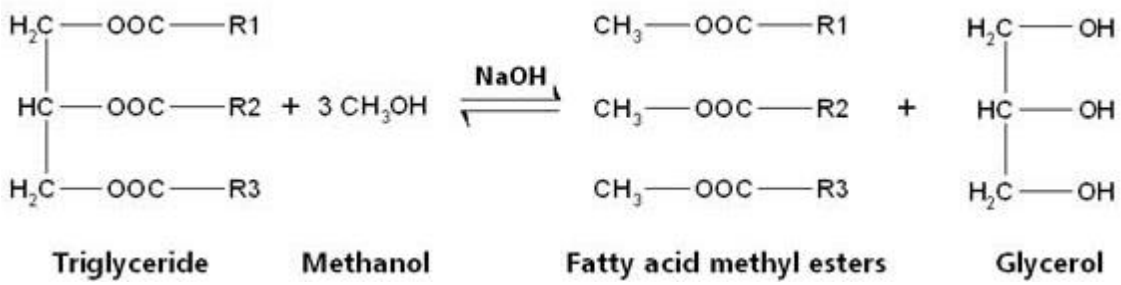

Figure 5 : Chemical transesterification reaction (Babcock et al., 2007)

2.4.4.3 Variables affecting the transesterification reaction

Meher *et al*. (2006) and Sharma *et al*. (2008) pointed out that there are many factors that affect transesterification. These are discussed below:

Free fatty acid and moisture

Factors affecting yield of FAME is the ratio of alcohol to vegetable oil and reaction temperature. An increase in temperature has a positive effect on the transesterification reaction, resulting in increased yields (Meher *et al*., 2006). High acidity value oil decreases conversion efficiency. Ideally triglycerides should have a low acid value. Increasing the level of sodium hydroxide catalyst to reduce acidity results in soap. This increases the viscosity and the condition allows gels to form (Anastopoulos *et al*., 2009). The gels affect the reaction and separation of glycerol, thereby reducing FAME yields (Mathiyazhagan and Ganapathi, 2011). Moisture and carbon dioxide reduces the catalytic effect (Sharma *et al*., 2008). Methanol and an alkaline catalyst are a popular combination for transesterification of edible oils. An excess or insufficient amount of catalyst results in soap formation (Anastopoulos *et al*., 2009; Mathiyazhagan and Ganapathi, 2011). Free fatty acids react with basic catalyst to form soap. This results in the catalyst being neutralised and losing its ability to transesterify the fatty acids (Sharma *et al*., 2008). To overcome this problem a two-step transesterification process has been developed. In the first step the free fatty acids are converted to fatty acid methyl esters by an acid catalysed pre-treatment. The second step completes the transesterification by using an alkaline catalyst to complete the reaction.

Catalyst type and concentration

The main catalysts that can be used for transesterification are alkali and acid (Schuchardt *et al*., 1998). However, enzymes or heterogeneous catalysts could also be used. Alkali catalysts such as sodium hydroxide, sodium methoxide, potassium hydroxide and potassium methoxide are the most effective. NaOH is better than NaOMe. Sodium methoxide causes formation of several by-products; mainly sodium salts, which are considered waste products. The catalyst chosen should be based on the characteristics of the oil (Sharma *et al*., 2008). An acid

catalyst should be used if an oil sample has a high free fatty acid content and water. Recommended acids are sulphuric acid, phosphoric acid, hydrochloric acid or organic sulphuric acid. An alkaline catalyst gives high conversion in a short reaction time (Karmakar *et al*., 2010). Meher *et al*. (2006) expressed concerns using alkalis as catalysts. The concerns regarding the alkaline catalyst process is that it is energy intensive. In addition glycerol recovery is difficult and the catalyst has to be removed from the product. Further, alkaline waste water requires treatment.

Enzyme catalysts such as lipase can catalyse the transesterification in aqueous or non-aqueous systems, therefore eliminating the concerns of Meher *et al*. (2006) mentioned above. The glycerol can easily be removed and free fatty acids can be converted to FAME (Meher *et al*., 2006; Karmakar *et al*., 2010). However, the production costs of lipase catalysts are greater than an alkaline catalyst (Karmakar *et al*., 2010).

Molar ratio of alcohol to oil and type of alcohol

Transesterification requires three moles of alcohol and one mole of triglyceride to yield three moles of fatty acid alkyl esters and one mole of glycerol (**Figure 5**) (Mathiyazhagan and Ganapathi, 2011). Transesterification is an equilibrium reaction. An excess of alcohol is necessary to drive the reaction to the right (Elam, 2008). A molar ratio of 6:1 between catalyst mixture and oil is most efficient for optimal conversion (Anastopoulos *et al*., 2009). The molar ratio has no effect on the oil characteristics such as acid, saponification and iodine values of methyl esters. High molar ratio of alcohol to vegetable oil hinders the separation of glycerol due to an increase in solubility (Anastopoulos *et al*., 2009). Glycerol that remains with the reactants drives the equilibrium to the left, reducing the yield of FAME (Meher *et al*., 2006).

Transesterification with methanol is referred to as methanolysis and transesterification with ethanol is referred to as ethanolysis (García *et al*., 2011). Methanol and ethanol are not miscible with triglycerides at ambient temperature; they have to be mechanically stirred to promote transfer. The stirring causes an emulsion. Emulsions from methanolysis break down rapidly thereby forming a glycerol layer and a FAME layer (Tan *et al*., 2012). Ethanolysis creates a stable emulsion causing the separation and purification of the esters to be

complicated (García *et al*., 2011). The emulsions are a result of the intermediate monoglycerides and diglycerides, which have both polar hydroxyl groups and non-polar hydrocarbon chains which are strong-surface active agents (Meher *et al*., 2006).

Effect of reaction time and temperature

Temperature affects transesterification yields and rates (Mathiyazhagan and Ganapathi, 2011). By increasing temperature the conversion rate increases and reaction time decreases (Sharma *et al*., 2008). The increase of yield was related to the dispersion of methanol.

Mixing intensity

Mixing during transesterification is essential since oils or fats are immiscible with the sodium hydroxide-methanol solution (Mathiyazhagan and Ganapathi, 2011). Once the two phases are mixed and the reaction has started, stirring is no longer needed. No reaction occurs without mixing but stirring speed has no effect on the transesterification reaction results (Meher *et al*., 2006; Sharma *et al*., 2008).

2.5 Plant Tissue Culture

Litsea glutinosa can only be harvested seasonally and this poses a problem because biodiesel as fuel is required through out the year. Plant tissue culture offers a solution to this problem. Therefore experiments were conducted to determine whether callus and hairy root cultures could be obtained from *Litsea glutinosa*. The callus and hairy roots obtained would then be analysed for production of fatty acids to produce FAME. If sufficient FAME are available, characterisation of the FAME would be conducted.

Plant tissue culture is important since disease free clones can be propagated and gene pool can be conserved (Rout *et al*., 2006). Plants and plant tissue cultures can be used to express

foreign proteins, antibodies, vaccines and other therapeutic agents (Shih and Doran, 2009). The advantages of plant tissue culture are:

- synthesis of specialised high quality compounds when plants are out of season,
- plant tissue cells proliferate rapidly when out of season,
- regular generation of plants from seeds are eliminated for production of hairy roots and
- production times of coumpounds are shorter and purification methods are simpler and cheaper.

Cultures are generally initiated from sterile explants (Ruyack *et al*., 1979). Explants are pieces of plant organs. Explants are different parts of the plant, which include: leaves, stems, roots, seeds and flowers. The explant affects the efficiency of culture initiation. Younger, more rapidly growing tissue or tissue at an early stage of development is most effective. There are several different culture types, which are:

- Callus,
- Cell-suspension cultures,
- Protoplasts,
- Shoot tip and meristem,
- Embryo culture and
- Hairy root cultures.

2.5.1 Callus

Callus is unorganised and undifferentiated tissue (Welter *et al*., 1995). It can be grown on media containing hormones such as auxins and cytokinin. Any plant tissue can be used as an explant (Ruyack *et al*., 1979) to start a callus culture. Proliferation of the callus can be maintained provided it is subcultured onto fresh media periodically. During callus formation there is a degree of dedifferentiation in morphology and metabolism. Dedifferentiation refers to the changes that occur during development of the callus. A consequence of dedifferentiation, according to Ożarowsk (2011) is that some plant cultures lose the ability to photosynthesise. Therefore vitamins and a carbon source are added to the culture media, in addition to the usual minerals and nutrients.

Callus culture is usually conducted in the dark because the callus lacks of photosynthetic capability and light can encourage differentiation of the callus. The callus culture may lose the requirement for auxins and / or cytokinins. This process is known as habituation (Ożarowsk, 2011).

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the media can lead to the development of shoots, roots, or somatic embryos from which whole plants are subsequently produced (Tariq *et al*., 2008). Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies (Phillips and Collins, 1981).

Previous research shows that fatty acids can be produced through callus suspension culture (Hirano *et al*., 1997). However, callus cultures has been more difficult to induce in tree species (Sahoo *et al*., 1997).

2.5.2 Cell-suspension cultures

According to Tadeo *et al*. (1995) callus cultures fall into one of two categories: compact or friable. In compact callus, the cells are densely aggregated, whereas in friable callus, the cells are loosely associated with each other and the callus becomes soft and breaks apart easily. Friable callus provides the inoculum to form cell-suspension cultures.

Explants from some plant species or particular cell types tend not to form friable callus. This makes it difficult to initiate cell suspension (Armstrong and Green, 1985). The friability of the callus can sometimes be improved by manipulating the media components or by repeated subculturing. The friability of the callus can be improved by culturing it on semi-solid media.

When friable callus is placed into a liquid media and then agitated, single cells or small clumps of cells are released into the media. Under the correct conditions, these released cells continue to grow and divide, producing a cell-suspension culture. A relatively large inoculum should be used when initiating cell suspensions to obtain rapid growth.

Cell suspensions can be maintained as batch cultures in conical flasks. They are continually cultured by repeated subculturing into fresh media. This results in dilution of the suspension and the initiation of another batch growth cycle.

2.5.3 Protoplasts

Protoplasts are plant cells with the cell wall removed. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions (Atanassov and Brown, 1984). The methods to removing the cell wall are mechanical or enzymatic isolation. Mechanical isolation results in low yields, poor quality, and poor performance in culture due to substances released from damaged cells. Enzymatic isolation is conducted in a salt solution with a high osmotic pressure and cell-wall-degrading enzymes (Vogelzang and Prins, 1992). Enzymatic degradation uses cellulase and pectinase, leading to high quality and purity in results.

Protoplasts are fragile and are easily damaged. Therefore they must be cultured carefully. Liquid media used for propoplasts is not agitated and a high osmotic potential is maintained. Protoplasts can be plated out on to solid media to produce callus. Whole plants can be regenerated by organogenesis or somatic embryogenesis from callus. Protoplasts are ideal for transformation.

2.5.4 Shoot tip and meristem culture

The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro*, producing clumps of shoots from either axillary or adventitious buds. This method can be used for propagation. Shoot meristem cultures are alternatives to the commonly used methods for serial regeneration since they are less genotype dependent and more efficient. Seedlings can be used as donor material.

2.5.5 Embryo culture

Embryos can be used as explants to generate callus cultures or somatic embryos (Özgen *et al.*, 1998). Immature and mature embryos can be used as explants. Immature, embryo-derived embryogenic callus is the most popular method of monocotyledon plant regeneration.

2.6 Hairy Root Culture

2.6.1 Introduction to transformation

Plants produce many useful compounds. Some of these useful compounds maybe produced in small volumes and it may not be viable to produce them commercially in large volumes. Availability of plant material is also a concern for obtaining compounds since plant material may only be collected seasonally or cultivated for supply. Scientists have developed methods to produce these compounds using tissue culture. A method that is cost effective and sustainable to supply these compounds regularly in large quantities is required. Zhoua and Wu (2006) stated that compounds can be extracted in mass production from tissue culture in bioreactors. Agrobacterium transformation is one type of tissue culture developed to produce secondary metabolites. Many useful secondary compounds that are synthesised by plants are terpenes, phenols and alkaloids from hairy roots (Zhoua and Wu, 2006). These compounds can be used in the pharmaceutical and nutraceutical industries. It can also be used as flavours, dyes, fragrances and pesticides.

Pharmaceutical compounds artemisinin is produced from the hairy roots of *Artemisia annua* (Komaraiah *et al*., 2003). Hairy roots of *Saussurea involucrate* produces syringin, rutin and hispidulin (Fu *et al*., 2006). The hairy roots of *Beta vulgaris* and *Polygonum tinctorium* produce dyes such as betaxanthin and indigo respectively (Komaraiah *et al*., 2003). Garlic flavour is produced from the hairy roots of *Allium sativum* (Rao and Ravishankar, 2002). Hairy roots of *Leontopodium alpinum* produce essential oil and fragrances (Karuppusamy, 2009).

Agrobacterium rhizogenes 15834 is a Gram negative soil bacterium that causes hairy root disease in dicotyledonous plants (Nilsson and Olsson, 1997). The hairy roots are caused by a virulence plasmid called Ri-plasmid which is similar to the Ti-plasmid found in *Agrobacterium tumefaciens*, which causes crown gall tumors of plants. The production and transport of T- DNA into the host cell depends on proteins that are encoded by a set of bacterial chromosomes (*chv*) and Ri-plasmid virulence (*vir*) genes (Tzfira and Citovsky, 2006).

The Ri–plasmid contains DNA which is transferred into the plant genome during infection. The transfer of the DNA to the plant genome is assisted by a plasmid called the virulence (*vir*) region. The transferred DNA (T-DNA) carries a set of oncogenes and opine catabolism genes; which causes neoplastic growth of the transformed tissue (Tzfira and Citovsky, 2006). It causes the transformed tissue to produce modified amino acids (opines), which are utilised by the inciting bacteria as their carbon, nitrogen and energy source (Mugnier, 1988). *Agrobacterium rhizogenes* is therefore a parasite.

2.6.2 The genetic transformation process

There are 10 steps for genetic transformation according to (Tzfira and Citovsky, 2006), as shown in **Figure 6**. (1) Agrobacterium recognises and attaches to its host. (2) The injured plant produces phenols that signal the *VirA*/*VirG* to commence transduction. (3) The *vir* gene region is activated. (4) A mobile copy of T- DNA is made by *VirD1*/*VirD2* protein complex. (5) The immature T-complex (*VirD2*-DNA) together with other *Vir* proteins enters the host cell. (6) The mature T-complex (T-strand and *VirE2*) travels through the host cell. (7) The T-complex is actively transported through the host cell. (8) Inside the nucleus the T-DNA is taken to the site of integration. (9) The escorting proteins are striped. (10) The T-DNA is integrated into the host cell. These ten steps can be seen in the **Figure 6**.

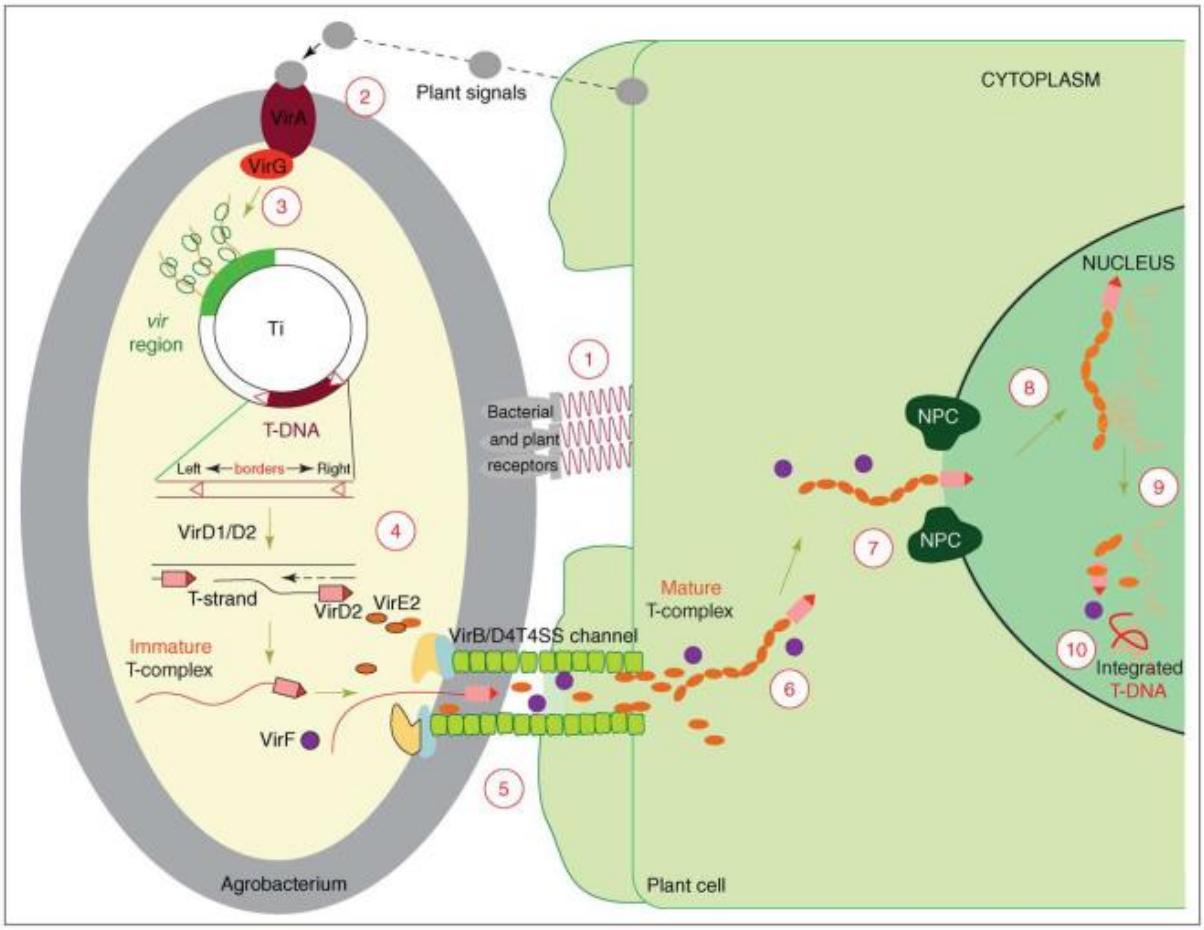

Figure 6: Genetic transformation of *Agrobacterium rhizogenes* (Tzfira and Citovsky, 2006)

2.6.3 Function and Advantages

Agrobacterium rhizogenes, is a soil pathogen used to obtain genetically transformed hairy roots which have many advantages (Chabaud *et al*., 2006). The neoplastic roots produced by *Agrobacterium rhizogenes* infection are characterised by a high growth rate (Gangopadhyay *et al*., 2008) and genetic stability (Pietrosiuk *et al*., 2007). The transformation creates "composite plants" which consists of a transgenic hairy root attached to non-transformed explants (Chabaud *et al*., 2006). The hairy roots can grow in hormone-free and low nutrient salt media (Sudha *et al*., 2003; Pietrosiuk *et al*., 2007). They grow faster and produce a higher yield of metabol *Agrobacterium rhizogenes* ites than cell suspension cultures (Gangopadhyay *et al*., 2008). Transformation *Agrobacterium rhizogenes* is a rapid and simple technique. Production of transformed roots takes a few weeks compared to other transformation methods that take months (Chabaud *et al*., 2006). Many strains are developed

for the transformation process to expand host range and maintain control to produce hairy roots (Tzfira and Citovsky, 2006).

The focus of this project is Characterisation of Biodiesel from *Litsea glutinosa*. Plant tissue culture has benefits of plant tissue cells proliferating rapidly, shorter production times and simpler cheaper purification methods. Further culture conditions can be manipulated and maintained to obtain high quality cultures. Hence the choice to experiment with hairy root culture and callus culture. Hairy root culture is favoured because it grows faster and produces a high yield of metabolites. If callus culture of *Litsea glutinosa* is successful it could be produced in a tank for production of FAMEs. Plant tissue culture could be investigated to assist in producing all year supply of FAMEs from *Litsea glutinosa*.

CHAPTER THREE: METHODOLOGY

3.1 Biodiesel Production

Figure 7 provides an overview of the production of biodiesel. The raw materials were collected and the oil was extracted. The oil was then transesterified to biodiesel and characterised. The biodiesel properties determined were iodine value, saponification number, acid value, viscosity, kinematic viscosity, density / specific gravity, fatty acid methyl ester profile, theromstability, distillation point and sulphur.

