School of Pharmacy

Pharmaceutical Evaluation of Western Australian Sandalwood Seed Oil

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Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Dhanushka Sugeeshwara Hettiarachchi

Signature: ……..

Date : ..…….

Women behind my success: *My late grandmothers, my mother and my wife.*

Man who inspired me: *My father*

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Best virtues of a gentleman appear while facing hardships, such as a mature log of Sandalwood spreads the aroma upon chopping.

- 15th century Sinhala poetry “Subhashitha” by Alagiyawanna Mukaweti

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Table of Contents

List of Figures

List of Tables

Abstract

Western Australian Sandalwood (*Santalum spicatum* R. Br.) is high value timber crop as its fragrant heartwood is used for perfumery and Asian religious products. In recent years many private and state sector plantations have been established in Western Australia. Sandalwood seeds are seen as a potential financial incentive for planters to retain the trees for the prolonged period to produce more heartwood. Seed kernels are rich in oil composed of oleic acid and an acetylenic ximenynic acid. This study aimed to evaluate this seed oil to be suitable as an excipient in formulating cosmetic and pharmaceutical formulations.

Seeds were evaluated as a raw material source for a future oil extraction industry. Seed production, kernel yield, oil content and fatty acid profiles were assessed for seeds from different regions of Western Australia. Seeds from the Wheatbelt region were superior to the natural stands of the Goldfields region and coastal Perth region based on the above parameters. The effect of host trees on sandalwood seed oil was studied based on earlier parameters on plantation trees in the Wheatbelt region. Three Acacia species (*Acacia acuminata*, *Acacia microbotrya* and *Acacia aneura*) and Sheoak (*Allocasuarina huegeliana*) were tested for their effects on seed oil and seed production. Overall seed and oil parameters between the Acacia species showed no significant difference; however sandalwood trees hosted by acacia were a better source of seeds than Sheoak. The recently commenced sandalwood seed oil industry could rely on plantations hosted by Acacia species in the Wheatbelt region of Western Australia.

A novel method has been developed to extract oil from the sandalwood seed kernels using supercritical carbon dioxide. Different extraction pressures and feed to carbon dioxide ratios were studied at 40^{o}C.The method was developed from a laboratory scale to a pilot scale and found the extraction pressure of 40 MPa at mass ratios of 50-55 CO_2 kg/feed kg was found to be technically feasible and yielded 48-52% w/w oil from the seed kernel. This extract was compared with hexane and isopropyl alcohol extracts of sandalwood seeds obtained as described in previous studies, and other unrefined oils used in cosmetic and pharmaceutical preparations. The supercritical extract was found to comply with limits specified for other oils. Fatty acid profiles of a solvent extract and the supercritical extract showed no significant

difference. The phytosterols compositions were similar to the other seed oils, but the tocopherol concentration was found to be lower.

Supercritical extracted sandalwood seed oil was subjected to stability testing as air protected and unprotected groups for 360 days at 40^{o}C. Protected samples showed a remarkable oxidative stability when compared to the unprotected sample exposed to air. The unprotected sample had a high peroxide value (<10 meq O_2 Kg^{-1} of oil) within the first 30 days while the protected sample remained within 2-3 meq O_2 Kg^{-1} of oil for the test period. Secondary oxidative products were formed in the unprotected sample, whereas the protected sample had negligible amounts. However, the protected and unprotected samples showed no significant difference in the fatty acid profile. A similar trend was seen in the hydrolysis of glyceride bonds, as the free fatty acid content showed no clear difference between the unprotected and protected oils.

Toxicity and irritancy of sandalwood seed oil was studied and its safety was established. Sandalwood seed oil was found to have no cytotoxicity as a high LD_{50} value of 1240 mg/kg bodyweight was observed on *in-vitro* acute toxicity studies conducted on 3T3 NRU mice fibroblasts. Skin irritation on reconstituted human epidermal cells resulted in a 116% cell viability suggesting no irritation. Ocular irritation and corrosiveness was measured using bovine corneas, *in-vitro* irritation score was <55.1 which indicated no irritation or corrosion on ocular tissue. These findings would further support the use of sandalwood seed oil as an excipient in cosmetic and pharmaceutical preparations.

Santalum spicatum seed oil was reported to contain three main fatty acids including ximenynic acid; triglyceride with all ximenynic acid was identified previously identified as triximenynin. A novel reverse phase HPLC method was developed to detect the triglycerides on a photodiode array detector. A column chromatography method was developed to isolate the triximenynin from the sandalwood seed oil. Isolated triximenynin was primarily identified for fatty acid composition by gas chromatography. Further characterisation by nuclear magnetic resonance and mass spectrometry confirmed the structure of triximenynin. This method was scaled up to isolate this triglyceride at a purity of 90%; which was used in biological studies.

Anti-inflammatory, cytotoxicity and anti-oxidant studies were conducted on sandalwood seed oil, triximenynin and ximenynic acid. The free ximenynic acid showed an anti-inflammatory effect by inhibiting prostaglandin E_2 and dose dependent cytotoxic activity. However, triximenynin or the sandalwood oil did not show any of the above activities.

Current study has discussed seed production, extraction of oil, physiochemical characterisation, stability and pharmaceutical testing. This study has established several key properties of sandalwood seed oil enabling the oil to be industrially processed and utilised as an excipient in cosmetic and pharmaceutical preparations.

List of Publications and Presentations

Hettiarachchi DS, Liu Y, Fox JED, Sunderland VB. Western Australian Sandalwood Seed Oil: New Opportunities. Lipid Technology. 2010;22(2):27-30

Hettiarachchi DS, Liu Y, Jose S, Boddy MR, Fox JED, Sunderland VB. Assessment of Western Australian sandalwood seeds for seed oil production. Australian Forestry. 2012;75(4):246-50.

Hettiarachchi DS, Liu YD, Boddy MR, Fox JED, Sunderland VB. Contents of Fatty Acids, Selected Lipids and Physicochemical Properties of Western Australian Sandalwood Seed Oil. Journal of the American Oil Chemists Society. 2013;90(2):285-90.

Hettiarachchi, DS, Ang ZY, Brand JE, Fox JED, Sunderland VB, Liu Y. Effects of Host Trees on Western Australian Sandalwood Seed Oil. Proceedings of the 1st International Sandalwood Symposium. 2012; University of Hawaii, Honolulu, Hawaii, USA. p.207-217.

Hettiarachchi, DS, Ang ZY, Jose S, Liu Y, Fox JED, Sunderland VB. Sandalwood Seeds for Oleochemical Industry: Sustainability in Sandalwood Agro-forestry. Proceedings of the 16th International Forestry and Environmental Symposium. 2011; Sri Jayewardenepura University, Nugegoda, Sri Lanka.

Abbreviations

^{13}C	Carbon 13 isotope
^{1}H	Proton
AV	Anisidine Value
BP	British Pharmacopeia
d	Doublet (in NMR)
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
EN	Endocarp
EP	Epicarp
FAMEs	Fatty acid methyl esters
FID	Flame Ionisation Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography with Mass Spectrometry
HPLC	High Performance Liquid Chromatography
IR	Infra Red (Spectrometry)
IUPAC	International Union of Pure and Applied Chemistry
LC-MS	Liquid Chromatography with Mass Spectrometry
m	Multiplet (in NMR)
m/z	Mass to charge ratio
ME	Mesocarp
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear Magnetic Resonance Spectrometry
NRU	Neutral Red Uptake (of 3T3 cells)
OECD	Organisation for Economic Co-operation and Development
ORAC	Oxygen Radical Absorbance Capacity
PGE_2	Prostaglandin E_2
PV	Peroxide Value
q	Quartet (in NMR)
R*f*	Retention factor
s	Singlet (in NMR)
SCFE	Supercritical Fluid Extraction
SK	Seed kernel
t	Triplet (in NMR)
TAG	Triacylglycerol (Triglyceride)
TLC	Thin Layer Chromatography
TNF-α	Tissue Necrosis Factor α
UV	Ultra Violet (Spectrometry)
WA	Western Australia
XYMA	Ximenynic acid

Chapter 1 Introduction

Western Australian Sandalwood (*Santalum spicatum* R.Br.) is an important timber crop highly valued for its fragrant heartwood. Commercially available natural sandalwood is obtained from several species of the Santalum genus, including *Santalum spicatum*. Sandalwood has played a significant role in the ecology, ethnobotany, science and commerce of Western Australia for many years[1]. This study of the pharmaceutical evaluation of its seed oil aims to facilitate the sustainability of a plantation based Western Australian sandalwood industry and develop a new product from rural Australia.