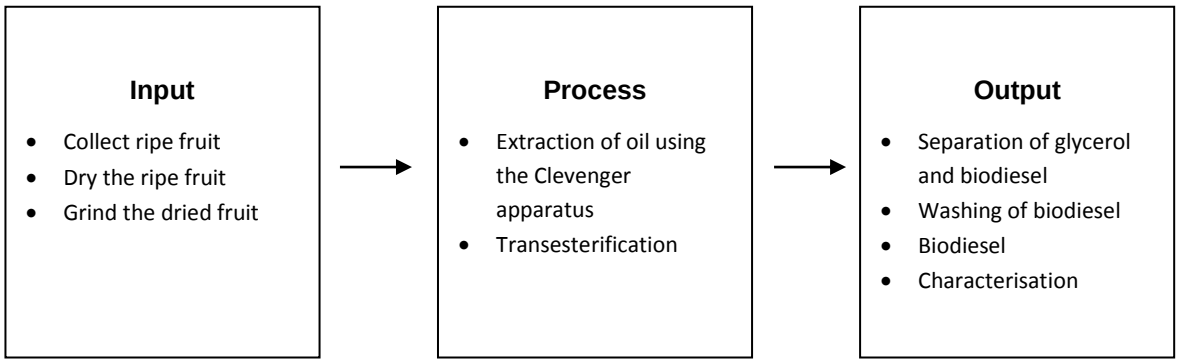

Figure 7: Figure outlining biodiesel production (Perumal, 2012)

3.2 Extraction of oil from *Litsea glutinosa*

The ripe fruits were collected during the months of February and March from mature trees found at the Currie's Fountain car park on M.L. Sultan Campus of the Durban University of Technology. The fruits were washed to remove sand and dirt.

The fruit flesh and the seeds were separated. Some of seeds were used to germinate sterile plants. The fruit flesh and balance of the seeds were dried. Both the dried fruit flesh and the dried seeds were crushed separately to obtain a coarse powder and stored at room temperature. The bulk of the whole fruit (fruit flesh and seeds) that was dried in the oven

were also crushed separately and stored at room temperature. Hence there were three sets of crushed coarse powder of plant material to extract oil from. To summarise there were:

- coarse powder from the dried fruit flesh;
- coarse powder from the dried seed;
- coarse powder from the dried whole fruit.

The research elaborates on the oil extraction from the course powder of the dried whole fruit and the transesterification thereof. The same method of oil extraction and transesterification was used for the powder of dried fruit flesh and dried seed.

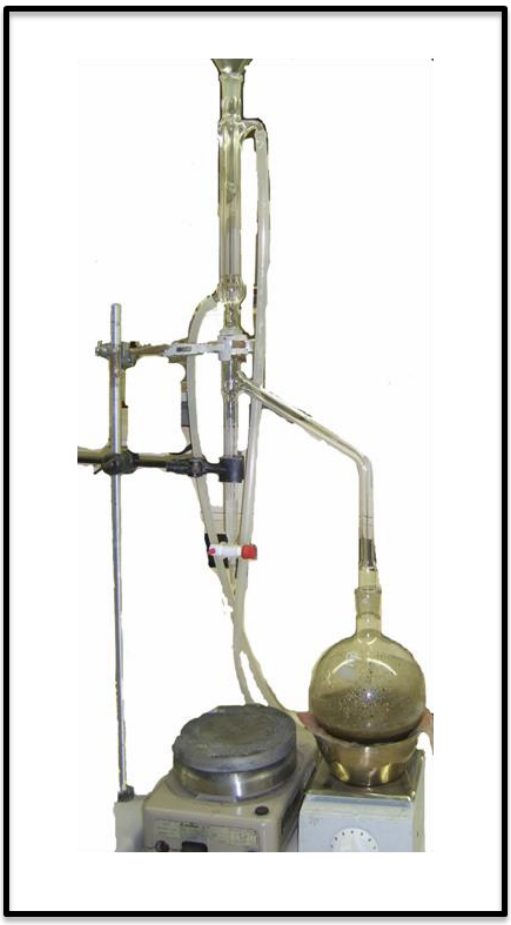

Figure 8: Clevenger apparatus (Perumal, 2009)

The fatty acids were extracted from the powder of the whole fruit using hydro-distillation. A mass of 300 g of powder was weighed out and placed in a 5 L round bottom flask with 350 ml of dichloromethane. The flask was then attached to a modified Clevenger apparatus (Esquível *et al*., 1998) shown in **Figure 8**. The temperature was maintained at 40°C for 1 hr. The

powdered plant material was continuously rinsed with the solvent (DCM) at three day intervals to remove all the oil.

The seeds that floated in the dichloromethane were removed and placed in a separating flask (**Figure 9**). The oil was extracted from the seeds by a washing procedure. The washing procedure was conducted by placing the seeds in a separating flask, shown in **Figure 9**. The seeds were rinsed by swirling in dichloromethane. The separating flask was left to stand until the seeds and the dichloromethane separated. The tap was opened and the dichloromethane was collected. The washing process was repeated until the dichloromethane remained clear. The oil samples were centrifuged in 50 ml centrifuge tubes at 4.4 rpm for 20 min to separate the oil and the plant material. The oil phase was collected and the excess solvent was removed in a rotary evaporator at 60°C at 220 rpm.

Figure 9: Washing the plant material to extract the oil (Perumal, 2011)

3.3 Transesterification

Transesterification of the oil was carried out in the presence of nitrogen and an excess of methanol. The reaction was conducted in a 250 ml flask with two openings. One opening

pumped nitrogen into the oil and the other opening allowed the gas to escape so as to prevent the pressure from building up. The method for the transesterification reaction is as follows:

A catalyst of methanol and NaOH in a ratio of 7 : 1 was prepared. An oil sample of 60 ml was weighed and placed in a 250 ml flask with 180 ml of the prepared catalyst mixture. This was in keeping with the molar ratio of three moles of alcohol to one mole of triglyceride. The reaction was conducted on a hot plate at 65°C with a stirrer bar in a bowl of liquid paraffin, for 4 hr at 600 rpm (Vicente *et al.*, 2003). The temperature was monitored with a thermometer placed into the liquid paraffin. The transesterification apparatus is shown in **Figure 10**.

Figure 10: Transesterification equipment (Perumal, 2011)

After transesterification the oil was placed in a separating funnel and the glycerol was allowed to settle out from the oil (**Figure 11**). The FAME, the glycerol and the catalyst mixture had to be separated before it could be tested or used. The FAME was washed using two methods; wet washing and dry washing (Demirba, 2002; Faccini *et al.*, 2011).

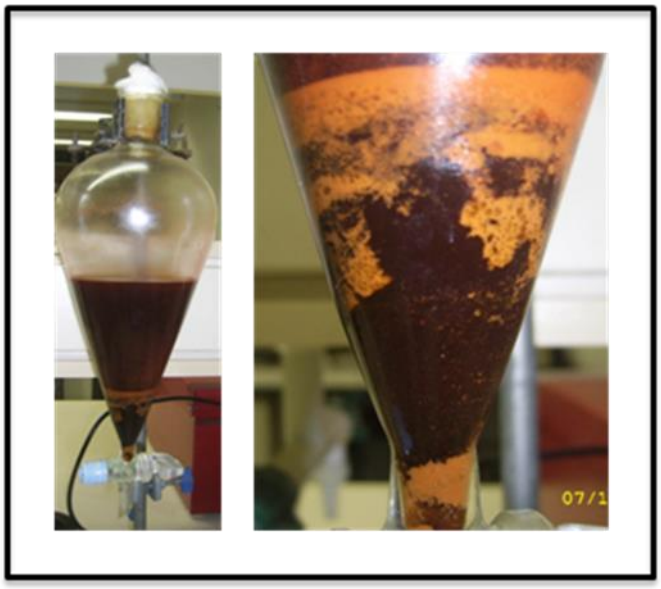

Figure 11: Biodiesel, glycerol and orange precipitate (Perumal, 2011)

Wet washing was carried, using a two step process, according to Faccini *et al*. (2011):

Firstly, a water wash solution (1 g of tannic acid per L of water) was added to the ester and gently agitated. The water solution was left to stand until the oil rose to the top of the mixture. Secondly, the waste solution and the oil were separated using a separating funnel (**Figure 12**).

Figure 12: Wet washing of the biodiesel (Perumal, 2011)

Dry washing was carried out according to the methods prescribed by Faccini *et al*. (2011). Amberlite, obtained from GC Biofuels, was placed in a separating funnel. The transesterified oil was poured into the separating funnel, shown in **Figure 13**. The biofuel was then placed in the rotary evaporator to remove the excess solvent from the biofuel. The rotary evaporator (Heidolph Laborata 400 efficient) with the water bath set at a temperature of 50°C and the flask rotated at 60 rpm.

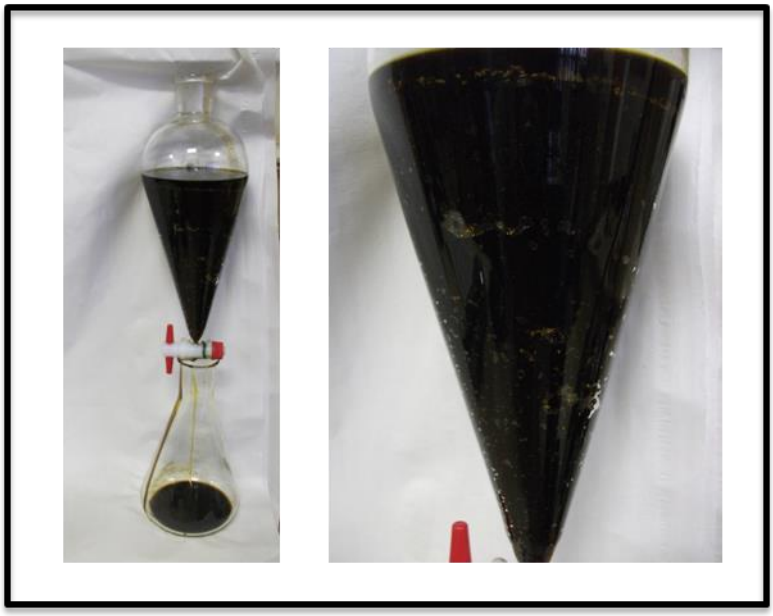

Figure 13: Dry washing of biodiesel (Perumal, 2011)

3.4 Characterisation of transesterified oil

Testing the FAME to determine biofuel characteristics were performed at:

- SAPREF (South African Petroleum Refinery) Durban,
- The Chemistry Department, M. L. Sutan Campus, Durban University of Technology,
- The Mechanical Engineering Department, Steve Biko Campus, Durban University of Technology and
- The Chemistry Department, Westville Campus, University of KwaZulu-Natal.

3.4.1 Iodine value

The iodine value was determined according to the method prescribed by Morio (1931). The oil sample of *Litsea glutinosa* was filtered into a flask using a funnel with Whatman No. 1 filter paper and anhydrous sodium sulphate (Na_2SO_4). This was done to remove solid debris and small amounts of water. Anhydrous sodium sulphate (Na_2SO_4) was placed on the filter paper to remove any water in the oil. The oil sample of *Litsea glutinosa* was then incubated in a water bath at 70°C for 30 min. A 1.0 g sample was weighed into an Ehrlenmeyer flask with a ground glass 24 / 40 joint. A volume of 20 ml of cyclohexane was added to the flask to dissolve the sample and 25 ml of Wijs Solution was pipetted into a flask. The flask was sealed with a glass stopper and placed in an incubator at 25 °C for 2 hr. A blank was prepared following the same method described above. However, the oil sample of *Litsea glutinosa* was not placed in the flask.

A 0.1 N solution of sodium thiosulphate was made by dissolving 15 g of KI in 100 ml distilled water in a volumetric flask. After 2 hr 20 ml of KI solution was added followed by 100 ml of distilled water into the Ehrlenmeyer flask. The solution was titrated with 0.1 N sodium thiosulphate. Soluble starch solution was used as an indicator. The indicator was added when the yellow-brown colour almost disappeared from the flask. The end point was reached when the blue / brown colour disappeared from the flask. The titration was conducted in triplicate. The volume of sodium thiosulphate was recorded.

The iodine value was calculated using **Equation 1**:

Equation 1: Iodine value equation

$$IV = \frac{(\text{ml } Na_2S_2O_3 \text{ for blank} - \text{ml } Na_2S_2O_3 \text{ for sample}) \times Na_2S_2O_3 \text{ normality } 12.69}{\text{sample weight (g)}}$$

The iodine value results are discussed in Section 4.3.1 in Chapter four.

3.4.2 Saponification value

The saponification value was calculated according to the method developed by Englis and Reinschreiber (1949). The oil sample of *Litsea glutinosa* was filtered using a funnel with Whatman No. 1 filter paper and anhydrous sodium sulphate (Na_2SO_4) into a flask. This was done to remove solid debris and small amounts of water. Anhydrous sodium sulphate (Na_2SO_4) was placed on the filter paper to remove any water in the oil. An oil sample of 5.000 g was weighed into a flask. A volume of 50 ml of methanolic KOH was added in the flask. A blank was prepared following the same method. However, the sample was not placed in the flask. The flasks were incubated in a water bath for 2 hr until saponification was complete. The indicator phenolphthalein was added to the flask after the samples were cooled. The samples were titrated with hydrochloric acid (HCl) until a pink colour was observed for 15 to 30 seconds, indicating that the end point was reached. The titration was conducted in triplicate. The volume of hydrochloric acid was noted.

The saponification value was calculated using **Equation 2**:

Equation 2: Saponification value equation

$$\text{Saponification value} = \frac{(\text{ml HCl for blank ml HCl for sample}) \times \text{HCl normality } 56.1}{\text{sample weight (g)}}$$

The saponification value results are discussed in Section 4.3.2 in Chapter four.

3.4.3 Acid value

The acid value was determined according to Mahajan *et al*. (2006). A volume of 100 ml of the oil sample of *Litsea glutinosa* was filtered using a funnel with Whatman No.1 filter paper and anhydrous sodium sulfate (Na_2SO_4) into a flask. This was done to remove solid debris and small amounts of water. An oil sample weighing 7.05 g of sample was weighed into a flask. The titration was conducted with neutralised methanolic. Phenolphthalein (a pH indicator) was added and the solution was swirled to obtain a homologous solution. The samples were titrated with sodium hydroxide solution until a pink colour was observed, indicating the end point. The titration was conducted in triplicate. The volume of sodium hydroxide was noted.

The acid value was calculated using **Equation 3**:

Equation 3: Acid value equation

$$\text{Acid Value} = \frac{\text{(ml NaOH) X NaOH normality}}{\text{sample weight (g)}}$$

The acid value results are discussed in Section 4.3.3 in Chapter four.

3.4.4 Viscosity

The viscosity was determined using a Cannon Digital Viscometer. This test method covers and specifies a procedure for the concurrent measurement of both the dynamic viscosity, η, and the density, ρ, of liquid petroleum products and crude oils, both transparent and opaque. SAPREF, Durban determined the viscosity of the oil sample of *Litsea glutinosa* following the ASTM method. The results are discussed in Section 4.3.4 in Chapter four.

3.4.5 Kinematic viscosity

The viscosity was determined using a Cannon Digital Viscometer. A test method that the supplier has developed specifies a procedure for the determination of the kinematic viscosity, *v*, of liquid petroleum products, both transparent and opaque, by measuring the time for a volume of liquid to flow under gravity through a calibrated glass capillary viscometer was used. The dynamic viscosity, η, can be obtained by multiplying the kinematic viscosity, *v*, by the density, ρ, of the liquid. The kinematic viscosity was determined at SAPREF, Durban using the oil sample of *Litsea glutinosa*. The results are discussed in Section 4.3.5 in Chapter four.

3.4.6 Density / Specific gravity

SAPREF, Durban used an Anton Paar Density Meter to determine density and specific gravity of the oil sample of *Litsea glutinosa*. This test determines the density, relative density and specific gravity of petroleum distillates. Its application is restricted to liquids with total vapour pressures typically below 100 kPa and viscosities typically below 15 000 $mm^2.s^{-1}$ at the temperature of the test. The total vapour pressure limitation, however, can be extended to greater than 100 kPa, provided that it is first ascertained that no bubbles form in the U-shaped, oscillating tube, which can affect the density determination. Some examples of products that may be tested by this procedure include: gasoline and gasoline-oxygenate blends, diesel, base stocks, waxes and lubricating oils. SAPREF, Durban determined the density and specific density following the ASTM method. The results of the density and specific gravity are discussed in Section 4.3.6 and 4.3.7 respectively in Chapter four.

3.4.7 Distillation point

Measurement of the distillation point determines the volatility of biofuel as temperatures increase. The distillation point test determines the atmospheric distillation of petroleum products using a Laboratory Batch Distillation Unit to quantify the quantitative and the boiling range characteristics of such products as light and middle distillates, automotive spark-ignition engine fuels with or without oxygenates, aviation gasoline, aviation turbine

fuels, diesel fuels, biodiesel blends up to 20%, marine fuels, special petroleum spirits, naphthas, white spirits, kerosene and grade one and two burner fuels. The distillation point was determined at SAPREF, Durban following the ASTM method. The results are discussed in Section 4.3.8 in Chapter four.

3.4.8 Thermostability

The themostability test covers relative stability of middle distillate fuels under high temperature aging conditions with limited air exposure. Thermographs indicate weight change, and exothermic and endothermic reactions as temperature increases. Thermogravimetric Analysis (TGA) of *Litsea glutinosa* oil sample was conducted in the, Mechanical Engineering Department, DUT. The Mechanical Engineering Department performed the analysis on the Analyzer SDT Q600. The oil sample of *Litsea glutinosa* was analysed in an alumina pan in dry air atmosphere at 100 ml. min^{-1}. A heating rate of 10.00°C. min^{-1} to 600.00°C. min^{-1} was selected. The temperature range was set between 0.00°C to 600.00°C (Jain and Sharma, 2012). The results are discussed in Section 4.3.9 in Chapter four.

3.4.9 Sulphur

High sulphur contents in fuel have been associated with negative heating effect and an increase service frequency of vehicles. Oxford Instrument XRF SULPHUR ANALYSER[1] was used to detect the amount of sulphur present in the oil sample of *Litsea glutinosa*. XRF represents X-Ray Fluorescence Spectrometry. The test is to determine the total sulphur in petroleum and petroleum products that are single-phase and either liquid at ambient conditions, liquefiable with moderate heat or soluble in hydrocarbon solvents. The test covers materials such as diesel fuel, jet fuel, kerosene, other distillate oil, naphtha, residual oil, lubrication base oil, hydraulic oil, crude oil, unleaded gasoline, gasohol, biodiesel and similar petroleum products. The sulphur test was conducted at SAPREF, Durban following their method. The results are discussed in Section 4.3.10 in Chapter four.

3.4.10 Gas Chromatography

The Gas-Chromatography (GC) analysis was conducted in the Chemistry Department, DUT on the ML Sultan Campus. A volume of 1 ml of the *Litsea glutinosa* oil sample was dissolved in 3 ml of hexane. The standards: stearic acid, arachadonic acid, oleic acid, lineolic acid and the 37 FAME mix standards, purchased from SIGMA, were liquid. The 37 FAME standard mix was required at a concentration of 10 mg. kg^{-1}. To obtain a 10 mg. kg^{-1} solution, 0.25 ml of the 37 FAME mix was dissolved into 5 ml of Dichloromethane. The same ratio was used to prepare the stearic FAME, arachadonic FAME, oleic FAME and lineolic FAME standards. The *Litsea glutinosa* oil sample and the prepared standards were analysed separately. The GC analysis was conducted under the following conditions. The column used was a SPTM – 2560, 100 m x 0.25 mm ID and 0.2 μm film thickness. The column was borrowed from SAPREF, Durban. The analysis was conducted on Varian 3800 GC, using Galaxy software. The oven was set at 140°C (5 min) to 240°C at 4°C . min^{-1}. Helium was the carrier gas used and a flow rate of 20 cm. s^{-1} was set. The detector used was FID, which was held at 260°C for the duration of the GC analysis. The injector was set at 1 μL at 260°C and a split 100 : 1. The results are presented in **Figure 24** and **Figure 25**. The results are discussed in Section 4.3.11 in Chapter Four.

3.4.11 Gas Chromatography-Mass Spectrometry (GC-MS)

The GC-MS analysis of the *Litsea glutinosa* oil sample was performed at Chemistry Department, UKZN on the Westville Campus, on an Agilent GC 6890 model gas chromatograph - 5973N model mass spectrometer equipped with a 7683 series auto-injector (Agilent, USA). A DB-5MS column (30 m × 0.25 mm × 0.25 μM film thickness) was used. Temperature program was set from 80°C to 280°C in 1 to 20 min. Injection volume was 1 μL and inlet pressure was 38.5 kPa. Helium was used as the carrier gas, with a linear velocity (u) of 31 cm. s^{-1}. Injection mode was split (75 : 5). MS interface temperature was 230°C. MS mode was EI, detector voltage was 1.66 Kv, mass range was 10 - 700 u, scan speed was 2.86 scan/s and interval was 0.01 min (20 Hz). The results are discussed in Section 4.3.11 in Chapter Four.

3.5 Fruit and Seeds

The fruit, including the seed, was used to extract oil for transesterification to produce FAMEs.

Transformation with *Agrobacterium rhizogenes* on field grown explants of *Litsea glutinosa* to obtain hairy roots, were attempted. This transformation was unsuccessful since the *Agrobacterium rhizogenes* was killed by a fungal contamination that survived the explant sterilising process. Sterile seedlings were grown to transform the plant. The long germination period for the seeds presented difficulties in obtaining sterile seedlings. The scarcity of sterile seedlings hampered both hairy root and callus tissue culture.

3.6 Germination of *Litsea glutinosa*

The seeds were removed from the flesh of the fruit. They were then washed, dried with a paper towel and air dried. The seeds were stored at room temperature. Germination of the seeds were was attempted by placing them in cotton wool soaked in distilled water, filter paper soaked in distilled water, distilled water, M.S media, Water agar and McCowns woody plant basal salt media.

3.6.1 Germination time frame

Germination of unsterilised seeds were conducted using three different methods: first on damp cotton wool, second on filter paper soaked in distilled water and third in distilled water to determine the germination time frame. Germination was attempted using seeds with the seed coat and seeds without the seed coat. Seeds were placed both in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$ and in natural light on the bench top. The plates with the seeds were place in different environments to determine factors that would result in optimum germination results. Special attention was focused on how the seeds reacted in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$.

3.6.2 Germination of sterile plants

Three media were prepared to germinate sterile seeds. Water agar was made up of 8 g of bacteriological agar to 1 L of distilled water. The second medium, MS media, which was purchased from SIGMA, was made by following the instructions on the package (12 g. L^{-1}). The Water Agar and MS Agar were sterilised in an autoclave and poured into sterilised culture bottles. The media was left to cool overnight.

The third medium, McCowns woody plant basal salt media, was also made and sterilised in an autoclave. 0.5 ml. L^{-1} of 6-benzylaminopurine (BAP), a plant growth hormone, was filter sterilised into the cooled sterilised media. The medium was then poured into the culture bottle, using standard aseptic techniques on the tissue culture lamina flow bench. The medium was left to cool overnight.

The seeds of *Litsea glutinosa* were sterilised in 30% NaClO, (Abdul – Baki, 1974; Baiyeri and Mbah, 2006). The 30% NaClO was made up of 30 ml sodium hypochlorite, 70 ml sterilised distilled water and 1 ml Tween 20. The seeds were placed in a 50 ml beaker and sterilised for 5 min, while being swirled periodically. The seeds were then rinsed three times in sterilised distilled water.

Three to four sterilised seeds were placed into each culture bottle. Some of the seeds were nicked with a sterile blade. The bottles were then sealed with parafilm. The culture bottles were placed in the 16 hr photoperiod growth chamber at 26^{o}C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}.m^{-2}$.

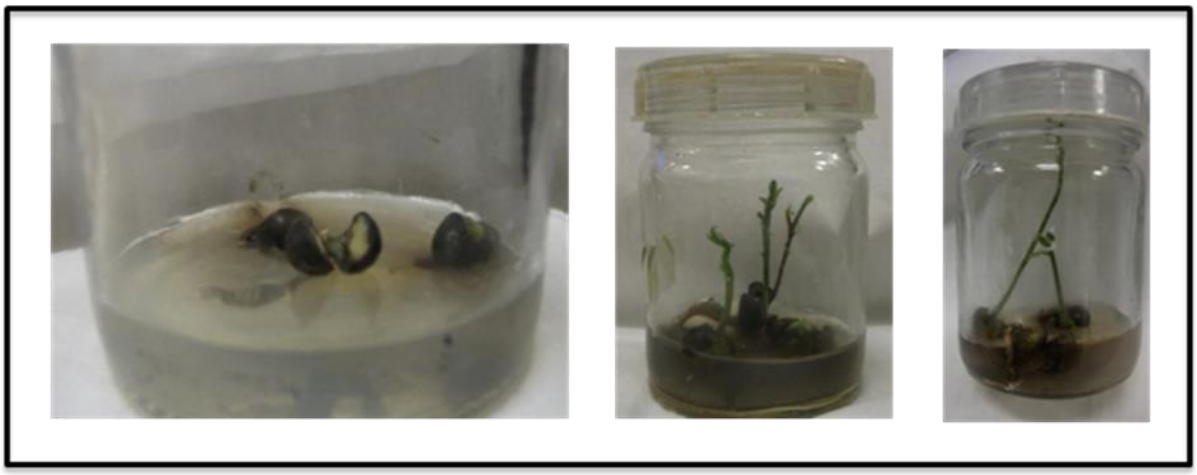

Figure 14: Germination of *Litsea glutinosa* (Perumal, 2012)

3.6.3 Germination of *Nicotiana tabacum*

Nicotiana tabacum was used as a control for this experiment. The seeds of *Nicotiana tabacum* were sterilised in 30% NaClO, (Abdul – Baki, 1974; Baiyeri and Mbah, 2006). The 30% NaClO was made up of 30 ml sodium hypochlorite, 70 ml sterilised distilled water and 1 ml Tween 20. The seeds were placed in a 50 ml beaker and sterilised for 5 min, while swirling periodically. The seeds were then rinsed three times in sterilised distilled water. The seeds were germinated on MS medium in petri plates (Amayaa *et al*., 1999) with pseudo-hormones. The Petri plates were then placed in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$.

3.7 Transformation

Transformation of the leaves of *Litsea glutinosa* was carried out using three methods adapted from Cao *et al*. (2008).

3.7.1 Induction of field grown *Litsea glutinosa*

The leaves were collected and the surface sterilised. The leaves were soaked in 30% NaClO for 15 min in a sterile beaker (Ramakrishna *et al*., 1991) as soaking for a shorter period thus far had not been effective in producing sterile leaves. The leaves were then washed in sterile H_2O.

Method One

Agrobacterium rhizogenes 15834 was cultured on Yeast Extract Peptone (YEP) Agar plates at 30 °C for two days (**Figure 15**). A Gram stain was conducted on the cultured *Agrobacterium rhizogenes* as a confirmation test; since it is Gram negative. The *Agrobacterium rhizogenes* was prepared for transformation by inoculating a loop of *Agrobacterium rhizogenes* into Yeast Extract Peptone (YEP) broth medium (de la Riva *et al*., 1998). The YEP broth inoculated with *Agrobacterium rhizogenes* was placed on a shake flask at 30°C at 180 rpm (Zhao *et al*., 2004) and allowed to grow overnight. Cao *et al*. (2008) and Vernade *et al.* (1988) indicated that at OD_{600} an absorbance of 0.2 of *Agrobacterium rhizogenes* broth yields the best transformation result. MS media was prepared by following the instructions on the packaging.

The scalpel and forceps were placed into 70% ethanol and flamed. The leaves and stems of the plant were infected by two approaches.

1. A scalpel was swabbed with the *Agrobacterium rhizogenes* dilution. The leaves were injured along the veins on the back of the leaf (Zhao *et al*., 2004) with the infected scalpel and placed on the prepared MS media.

2. The veins on the back of the leaves were injured with a sterile scalpel. A swab was placed in the *Agrobacterium rhizogenes* dilution. The stems and leaves were then infected with the swab and then placed on the prepared MS media.

The leaves were incubated in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$.

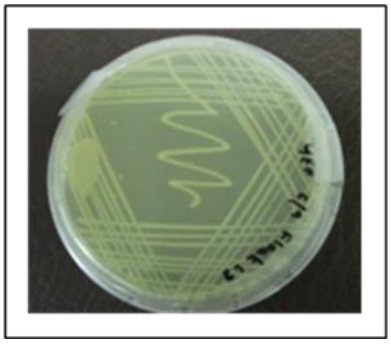

Figure 15: ***Agrobacterium rhizogenes on MS agar*** **(Perumal, 2012)**

Method Two

In Method Two *Agrobacterium rhizogenes* was prepared according to Method One for the transformation of explants of *Litsea glutinosa*. However, the *Agrobacterium rhizogenes* inoculated Yeast Extract Peptone (YEP) broth media (de la Riva *et al*., 1998) was supplemented with acetosyringone. Acetosyringone is a phenolic compound that activates the transformation gene. Vernade *et al*. (1988); Fortin *et al*. (1992) and Loubens *et al*. (1997) suggested that acetosyringone can enhance the transformation. The acetosyringone concentration of 10^{-4} was supplemented into the YEP broth. A stock of acetosyringone was prepared by diluting 10 mg of acetosyringone to 10 ml of dimethyl sulfoxide (DMSO) (Kumar *et al*., 2005). A concentration of 10^{-4} was achieved by adding 5 µl of acetosyringone stock to 50 ml of YEP broth. MS media was prepared by following the instructions on the packaging. The explant infected with the *Agrobacterium rhizogenes* broth was place on the prepared MS media. The transformation procedure was conducted according to the two approaches in Method One stated in 3.7.1 above. In addition two different absorbances were used to measure for transformation. An absorbance of 0.209 was used as suggested by Vernade *et al*. (1988) and Cao *et al*. (2008) as well as an absorbance of 0.130 at OD_{600}. The leaves were placed on MS media plates and incubated in the growth chamber at 16 hr photoperiod at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$.

Method Three

In Method Three *Agrobacterium rhizogenes* was prepared according to Method One for the transformation of explants of *Litsea glutinosa* and acetosyringone stock prepared as per Method Two above. The MS media was prepared and supplemented with different concentration of acetosyringone stock to MS media; 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A volume of 50 ml of MS media was made for each concentration. A concentration of 10^{-1} was achieved by adding 5 ml of acetosyringone stock to 50 ml of MS media. A concentration of 10^{-2} was achieved by adding 0.5 ml of acetosyringone stock to 50 ml of MS media. A concentration of 10^{-3} was achieved by adding 50 µl of acetosyringone stock to 50 ml of MS media. A concentration of 10^{-4} was achieved by adding 5 µl of acetosyringone to 50 ml of MS media. The MS media was left to set overnight. The transformation procedure was conducted according to the approach one in Method One described in 3.7.1 as above. The leaves were placed on MS media plates and left in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 µmols^{-1}. m^{-2}.

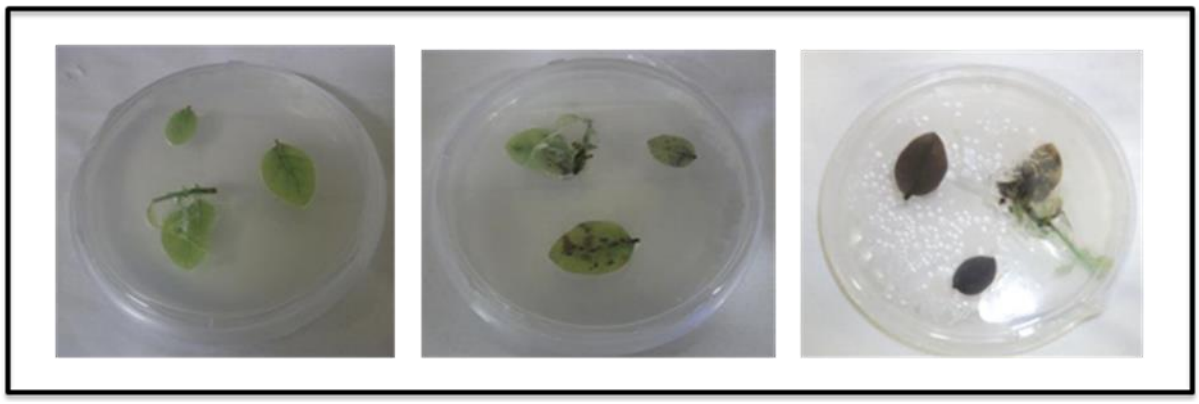

Figure 16: Failed transformation of *Litsea glutinosa* (Perumal, 2012)

3.7.2 Induction of hairy roots from *Nicotiana tabacum* seedling grown *in-vitro*.

Nicotiana tabacum was grown *in-vitro*. The leaves were transformed in the same manner the leaves of *Litsea glutinosa* were transformed in Section 3.7.1 above. However the leaves did

not need to be sterilised before inoculation because the plants were grown *in-vitro*. An example of sucessful transformation is shown in **Figure 17**.

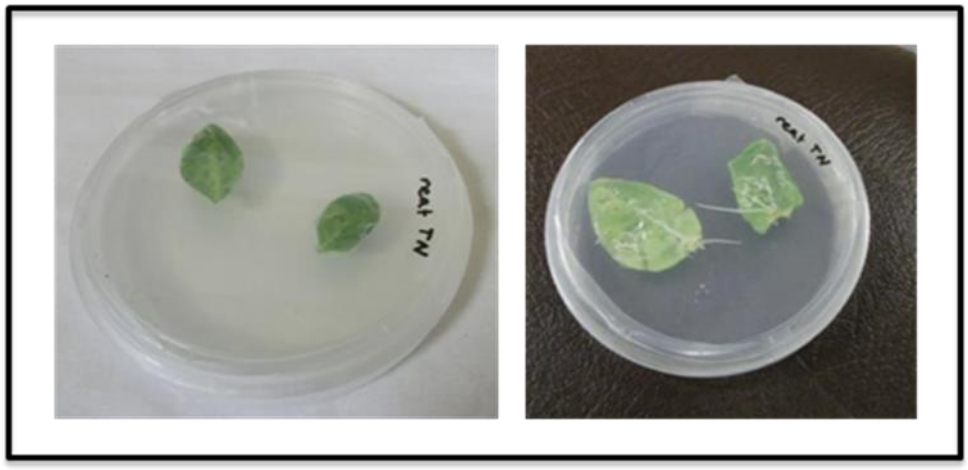

Figure 17: Successful transformation of *Nicotiana tabacum* (Perumal, 2012)

3.7.3 Maintenance of hairy roots

Hairy roots must be moved to antibiotic media which contains cefotaxime, to prevent the bacteria from proliferating and competing with the hairy roots for nutrients. The cefotaxime is heat sensitive and therefore it was filter sterilised into the MS media before being poured onto the plates. The hairy roots were maintained in half strength MS salts liquid media supplemented with 0.5 mg 1 - 1 indole-3-acetic acid (IAA) and 30 g 1 - 1sucrose, with 16 hr daily light (45 μmol $.m^{-2}$ $.s^{-1}$) at 25°C, on a rotary shaker (90 rpm).

3.8 Callus

Seven methods were used to induce callus culture. The reason for this is explained in the discussion in Section 4.6 in Chapter four.

3.8.1 Method One

MS media was prepared and supplemented with 1 mg 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 mg 6-benzylaminopurine (BAP) into 1 L of MS medium (Chakravarty and Goswami, 1999). The two chemicals were filter sterilised into the MS medium after the media was autoclaved and cooled. The *in vitro* leaves of *Litsea glutinosa* were used. The leaves were cut and placed on the prepared media and incubated in a 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$. The callus obtained was divided and sub-cultured onto fresh media regularly (Thomas and Maseena, 2006). Some plates were covered in foil and some plates were not foiled, to determine whether callus induction was affected by light. The calli obtained were grown in a broth media, known as cell suspension culture.

3.8.2 Method Two

Woody plant media was prepared and supplemented with 1 mg 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 mg 6-benzylaminopurine (BAP) into 1 L woody plant media (Chakravarty and Goswami, 1999). The two chemicals were filter sterilised into the media after the media was autoclaved and cooled. The *in vitro* leaves of *Litsea glutinosa* were cut and placed on the prepared media and incubated in a 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$. The callus obtained was divided and sub-cultured on fresh media regularly (Thomas and Maseena, 2006). Some plates were covered in foil and some plates were not foiled to determine whether callus induction was affected by light. The calli was maintained in a broth media, known as cell suspension culture.

3.8.3 Method Three

MS media and woody plant media were prepared separately and supplemented with 1 mg 2,4-dichlorophenoxy acetic acid (2,4-D) and 1 mg 6-benzylaminopurine (BAP) to produce 1 L of MS media and 1 L woody plant media (Chakravarty and Goswami, 1999). The two chemicals were filter sterilised into the media after the media was autoclaved and cooled. A

germinated seed was placed in the MS media plates and woody plant media plates and left under the fluorescent light in the tissue culture laboratory to determine whether the media would promote callus induction with the explants utilised.

3.8.4 Method Four

Plates of MS medium and woody plant medium were prepared as per Method Three in Section 3.8.3. However, seeds with their coats were used in Method Four as compared to germinated seeds used in Method Three. The seeds were sterilised in 30% NaClO for 5 min. The seeds were placed on the MS medium plates and woody plant medium plates. The plates were placed under the fluorescent light in the tissue culture laboratory to determine whether the media would promote callus induction with explants utilised.

3.8.5 Method Five

Plates of MS medium and woody plant medium were prepared as per Method Three in Section 3.8.3. Field grown leaves were used. The leaves were sterilised following the method used to sterilise the seed in Method Four. However, the sterilisation time was increased to 5 min in 30% NaClO. The plates were placed under the fluorescent light in the tissue culture laboratory to determine whether the media would promote callus induction with explants utilised.

3.8.6 Method Six

Plates of MS medium and woody plant medium were prepared as per Method Three in Section 3.8.3. Field grown explants were used. The leaves were sterilised following the method used to sterilise the seed, explained in Method Four. However the concentration of NaClO was increased to 50% and the sterilisation time was increased to 35 min, in an attempt to overcome the fungal contamination. The leaves were further left in a 0.1% amphotericin B solution overnight in a further attempt to reduce fungal contamination. The plates were

placed under the fluorescent light in the tissue culture laboratory to determine whether the medium would promote callus induction with explants utilised.

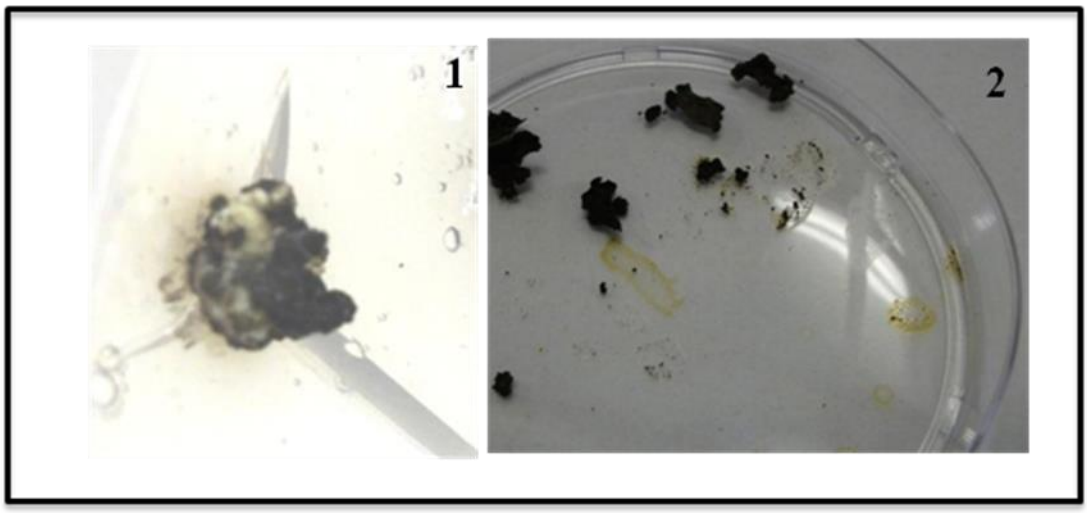

Figure 18: 1. Callus from *Litsea glutinosa* 2. Oil from dried callus of *Litsea glutinosa* (Perumal, 2012)

3.8.7 Method Seven

Plates of MS medium and woody plant medium were prepared as per Method Three in Section 3.8.3. In addition 1 ml amphotericin B was added to each 1 L of media. Field grown explants were used. The leaves were sterilised following the method used to sterilise the seed, explained in Method Six. The plates were placed under the fluorescent light in the tissue culture laboratory to determine whether the medium would promote callus induction with the explants utilised.

Table 6: Different methods to obtain callus

Method	Fruit	
Method One	Media :	MS medium
	Supplement:	2,4-D and BAP
	Explants:	*in-vitro* leaves
	Sterilisation:	N/A
Method Two	Media:	Woody plant medium
	Supplement:	2,4-D and BAP
	Explant:	*in-vitro* leaves
	Sterilisation:	N/A
Method Three	Media:	MS medium and Woody plant medium
	Supplement:	2,4-D and BAP
	Explant:	Germinated seeds
	Sterilisation:	N/A
Method Four	Media:	MS medium and woody plant medium
	Supplement:	2,4-D and BAP
	Explant:	Seeds
	Sterilisation:	30% NaClO for 5 min
Method Five	Media:	MS medium and Woody plant medium
	Supplement:	2,4-D and BAP
	Explant:	Field grown leaves
	Sterilisation:	30% NaClO for 5 min
Method Six	Media:	MS medium and Woody plant medium
	Supplement:	2,4-D and BAP
	Explant:	Field grown leaves
	Sterilisation:	50% NaClO for 35 min
	Additional:	0.1% amphotericin B -overnight
Method Seven	Media:	MS medium and Woody plant medium
	Supplement:	2,4-D and BAP and Amphotericin B
	Explant:	Field grown leaves
	Sterilisation:	50% NaClO for 35 min
	Additional:	0.1% amphotericin B - overnight

CHAPTER FOUR: RESULTS AND DISCUSSION

4.1 Oil Extraction

The oil extracted from *Litsea glutinosa* was dark red-brown colour (**Figure 11**). **Table 7** shows 4.013 kg of dried fruit yielded 2 480 ml of oil and 1 050 ml of FAME. The yield from crude oil to FAME was 42.34%.