1.1 Western Australian Sandalwood

1.1.1 Botanical Background

Western Australian sandalwood is one among seventeen members identified for the genus *Santalum* of the family Santalaceae[2] (Figure 1.1). A phylogeny study on the genus has found that *S .spicatum* is not closely related to tropical sandalwood species[3]. There are six species of the Santalum genus endemic to Australia, *Santalum spicatum* is mostly found in the arid and semi-arid areas of Western Australia and some areas of South Australia[4, 5] (Figure 1.2). This geographical distribution is shared between *S. accuminatum* (Sweet Quangdong), *S. murryanum* (Bitter Quangdong) and *S. leptocladum*, also they show morphological and phylogenic relationships with *S. spicatum*. However *S. lanceolatum* is a tropical variety which is found in the northern tropical parts of Australia and closer to other tropical sandalwood species than the above said Australian cousins. Santalaceae is considered as a family that originated from ancient Gondwanaland[3, 5].

Western Australian Sandalwood is a root hemi-parasite and depends upon multiple hosts throughout its life time. Nutrition and moisture is taken from the host trees through specially formed haustoria. Due to the parasitic nature they are found in topographical locations suited to their hosts from granite cliffs to fertile meadows. A number of hosts mainly Acacia species are preferred by *S.spicatum*, but it shows a diversity in host selection[6].

S.spicatum is a native perennial shrub or a medium size tree reaching a maximum height of 8 m with thin foliage and complex ascending branches covering an area of about 3-12 m^2. The trunk of the tree would reach a maximum of 40 cm diameter at the base, with a dark greyish brown fissured bark (Figure 1.3). The leaves are leathery and greyish green ovate shaped obtuse end with opposite arrangement.

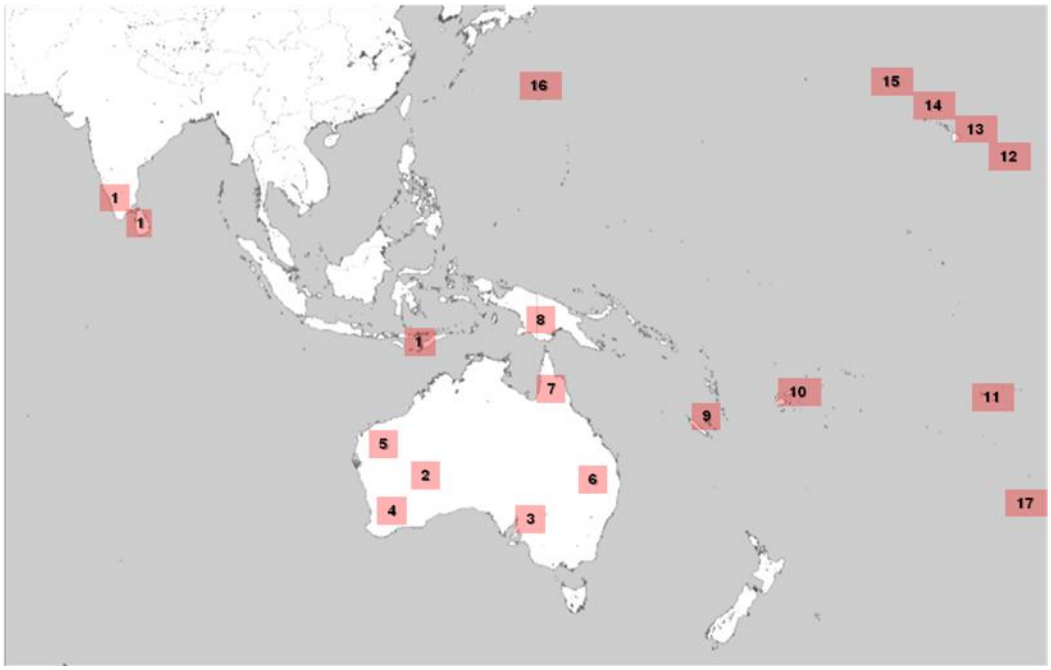

Figure 1.1: Global distribution of Santalum species[7]

1. *Santalum album* Southern India, Sri Lanka, Nusa Tenggara (Indonesia);
2. *Santalum spicatum* Western Australia/ South Australia;
3. *Santalum acuminatum* Southern regions, Australia;
4. *Santalum murrayanum* Southern regions, Australia
5. *Satalum leptocladum* Western Australia
6. *Santalum obtusifolium* Eastern Australia
7. *Santalum lanceolatum* Northern Australia
8. *Santalum macgregorii* Papua New Guinea, Iran Jaya (Indonesia)
9. *Santalum austrocaledonicum* Vanuatu, New Caledonia
10. *Santalum yasi* Fiji, Tonga
11. *Santalum inslare* Marques and Cook Islands
12. *Santalum paniculatum* Hawai', Hawaiian Islands (USA)
13. *Santalum ellipticum* O'ahu, Hawaiian Islands (USA)
14. *Santalum freycinetianum* O'ahu, Hawaiian Islands (USA)
15. *Santalum haleakalae* Maui, Hawaiian Islands (USA)
16. *Santalum boninense* Bonin Islands (Japan)
17. *Santalum fernandezianum* Juan Fernandez Islands (Chile)

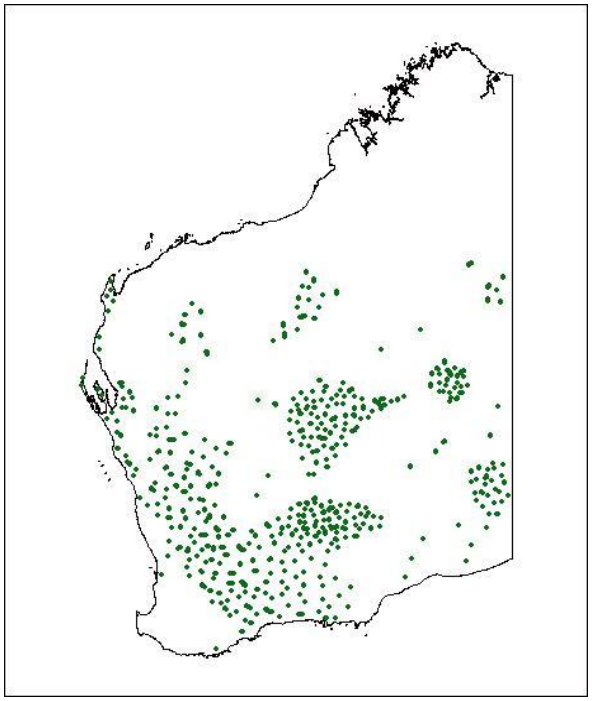

Figure 1.2: Distribution of *S. spicatum* in Western Australia based on spot identifications[8].

Figure 1.3: A – Rare large *S.spicatum* tree in the Wheatbelt region of Western Australia; B- harvesting of a *S.spicatum* tree in the Goldfields region of Western Australia[7]

Figure 1.4: *Santalum spicatum* Flowers; ripened fruit; fallen fruit with nuts separated from husk; tree bearing fruit in early summer[7]

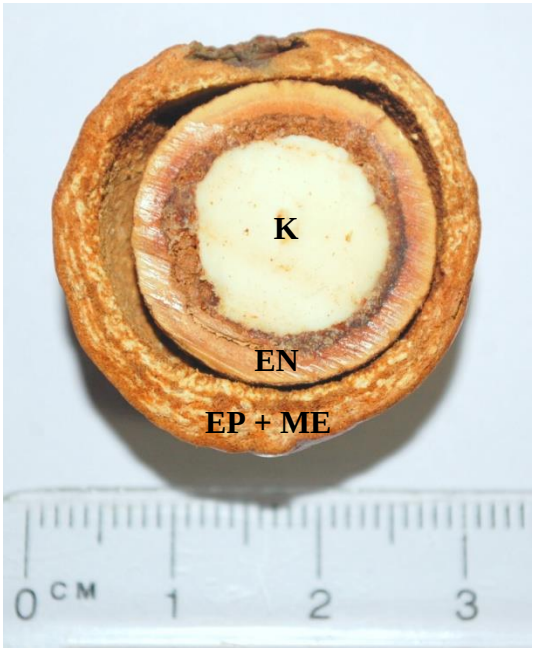

Figure 1.5: Transverse section of *Santalum spicatum* fruit.
EP- epicarp, ME- mesocarp (EP+ME together known as husk), EN – endocarp (Shell), K- kernel , K with EN henceforth mentioned as the seed [9]

S.spicatum bears flowers during the summer from the age of three years, the flowers are about 4-6 mm long, bisexual and brownish purple in colour; perianth and campanulate. There are four stamens, exerted, alternating with four rounded obtuse scales, inflorescence terminal or axillary, paniculate cyme [4, 6](Figure 1.4). Fox *et al.* have described the odour of the flower to be mildly carrion scented and necatariferous[1]. *S.spicatum* bears a drupe with a fleshy but leathery exocarp and hard-shelled endocarp (Figure 1.5). Once the fruit has fallen on the ground after maturation the exocarp easily disintegrates exposing the hard-shelled nut (Figure 1.4). This nut remains intact until germination. Seeds with shells which vary from 12-25 mm in diameter and the kernel within is oil rich, white and weighs one third of the total weight of the nut. A tree aged over three years could bear a crop of seeds weighing around a kilogram[9]. There are no major fauna dependent upon these seeds, it has been recorded that a highly endangered marsupial Woylie (*Bettongia penicillata*) consumed the seed kernel and also contributed to the propagation of seeds[10]. Tropical varieties of sandalwood produce a smaller drupe with a seed with a soft endocarp. These fruits and seeds are recorded to be consumed by different types of birds and mammals[11].