Table 7: Table showing oil yield

Dry Weight	Extracted oil	FAME	Percentage yield
4.013 kg	2 480 ml	1 050 ml	42.34%

The oil extracts were kept in the fridge. Solid particles formed in the FAME in the fridge. These particles dissolved at room temperature and in a hot water bath at 30°C. These solid particles are called lipids which solidify at low temperature (Imahara *et al*., 2006). This is a biodiesel characteristic referred to as the cloud point. The cloud point can be altered by a technique known as blending (Imahara *et al*., 2006).

4.2 Transesterification

Transesterification is the chemical reaction whereby the glycerol is removed from the triglyceride (vegetable oil) by reacting it with an alcohol to form an ester (biodiesel) and glycerol as a by-product. If methanol is used in the reaction, the resulting product is a methyl ester, whereas ethanol will produce an ethyl ester.

Regardless of which alcohol is used, a catalyst has to be present in order to achieve good yields under relatively mild conditions. The two most common processes are either acid or alkali catalysed transesterification reaction.

The presence of glycerol in **Figure 11** indicated that transesterification was successful. Glycerol accumulates at the bottom of the flask as it has a higher density than biodiesel

(**Figure 11**). Visibility of the precipitate indicates that it did not mix with the glycerol. An orange emulsion formed with the precipitate when water was added. Hence the precipitate was miscible with water. The orange precipitate may have be the result of saponification as suggested by Sharma *et al*. (2008).

4.2.1 Biodiesel Washing Results

Wet washing and dry washing were used. Wet washing was time consuming and affected the water content. Two products were expected to form after transesterification, namely glycerol and biodiesel. However, an additional orange red precipitate was observed as well. **Figure 12** shows two layers, an orange yellow emulsion at the bottom, a result from wet washing, and an oily emulsion layer formed at the top. The top layer was a mixture of water and oil. Difficulty was experienced when the FAME's were being removed from the oily layer emulsion. A period of 45 days were spent on attempting to remove FAME from the emulsion. The water used in wet washing could not be reused. The water changed colour. The change in the colour of the water could have been the result of the red orange colour of the precipitate, shown in **Figure 12**. Dry washing was a more efficient method of purifying biodiesel. The FAME's were easily obtained through dry washing with amberlite (**Figure 13**). The amberlite absorbed all the impurities and it was discarded easily. However, the amberlite could not be reused.

4.3 Biodiesel Properties

The biodiesel produced from *Litsea glutinosa* was tested and the results were compared against the ASTM D6751-08 and EN 14214 standards to determine if the biodiesel could be produced from *Litsea glutinosa*. *Jatropha curcas* has been listed as a potential biodiesel source (Mohibbe *et al*., 2005; Bora and Baruah, 2012 and Tan *et al*., 2012). The biodiesel of *Litsea glutinosa* was compared against the biodiesel from *Jatropha curcas*, since literature on *Litsea glutinosa* was scarce. Many tests have been conducted on biodiesel from *Jatropha curcas* and therefore its specification provided a good indication of what was acceptable in biodiesel. It is important to note that biodiesel may be used for different applications, which may have different requirements. The expected outcome of the fuel is dependent on the task

and equipment. Biodiesel from one source may be ideal for an automobile engine but may be detrimental to an engine used for another application. The properties of biodiesel are dependent on the FAME content of the oil (Hoekman *et al*., 2012). The iodine and saponification value are dependent on the FAME profile. These values do not have a significant direct effect on the engine; however, they provide a guideline on the expected fuel properties.

Table 8: ASTM D 6751 biodiesel specification adapted from Ramadhas *et al*. (2009)

Property	Test Method ASTM	Limits
Flash point	D 93	Min. 130°C
Water and sediment	D 2709	Max. 0.05 vol %
Sulphated ash	D 874	Max. 0.02 wt %
Kinematic viscosity	D 445	1.9 - 6.0 $mm^2.s^{-1}$
Total sulphur	D 5453	Max. 0.0015 mg. kg^{-1}
Carbon residue	D 4530	Max. 0.05 wt %
Cetane number	D 613	Min. 47
Acid no.	D 664	Max. 0.8 mg KOH/g
Copper Strip corrosion	D 130	Max. No. 3°C
Free glycerol	D 6584	0.02 wt %
Total glycerol	D 6584	Max 0.240 wt %
Phosphorus content	D 4951	Max. 0.001 mg. kg^{-1}
Distillation temperature	D 1160	Max. 600°C
Viscosity	D445	1.9 - 4.1 $mm^2.s^{-1}$

Table 9: Biodiesel characteristics that were determined for *Litsea glutinosa* and compared to *Jatropha curcas*

Characteristics	*Litsea glutinosa*	*Jatropha curcas*	Diesel (petroleum)
Iodine value	6.3 (DUT)	93.0 (Mohibbe *et al*., 2005)	/
Saponification value	274 (DUT)	202.6 (Mohibbe *et al*., 2005)	/
Acid value	0.45 mg KOH. g^{-1} (DUT)	0.40 mg KOH. g^{-1} (Bora and Baruah, 2012)	0.35 mg KOH. g^{-1} (Mofijur *et al*., 2012)
Viscosity	22.48 $mm^2.s^{-1}$ (50°C) (SAPREF)	4.8 $mm^2.s^{-1}$ (40°C) (Tan *et al*., 2012)	1.9-6.0 $mm^2.s^{-1}$ (Hoekman *et al*., 2012)
Kinematic viscosity	23.84 mm^2. s^{-1} (SAPREF)	4.34 mm^2. s^{-1} (Tan *et al*., 2012)	3.30 mm^2. s^{-1} (Tan *et al*., 2012)
Density	942.69 kg. m^{-3} (40°C) (SAPREF)	880 kg. m^{-3} (15°C) (Bora and Baruah, 2012)	824.5 kg. m^{-3} (Tan *et al*., 2012)
Specific gravity	0.9444 g. cm^{-3} (40 ºC) (SAPREF)	4.40 g. cm^{-3} (40°C) (Bora and Baruah, 2012)	/
Distillation point	52.2 – 610.2°C (SAPREF)	/	320°C (Tan *et al*., 2012)
Sulphur	383 mg. kg^{-1} (SAPREF)	10 000 mg. kg^{-1} (Tan *et al*., 2012)	449 mg. kg^{-1} (Tan *et al*., 2012)
Calorific value (MJ/kg)	/	39.23 MJ. kg^{-1} (Bora and Baruah, 2012)	/
V50	23.8 (SAPREF)	13.5 (SAPREF)	/
Main Fatty Acids	Stearic acid (36.38%) Lauric Acid (39.177%) Palmitic acid (13.962%) (UKZN)	Palmitic acid (16.8%) Oleic acid (39.1%) Linoleic acid (36.0%) (Jain and Sharma, 2012)	/
Cetane value	64.79 (Mohibbe *et al*., 2005)	57.1 (Bora and Baruah, 2012)	50.5 (Tan *et al*., 2012)
pH	6.85	/	/

4.3.1 Iodine value

The iodine value or iodine number (IV) for oil sample of *Litsea glutinosa* was found to be 6.5 (**Table 10**), compared to *Jatropha curcas* which is 93.0 (**Table 9**). The EN 14111 standard limits this value to 120 (**Appendix 3**). Iodine value is inversely proportional to cetane number. Biodiesel from *Litsea glutinosa* and *Jatropha curcas* are both within the standard specification. However, the iodine number of the *Litsea glutinosa* biodiesel is lower than that from *Jatropha curcas*. Oil from *Litsea glutinosa* has a higher CN value and carries a low IV value, and oil from *Jatropha curcas* has a lower CN value (compared to *Litsea glutinosa*) and carries a higher IV value. The cetane number of *Litsea glutinosa* biodiesel is 64.7, compared to that from *Jatropha curcas* of 57.1. This confirms the literature review that the iodine value is inversely proportional to the cetane value. It can be concluded that the oil produced from *Litsea glutinosa* is better than *Jatropha curcas*, in terms of iodine value and cetane number.

Table 10: Iodine titration and calculation

Sample	Blank (ml)	Sample (ml)	Iodine number
1	27.60	22.40	6.7
2	27.60	22.35	6.5
3	27.60	22.40	6.5
			6.5

4.3.2 Saponification value

The saponification value or saponification number (SV) for are oil sample of *Litsea glutinosa* was found to be 278.443 **(Table 11)**, compared to oil from *Jatropha curcas* which was found to be 202.6 (**Table 9**). The saponification number is an indication of free fatty acids present in the fuel, which results in saponification during transesterification, thereby decreasing biodiesel production. The saponification number of oil from *Litsea glutinosa* is higher than that of oil from *Jatropha curcas*. This indicates that there are more free fatty acids in the oil from *Litsea glutinosa* than that from *Jatropha curcas*. The transesterification reaction to convert fatty acids to FAME for oil from *Litsea glutinosa* will require more pre-treatments,

compared to oil from *Jatropha curcas,* to ensure a complete reaction. Therefore, *Jatropha curcas* is a better source of biodiesel when compared to *Litsea glutinosa* with regards to saponification number.

Table 11: Saponification titration and calculation

Sample	Blank (ml)	Sample (ml)	Saponification value
1	86.80	37.20	278.256
2	86.80	37.10	278.817
3	86.80	37.20	278.256
			278.443

4.3.3 Acid value

The acid number for the oil sample from *Litsea glutinosa* was found to be 0.45 mg. KOH. g^{-1} **(Table 12)** compared to oil from *Jatropha curcas*, which was found to be 0.40 mg. KOH. g^{-1} (**Table 9**). The ASTM standards require a maximum value of 0.8 mg. KOH. g^{-1} (**Table 8**). Oil from *Litsea glutinosa* and *Jatropha curcas* meet the ASTM standards for acid value. The acid value indicates the stability and corrosiveness of the fuel. The lower the acid number the higher the biodiesel quality. The difference between the acid numbers of the two biofuel sources being discussed is negligible.

Table 12: Acid titration and calculation

Sample	Sample (ml)	Acid value (mg. KOH. g^{-1})
1	3.17	0.45
2	3.17	0.45
3	3.17	0.45
		0.45

4.3.4 Viscosity

The viscosity of oil sample of *Litsea glutinosa* was found to be 22.48 $mm^2. s^{-1}$ at 50°C at the laboratories of SAPREF (**Table 9**), Durban. Oil from *Jatropha curcas* has a viscosity of 4.8 $mm^2. s^{-1}$ at 40°C (**Table 9**). The viscosities of both biodiesels were measured at different temperatures. This makes comparison between the sources of the biodiesel for viscosity difficult. The viscosity requirement for biofuel is 1.9 - 4.1 $mm^2. s^{-1}$ (**Table 8**). The petroleum industry determines the V50 to standardise the viscosity for comparison purposes. SAPREF, Durban calculated the V50 for biodiesel from *Litsea glutinosa* to be 22.48 $mm^2. s^{-1}$ (**Table 9**). The viscosity of biodiesel from *Jatropha curcas* to be 4.8 $mm^2. s^{-1}$ (**Table 9**). It can be seen that the V50 value for *Litsea glutinosa* is higher than *Jatropha curcas. Litsea glutinosa* and *Jatropha curcas* did not meet the viscosity requirement.

Viscosity is the property of fluids resistance to flow (Alptekin and Canakci, 2011). The viscosity of biodiesel is higher than petroleum-diesel (1.9 - 6.0 $mm^2. s^{-1}$ – **Table 9**). Tesfa *et al*. (2010) stated that the viscosity of biodiesel can be 1.6 times that of diesel at 40°C. They noted that the ratio increases particularly when temperatures are below 25°C. Viscosity affects the performance of the fuel supply system. It affects the fuel pump, fuel filter and air-fuel mixing behaviour (Alptekin and Canakci, 2011; Hoekman *et al*., 2012). Viscosity has a direct effect on the atomisation process during combustion (Ramírez-Verduzco *et al*., 2012). The viscosity of the fuel affects the atomisation quality, size of drop and penetration. High viscosity tends to form larger droplets on fuel injection, causing the problems listed below:

- Poor fuel atomisation during the spray;
- The engine deposits increase;
- More energy is needed to pump the fuel and
- The fuel pump elements and injectors become worn more quickly.

High viscosity subsequently leads to poor combustion, increased exhaust smoke and emissions (Ramírez-Verduzco *et al.*, 2012). The higher viscosity means lower flow rate of fuel and lower engine power (Tesfa *et al*., 2010). The viscosity of biodiesel can be improved by blending with pertroleum fuel (Alptekin and Canakci, 2011).

4.3.5 Kinematic viscosity

The kinematic viscosity of the oil sample of *Litsea glutinosa* was found by SAPREF, Durban to be 23.84 $mm^2. s^{-1}$ (**Table 9**) compared to oil from *Jatropha curcas* which is 4.34 $mm^2. s^{-1}$ (**Table 9**). The ASTM requires a value between 1.9 - 6.0 $mm^2. s^{-1}$ (**Table 8**). The kinematic viscosity of oil of *Litsea glutinosa* is higher than oil from *Jatropha curcas*, which meets the ASTM standard requirement for kinematic viscosity. Oil from *Litsea glutinosa* failed to meet the ASTM specification of 1.9 – 6.0 $mm^2. s^{-1}$ for kinematic viscosity.

The kinematic viscosity of the biodiesel decreases with the increase in temperature (Tesfa *et al*., 2010). When kinematic viscosity and surface tension of the fuel increase, the cohesion and the surface viscosity between fuel molecules increases. The Sauter Mean Diameter (SMD) of the cylinder chamber increases and consequently the surface area of the fuel drop is reduced. As a result the tendency of evaporation of fuel decreases and this might decrease the performance of the engine and increase the emissions (Tesfa *et al*., 2010).

As engine speed increases the SMD of the fuel in the cylinder decreases. SMD of the biodiesel decreases with increase in the injected fuel temperature. As the temperature increases the inter-molecular forces of the biodiesel decrease. This results in the SMD decreasing in the engine cylinder. This facilitates the evaporation of the fuel and combustion rate in the engine cylinder.

4.3.6 Density

The density of the oil sample of *Litsea glutinosa* as found by SAPREF, Durban was 942.69 kg. m^{-3} (40°C) (**Table 9**). The density of oil from *Jatropha curcas* is 880 kg. m^{-3} (15°C) (**Table 9**). Density is inversely proportional to temperature. *Litsea glutinosa* oil has a higher fuel density than *Jatropha curcas* oil. Fuel density is a key property and affects engine performance (Hoekman *et al*., 2012). Density affects the mass of fuel injected into the combustion chamber and the air-fuel ratio. Fuel injection pump meters fuel by volume, and not by mass. A denser fuel will have a larger mass of fuel without a change in volume

(Hoekman *et al*., 2012; Ramírez-Verduzco *et al*., 2012). Fuel with a higher density is preferred.

4.3.7 Specific gravity

SAPREF, Durban found the specific gravity of the oil sample from *Litsea glutinosa* to be 0.9444 g. cm^{-3} (0.000944 kg. L^{-1}) (40°C) (**Table 9**). The specific gravity of oil from *Jatropha curcas* is 4.40 g. cm^{-3} (0.0044 kg. L^{-1}) (40°C) (**Table 9**). The required ASTM standard for specific gravity of biofuel is 0.88 kg. L^{-1} (**Table 1**). Oil from both *Litsea glutinosa* and *Jatropha curcas* meet the ASTM requirement for specific gravity. Specific gravity is defined as a ratio of the density of a given solid or liquid substance to the density of water at a specific temperature and pressure (Tesfa *et al*., 2010). Specific gravity increases by approximately 0.0033 units for each 5°C decrease in temperature (Ramírez-Verduzco *et al*., 2012). Specific gravity demonstrates temperature dependent behavior (Tesfa *et al*., 2010). A low value of the specific gravity indicates the completion of the transesterification reaction and removal of heavy glycerol (Sharma *et al*., 2008). The specific gravity value of biodiesel from *Litsea glutinosa* is lower than biodiesel from *Jatropha curcas*. The specific gravity value of the biofuel sample obtained from *Litsea glutinosa* confirmed that the transesterification reaction was complete and successful. The specific gravity of biodiesel from *Litsea glutinosa* is better than biodiesel from *Jatropha curcas*.

4.3.8 Distillation point

The distillation temperature of the oil sample from *Litsea glutinosa* was found at SAPREF, Durban to be from 52.2°C to 610.2°C (**Table 9**). A distillation temperature range is unavailable for *Jatropha curcas*. The ASTM distillation point temperature standard for biodiesel has a maximum 600°C (**Table 8**). *Litsea glutinosa* meets the required standard for distillation point temperature.

Distillation point is a temperature range, not a single value. When the distillation temperature increases, the volatility of a biofuel decreases (Arpa *et al*., 2010). Biodiesel exists in three states namely solid, liquid and vapour. The FAME composition affects many

fuel characteristics (Bora and Baruah, 2012). In order to comprehend how the FAME composition affects distillation temperature an understanding of how the fuel is utilised in an engine is needed. Fuel is utilised in an engine in its vaporised state. Vaporisation that occurs at a very narrow temperature band results in rapid combustion which is dangerous. The rapid burning of fuel increases the engine temperature, subsequently leading to increased pressure in the combustion chamber. High pressures have detrimental effects on engine components (Arpa *et al*., 2010). Therefore, fuel should burn gradually in a combustion chamber of an engine. The distillation points of the fuel shows the different temperatures that the fuel vaporises and can be used in the engine, preventing damage to the engine. The distillation value can be seen in **Figures 19** and **20**. It shows the range at which the oil vaporises. Graphs depicting the thermostability of the oil is shown in **Figures 21, 22** and **23**.