1.1.2 Sandalwood Essential Oil

Sandalwood heartwood is valued for its essential oil content; this essential oil is embedded within the xylem tissue of the tree[1]. There are two major sesquiterpene alcohols responsible for the distinct aroma of sandalwood, namely alpha and beta santalol. Each sandalwood species has its own composition of these two aroma components and other sesquiterpenes which are generated in a similar biosynthetic pathway. The quality of the essential oil depends upon the concentration of alpha and beta santalols[12]. There are numerous multidisciplinary studies been conducted on essential oil chemistry, genetics and forest ecology. Biosynthesis of sesquiterpenes in sandalwood heartwood is based on the genetics of the species or a particular strain which regulates the enzyme dependent process. These sesquiterpenes have never been reported from any tissues other than xylem[13, 14]. Indian sandalwood (*S. album*) is regarded to have the highest levels of these sesquiterpenes. Western Australian sandalwood has comparatively lower levels of the santalols but comprised of other sesquiterpenal compounds[12]. Typically Western Australian sandalwood has 15-25%

alpha santalol and 5-15% beta santalol. Apart from the santalols, Western Australian sandalwood contains considerable quantities of trans- farnesol, alpha-bisabalol, cis-nuciferol, beta and gamma curcumen-12-ol[15].

This essential oil was once used as a cure for topical bacterial infections prior to the discovery of antibiotics. Currently Western Australian sandalwood is used in perfume and traditional industries[7].

1.1.3 Sandalwood Industry in Western Australia

Sandalwood is a highly prized commodity in oriental cultures, being used as a cosmetic, medicine and in religious rituals. However sandalwood did not play a very prominent role in the indigenous culture in Western Australia, hence the resource was not exploited, anecdotal evidence of burning sandalwood as an effective flying insect repellent has been recorded[7].

European settlers identified sandalwood in the Western Australian wheatbelt region while clearing land for sheep and wheat farming. The first shipment of sandalwood left Fremantle port bound for Calcutta, India in 1845; hence starting a new export from a previously impoverished Swan River colony. During this time all of the European colonial powers were trying to trade goods with China, however the Chinese wanted only a handful of foreign items in exchange and sandalwood was the highest priority among them. By the time sandalwood was found in Australia different colonial powers were controlling the sandalwood trade in the pacific and the traditional sandalwood trade was between India and Indonesia. Western Australian sandalwood was seen as a more abundant resource and many ship loads started to trade via Hong Kong and Singapore[16].

Sandalwood was the first export of Western Australia and the highest income earner for the new colony for several decades. The search for sandalwood and the income from clearing the land established the European settlement and facilitated the exploration of the western half of Australia. It was a sandalwood harvester who first found gold in Kalgoorlie in the late 19th century. Soon after establishing Western Australia as a state, the government envisaged a future scarcity of sandalwood and drafted a Sandalwood Act in 1929 claiming Sandalwood as a Crown Property, henceforth anyone harvesting or selling sandalwood required a permit from the State

Government authorities. The sandalwood industry declined owing to the development of a mining and agriculture based economy and also the influx of better quality wood from the Pacific islands, Indonesia and India. Arrival of synthetic sandalwood analogues initially challenged the industry however it was evident that other than low grade products the remainder of the market demanded natural sandalwood. It was of concern whether the natural standing sandalwood would be sufficient to cater for the demand sustainably[7].

1.1.4 Sandalwood Plantations

In recent times a considerable amount of work has occurred in India and Western Australia to establish sandalwood as a plantation agro-forestry crop. Numerous studies have been conducted at Curtin University since 1975 and have lead this research. Under the flagship of the Mulga Research Institute at Curtin University emphasis was given to the establishment of plantations of sandalwood in semi-arid farm lands and reintroducing sandalwood to arid regions in Western Australia[6, 11]. Knowledge and expertise developed by this group of researchers was widely used in other parts of the world to establish sandalwood as a commercial crop. In Western Australia academic and state sponsorship has initiated the plantations; farmers have since joined the researchers to reintroduce sandalwood back to the Wheatbelt region. Currently there are over seventeen thousand hectares of *Santalum spicatum* plantations in the Wheatbelt region of Western Australia (Figure 1.6). Tree density is 350-400 sandalwood trees per hectare, which are usually hosted by few host tree species. There are private organisations involved in the plantations and several state owned plantations. A majority of the cultivated land is private farms, where farmers have formed the Australian Sandalwood Network which engages researchers from all disciplines to conduct research related to *S. spicatum*. This research is also coordinated with the ongoing re-forestation and sustainable harvesting research on natural sandalwood from arid regions of Western Australia[17].

Sandalwood is a slow growing tree, which takes approximately twenty years to produce a profitable amount of heartwood from cultivated trees. It was envisaged that plantations would not be successful without an incentive during the long growth period. The only product during this time would be the seeds which are produced

from the age of three years are easy to collect through the summer months. It is approximated that over 700-1000 tons of seeds could be collected from plantations in Western Australia; therefore potentially add value to sandalwood plantations and the industry[17, 18].

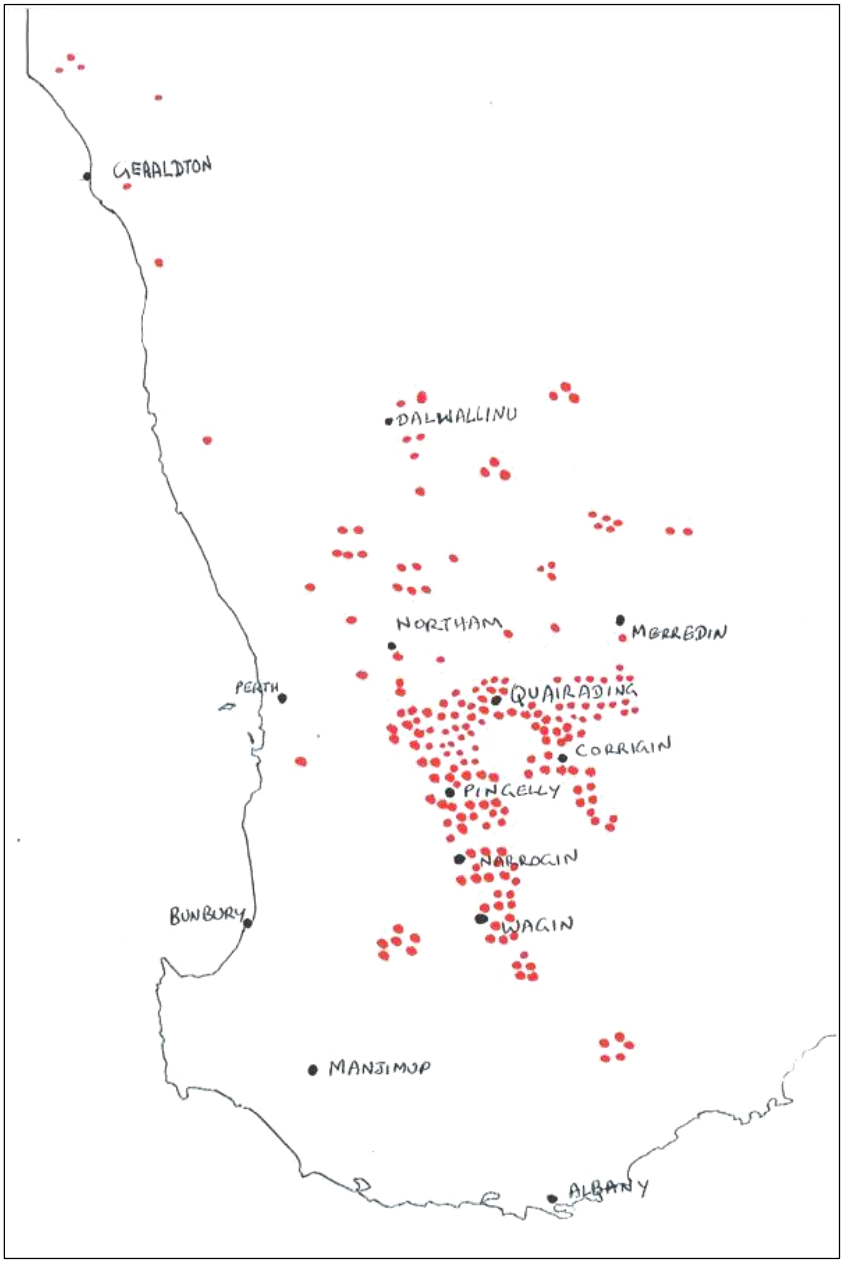

Figure 1.6: Geographical distribution of *S. spicatum* plantations in South Western Australia; Red dots represents plantations areas larger than 5 hectares (approximately 300 sandalwood trees per hectare)[17]

1.2 Plant Lipids

1.2.1 Lipid Components

The main component of plant lipids are the fatty acids commonly found as triglycerides. Lipids are complex mixtures of biological non-polar organic components, which includes phospholipids, sphingolipids, glycolipids, waxes, sterols, tocopherols, carotenoids and squalenes[19, 20]. These different lipid classes produce unique biological and chemical properties when isolated from plant tissue. Plant fatty acids have short to very long chain lengths. Common fatty acids found in plants are of C10, C12, C16 and C18 chain lengths, sometimes containing one or more unsaturated bonds[19, 20].

Fatty acids are synthesised in living cells, in green plant cells the fatty acid synthesis starts with acetyl-CoA originating from stroma of plastids. This follows by condensation of malonyl groups elongating the chain through an enzyme catalysed reaction. An enzyme known as stearoyl acyl carrier protein is responsible for the desaturation of these chains to form the unsaturated bonds. Also at the endoplasmic reticulum of plant cells the fatty acids will undergo further elongation and desaturation. As these enzymes are substrate specific, synthesis of different fatty acids is mostly governed by mitochondrial sequencing for selectivity. The above is known as primary metabolism and produces the more common fatty acids with functional properties in living tissue. Fatty acid synthase would catalyse acetyl CoA and malonyl CoA to form stearic CoA which is transformed to oleic by delta 9-desaturase desaturating the bond between carbon 9 and 10. This is further unsaturated at the 12 and 13 carbons by delta12 –desaturase. Further secondary metabolism occurs in plant cells which produce different types of fatty acids or derivatives. Among them linolenic acid isomers in many plants and ricinoleic acid production in castor oil seeds have been identified[21]. Triglycerols are synthesised in the endoplasmic reticulum of plant cells. Glycerol-3- monophosphate undergoes an acylation reaction with fatty acids-CoA. This would further undergos acylation at *sn*-2 and *sn*-3 positions to form triglycerides. Most of these triglycerides are stored within the cell as oil bodies[20].

Sterols are synthesised in plant cells via an isoprene synthesis pathway, squalene is an intermediate of sterol synthesis[22]. Commonly found plant sterols in seeds are campesterols, stigmasterols, β-sitosterol, δ-5-avenasterol and certain levels of cholesterol, brassiacasterols and ergosterols are also reported from plant seeds[23]. The most abundant tocopherol in plant seeds is γ-tocopherol, other main tocopherols are α and δ tocopherol[24]. However not all the seeds are rich in tocopherols, walnuts are found to have very low concentrations of tocopherols whereas almonds have a higher concentration[25].

Seeds of plants are generally high in oil, hence are used as a major source of lipids. Certain fruits, leaves and barks are harvested for their lipid components. Isolated phospholipids are used as emulsifying agents, however they are removed from the oils during extraction. Inclusion of plant sterols, tocopherols, carotenoids and squalenes in oils are beneficial for health as well as the stability of the oil. These are minor components in oils and they are synthesised via different pathways to fatty acids[19].

1.2.2 Lipid Analysis

Qualitative and quantitative analysis is an essential component in plant lipid research. Different classes of lipids and their composition are measured by diverse chemical and physical means. Lipids from plant sources are isolated from the tissues removing other undesired components by different methods. Plant lipid extraction has developed with technology to isolate and characterise specific lipid classes[26, 27]. A detailed account of lipid extraction from plants is given in Chapter 4.

Plant lipids are primarily analysed for their fatty acid composition; the unique physicochemical nature of oils depends upon its fatty acids. Triglycerides need to be hydrolysed first to obtain free fatty acids. In the majority of the plants these free fatty acids are similar in chemical nature, thus more specific methods are necessary to separate and identify them[20]. The common method is to transesterify the fatty acids to their methyl esters in the presence of a strong acid or a base. These fatty acid methyl esters are volatile compounds which can be analysed by gas chromatography. Mass spectrometry is an essential tool in identification of fatty acids of different

chain length and saturation. Detailed studies on fatty acids using mass spectrometry requires free fatty acids to be 4,4-dimethyloxazolinyl derivatives. These derivatives would help to identify the mass fragmentation patterns more suitable to determine the spatial arrangement of fatty acid chains[27].

Triglycerides are separated by different chromatographic methods such as thin layer chromatography and liquid column chromatography. Several HPLC methods have been developed to separate and quantify triglycerides in low concentrations. Use of silver ion with a silica stationary phase are widely used to separate unsaturated triglycerides[28, 29]. More detailed information on triglyceride isolation and separation is given in Chapter 6.

Plant sterols have been studied by conversion to their respective trimethylsilyl derivatives. This derivatisation would make sterols volatile to be analysed by GC[30]. There are several standard methods specified for derivatising and GC analysis[31]. Tocopherols are conveniently assayed by HPLC using the unsaponificable fraction of oil[24]. A mild method needs to be utilised for the saponification as tocopherols could undergo oxidation easily, there are several methods specified to assay tocopherols directly from the oils or lipid containing materials using mass spectrometry analysis[24]. Total lipid class analysis is conducted for certain oils and other plant materials. Plant extract or oil is often separated by column chromatography using a gradient of different solvents and gravimetric measurement of eluent composition as a commonly published method[26]. Thin layer chromatography is used for this purpose where the isolated bands representing different lipid classes which are measured by densitometry.

An important part of lipid analysis is the assay of stability; oxidation of the fatty acids is seen as the major cause for fatty acid degradation[32, 33]. Chemical changes will occur in the oil upon degradation which could be detected by simple chemical analysis. Hydrolysis of triglycerides is detected by increased free fatty acids which could be assayed by a simple acid base titration[34]. Formation of peroxide as the first step of oxidation can be iodometrically assayed. Further breakdown of fatty acids upon oxidation and formation of carbonyl compounds is detected by complexation

reactions with p-anisidine[33]. Most of these methods are standard methods for lipid analysis[31, 35]. Lipid oxidation and analysis is further discussed on Chapter 5.

1.2.3 Plant Lipids in Pharmaceutical and Cosmetic Preparations

Plant lipids have been used as drugs and cosmetics since recorded history. Most oils were used as nutritional supplements and also for laxative, anthelmintic, liver protective and anti-inflammatory properties. Essential fatty acids are needed for functioning of the human body, which is sourced only by consumption as they cannot be synthesised. A number of therapeutic agents are either extracted or dissolved in plant oils as a suitable vehicle. Oils are emollient on the skin helping to retain moisture and also used in skin disorders[36]. Topical preparations used as cosmetics and medications often contain an oleaginous phase. These oils are of petroleum or plant based origin. The oil phase of a topical preparation is often considered an excipient which creates a matrix for suitable dosage forms also acting as a vehicle for hydrophobic drugs; however in certain cases the active ingredient itself is the oil exerting a therapeutic activity on skin[37]. Another use of plant lipids is to derive oleochemicals, these are isolated single compounds or their derivatives from plant lipids. Free fatty acids are used for their formulation and as active ingredients; their alcohol esters, amides, salts are commonly used in day to day personal care formulations. Other lipid components such as phospholipids, sterols and tocopherols are isolated form plant oils and used extensively in the cosmetic, food and pharmaceutical industries[38].

Currently over a dozen oils are used in the above applications and novel oils are trialled and introduced to claim benefits over existing oils. Pharmacopoeias and formulation handbooks have listed monographs and required standards of several virgin grade and refined grade oils. Frequently used plant oils in pharmaceutical formulations are refined soybean oil, olive oil, linseed oil, castor oil and almond oil. The oils commonly used in cosmetic formulation apart from the above are; coconut, jojoba, wheatgerm, macadamia nut, shea butter and grape seed[39]. There are numerous other oils used in specific cosmetics and complementary medicine formulations, such as sesame seed oil in Ayurvedic formulations and safflower oil in traditional Chinese medicine[40 41]. These oils cater for different uses and strict

parameters are drawn to maintain the standards of these oils. Toxicity, stability and appearance are highly regulated, and the methods of extraction, storage and refining also play an important role[31]. When a novel oil enters the market it should address the above criteria to be deemed suitable for use as a pharmaceutical or a cosmetic agent. Extraction with a solvent free method, highly stable, free from toxins and allergens are seen as ideal apart from the standard chemical and physical suitability. Expecting added benefits from oils used as a matrix or a vehicle is seen as a current trend in many cosmetics. A tendency to use novel plant based products has been observed during the past decades and currently on the rise. The competition to provide this market is intensive, numerous research studies are underway to develop novel oils to cater for these different needs, while manufactures are seeking new oils to add novelty to their products[31, 35].