Instrument 1 ASTM High temperature 1

Sample name	: BIODIESEL_LG		
Acquired on	: 5/3/2012 12:51:03 PM	Vial	: 51
Processed on	: 5/3/2012 1:42:56 PM	Injection	: 1
Sample type	: TBP	Sample (g)	: 0.0327
Method name	: CRYO_DUAL	Solvent (g)	: 1.9260
Operator	: PRAVIND	ISTD (g)	: 0.0000
Sequence name	: C:\Chem32\1\DATA\3MAY12d\3MAY12D 2012-05-03 12-43-53\3MAY12D.S		
Data File	: 3MAY12D\3MAY12D 2012-05-03 12-43-53\051B0101.D\		

General Variables

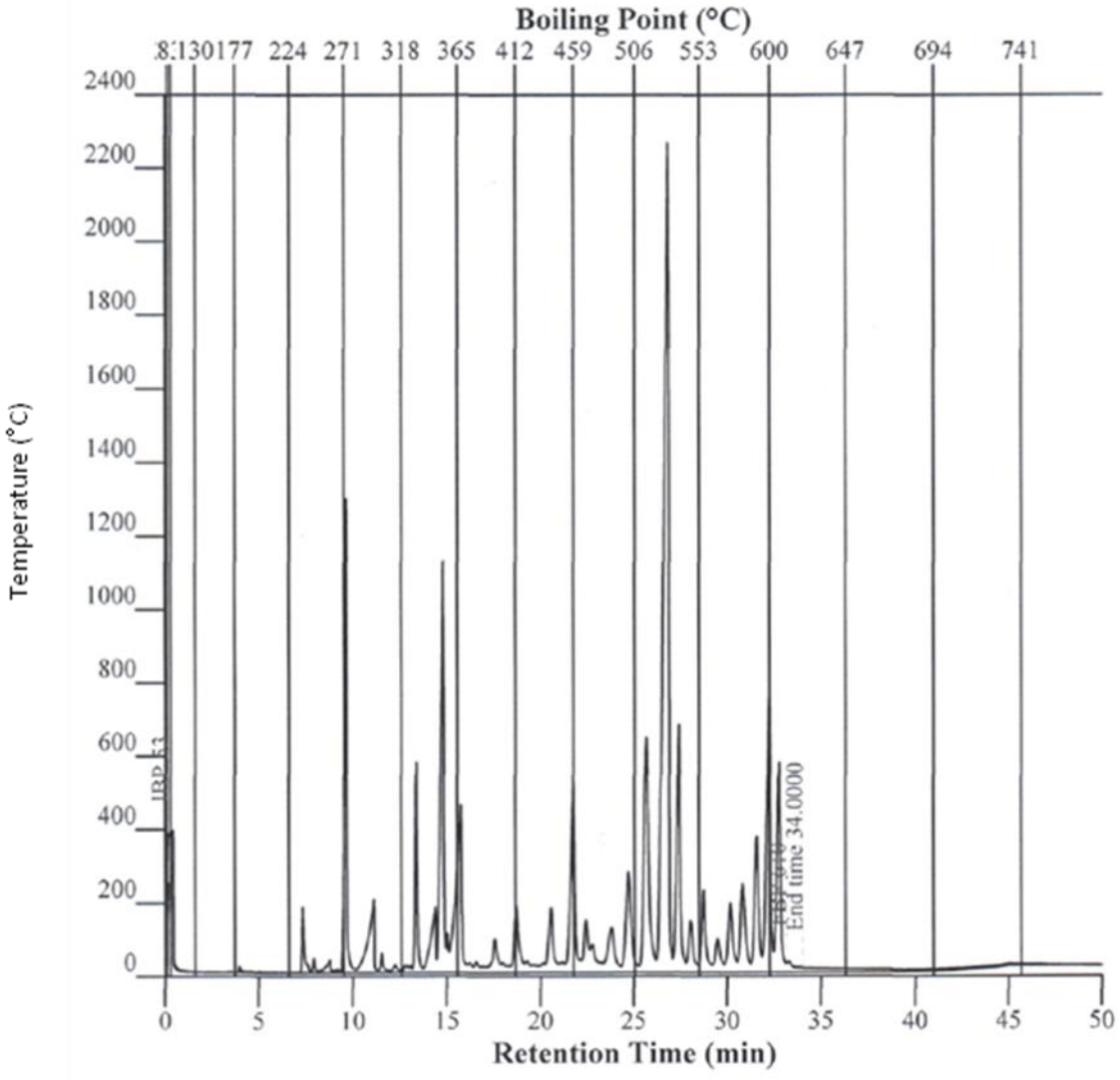

Figure 19: Distillation graph

Instrument 1 ASTM High temperature 2

Sample name	: BIODIESEL_LG		
Acquired on	: 5/3/2012 12:51:03 PM	Vial	: 51
Processed on	: 5/3/2012 1:42:56 PM	Injection	: 1
Data File	: 3MAY12D\3MAY12D 2012-05-03 12-43-53\051B0101.D\		

BP Distribution table - Percent

Recovered mass%	BP °C	Recovered mass%	BP °C	Recovered mass%	BP °C	Recovered mass%	BP °C
IBP	52.6	30.0	391.2	60.0	528.4	90.0	596.8
5.0	266.6	35.0	444.2	65.0	529.6	95.0	601.0
10.0	274.4	40.0	468.4	70.0	530.8	FBP	610.2
15.0	331.4	45.0	502.0	75.0	536.4		
20.0	352.2	50.0	515.6	80.0	549.2		
25.0	361.6	55.0	526.2	85.0	581.4		

Boiling point distribution plot

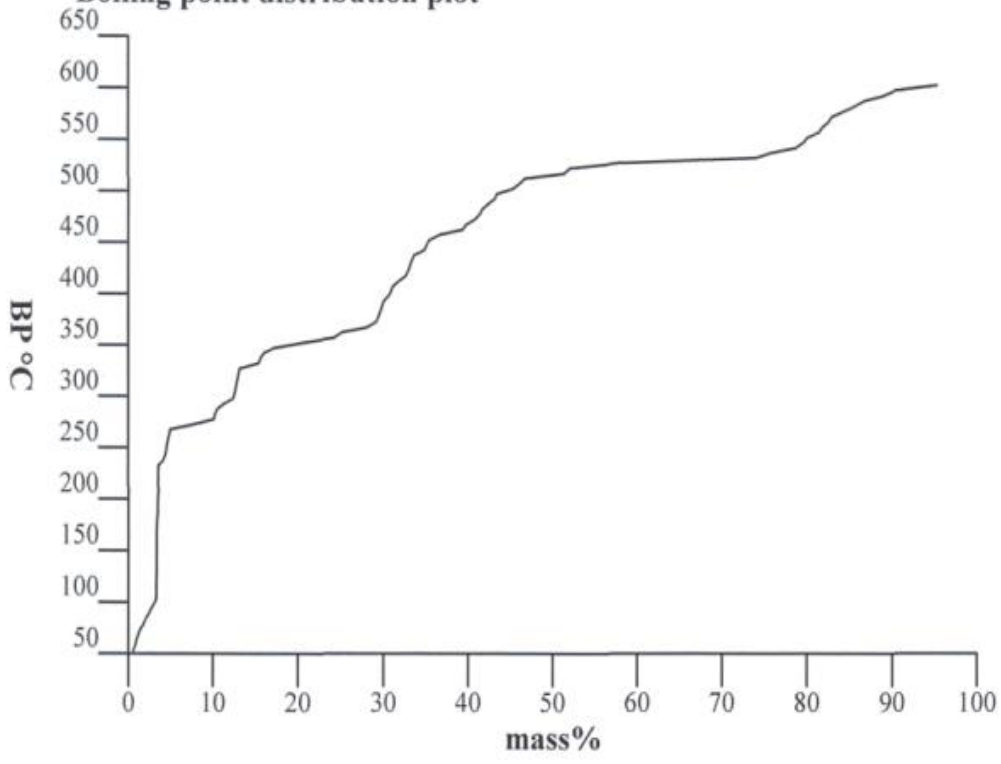

Figure 20: Distillation plot

4.3.9 Thermo gravimetric analysis

The thermographs from the thermostability test of the oil sample from *Litsea glutinosa* are presented in **Figures 21**, **22** and **23**.

Thermo Gravimetric Analysis (TGA) determines the thermal stability of a variety of substances. The thermal properties of the oil sample of *Litsea glutinosa* were measured under various conditions of temperature and time. TGA analyses the weight change of the oil depending on the temperature change. Three weight changes were observed on the oil sample of *Litsea glutinosa* in **Figure 23**. The weight change can be attributed to decomposition, either to evaporation or to combustion of the FAME (Jain and Sharma, 2012).

Figure 21 is a thermograph that shows heat flow versus temperature. **Figure 21** shows the weight of the oil of *Litsea glutinosa* decreasing as temperature increases. This was expected since the oil was undergoing combustion to produce energy. The graph in **Figure 21** illustrates the different weight losses at different temperatures. The fatty acid composition accounts for this, since different fatty acids will undergo combustion at different temperatures. Most of the oil weight is reduced between the temperature ranges of 62.58°C to 408.63°C. Hence it can be deduced that the temperature range between 62.58°C to 408.63°C is recommended for the use of biodiesel from *Litsea glutinosa*.

Figure 23 shows the weight of the oil of *Litsea glutinosa* as a function of temperature. The graph in **Figure 23** shows the change in weight of the oil as the temperature increased. A three phase weight loss was noted from 0°C to 600°C.

Figure 22 shows super-imposed thermographs of **Figure 21 and 23**. A heat derived graph is depicted on **Figure 22.** A heat derived graph shows an endothermic and exothermic reaction. An endothermic reaction involved heat / energy being absorbed whereas an exothermic reaction involves energy being released. An exothermic reaction can be seen on the brown graph in **Figure 22**. This was expected of biodiesel since it is intended to generate energy.

The thermographs of biodiesel from *Litsea glutinosa* (**Figure 21, 22 and 23**) and *Jatropha curcas* (**Appendix 2**) were compared and observations were made. The thermograph of

biodiesel from *Litsea glutinosa* (**Figure 22 and 23**) shows three phases. In the first phase a 41.92% weight loss at 350°C was noted. The weight loss in the second phase was similar to the first phase, i.e. 41.80% weight loss in a temperature range from 350°C to 400°C. The third phase had the highest temperature range from 400°C to 600°C and a 21.35% weight loss was noted. The graph leveled at 60°C. This could be attributed to the vaporisation or combustion ability of the biodiesel from *Litsea glutinosa*.

The thermograph of biodiesel from *Jatropha curcas* (**Appendix 2**) consists of three phases. In the first phase, a 10% weight loss at 113°C was noted. The weight loss in the second phase was significantly larger (about 90%) in a temperature range from 113°C to 198°C. The third phase weight loss occurred at the highest temperature range from 318°C to 700°C. The graph leveled at 700°C. This observation may be attributed to the vaporisation or combustion ability of the biodiesel from *Jatropha curcas*.

The weight loss of both fuels at 100°C is practically the same (refer to **Figure 23** and **Appendix 2**). An exothermic reaction results in energy being released in the form of heat. Thermal stability of biodiesel is its resistance to thermal degradation. Biodiesel becomes oxidatively unstable at high temperatures causing the thermal stability of biofuel decreases. The higher the temperature, the faster is the oxidation process of biofuel, i.e. the higher the rate of degradation of biodiesel. Oxidative degradation compromises the kinematic viscosity, the cetane number and the acid value of biodiesel. Biodiesel is subjected to high temperatures in an engine. These conditions encourage deterioration of fuel. The filter pipelines and fuel pump pipelines can be blocked by oil deposits resulting from the degradation of fuel. The combustion process is less efficient since the combustion conditions are affected. Engine efficiency and engine life decreases as a result (Jain and Sharma, 2012).

Freire *et al*. (2009) reported the effect of antioxidants on the thermal stability of palm oil. The antioxidants had no significant effect on boiling point. However, the antioxidants increased resistance to thermal degradation of the biofuel.

4.3.10 Sulphur

SAPREF, Durban found the sulphur content of the oil sample from *Litsea glutinosa* to be 383 mg. kg^{-1} (**Table 9**). Oil from *Jatropha curcas* has a sulphur content of 10 mg. kg^{-1} (**Table 9**). The ASTM standard allows a maximum of 0.0015 mg. kg^{-1} for sulfur content (**Table 8**). *Litsea glutinosa* and *Jatropha curcas* do not meet the ASTM standard requirement for sulfur. However, the sulphur content of oil from *Litsea glutinosa* is lower than that from *Jatropha curcas*.

The sulphur content is of environmental concern (Lapuerta *et al*., 2008). However, a decrease in the level of of sulphur leads to a decrease in fuel lubricity (Muñoz *et al*., 2011). Lubrication is defined as the capacity of a fluid to prevent wear due to adhesion between surfaces in contact.

The sulphur found in oils precipitates via a chemical reaction with metal oxides, such as zinc oxide (ZnO), iron oxide (Fe_2O_3) and copper oxide (CuO) at a certain temperature, results in sulphuric acid being decomposed in the engine (Arpa *et al*., 2010). The resulting sulphuric acid would result in damage to the engine parts and created particulate matter emissions. Lin *et al*. (2011) stated that particulate matter emissions from engines have three major components:

1. Soot from combustion;
2. Heavy hydrocarbon condensed or absorbed on the soot; and
3. Sulphates.

These emissions pose health and environmental concerns.

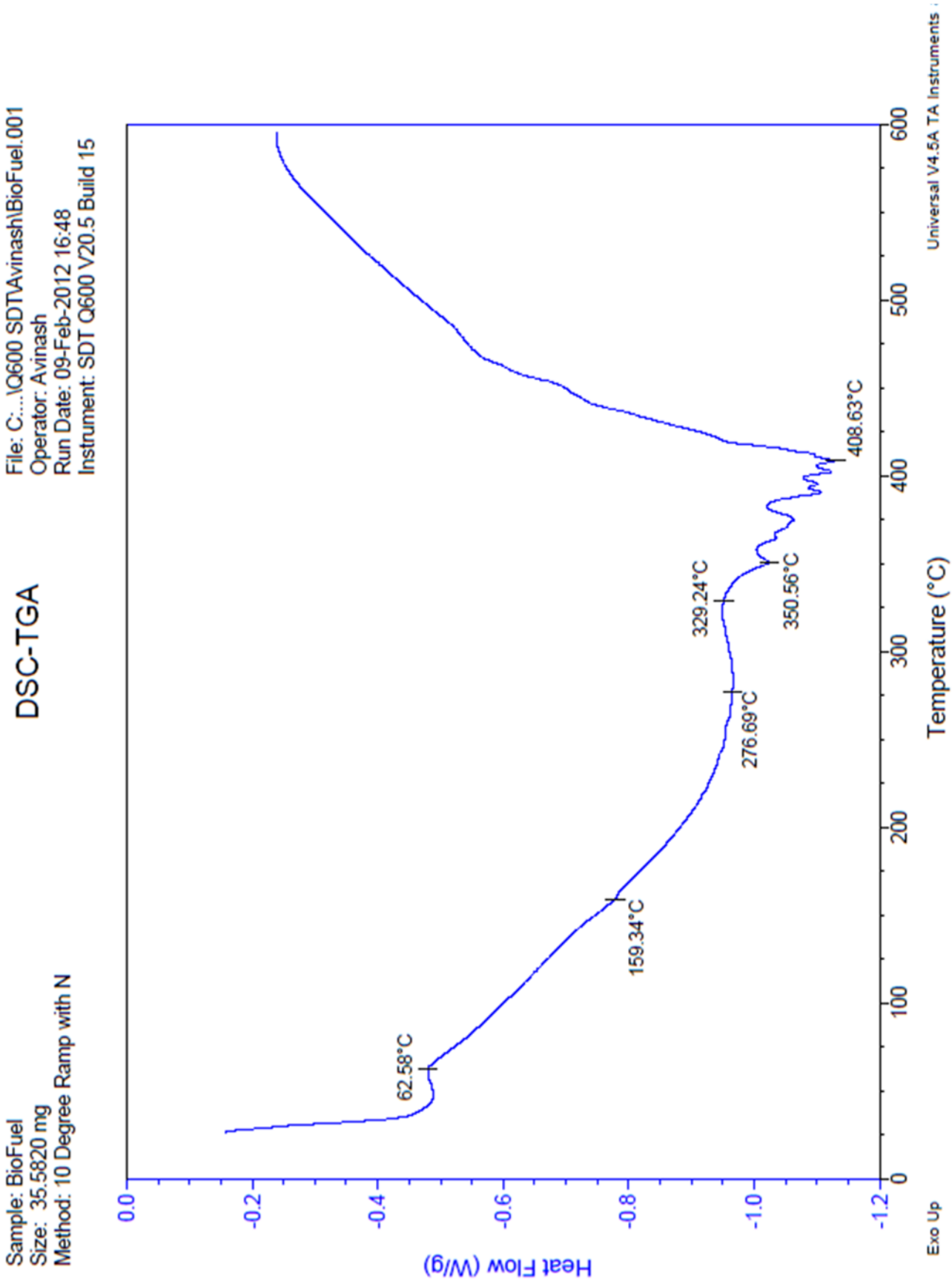

Figure 21: Thermograph showing heat flow vs temperature

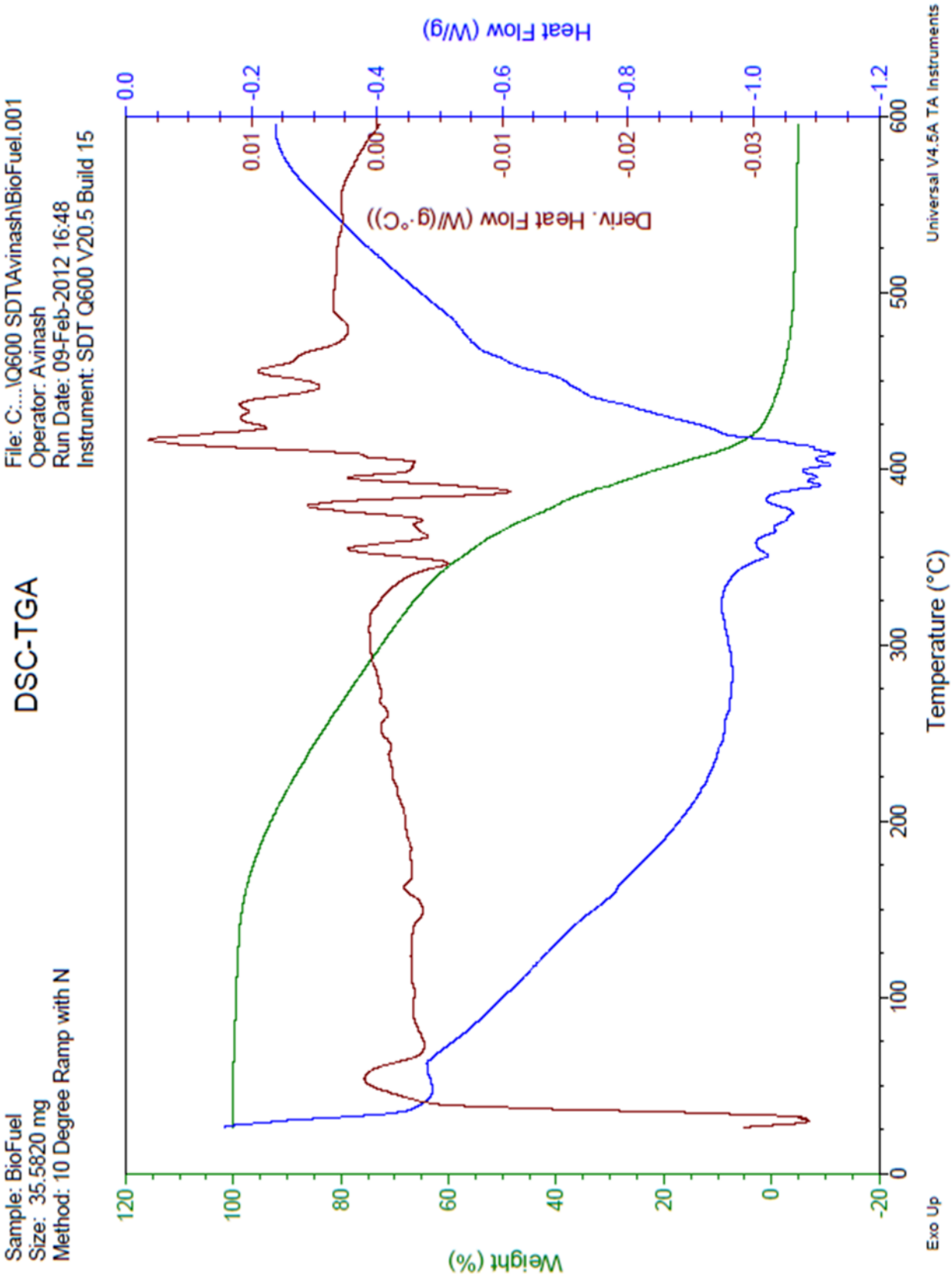

Figure 22: Thermograph showing weight vs heat flow vs temperature

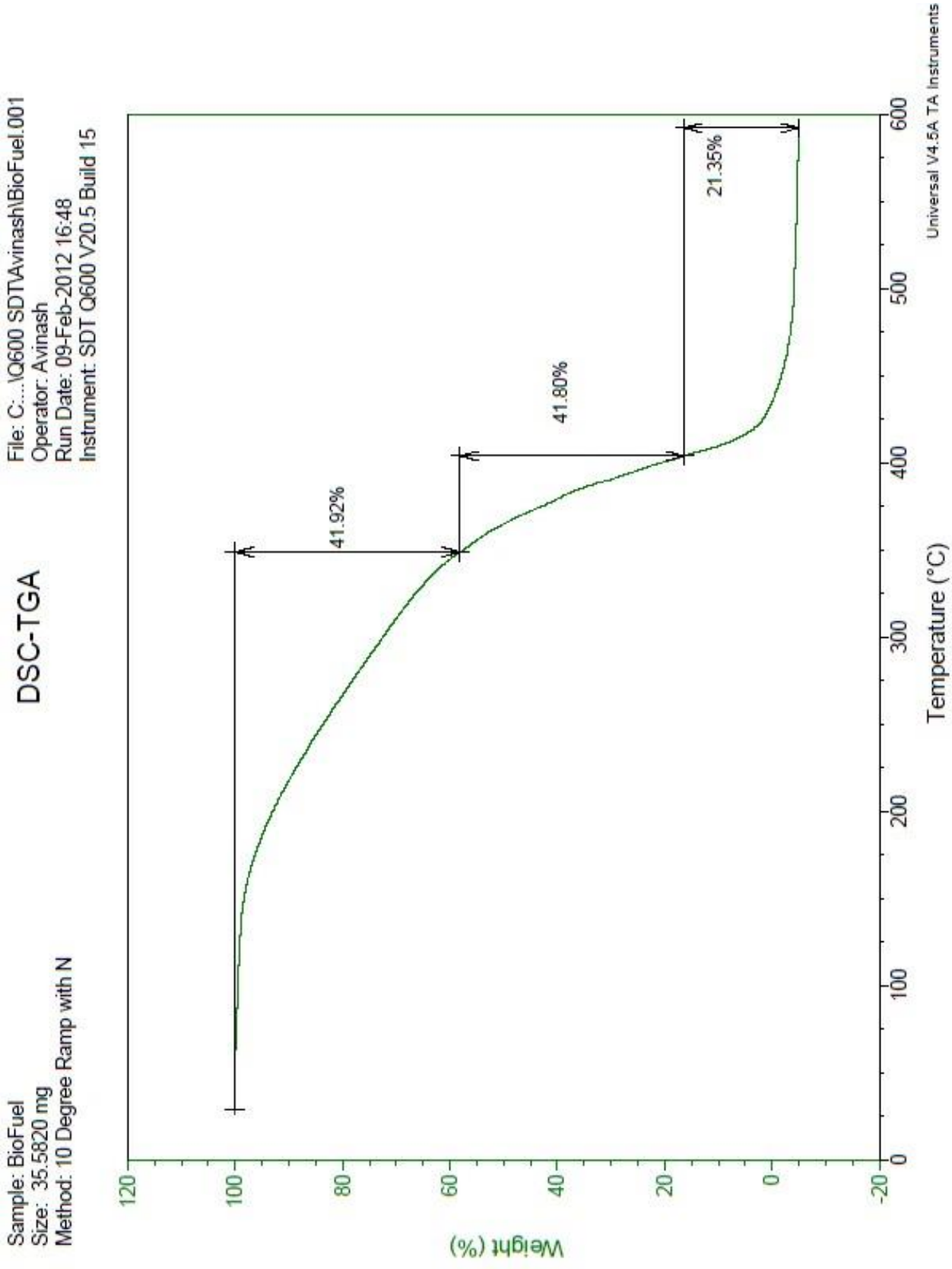

Figure 23: Thermograph showing weight vs temperature

4.3.11 Fatty acid methyl ester (FAME) oil analysis

The Gas Chromatography-Mass Spectrometry (GC-MS) results can be seen in **Tables 16, 17**, **Figures 26** and **27**. Common fatty acids between the fruit flesh and the seed are highlighted in **Table 16** and **17**. They are: caryophyllene, n-hexadecanoic acid and 9-octadecanoic acid.