1.3 Sandalwood Seed Oil

1.3.1 Chemical Composition

The seed is typically rich in a drying fixed oil (50-60%), which contains a high percentage of an unusual acetylenic fatty acid identified as ximenynic acid discussed below[42-44]. Oil from sandalwood seeds (*S. spicatum*) has been reported to contain many other fatty acids along with ximenynic acid which are listed with their average composition in Table 1.1.

Table 1.1: Fatty acid composition as relative percentage of oil of *Santalum spicatum* seeds[45]

Fatty acid	Notation	Relative percentage of Oil
Palmitic acid	16:0	3.40
Palmitoleic acid	16:1 (n-7)	0.70
Stearic acid	18:0	2.70
Oleic acid	18:1 (n-9)	52.65
Linoleic acid	18:2 (n-6)	1.15
Linolenic acid	18:3 (n-3)	1.25
Stearolic acid	18:1 (9a)	0.95
Ximenynic acid	18:1 (9a,11t)	30.9
others		6.3

A study was conducted on the developing seeds of *Santalum spicatum* to research differences in lipid content from 160 days after flowering to seed maturation. Seeds reported less than 5% lipid of total wet weight of kernel until 100 days, and then an exponential growth increased the lipid content to 40% in the last 60 days. After 80 days of flowering palmatic and linoleic content dropped notably while oleic and ximenynic acids increased. This could be well explained by its proposed biosynthesis in the following section[46].

Seed oils of seven species of sandalwood have been reported from several studies (Table 1.2). It is clearly evident that the tropical varieties have a higher content of ximenynic acid compared to oleic acid, this is contrary to arid sandalwood spices where ximenynic content is lower than the oleic acid content[47]. Liu *et al.* has identified natural triglycerides containing ximenynic acid. It was found that triximenynic glyceride, monooleo diximenynic glyceride, monoximenynic dioleo glyceride and trioleoglyceride to be the main forms found in sandalwood seed oil[48]. There are no reports on the other lipid compounds such as phospholipids, tocopherols, sterols or the oxidative stability of sandalwood seed oil.

Table 1.2: Fatty acid composition as relative percentage of oil reported for some Santalum species[36, 47]

Fatty acids	Relative percentage of Oil					
	S. album	*S. obtusifolium*	*S. insulare*	*S. lanceolatum*	*S. accuminatum*	*S. murrayanum*
Palmatic acid	0.8	0.6	1.0	2.3	2.9	2.4
Palmitoic acid	0.6	0.4	0.6	1.3	2.7	0.3
Stearic acid	0.4	1.2	1.0	2.7	2.3	2.1
Oleic acid	18	14.3	1.8	26	57.7	54.8
Linoleic acid	0.7	0.7	0.5	NA	1.4	1.4
Linolenic acid	0.5	3.2	1.0	NA	2.5	2.3
Ximenynic acid	79	71.5	74.5	45	46.2	35.5

1.3.2 Ximenynic Acid

Ximenynic acid (*E*-11-octadecen-9-ynoic acid) is also known as santalbic acid (Figure 1.7) is a rare acetylenic fatty acid which is distributed among the plants of the order Santalales comprised of families Santalaceae, Olacaceae and Opiliaceae[49]. There are several genera of these families found to contain no ximenynic acid, but most of them have this fatty acid as a lipid component in the seed kernel, bark and leaves.

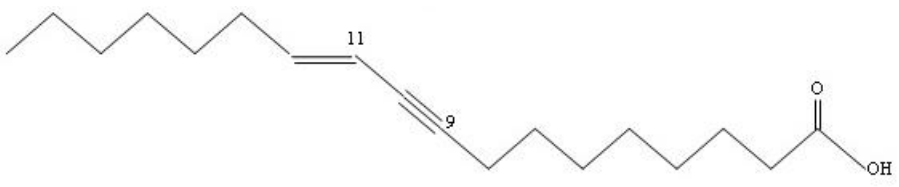

Figure 1.7: Structure of ximenynic acid (*E*-11-octadecen-9-ynoic acid)[50]

This fatty acid was first identified from *Ximenia* species in South Africa and later reported in the *Santalum* species in Australia followed by the Indian and Pacific species[51, 52]. Ximenynic acid is an eighteen carbon chain fatty acid with a triple bond at the 9th position and a double bond at the 11th. It is found abundantly as the *trans* isomer while a very low proportion of *cis* isomer was identified when fatty acids were derivatised with dimethyloxazoline[45].

There are two published methods on total synthesis of ximenynic acid using ricinoleic acid from castor oil. In these experiments both the *cis* and *trans* isomers have been synthesised[53]. Isolation of *E* -ximenynic acid from sandalwood seeds has been reported using different methods[52, 53]. A simple freezing method was introduced by Liu et al[45], while a more elaborate and precise urea adduct method was used by Likenji *et al.*[53] other approach include free fatty acid separation using supercritical fluids[54]. Hydrogenation of XYMA produces conjugated linolenic acid (CLA) which has biochemical importance[50]. Ximenynic acid (XMYA) has been reported to have good foaming capacity with diethanolamine or alkali-hydrolysates of gelatin[55].

Acetylenic acids are synthesised in plant tissue following separate pathways, thus their chemical structures are considerably different. Crepenynic acid of *Ixiolaena*

brevicompta (Asteraceae) is derived from linoleic acid which will undergo acetylenation at the 12th position to form a triple bond. However ximenynic acid is synthesised via a stearolic acid pathway, where oleic acid undergoes acetylenation at the 9th position before completing the primary metabolism and forming stearolic acid, this would undergo desaturation at the 11-12 carbon by delta 11 desaturase[21] (Figure 1.8). A recent study has identified rare fatty acid desaturase enzyme group in Santalaceae family[56]. These Δ12-desaturases unsaturate the 12-13 position of stearolic acid to form 12-octadecen-9-ynoic acid, which then isomerised to form ximenynic acid. These reactions could undergo further desaturation and acetylation to form poly-acetylenic compounds. A recent study has identified the genes responsible for poly-acetylenic 8Z-dihydromatricaria acid in soldier beetles. It has been indicated that acetylenic fatty acids play a defensive role in the organism and a significant role as defence in the seeds, roots and bark of trees[49].

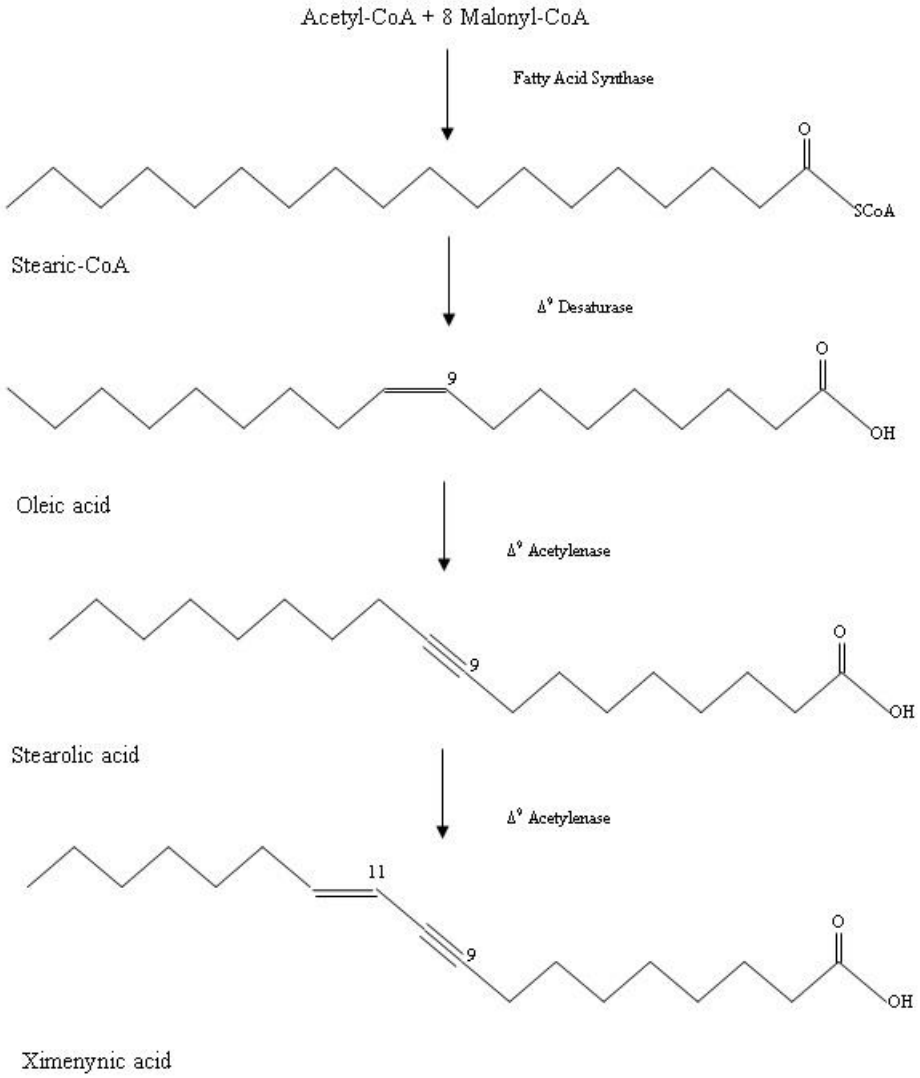

Figure 1.8: Acetylenic fatty acids biosynthesis from stearolic acid pathway[21]

1.3.3 Pharmacological Studies

The pharmacological effects of sandalwood seed oil and ximenynic acid isolated from different Santalum species have been studied previously. Sandalwood seed oil modifies the fatty acid composition of adipose tissue, liver and brain of mice[57]. This study was conducted in comparison to a standard laboratory meal and canola oil, where mice fed with sandalwood seed oil gained the least weight during the eight weeks observation period. Concentrations of ximenynic acid in the above tissues were lower than expected (0.3-3% of total fatty acids), there were no traces of ximenynic acid in the brain tissue. There were no effects on the content of other fatty acids, hence confirming that ximenynic acid has no anti-metabolic effects. Upon analysing the adipose tissue the unsaturated to saturated ratio (18:1/18:0 and 16:1/16:0) had markedly increased[57]. Increase of oleic acid in the tissue suggested that ximenynic acid is principally bio transformed to oleic acid. Docosahexaenoic acid content in the tissue of sandalwood seed oil fed mice was higher than in the control group. The above two observations suggest that ximenynic acid undergoes biohydrogenation as a metabolic step and may be producing conjugated linolenic acid isomers as intermediates. Increased docosahexaenoic acid suggests that ximenynic acid has inhibited the arachidonic acid pathway, thus inhibiting the arachidonic acid derived inflammatory mediators[50]. The researchers suggested that ximenynic acid might stimulate the Δ9-desaturase enzyme as per the above observations[57]. Mice fed with sandalwood seed oil were reported to lose whiskers in the third week. However histopathological examinations of liver, kidney, brain and adipose tissue showed no pathological difference upon feeding on sandalwood seed oil. The serum aspartate aminotransferase enzyme levels in mice fed with sandalwood seed oil were found to be higher than the control but much lower than the canola oil fed group[57].

A recent study has compared the tissue fatty acids and inflammatory factors of *S. spicatum* seed oil with other dietary oils in rats[58]. They have found that ximenynic acid is predominantly found in the adipose tissue but least in the liver. Increase of docosahexaenoic acid level in tissue was similar to the group fed with linseed oil, which is a known dietary supplement for polyunsaturated fatty acids. Remarkably the n6:n3 ratio had decreased, this could be correlated with the n-6 mediated pro-

inflammatory cytokines like prostaglandin F2α, E2, thromboxine B2, leukotriene B4, tissue necrosis factor-α (TNFα) and interleukin-1β. The researchers have concluded that sandalwood seed oil increased the tissue n3 polyunsaturated fatty acids while decreasing the n-6 thus reducing chronic inflammatory conditions[58]. In another study rats were fed with *Santalum accuminatum* (Sweet Quangdon) seed kernel and seed oil rich diet. Effects on tissue lipids, hepatic P450 and tissue histology of the above diet have been investigated[59]. It was found that 90% of the ximenynic acid was absorbed from the seed oil to the system. Rats fed with quangdong seed oil were found to have significantly elevated hepatic cytochrome P450 and specifically the 4A subgroup of that enzyme family[59]. Complying with the earlier study of *S. spicatum* seed oil on mice, no histopathological damage was recorded. Researchers have further tested the effects of ximenynic acid (santalbic acid) methyl esters on rats[59]. Hepatic and kidney cytochrome P450 4A levels were significantly elevated as with the seed oil study. Distribution of ximenynic acid was wide except in the brain. This study has concluded that ximenynic acid is metabolised differently from other fatty acids and the consumption of ximenynic acid could interrupt normal fatty acid biochemistry[59]. A broader claim of the nutritional benefits of Santalum species oils with ximenynic acid triglycerides and free acid has been made as a patent[60].

Seed extracts of *Santalum accuminatum* and the saponified fraction were found to be active on actylcholine esterase, peroxisome proliferator activate receptor (PPARα) and pancreatic lipase, also calcium metabolism had also improved[60]. Based on these activities inventors have claimed that food products containing triglycerides and free ximenynic acid could prevent diabetes, cancer, Alzheimer's disease and be beneficial on the skin and reduce body weight[60].

Though no toxicity data for ximenynic acid have been reported, crepenynic acid (12-octadecoynoic acid) from *Ixiolaena brevicompta* was found to be poisonous to sheep[61]. A study was conducted on the effects of ximenynic acid and crepenynic acid on leukotrien B4 and thromboxane B2 in rat peritoneal leukocytes in comparison with linoleic and ricinolic acid. Ximenynic acid inhibited leukotrien B4, thromboxane B2 and 6-ketoprostaglandin F1α. Ximenynic acid was found to be a better inhibiter of leukotrienes than cyclooxygenase. Both the acetylenic fatty acids have inhibited the phopholipase activity up 40-50% at higher concentrations

(>10µM). It was found that the inhibition pathway of the two fatty acids is different, this may be due to the positioning of the acetylenic bond or the presence of unsaturated bond at the 11th position[61]. Even though no toxicity has been reported it is clear that ximenynic acid is exerting xenobiotic effects, thus use of this seed as a dietary component needs further studies. The ethyl ester of ximenynic acid was found to produce vasoconstrictor effects by inhibiting the cytochrome P450-monooxygenase system resulting in a decrease of potent vasodilator epoxyeicosatrienoic acid[62]. This activity was positively tested to treat patients suffering from venous insufficiency cellulitis. This finding was further developed as a formulation with other known vasoactive natural ingredients as a treatment for hair loss evaluated by measuring the vascular capillary flow[63]. A different approach was taken in another patent where free ximenynic acid was added to a poly-herbal formula to promote hair growth. In this study inventors have measured in-vivo secretion of vascular endothelial growth factor and keratinocyte growth factor on isolated dermal papilla cells[64]. Dermal formulations have claimed the stimulation of microcirculation from its ximenynic acid composition when used from 0.01 to 5% of total weight[64]. In general acetylenic fatty acids could play an anti-inflammatory role by inhibiting prostaglandin, thromboxane and leukotriene synthesis. Terminal acetylenic fatty acids, 11-dodecoynoic acid and 10-undecoynoic acid found to inactivate hepatic cytochrome P-450 isozymes resulting in the termination of omega and omega -1 hydroxylation of fatty acids. This process has not shown any effect on increased P-450 levels of the rat hepatic microsomes[65, 66].

1.3.4 Ethnobotanical Evidence

There are several records from Aboriginal communities that sandalwood seeds have been part of the diet, nevertheless it would have been a supplement rather than a food source[67-69]. The most important findings are from personal communication by Dr. R. Longmore recorded where certain aboriginal communities in Western Australia have taken seed kernels as a remedy for arthritic pains and joint stiffness. It has also recorded that the kernel was applied on skin lesions and bruises. A recent communication with Mr. Clinton Farmer of Kutkabubba Martu community in Wiluna, WA has explained the current use of seeds. Seed kernels were made into a paste with water and boiled for a considerable time, then the paste is applied over

sores, bruises, lesions and aching joints and muscles. In certain places in Australia where the local knowledge was passed on by aboriginal communities, European settlers have consumed locally available *S. spicatum* and *S. accuminatum* seeds for arthritic pain[69]. These uses could be understood based on the above discussed anti-inflammatory properties of ximenynic acid.

1.3.5 Future Applications of Sandalwood Seed Oil

Sandalwood seed oil could be seen as a novel specialty oil with many different applications, such as food, nutrition, cosmetic and therapeutic. Currently there are several animal and isolated organ studies that have confirmed biological effects of the seed oil and ximenynic acid. Though some of these effects are seen as beneficial on conditions like inflammation, fatty acid metabolism and microvascular activity, it has also shown an increase in hepatic enzymes and disruption to fatty acid metabolism. Until full scale clinical studies confirm the pharmaco-dynamics and kinetics of the unusual fatty acid present in this oil it is uncertain whether this oil could be used as a food or a nutritional supplement to exert the benefits discussed above[70].