Fatty acids of *Litsea glutinosa* are stearic acid ($C_{18}H_{36}O_2$, BP-383°C - 36.4%), lauric acid ($C_{12}H_{24}O_2$, BP-298.9°C - 39.2%) and palmitic acid ($C_{16}H_{32}O_2$, BP-351°C - 14.0%) (**Table 9**). Fatty acids of *Jatropha curcas* are palmitic acid ($C_{16}H_{32}O_2$, BP- 351°C - 16.8%), oleic acid ($C_{18}H_{34}O_2$, BP-360°C - 39.1%) and linoleic acid ($C_{18}H_{32}O_2$, BP-230°C - 36.0%) (**Table 9**). Hoekman *et al*. (2012) mentioned steric acid, lauric acid and plamitic acid are common fatty acids found in biodiesels (**Table 14**). The FAME composition of each plant oil is crucial, since it has a direct impact on the characteristics and quality of the fuel. Many programs and equations have been developed to determine the fuel properties based on the FAME composition. The dominating FAME of *Litsea glutinosa* is from stearic acid which has a boiling point of 383°C and it makes up 65.4% of the oil (**Table 16**). The dominating FAME of *Jatropha curcas* is from oleic acid with a boiling point of 360 °C and it makes up 39.1% of the oil (**Table 9**). Palmitic acid provides the common FAME between *Litsea glutinosa* and *Jatropha curcas*. However, it is present in small quantities, 13.9% and 16.8% in *Litsea glutinosa* and *Jatropha curcas* respectively. The FAME dominating *Litsea glutinosa* has a higher boiling point temperature than the FAME dominating *Jatropha curcas*. The dominating FAME of *Litsea glutinosa*, i.e. from stearic acid, is more than 50% of its FAME component when compared to the dominating FAME of *Jatropha curcas*. These factors play a role in the characteristics of fuel properties.

Gas Chromatography–Mass Spectrometry (GC-MS) was conducted on both the oil sample from the fruit flesh of *Litsea glutinosa* and the oil sample from the seeds of *Litsea glutinosa*. The test results of the oil samples from the fruit flesh and the seeds of *Litsea glutinosa* are reflected in **Tables 16 and 17** respectively. A total of 47 compounds were identified in the oil of the fruit flesh (**Table 16**) and 24 compounds were identified in the oil of the seeds (**Table 17**). There were 23 more fatty acid compounds in the oil of the fruit flesh than in the seeds. The data was compared with information in the literature review to find similar compounds to those found in the fruit and seeds of *Litsea glutinosa* (**Table 2**). Here we found

caryophyllene, lauric acid and n-hexadecanoic compounds in the seeds and fruit flesh of *Litsea glutinosa* (**Table 16** and **17**) and these findings correlates to the findings of Chowdhury *et al*. (2008) (**Table 2**). The compounds found in the fruit were also compared to the compounds found in the seeds. There were three dominating compounds found which were common in the seeds and fruit of *Litsea glutinosa* (**Table 13**). These dominating compounds were octadecanoic acid, caryophyllene and hexadecanoic acid.

Table 13: Common dominating fatty acids found in *L. glutinosa*

Seeds	Fruit	Common Names
octadecanoic acid (36.38%)	octadecanoic acid (65.43%)	stearic acid
caryophyllene	caryophyllene	
dodecanoic acid (39.17%)		lauric acid
hexadecanoic acid (13.96%)	hexadecanoic acid (13.68%)	palmitic acid

Hoekman *et al.* (2012), compiled a table showing fatty acids typically present in biodiesel (**Table 14**). The blue highlighted FAMEs, in **Table 14**, from hexadecanoic acid, octadecanoic acid, *cis*-9-octadecenoic acid, eicosanoic acid and *cis*-9, 12-octadecadiennoic acid are the common biodiesel FAMEs. The common dominating compounds hexadecanoic acid and octadecanoic acid, found in the fruit flesh and the seeds of *Litsea glutinosa* are also found in biodiesel (**Table 14**).

Gas Chromatography (GC) was conducted on the oil sample extracted from the whole fruit of *Litsea glutinosa.* **Table 15** shows the fatty acids identified in the whole fruit of *Litsea glutinosa* from GC testing at DUT. **Table 15** was compiled by comparing the gas chromatogram of the standards to the gas chromatograms of the *Litsea glutinosa* oil sample (**Figure 24** to **Figure 25**). The GC-MS results (**Table 16** and **17**) and the GC results (**Table 15**) confirmed the presence of lauric acid, palmitic acid, oleic acid, myristoleic acid and pentadecanoic acid in the oil of *Litsea glutinosa*.

Hoekman *et al*. (2012) identified lauric acid, palmitic acid, oleic acid and myristoleic acids as common fatty acid present in biodiesel (**Table 14**). Myristoleric acid was found in the oil of the whole fruit and seeds only (**Table 15** and **17**). Pentadecanoic acid found in biodiesel was

detected in the whole fruit of *Litsea glutinosa* by the GC testing (**Table 15**) and in the fruit flesh of *Litsea glutinosa* by GC-MS testing (**Table 16**). This was not a common fatty acid in biodiesel.

Table 14: Common fatty acids found in biodiesel (Hoekman *et al*., 2012)

Common name	Formal name	Abbreviation	Molecular formula	Molecular weight
lauric acid	**dodecanoic acid**	**12:0**	**$C_{12}H_{24}O_2$**	**200.32**
myristic acid	**tetradecanoic acid**	**14:0**	**$C_{14}H_{28}O_2$**	**228.38**
myristoleic acid	*cis*-9-tetradecenoic acid	14:1	$C_{14}H_{26}O_2$	226.43
palmitic acid	**hexadecanoic acid**	**16:0**	**$C_{16}H_{32}O_2$**	**256.43**
palmitoleic acid	*cis*-9-hexadecanoic acid	16:1	$C_{16}H_{30}O_2$	254.42
stearic acid	**octadecanoic Acid**	**18:0**	**$C_{18}H_{36}O_2$**	**284.48**
oleic acid	***cis*-9-octadecenoic acid**	**18:1**	**$C_{18}H_{34}O_2$**	**282.47**
linoleic acid	*cis*-9,12-octadecadiennoic acid	18:2	$C_{18}H_{32}O_2$	280.46
arachidic acid	eicosanoic acid	20:0	$C_{20}H_{40}O_2$	312.54
gondoic acid	*cis*-11-eicosenoic acid	20:1	$C_{20}H_{38}O_2$	310.53
behenic acid	**docosanoic acid**	**22:0**	**$C_{22}H_{44}O_2$**	**340.60**
lauric acid	dodecanoic acid	12:0	$C_{12}H_{24}O_2$	200.32
myristic acid	tetradecanoic acid	14:0	$C_{14}H_{28}O_2$	228.38
myristoleic acid	*cis*-9-tetradecenoic acid	14:1	$C_{14}H_{26}O_2$	226.43

Table 15: Table showing the fatty acids identified in sample whole fruit of *Litsea glutinosa* against the standard by Gas-Chromatography (GC) analysis

Peak	Retention time	Area	Compound name
2	8.267	28985020	butyric acid methyl ester (C4:0)
4	9.126	116971	caproic acid methyl ester (C6:0)
5	11.108	325313	caprylic acid methyl ester (C8:0)
6	15.233	320602	lauric acid methyl ester (C12:0)
7	19.682	24481	myristoleic acid methyl ester (C14:1)
8	21.148	279004	
9	21.384	10270	cis-10-pentadecenoic acid methyl ester (C15:1)
10	21.653	10442	
11	21.929	15671	
12	24.348	128471	plamitic acid methyl ester (C16:0)
13	26.037	29159	elaidic acid methyl ester (C18:1n9t)
14	27.181	21040	oleic acid methyl ester (C18:1n9c)
15	27.530	249619	linolelaidic acid methyl ester (C18:2n6t)
16	30.622	116788	linolenic acid methyl ester (C18:3n3)

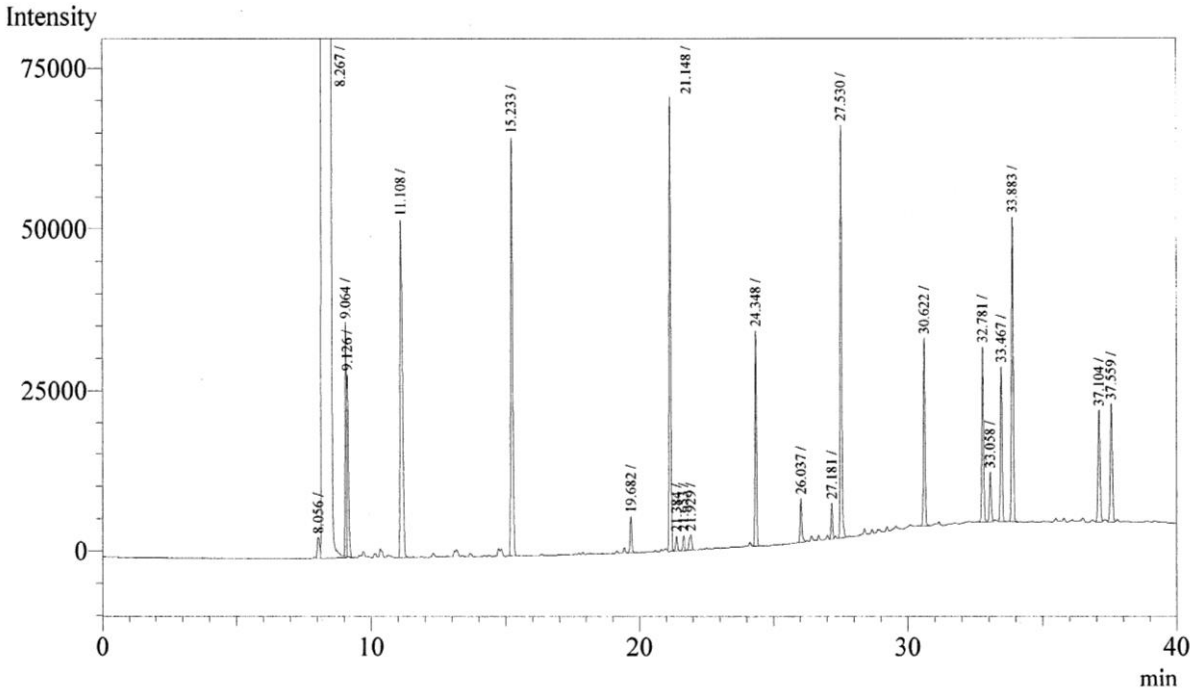

Figure 24: Gas Chromatogram of the sample oil of the whole fruit of *Litsea glutinosa*

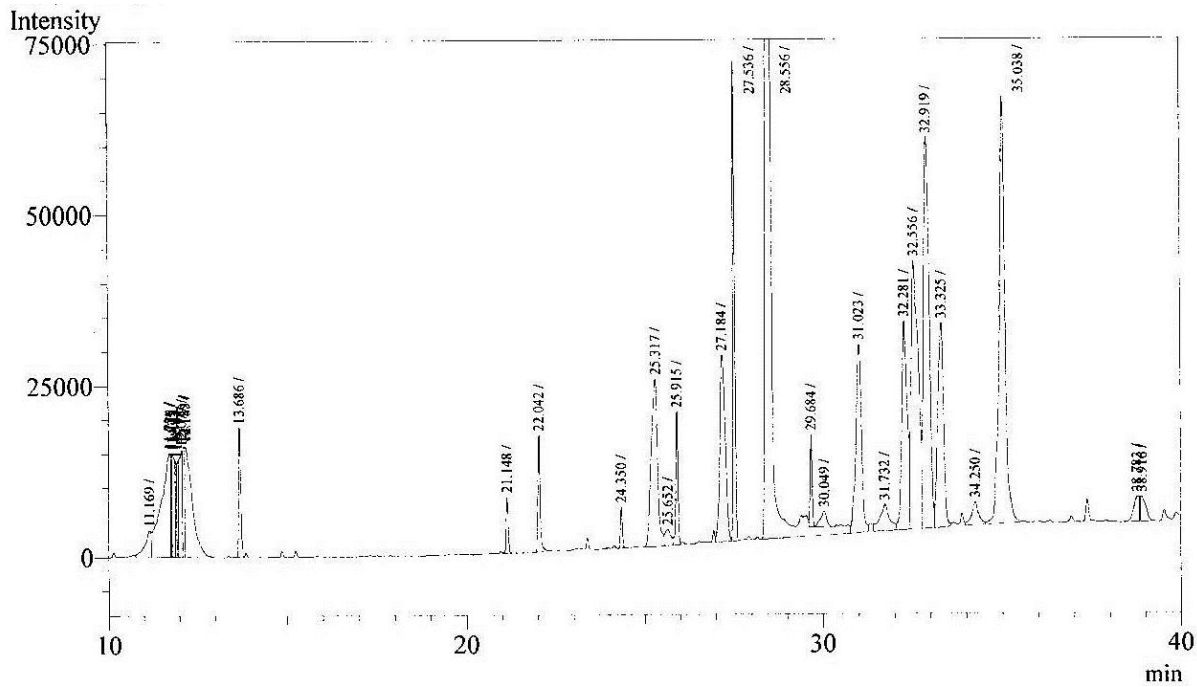

Figure 25: Gas chromatogram of the standards

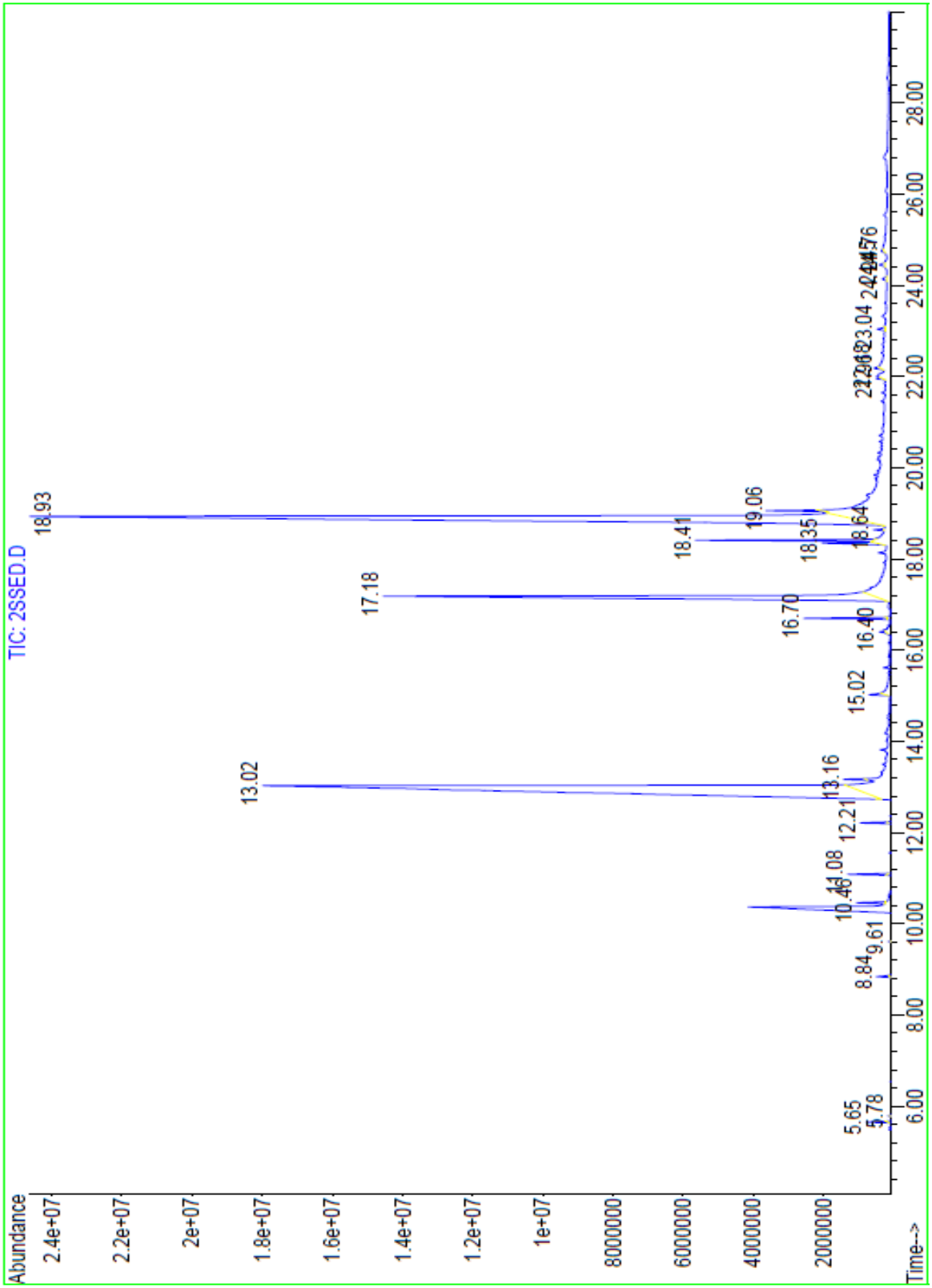

Figure 26: GC-MS Chromatogram of the oil from the skin of *Litsea glutinosa*

Table 16: Table showing compounds found in the fruit flesh of *Litsea glutinosa* Gas Chromatography-Mass Spectrometry (GC-MS)

Peak	Ret. Time	Area	Compound Name
1	4.849	0.011%	N-(n-propyl)-1,3-propanediamine
2	4.878	0.005%	carbamic acid, (3,4,4-trimethyl-1,2-dioxetan-3-yl)methyl ester
3	4.943	0.004%	1,2,4-trioxolane, 3,5-dipropyl-
4	5.072	0.131%	guanidine, methyl-
5	5.313	0.037%	acetyl-l-arginine
6	5.495	0.212%	acetamide, N-butyl-
7	5.578	0.055%	2,4-dimethyl-3-pentanol acetate
8	5.660	0.043%	cyclopentene, 3-isopropenyl-5,5-dimethyl-
9	5.707	0.058%	propane, 2-isothiocyanato-2-methyl-
10	5.795	0.093%	4-methyl-1,5-heptadiene
11	5.936	0.072%	1,3-dioxolane, 2-methoxy-2,4,5-trimethyl-
12	6.059	0.041%	1-octyn-3-ol, 4-ethyl-
13	6.206	0.159%	1-dodecanamine
14	6.565	0.021%	guanidineacetic acid
15	6.635	0.002%	2,4-dimethylamphetamine
16	6.717	0.003%	1,2,4-trioxolane, 3,5-dipropyl-
17	6.753	0.002%	benzenepropanamine, N-(1,1-dimethylethyl)-.alpha.-methyl-.gamma.-phenyl-
18	8.034	0.004%	1,4-butanediamine, N-(3-aminopropyl)-
19	8.157	0.007%	hexanal
20	8.274	0.003%	butanal
21	8.604	0.046%	1-undecene, 9-methyl-
22	8.880	0.264%	cyclohexasiloxane, dodecamethyl-
23	9.150	0.024%	benzenemethanol, .alpha.-(1-aminoethyl)-2,5-dimethoxy-
24	9.215	0.002%	histidine, 1,N-dimethyl-4-nitro-
25	9.244	0.003%	piperazine, 2-methyl-
26	9.273	0.013%	3-butenamide
27	9.391	0.027%	1-octadecanamine, N-methyl-
28	9.450	0.002%	fluoxetine
29	9.508	0.013%	caryophyllene
30	11.077	0.127%	cycloheptasiloxane, tetradecamethyl-
31	11.353	0.143%	cyclooctene, 3-(1-methylethenyl)-
32	13.163	0.286%	silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene]bis(oxy)]bis[trimethyl-
33	13.327	0.199%	cyclononasiloxane, octadecamethyl-
34	15.025	0.140%	cyclononasiloxane, octadecamethyl-
35	16.694	1.289%	pentadecanoic acid, 14-methyl-, methyl ester
36	17.094	13.675%	n-hexadecanoic acid (palmitic acid)
37	18.345	0.582%	10,13-octadecadienoic acid, methyl ester
38	18.404	2.850%	9-octadecenoic acid (Z)-, methyl ester
39	18.815	65.430%	9-octadecenoic acid, (E)- (stearic acid)
40	19.015	7.269%	octadec-9-enoic acid
41	19.162	3.205%	cyclononasiloxane, octadecamethyl-
42	20.325	0.442%	silane, [[(16.beta.,17.beta.)-estra-1,3,5(10)-triene-3,16,17-triyl]tris(oxy)]tris[trimethyl-
43	21.641	0.705%	cyclodecasiloxane, eicosamethyl-
44	23.316	0.778%	propanoic acid, 3-[bis[(trimethylsilyl)oxy]phosphinyl]-, trimethylsilyl ester
45	24.438	0.577%	indan, 1-methyl-3-nonyl-
46	24.726	0.244%	octadecanoic acid, trimethylsilyl ester
47	25.537	0.702%	Cyclononasiloxane, octadecamethyl-

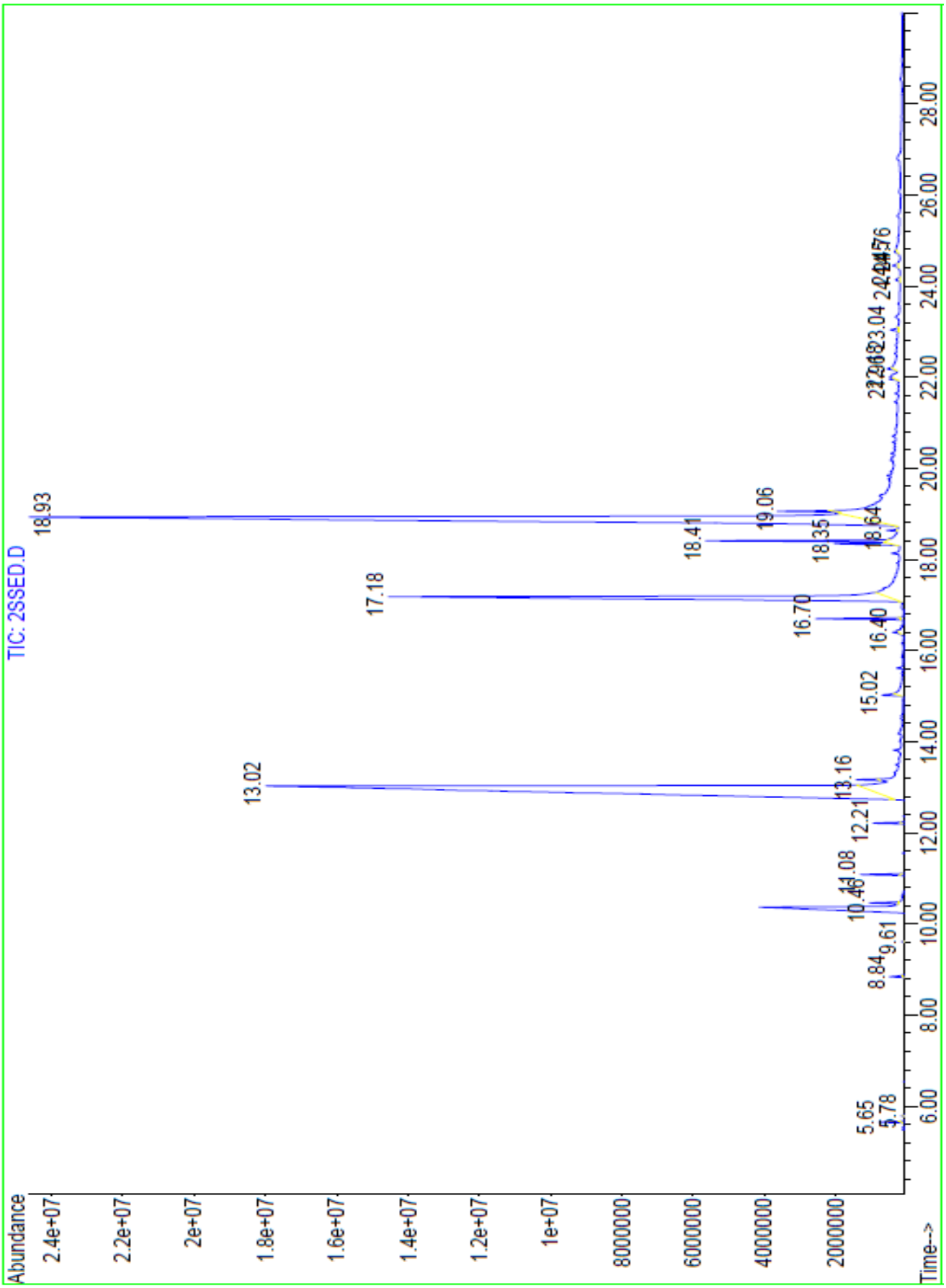

Figure 27: GC-MS chromatogram of the oil from the seeds of *Litsea glutinosa*

Table 17: Table showing compounds by Gas Chromatography-Mass Spectrometry (GC-MS) found in the seeds of *Litsea glutinosa*

Peak	Ret. Time	Area	Compound Name
1	5.648	0.195%	1,3,6-octatriene, 3,7-dimethyl-, (Z)-
2	5.783	0.014%	1,5-heptadiene, 2,3,6-trimethyl-
3	8.838	0.138%	2-decenal, (E)-
4	9.608	0.010%	decanoic acid, methyl ester
5	10.366	3.852%	n-decanoic acid
6	10.460	0.314%	copaene
7	11.083	0.393%	caryophyllene
8	12.211	0.268%	dodecanoic acid, methyl ester
9	13.028	39.177%	dodecanoic acid (lauric acid)
10	13.163	0.208%	caryophyllene oxide
11	15.019	0.225%	tetradecanoic acid (myristoleic acid)
12	16.400	0.084%	2-dibenzofuranamine
13	16.694	0.905%	hexadecanoic acid, methyl ester
14	17.182	13.962%	n-hexadecanoic acid (palmitic acid)
15	18.351	0.795%	8,11-octadecadienoic acid, methyl ester
16	18.410	1.841%	7-octadecenoic acid, methyl ester
17	18.639	0.106%	octadecanoic acid, methyl ester (oleic acid)
18	18.933	36.384%	9-octadecenoic acid, (E)- (stearic acid)
19	19.056	0.480%	octadecanoic acid
20	21.959	0.084%	floxuridine
21	22.182	0.110%	3-phenylbut-1-ene
22	23.040	0.163%	1. 2,5,5,6,1a-pentamethyl-cis-1a,4a,5,6,7,8-hexahydro-gamma-chromene 2. 2,6,10,14-hexadecatetraenoic acid,3,7,11,15-tetramethyl-, methyl ester, (E,E,E)-
23	24.138	0.106%	1H-indole, 1-ethyl-5-methoxy-2-(4-methoxyphenyl)-3-methyl-
24	24.450	0.115%	benzene, (2-chloro-2-butenyl)-

4.3.12 Cetane number

Importance of cetane number

The cetane number is a significant property to specify the ignition quality of a fuel for use in a diesel engine (Gopinath *et al*., 2009). The cetane number of a biodiesel fuel is influenced by the FAME composition of the oil (Gopinath *et al*., 2009). The determination of cetane number of biodiesel is an expensive and a time-consuming process (Gopinath *et al*., 2009). The higher the value, the better the ignition quality of fuel (Mohibbe *et al*., 2005). The cetane number of *Litsea glutinosa* is 64.79 (Mohibbe *et al*., 2005; Gopinath *et al*., 2009; Tong *et al*., 2011).

Appendix 1 highlights 17 plants with a cetane number above 60. *Litsea glutinosa* has the 7th highest cetane number out of 75 plants from the study according to Mohibbe *et al*. (2005) (**Appendix 1**). The biodiesel from different sources can be manipulated to obtain the desired cetane number. However, these treatments are expensive with many disadvantages, as discussed, in Section 2.3.2.5 above. This provides support to the choice of *Litsea glutinosa* as a potential source of biodiesel due to its excellent ignition quality that requires no additional treatment.

4.4 Germination

The seed of *Litsea glutinosa* was germinated *in-vitro* due to the contamination observed on the field grown explants after transformation was attempted.

4.4.1 Germination of *Litsea glutinosa*

Table 18 shows that germination of sterile *Litsea glutinosa* seeds were not successful on all three different media. The unsterilised seeds germinated on the damp cotton wool. The seeds did not germinate on damp filter paper in a petri plate and in distilled water. The germination time on each media was different. Germination was achieved on damp cotton wool and MS media after two months (**Table 18**). The seeds on Water agar (**Table 18**) and woody plant medium germinated in 54 and 16 days respectively. Wood plant medium was the most effective for the germination of seeds of *Litsea glutinosa*. The seeds without the seed coats did not germinate, while the seeds with seed coats took longer to germinate compared to the seeds which had their seed coats nicked.

4.4.2 Germination of *Litsea glutinosa* on MS medium

Table 18 shows that germination was achieved after two months on MS medium. Suitable leaves were obtained after eight months.

4.4.3 Germination of *Litsea glutinosa* on Water agar

The seeds on Water agar began to swell after 23 days (**Figure 14** and **Table 18**). Two plates were removed from the growth chamber. The first plate was placed inside the cupboard, to determine if light would affect germination and the second plate was left on the bench top to determine whether natural light and dark phase would affect the germination process. The temperature of the laboratory was maintained at ± 25°C. The conditions in the growth chamber was a 16 hr photoperiod maintained at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$. It was observed that all the seeds split open after 54 days. The plates outside the growth chamber got contaminated with fungi. This was a result of the plates being exposed to the laboratory environment. The plates in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$ were not contaminated since it was kept in a controlled enviroment. It was concluded that for this project the seeds would be germinated in the growth chamber since it was a more controlled environment and it took the same time to germinate compared to the plates left on the bench top and in the cupboard. Special note must be made that the contamination was found on the agar and not on the seed.

4.4.4 Germination of *Litsea glutinosa* on McCowns woody plant basal salt medium

The seeds began to germinate 16 days after it was placed onto McCown's woody plant medium. The shells that were nicked to break dormancy germinated faster. The medium turned brown since it reacted with the phenols produced when the seed was injured. Suitable leaves were obtained six months after germination.

4.4.5 Germination of *Nicotiana tabacum*

The seeds of *Nicotiana tabacum* germinated within a week. It was noted that the tobacco plant had many healthy leaves within one month compared to *Litsea glutinosa* which took from 16 to 54 days to germinate.

4.4.6 Summary of germination

Table 18 illustrates that four out of six methods used to induce germination were successful. Germination was achieved faster in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$ compared to natural light. Seeds with the seed coats germinated whereas the seeds without seed coats did not germinate. Seeds that were nicked germinated fastest. The most efficient method to germinate *Litsea glutinosa* was on McCown's woody plant basal salt medium supplemented with BAP, which is an artificial plant hormone. The germination time with McCowns woody plant basal salt medium was 16 days. Germination was achieved with Water agar in 54 days followed by germination in cotton wool which took 2 months.

Suitable leaves were obtained from the MS medium and Water agar after eight months, whereas suitable leaves were obtained from McCowns woody plant basal salt medium in six months. The McCowns woody plant basal salt medium was supplemented with BAP an artifical plant hormone that encourages shoot formation. Since *Litsea glutinosa* was a woody plant, the woody plant medium was the most effective media since it had the necessary compounds required for the growth of a woody plant.

Table 18: Germination of *L. glutinosa*

Method	Germination	Time frame
Cotton wool (unsterilised)	Germination	2 months
Filter paper (unsterilised)	No Germination	N/A
Distilled water (unsterilised)	No Germination	N/A
MS medium (sterilised)	Germination	2 months
Water Agar (sterilised)	Germination	54 Days
McCown's Woody Plant Basal Salt medium (sterilised)	Germination	16 Days

4.5 Transformation

Hairy root transformation of field grown explants of *Litsea glutinosa* failed due to fungal contamination. Hence transformation with leaves grown *in vitro* was attempted. *Nicotiana tabacum* produced hairy roots ranging from 0.3 mm to 1.1 mm in four weeks (**Table 19, 20** and **21**). The length of hairy root (**Figure 17**) developed is placed in brackets for **Tables 19, 20** and **21**. The leaves of *Litsea glutinosa* failed to transform (**Tables 19, 20** and **21**). The researcher believes that since the *Litsea glutinosa* plants were grown in culture bottles, it was not able to grow to its full potential. The small young leaves of *Litsea glutinosa* showed browning after infection (**Figure 16**). A possible reason for the browning and death of the *Litsea glutinosa* explant could have been the strain of *Agrobacterium rhizogenes* and the age and type of the explants used.

There was expectation of transformation since others reported that *Agrobacterium rhizogenes* broth with absorption of 0.2 at OD_{600} produced optimum results (Vernade *et al*., 1988 and Cao *et al*., 2008). Different calibrations of absorbances of *Agrobacterium rhizogenes* was attempted to transform *Litsea glutinosa*. Attempts to transform with an absorbance of 0.2 *Agrobacterium rhizogenes* at OD_{600} was not successful. An *Agrobacterium rhizogenes* broth with an absorbance of 0.130 at OD_{600} was also attempted to produce hairy roots and this too proved unsuccessful. The absorbance of 0.209 and 0.130 at OD_{600} was still too concentrated and consequently the explants died. **Table 19** and **20** illustrate the results with an absorbance of 0.130 and 0.209 at OD_{600} respectively.

Nicotiana tabacum was used as a control. Acetosyringone "triggers" transformation (Loubens *et al*., 1997). It was easy to transform *Nicotiana tabacum* due to the high concentration of natural acetosyringone in *Nicotiana tabacum* (Vernada *et al*., 1988). **Figure 17** shows successful transformation of *Nicotiana tabacum*.

Since the transformation of *Nicotiana tabacum* was successful with acetosyringone, transformation of *Litsea glutinosa* was attempted with the phenol supplement, acetosyringone, as suggested by Fortin *et al*. (1992). Acetosyringone, a monocyclic phenolic compound which encourages the *vir* gene to induce transformation, was purchased from Sigma.Vernade

et al. (1988); Fortin *et al*. (1992) and Loubens *et al*. (1997) all found that phenolic compounds are *vir* gene activators. Acetosyringone was added to Yeast Extract Peptone (YEP) broth at 10^{-4} concentration to activate the *Agrobacterium rhizogenes vir* gene. Thereafter the leaves were inoculated with the activated *Agrobacterium rhizogenes* and placed on MS medium. This procedure proved successful for *Nicotiana tabacum* but not for *Litsea glutinosa*.

Further attempt to transform *Litsea glutinosa* with acetosyringone supplemented to Yeast Extract Peptone (YEP) broth at a concentration of 10^{-4} was used. MS medium with an acetosyringone concentration of 10^{-4}, 10^{-3}, 10^{-2} and 10^{-1} were also used to transform *Litsea glutinosa*. This is illustrated in **Table 21**. *Nicotiana tabacum*, the control plant, was successfully transformed but *Litsea glutinosa* failed to transform.

Not many concentrations of acetosyringone could be tried since there were not enough sterile *Litsea glutinosa* leaves. *Litsea glutinosa* is a woody plant, which was difficult to transform. The researcher concurs with Sahoo *et al.* (1997) that callus cultures have been more difficult to induce in tree species. Bensaddek *et al*. (2008) initiated the idea that not all plant species can be transformed, and that the strain of *Agrobacterium rhizogenes* affects transformation.

Table 19: Transformation with absorbance of 0.130 at OD_{600} Acetosyringone 10^{-4} YEP (Method 2)

Absorbance of 0.130 at OD_{600} Acetosyringone 10^{-4}	Hairy roots
Litsea glutinosa	-
Litsea glutinosa	-
Litsea glutinosa	-
Nicotiana tabacum	+ (0.3mm) - 4 weeks
Nicotiana tabacum	+
Nicotiana tabacum	+

****Table 20: Transformation with absorbance of 0.209 at OD_{600} Acetosyringone 10^{-4} in YEP (Method 2)**

Absorbance of 0.209 at OD_{600} Acetosyringone 10^{-4}	Time frame
Litsea glutinosa	-
Litsea glutinosa	-
Litsea glutinosa	-
Nicotiana tabacum	+ (0.7mm) - 4 weeks
Nicotiana tabacum	+ (1.1mm) - 4 weeks
Nicotiana tabacum	+ (0.5mm) - 4 weeks

****Table 21: Summary of results of different concentrations Acetosyringone in media (Method 3)**

Concentrations Acetosyringone	*Litsea glutinosa*	*Nicotiana tabacum*
10^{-4}	Failed	Successfully
10^{-3}	Failed	Successfully
10^{-2}	Failed	Successfully
10^{-1}	Failed	Successfully

****Key for tables: + means successful results, - means unsuccessful/failed**

4.6 Callus

Other studies have shown that callus culture is best induced on MS medium supplemented with a plant artificial hormone mixture of 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) (Tariq *et al*., 2008, Ożarowsk, 2011). Callus plant tissue culture is very sensitive and attention must be paid to the hormone concentration in the medium (Tariq *et al*., 2008). The medium used must contain the correct balance of plant hormones and nutrients to induce and support callus growth (Tariq *et al*., 2008). Media, light and temperature are important variable factors that determine the induction of callus. The type of plant used also determines callus induction. Woody plants are more difficult to induce callus from than herbaceous plants. Not much literature and research is available on the callus culture of woody plants. Woody plants takes longer to germinate and to callus when compared to the control plant, *Nicotiana tabacum*. This was a drawback of this research. However it is not impossible to germinate and callus a woody plant. *Litsea glutinosa* presented many difficulties to germinate and induce callus for this research. Many factors had to be manipulated, as illustrated in **Table 6**, in an attempt to obtain callus culture.

Table 6 showed seven methods that were used to obtain callus culture from *Litsea glutinosa*. Method One used MS medium in the 16 hr photoperiod growth chamber at 26°C, under a standard cool white fluorescent light with a flux rate of 35 $\mu mols^{-1}. m^{-2}$. Four attempts to induce callus was conducted using Method One. The second and third attempts were successful. Bigger leaves were used in Attempts Two and Three. The failure of Attempts One and Four could be attributed to the smaller leaves being used. The results obtained for

Method One are shown on **Table 22**. The appearance of callus on larger leaves supported the literature that the callus formation depends on the type and age of the explants (Ożarowsk, 2011). The researcher expected callus to be induced within one to two weeks (Ahloowalia, 1982). Since *Litsea glutinosa* showed no callusing after 21 days, the researcher attempted Methods Two to Six concurrently as the researcher began to doubt Method One.

Table 6 lists Methods One to Seven and the variations between between Methods One to Seven to induce callus from *Litsea glutinosa*. The variations were in the medium, type of explants and sterilisation techniques. Woody plant media was also used since germination was achieved the fastest on woody plant media (**Table 18**). *Litsea glutinosa* is a woody plant and McCowns woody plant basal salt medium has the adequate nutrients required for the growth and support of woody plants. McCowns woody plant basal salt medium was used in Method Two. However McCowns woody plant basal salt medium did not support the formation of callus from leaves (**Table 23**).

During these trials, technical problems were experienced with the growth chamber. As a result the growth chamber over heated and all the callus trials and *in vi*tro plants were killed. The callus obtained from the MS media were removed from the media and dried in an oven. Oil stains were noted on the plate. It was concluded that the callus was successfully producing oil. Analysis of the oil could not be conducted due to insufficient volumes. Since it would take approximately 7 to 8 months to obtain leaves of *Litsea glutinosa* suitable for callus formation, other explants were considered.

Method Three and Four used seed explants of *Litsea glutinosa* to induce callus formation. Seed explants of *Litsea glutinosa* were used to induce callus formation in Method Three and Four. The difference between Method Three and Four was in the type of seed used for callus formation. Method Three used germinated seeds and Method Four used ungerminated seeds with seeds coats. The seeds in Method Three did not need to be sterilised, since the seeds were sterilised before germination. The seeds in Method Four were sterilised. Two types of media were used for Method Three and Four: MS medium and woody plant medium. These plates were left under the florescent light in the tissue culture laboratory. **Table 24 and 25** shows that callus formation was successful for both Methods Three and Four. The callus was soft, friable and cream in colour. However it turned brown as time progressed. The callus

was then sub-cultured onto plates and left under the florescent light. The callus then turned black and stopped growing. Condensation on the plates was seen. The researcher believes this was the result of the temperature under the florescent light being too hot. This resulted in the hormone concentration of the media to change due to the water evaporating. Some of the plates even dried out. Callus culture is very sensitive and needs to grow under very specific conditions.