However this oil could be used as a vehicle or an excipient in pharmaceutical and cosmetic preparations. Sandalwood seed oil would be part of the existing recognition sandalwood has created for centuries in cosmetic markets. This would also act as a platform for wider use of this oil; hence support the future applications as a nutritional and therapeutic agent. However in order to be used in topical agents there are certain criteria as discussed previously for any novel plant oil. More studies need to be conducted on addressing the need of oils used topically which could gain the approval and recognition needed to permit the use of sandalwood seed oil[70].

1.4 Current Study

The current study has been structured to produce oil from sandalwood (*Santalum spicatum*) seeds which could be used in topical formulations for cosmetic and pharmaceutical purposes. Use of the oil from seeds would create a novel industry from Western Australia; this would encourage the sustainable cultivation of *Santalum spicatum* in arid and semi arid areas producing high grade sandalwood to cater for the world demand. Therefore this study is not only assisting to create a new industry but sustaining an existing industry of rural communities.

Thus the aims of the study are to address the following criteria;

- Evaluating sources of seeds for high oil and ximenynic acid contents.
- Development and evaluation of a supercritical carbon dioxide extraction method to obtain oil from seeds.
- Comparative study of supercritical fluid and solvent extraction methods on oil characteristics.
- Evaluate the stability of the oil on a long term basis for change in chemical parameters under aerobic and anaerobic conditions following international guideline requirements.
- Isolation, purification and characterisation of triximenynic glyceride from sandalwood seed oil.
- Comparatively evaluate sandalwood seed oil, triximenynin glyceride, ximenynic acid on cytotoxicity, antioxidant and anti-inflammatory effects.
- Evaluate the toxicity of the sandalwood seed oil on dermal cells.

Chapter 2 General Methodology

2.1 Materials

2.1.1 Reagents and Standards

Solvents used were of HPLC grade from Fischer Scientific (Fair Lawn, NJ, USA). All standards and reagents used for derivatisation reactions and chromatographic identification were sourced from Sigma-Aldrich (St. Louis, MO, USA).

Chemicals and reagents for specific studies were obtained from different sources; which are included in the respective sections. Water used in chemical analysis was purified water obtained from a Milli-Q filtration system (Millipore Co., MA, USA). It had a conductivity of less than 0.1 $\mu S.cm^{-1}$

2.1.2 Seed Material

S. spicatum seeds were provided by Wescorp Sandalwood Ltd. Seeds for this study were collected from sandalwood plantations in the Quairading area (Latitude 32.01197°, Longitude 117.39554°) situated in the Wheatbelt region of Western Australia. Collection was conducted in December 2009 (Batch: GT/QD/1209) and December 2010 (Batch: GT/QD/1210). Seeds were graded to sizes between 15mm and 25mm in diameter with shell; then stored under dry room temperature conditions away from sunlight.

2.1.3 Seed Oil

An authentic sample of seed oil extracted using supercritical fluid was provided by Wescorp Sandalwood Ltd (WS/SWSO2010A2). This oil was used for comparison studies and also for biological assays.

2.2 Instruments and Facilities

2.2.1 Gas Chromatography

An Agilent technologies gas chromatograph series 6980 with a mass selective detector (Series 5971) was used. Ultra high purity grade helium was used as the

carrier gas (BOC scientific gases, Australia). Compounds were identified by an on-line Wileys7 library using Chemstation software (Agilent Technologies, USA).

2.2.2 High Performance Liquid Chromatography

A reversed phase (RP) column (C18, 150 × 4.6 mm ID, 5 µm, Alltech, USA) was used on a Waters system (510 pump 717 auto sampler, Waters Corporation, USA). Ultraviolet absorption was detected using a dual wavelength photodiode array detector (series 2487, Waters Corporation, USA).

2.2.3 Thin Layer Chromatography

Silicagel G254 aluminium backed plates were used for analytical studies (20 x 20 cm, Macherey-Nagel GmbH & Co.KG, Germany). Preparative plates used were 2000 µm thickness and 20 × 20 cm in area. Samples were introduced using a Linomat IV autosampler with Camag 500µL and 10µL syringes. Developed plates were observed under 254 and 360 nm wavelength (CM10, Spectroline UV cabinet, Alltech, USA).

2.2.4 Column Chromatography

A glass column (40 mm diameter) with sintered glass filter and a PTFE stop cock. Stationary phase (170 mm height) reverse phase C18 silica of 60 Å pore size (710NC18E, Davisil Media, W. R. Grace and Co., MD, USA).

2.2.5 Liquid Chromatography – Mass Spectrometry

A single-quad LC-MS (model 2020, Shimadzu Scientific, Kyoto Japan) consisting of a binary pump (20AD), vacuum degasser, thermostatic auto sampler (CTO 20A), photodiode detector (SPD M20A) and mass analyser with both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) systems.

2.2.6 Nuclear Magnetic Resonance Spectrometry

1H and 13C NMR spectra were obtained by a Bruker Advance II 500MHz NMR spectrometer (Bruker Corp., Germany). Data were acquired, processed and analysed using the Topspin™ software (Bruker Corp., Germany).

2.3 Methods

2.3.1 Solvent Extraction Method

The seeds were de-shelled and the kernels pulverised using a domestic cutter mill to particles less than 2 mm diameter, the oil was extracted using hexane and isopropanol (3:2) admixture at room temperature for 18 hours in a screw capped glass bottle rotated in a vertical plane[71]. Larger extractions were conducted in larger reagent bottles which were placed in a flask shaker. Extracts were dried with anhydrous sodium sulfate and filtered under vacuum though Whatman paper. Larger extracts were filtered through suitable filter cloths before clarification. The solvent mixture was evaporated under reduced pressure at 50^{o}C and the oil obtained was bubbled with nitrogen under reduced pressure to remove residual solvents. Samples were stored in a vacuum chamber for 18 hr to remove residual traces of solvents.

2.3.2 Residual Solvent Analysis

Residual solvents were analysed using the method described in the British Pharmacopoeia[31]. A 0.2 g oil sample was dissolved in 20 mL of N,N-dimethylformamide . The solution was introduced to the GC-MS instrument described in Section 2.2.1 to deliver 1µL to a 50% poly-cyano siloxane column (30 m×0.25 mm×0.25 µm SP-225, SPE, Australia). Oven conditions were maintained at 40^{o}C for 20 min, then 10 ^{o}C per min to 200 ^{o}C, which was maintained for a further 20 min. Injector temperature was kept at 100 ^{o}C to avoid non-volatile lipids entering the column. Injection was performed split-less to a flow of 30 cm/s linear velocity. The mass spectra interface was kept at 220 ^{o}C and signals were measured as m/z in 40 ms intervals, using scan mode. Injector insert packing was changed after each test.

2.3.3 Supercritical Extraction

Development of a supercritical fluid extraction method for sandalwood seed oil is discussed in detail in Chapter 4.

2.3.4 Isolation of Ximenynic Acid

The seed oil was saponified with aqueous 0.5 M KOH solution containing 2% ascorbic acid by refluxing for 30 minutes. The contents were washed with deionised water and the unsaponifiable material separated by extracting with two aliquots of *n*-hexane. The aqueous fraction was acidified with 6 M hydrochloric solution until the pH was lower than 7. The opaque solution was then extracted using three aliquots of hexane in a separating funnel. Solvent layers were combined and dried with anhydrous sodium sulfate and filtered through a grade 5 Whatman filter paper. The solvent fraction was then stored at -20^{o}C for 48 hours until the ximenynic acid precipitated. The filtration apparatus and paper were kept at the same frozen temperature for 2 hours and then the contents were filtered immediately. Later a small quantity of hexane was passed through the filter at room temperature and collected in a smaller beaker. It was then frozen at -20 ^{o}C for 12 hours and the filtration repeated. Free ximenynic acid was collected by the filter paper as flakes and then kept in a vacuum chamber to remove residual solvent. Samples were stored at -20 ^{o}C free from moisture and protected from light[45].

2.3.5 Fatty Acid Analysis

2.3.5.1 Fatty Acid Methyl Esters

The fatty acids were converted to fatty acid methyl esters (FAMEs) and analysed by gas chromatography. The oil was dissolved in 10mL of n-hexane (10 mg/mL), then 500 µL of 2 M methanolic potassium hydroxide was added and vortexed for 2 minutes. When two clear layers were visible 25 mL of deionised water was added and the mixture shaken thoroughly and set to separate into two layers. The top hexane layer was aspirated and dried over anhydrous sodium sulfate[45, 72].