Method Five, Six and Seven used field grown leaves of *Litsea glutinosa* as explants with MS media and wood plant media (**Table 6**). Younger soft field grown leaves were used. Field grown leaves were susceptible to fungal contamination which competed with the explants for nutrients on the media and consequently killed the leaves. Hence different sterilising methods were used. The leaves were sterilised for 5 min in 30% NaClO in Method Five. Callusing was seen with Method Five. However fungal contamination was also seen proliferating around the leaf and soon the entire plate was covered in fungal growth. The fungal contamination prevented the callus from being sub-cultured into cell suspension culture.

In Method Six sterilisation followed Method Five but the concentration and time of sterilisation was changed. In addition the leaves were soaked in an anti-fungal solution of 1% amphotericin B overnight. Callus was obtained in Method Six. It was observed that it took a longer time for the fungal contamination to manifest. It was concluded that the sterilising method did yield a level of success. This success was attributed to the use of amphotericin B.

Method Seven was developed using the evidence based on Method Five and Six. Field grown leaves were sterilised following Method Six. The difference between Method Six and Seven was that the 1% amphotericin B was added to the media. Amphotericin B inhibits fungal growth. **Table 28** illustrates that callus was obtained. Unfortunately the amphotericin B slowed the growth of the fungi but did not prevent it. Further investigations could not be pursued due to time constraints. However calluses were obtained. The callus was friable, soft and crumbly which is good for cell suspensions culture. The callus culture produced oil (**Figure 18**). If the quantity of callus could be increased in future studies analysis of the callus of *Litsea glutinosa* could be made to determine the fatty acids present.

This research highlighted the importance of correct culture conditions and the sensitivity of callus induction and maintenance for *Litsea glutinosa*. The main problem experience was fungal contamination and environmental conditions. Recommendation for future work would be to grow the plants in bigger culture bottles to obtain bigger leaves to induce callus. The media used in this research induced callus effectively. However further research can be done by adjusting the hormone concentration to speed the induction time frame. Investigation could also be conducted why the callus formed by the seeds turned black and stopped growing.

****Table 22: Method 1 (Callus formation)**

***in vitro* leaves**

Supplemented 2.4 D and BAP

Method	MS media
Attempts	
1	-
2	+
3	+
4	-

****Table 23: Method 2 (Callus formation)**

***in vitro* leaves**

Supplemented 2.4 D and BAP

Method	Woody Plant
Attempts	
1	-
2	-
3	-
4	-

****Table 24: Method 3 (Callus formation)**

Germinated Seeds

Method	MS media	Woody Plant
Attempts	Light	Light
1	+	+
2	+	+
3	+	+
4	+	+

****Table 25: Method 4 (Callus formation)**

Seeds

5 minutes in 30% NaClO

Method	MS media	Woody plant
Attempts	Light	Light
1	+	+
2	+	+
3	-	-
4	+	+

****Table 26: Method 5 (Callus formation)**

Field Grown Leaves

5 minutes in 30% NaClO

Method	MS media	Woody plant	
Attempts	Light	Light	Contamination
1	+	+	Fungal
2	+	-	Fungal
3	-	-	Fungal
Attempts	+	-	Contamination

****Table 27: Method 6 (Callus formation)**

Field Grown Leaves

35 minutes in 50% NaClO,

Amphotericin B solution overnight

Method	MS media	Woody plant	
Attempts	Light	Light	Contamination
1	+	-	Fungal
2	+	+	Fungal
3	+	-	Fungal
4	+	-	Fungal

****Table 28: Method 7 (Callus formation)**

Field Grown Leaves

35 minutes in 50% NaClO,

Amphotericin B solution overnight

Amphotericin B media

Method	MS media	Woody plant	
Attempts	Light	Light	Contamination
1	+	+	Fungal
2	+	+	Fungal
3	+	-	Fungal
4	-	-	Fungal

****Key for tables: + means successful results, - means unsuccessful**

CHAPTER 5: CONCLUSION

The energy crises that the world faces is the dependence that was created by using the petroleum as the sole source of energy. Petroleum was cheap and available. However, the constant increase in crude oil prices, limited fossil fuel resources (Tan *et al*., 2012) and environmental concerns (Lapuerta *et al*., 2008) has renewed the worlds focus on producing biodiesel from vegetable oils and animal fats (Fangrui and Milford, 1999).

Alternate fuel sources are being investigated more intensely to avoid further world energy crises. Many reasons have contributed to research into biofuel. The major reason is the need to save the environment while maintaining the standard of living.

This study aimed to determine the characteristics of the biodiesel from *Litsea glutinosa*. This research obtained biodiesel from *Litsea glutinosa* and successfully characterised it. The quality of the biodiesel from *Litsea glutinosa* is reflected by its characteristics.

Characterisation was done by comparing the biodiesel of *Litsea glutinosa* against the ASTM standards **(Table 8)**. Biodiesel from *Litsea glutinosa* was also compared to biodiesel from *Jatropha curcas* as *Jatropha curcas* was a biodiesel source with documented characteristics **(Table 9)**. It was found that *Litsea glutinosa* has better characteristics than *Jatropha curcas* in terms of iodine, density, sulphur and cetane number. Fuel with a higher density is preferred. *Litsea glutinosa* was more dense that *Jatropha curcas*. Cetane value of *Litsea glutinosa* is 64.79 compared to *Jatropha curcas* which has a cetane value of 57.1 (**Table 9**). The ASTM standard requires a minimum cetane value of 47 (**Table 8**). The fuels from *Litsea glutinosa* and *Jatropha curcas* meet the requirements of the ASTM cetane value standard for biofuel. Special attention must be paid to the cetane number. The cetane number of *Jatropha curcas* is very close to the minimum ASTM requirement of 47 compared to CN value of *Litsea glutinosa* which is 64.79. The higher the cetane number the better the fuel. Therefore biodiesel from *Litsea glutinosa* is more favourable than the biodiesel from *Jatropha curcas* with regard to cetane value.

Jatropha curcas has better characteristics compared to *Litsea glutinosa* with regards to saponification number, acid numbers, kinematic viscosity and viscosity. *Jatropha curcas* produces a better biofuel in terms of saponification number. Low saponification number indicates lower free fatty acid. Lower free fatty acid requires little or no pre-treatments before transesterification to obtain maximum yield of FAME. The acid number between *Jatropha curcas* and *Litsea glutinosa* differ by 0.05. This is a small difference. The smaller acid value of *Jatropha curcas* makes it a better fuel option.

A low specific gravity indicated a complete transesterification reaction. The specific gravity value of *Litsea glutinosa* is lower than *Jatropha curcas*. Therefore the transesterification reaction for *Litsea glutinosa* was complete and successful.

Fatty acids of *Litsea glutinosa* are stearic acid, lauric acid and palmitic acid. Fatty acids of *Jatropha curcas* are palmitic acid, oleic acid and linoleic acid. Palmitic acid is the common fatty acid between the two biodiesel sources. The different FAME profile results in different fuel characteristics.

The characteristics of the potential biodiesel from *Litsea glutinosa* compares favorably with the characteristics of biodiesel currently produced from *Jatropha curcas*. The slight variation in the biodiesel characteristics is acceptable due to the biological derivation of the fuel.

The distillation range of *Litsea glutinosa* is 56.2°C to 610.2°C. The biodiesel of *Litsea glutinosa* is operational at a wide range of temperatures.

This study revealed that *Litsea glutinosa* and *Jatropha curcas* have different yet relevant biofuel characteristics. Cetane number of biodiesel is used to determine the quality of the fuel (Van Gerpen, 1996; Bamgboyet and Hansen, 2008; Bello *et al*., 2013 and Oliveira and Da Silva, 2013). *Litsea glutinosa* can be considered as a potential feedstock for biodiesel because its cetane value is high. Although the biodiesel from *Litsea glutinosa* has very poor viscosity properties. This poor viscosity could be remedied by blending the biofuel from *Litsea glutinosa* with fuels from other souces.

Biodiesel is produced from animal and plant material. Biodiesel that uses plant material as a feedstock would be dependent on the agricultural industry. However climate uncertainty due to global warming, which ironically was caused by petroleum based fuel, suggests that the availability of plant material would be unreliable. Plant feedstock would be available seasonally and could be climate dependent.

Production of biodiesel from plant tissue culture can be further investigated for the possibility of obtaining FAME for biodiesel. This study had shown that hairy root transformation of *Litsea glutinosa* was unsuccessful. However callus, culture from *Litsea glutinosa* was successful. Traces of oil was successfully extracted from callus culture of *Litsea glutinosa*. A suggestion for future work on the transformation of *Litsea glutinosa* would be to use a different strain of *Agrobacterium rhizogenes*. Further, research on transformation of callus could also be investigated. More research would be required in plant tissue culture of woody to establish if FAME's can be obtained from plant tissue culture. However it must be noted that plant tissue culture is expensive and this would be reflected in the cost of the biodiesel.

This thesis has presented that when the biodiesel from *Jatropha curcas* was compared to biodiesel from *Litsea glutinosa* different biodiesel characteristics which complemented each other were established. This is the consequence of fatty acid composition of the oil dictating the fuel characteristics of the biofuel. It is suggested that biodiesel be produced from diffrent feedstocks and blend the biofuel to ensure that the biofuel would meet the specifications of a good biodiesel.

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APPENDIX

Appendix 1
CETANE NUMBER

Table showing the different cetane number of different biodiesel sources, according to Mohibbe *et al*. (2005)

Source	Cetane Number
Anacardiaceae	
1.Rhus succedanea Linn	52.22
2. Annona reticulata Linn	53.47
Apocynacea	
3. Ervatamia coronaria Stapf	56.33
4Thevetia peruviana Merrill	57.48
5. Vallaris solanacea Kuntze	50.26
Balanitaceae	
6. Balanites roxburghii Planch	50.46
7. Basella rubra Linn	54.0
Burseraceae	
8. Canarium commune Linn	55.58
Cannabinaceae	
9. Cannabis sativa Linn	36.40
Celastraceae	
10. Celastrus paniculatus Linn	51.9
11. Euonymus hamiltonianuis Wall	45.45
Combretaceae	
12. Terminalia bellirica Roxb	56.24
13. Terminalia chebula Retz	49.6
Compositaceae	
14. Vernonia cinerea Less	68.5
Corylaceae	
15. Corylus avellana	54.50
Cucurbitaceae	
16. Momordica dioica Rox	35.95
Euphorbaceae	
17. Aleurites fordii Hemsl	36.25
18. Aleurites moluccana Wild	34.18
19. Aleurites montana Wils	20.56
20. Croton tiglium Linn	49.9
21. Euphorbia helioscopia Linn	34.25
22. Jatropa curcas Linn	52.31
23. Joannesia princeps Vell	45.20
24. Mallotus phillippinensis Arg	36.34

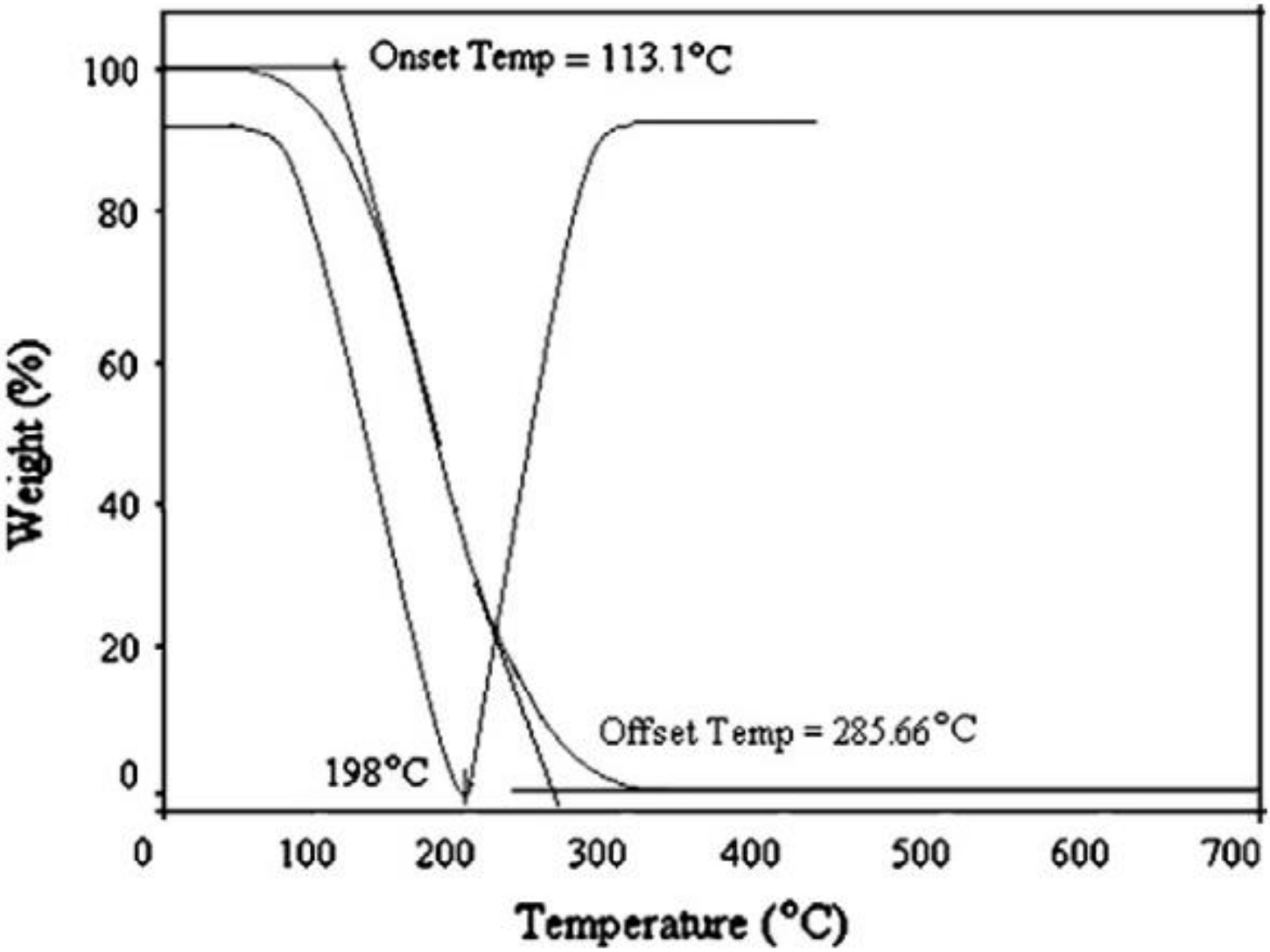
25. Putranjiva roxburghii	54.99
26. Sapium sebiferum Roxb	30.72
Flacourtiaceae	
27. Hydnocarpus kurzii Warb	47.89
28. Hydnocarpus wightiana Blume	49.25
Guttiferae	
29. Calophyllum apetalum Wild	51.57
30. Calophyllum inophyllum Linn	57.3
31. Garcinia combogia Desr	61.50
32. Garcinia indica Choisy	65.16
33. Garcinia echinocarpa Thw	63.10
34. Garcinia morella Desr	63.52
35. Mesua ferrea Linn	55.10
Icacinaceae	
36. Mappia foetida Milers	50.70
Illiciceae	
37. Illicium verum Hook	50.71
Labiatae	
38. Saturega hortensis Linn	25.46
39. Perilla frutescens Britton	30.09
Lauraceae	
40. Actinodaphne angustifolia	63.20
41. Litsea glutinosa Robins	64.79
42. Neolitsea cassia Linn	64.05
43. Neolitsea umbrosa Gamble	60.77
Magnoliaceae	
44. Michelia champaca Linn	50.28
Malpighiaceae	
45. Hiptage benghalensis Kurz	43.65
Meliaceae	
46. Aphanamixis polystachya Park	48.52
47. Azadirachta indica	57.83
48. Melia azadirach Linn	41.37
49. Swietenia mahagoni Jacq	52.26
Menispermaceae	
50. Anamirta cocculus Wight & Hrn	64.26
Moraceae	
51. Broussonetia papyrifera Vent	41.25
Moringaceae	
52. Moringa concanensis Nimmo	56.32
53. Moringa oleifera Lam	56.66
Myristicaceae	
54. Myristica malabarica Lam	61.81
Papaveraceae	

55. Argemone mexicana	44.45
Papilionaceae	
56. Pongamia pinnata Pierre	55.84
Rhamnaceae	
57. Ziziphus mauritiana Lam	55.37
Rosaceae	
58. Princepia utilis Royle	48.94
Rubiaceae	
59. Meyna laxiflora Robyns	50.42
Rutaceae	
60. Aegle marmelos correa Roxb	48.30
Salvadoraceae	
61. Salvadora oleoides Decne	66.13
62. Salvadora persica Linn	67.47
Santalaceae	
63 Santalum album Linn	42.88
Sapindaceae	
64. Nephelium lappaceum Linn	64.86
65. Sapindus trifoliatus Linn	59.77
66. Schleichera oleosa Oken	61.55
Sapotaceae	
67. Madhuca butyracea Mac	65.27
68. Madhuca indica JF Gmel	56.61
69. Mimusops hexendra Roxb	59.32
Simaroubaceae	
70. Quassia indica Nooleboom	46.74
71. Ximenia americana Linn	61.39
Sterculaceae	
72. Pterygota alata Rbr	51.09
Ulmaceae	
73. Holoptelia integrifolia	61.22
Urticaceae	
74. Urtica dioica Linn	38.73
Verbenaceae	
75. Tectona grandis Linn	48.31

Appendix 2

THERMO GRAVIMETRIC ANALYSIS

Thermogram of *Jatropha curcas* adapted from (Jain and Sharma, 2012).



Appendix 3

Biodiesel standards

Table showing Biodiesel properties adapted from Hoekman *et al*., 2012

Property	Biodiesel blendstock (B100)				B6–B20 Blends	
	U.S. (ASTM D6751-08)		Europe (EN 14214)		U.S. (ASTM D7467-08)	
	Limits	Method	Limits	Method	Limits	Method
Water and sediment (vol.%, max)	0.05	D 2709	0.05	EN 12937[g]	0.05	D 2709
Total contamination (mg/kg, max.)			24	EN 12662		
Kinematic viscosity @ 40 °C (mm^2/s)	1.9–6.0	D 445	3.5–5.0	EN 3104/3105	1.9–4.1	D 445
Flash point, closed cup (°C, min)	93	D 93	101	EN 3679	52	D 93
Methanol (wt.%, max.)	0.20[a]	EN 14110	0.20	EN 14110		
Cetane no. (min)	47	D 613	51	EN 5165	40	D 613
Cloud point (°C)	Report[d]	D 2500	Country Specific[d]		Report[d]	D 2500
Sulfated ash (wt.%, max.)	0.020	D 874	0.020	EN 3987		
Total ash (wt.%, max.)					0.01	D 482
Gp I metals Na + K (mg/kg, max.)	5.0	EN 14538	5.0	EN 14108/14109		
Gp II Metals Ca + Mg (mg/kg, max.)	5.0	EN 14538	5.0	EN 14538		
Total Sulfur (ppm, max.)	15[b]	D 5453	10	EN 20846	15	D 5453
Phosphorous (ppm, max.)	10	D 4951	4	EN 14107		
Acid no. (mg KOH/g, max.)	0.50	D 664	0.50	EN 14104	0.3	D 664
Carbon residue (wt.%, max)	0.05	D 4530	0.30[e]	EN 10370	0.35[e]	D 524
Free glycerin (wt.%, max.)	0.02	D 6584	0.02	EN 14105/14106		
Total glycerin (wt.%, max.)	0.24	D 6584	0.25	EN 14105		
Mono glyceride (wt.%, max)			0.80	EN 14105		
Diglyceride (wt.%, max)			0.20	EN 14105		
Triglyceride (wt.%, max)			0.20	EN 14105		
Distillation (T_{90} °C, max.)	36[c]	D 1160			343	D 86
Copper strip corrosion (3-h at 50° C, max.)	No. 3	D 130	No. 1	EN 2160	No. 3	D 130
Oxidation Stability (h @ 110 °C, min)	3.0	EN 14112	6.0	EN 14112	6	EN 14112
Linolenic acid methyl ester (wt.%, max)			12.0	EN 14103		
Polyunsaturated acid methyl esters (wt.%, max)			1.0	prEN 15799		
Ester Content (wt.%, min)			96.5	EN 14103	6–20 vol.%	D 7371
Iodine Value (g I_2/100 g, max.)			120	EN 14111		
Density (kg/m^3)			860–900	EN 3675		
Lubricity @ 60 °C, WSD, microns (max.)					520	D 6079
Cold Soak Filterability (seconds, max.)	360[f]	D 7501				