2.3.5.2 GC-MS Method for FAMEs Analysis

Nonadecanoic acid (C19) methyl ester (Sigma-Aldrich Chemicals, USA) was used as the internal standard at 10mg/mL in n-hexane. Samples for analysis were made with 200µL of the test solution and 200µL of internal standard and the volume was made up to 20mL with n-hexane. A C8-C24 series standard FAMEs (Sigma-Aldrich Chemicals, USA) was used to confirm the identification. The separation was carried out on a 50% poly-cyano siloxane column (30 m×0.25 mm×0.25 µm SP-225, SPE, Australia) at 50 ^{o}C for 5 min, then 5 ^{o}C per min to 120 ^{o}C, then 2 ^{o}C per min to 180 ^{o}C, which was maintained for a further 10 min. Injector temperature was kept at 220 ^{o}C and injected as split-less to a flow of 30 cm/s linear velocity. The mass spectra interface was kept at 220 ^{o}C and signals were measured as m/z in 40 ms intervals, using scan mode[73].

2.3.6 Phytosterols, Tocopherols and Squalenes

2.3.6.1 Saponification

The oil was saponified using 0.5 M ethanolic KOH with ascorbic acid (2% w/v) in screw cap vials at 70^{o}C for 30 min. The vials were protected from light, and their headspace filled with nitrogen gas. Unsaponified material was extracted into *n*-hexane and evaporated under vacuum at room temperature.

2.3.6.2 Silyl Derivatives of Phytosterols

This unsaponifed fraction (20 mg) was derivatised into tri-methyl silyl derivates using 200 µL of N,O-bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) 99:1 admixture (Sigma-Aldrich Chemicals, USA) dissolved in 200 µL of Pyridine (Sigma-Aldrich Chemicals, USA) and placed in screw capped glass vials. Vials were heated at 70 ^{o}C in a heating block for 30 minutes and then brought to room temperature before solvents were removed under nitrogen flow[30, 31].

2.3.6.3 GC-MS Method for Phytosterol Analysis

Derivatised samples (100 µg/mL) were dissolved in ethyl acetate with 5α-cholestene (Sigma-Aldrich Chemicals, USA) 50 µg/mL as the internal standard. The upper solution (1µL) was injected on to the column while maintaining the injector port at

250 ^{o}C. Carrier gas helium flow was at a linear velocity of 25 cm/sec with a split ratio of 1:10. Column conditions were initially at 120 ^{o}C and increased to 250 ^{o}C at 5 ^{o}C/min. Detector temperature was constant at 300 ^{o}C and signals were acquired at 40 ms intervals[30].

2.3.7 GC-MS Method for Squalene Analysis

The unsaponified fraction was dissolved in ethyl acetate with 5α-cholestene as the internal standard. Sample solution of 1 µL (100 µg/mL with 50 µg/mL 5α-cholestene as internal standard) was injected on to the column while maintaining the injector port at 250 ^{o}C. Carrier gas helium flow rate was at a linear velocity of 25cm/sec with a split ratio of 1:10. Column conditions were initially at 120 ^{o}C and increased to 250 ^{o}C at 5 ^{o}C/min. Detector temperature was constant at 300 ^{o}C and signals were acquired at 40 ms intervals[27].

2.3.8 Tocopherol Analysis by HPLC

Tocopherols were analysed by reversed phase HPLC using the unsaponified fraction (1mg/mL). Standards of α, γ and δ -tocopherols (Sigma-Aldrich Chemicals, USA) were used to develop a calibration curve. Spiking with standards of α, γ and δ-tocopherols were used to verify the identification based on retention time. A control of wheat germ oil was employed in order to confirm the assay methods. Mobile phase showed no absorption at 292 nm. Methanol and water (97:3) was the mobile phase at a flow rate of 1.5 mL/min[24, 27].

2.3.9 Triglyceride Analysis

Chapter 6 of the thesis elaborates the methods described in this section

2.3.9.1 Triglyceride Separation by TLC

TLC plates were developed in *n*-hexane, diethylether and glacial acetic acid (70:30:1). Samples for analysis were dissolved at 50 mg/mL concentration while preparative samples were at 250 mg/mL[26].

2.3.9.2 Flash Chromatographic Method for Triglyceride Separation

The mobile phase was acetone and isopropyl alcohol (65:35). Oil was dissolved to a concentration of 200 mg/mL in *n*-hexane. The solvent mixture was fed to the top of the column described in Section. 2.2.4 and a positive pressure was created using nitrogen gas at 50 kPa producing a steady flow of 5 mL/min. Each fraction volume was kept constant at 25 mL, while the number of fractions collected depended upon the mass of oil used[28, 74].

2.3.9.3 Triglyceride Analysis by HPLC

The mobile phase used for triglyceride separation was acetonitrile and propan-2-ol (65:35) at 2.5 mL/min for 30 minutes[28, 48, 74].

2.3.9.4 Mass Spectrometry Method for Triglyceride Characterisation

Isolated triglycerides were dissolved in a chloroform and methanol (1:1) admixture at a concentration of 100 µg/mL and 5µL was injected through the auto sampler to the LC-MS system. The sample was passed to the PDA and the mass selector without a separation column. Ions were scanned for 1-1000 m/z in ESI positive mode. Interference voltage was 4.5kV and detection was made at 1.2 kV. Heat block temperature was maintained at 300 ^{o}C, dissolution gas temperature 200 ^{o}C. Nitrogen was used as the nebulizer gas at the flow rate of 1.5L/min and dry gas flow of 10 L/min.

2.3.9.5 Nuclear Magnetic Resonance for Triglycerides Characterisation

Samples were analysed by Dr. Lindsay Byrne at School of Chemistry and Biochemistry at the University of Western Australia. Isolated triglycerides were dissolved in d-chloroform and the 1H and 13C NMR spectra were generated using a 5mm dual probe.

2.3.10 Physicochemical analysis

2.3.10.1 Peroxide Value

Peroxide value is defined as the milliequivalents of peroxide or active oxygen free radicals per kilogram of oil[31]. This is a representation of oxidation or rancidity of oils and fats, in this study the British Pharmacopoeial method was used[31]. An oil sample

of 5g was dissolved in 30 mL of dichloromethane acetic acid mixture (3:2) in an Iodine flask. A 0.5mL aliquot of saturated potassium iodide reagent was added and shaken for 2 minutes followed by adding 30mL of water. This was then titrated with 0.01M sodium thiosulfate until the yellow colour disappeared. When no further colour was observed, then 0.3g of Vitex powder (Ajax Fine Chemicals, NSW, Australia) was added and shaken well until the contents of the flask turned blue. It was then titrated until the blue colour just disappeared. A blank was performed and the titre value for the blank must not exceed 0.1mL. Peroxide value is calculated using Equation 2.1, tests were conducted in triplicate.

Equation 2.1

P_i= 10 (test titre value – blank titre value) / weight of the oil taken (g)

2.3.10.2 Acid Value

Acid value is defined as the milligrams of potassium hydroxide needed to neutralise the free fatty acids in 1g of oil[31]. A 100 mL mixture of ethanol and n-hexane (1:1) was prepared and neutralized with 0.1 M sodium hydroxide solution using few drops of phenolphthalein solution as indicator. This was terminated when a light pink colour persisted. A 1 g sample of oil in 20 mL of the previously neutralized solution was prepared and titrated with 0.1 M sodium hydroxide using a few drops of the same indicator until the light pink colour persisted. Acid value was calculated using Equation 2.2, tests were conducted in triplicate.

Equation 2.2

A_i= 5.610 × titre value (mL) / wt of oil taken (g)

2.3.10.3 Iodine Value

Iodine value is defined as the amount of iodine in grams taken up by 100 g of oil[31]. Iodine values increase with the number of double bonds in a fatty acid[31]. A 0.25 g sample of oil was dissolved in 15 mL of dichloromethane acetic acid mixture (3:2) in an Iodine flask, followed by 20 mL of iodine-chloride solution. The flask was closed and kept in the dark for 30 min, followed by shaking and adding 10 mL of 10%

potassium iodide solution and 100 mL of water. It was then titrated with 0.1M sodium thiosulfate solution until a yellow colour disappeared after which 0.3 g of Vitex powder was added then a blue colour is observed, titrate further until blue colour disappears. A blank assay followed the same procedure. The Iodine value was calculated as given in Equation 2.3, tests were conducted in triplicate.

Equation 2.3

$$I_i = 1.296\ (\text{Blank Titre} - \text{Test Titre}) / \text{weight of oil (g)}$$

2.3.10.4 p-Anisidine Value

This method is used to identify secondary oxidative products. p-Anisidine reacts with carbonyl groups and form chromogenic compounds which can be measured by UV/visible photospectrometry[31, 35]. A 0.5 g sample of oil was made up to 25 mL with trimethylpentane and designated Solution A. The absorbance at 350nm using trimethylpentane as the blank. To a 5 mL of A added with 1 mL of p-anisidine solution (0.25% w/v in glacial acetic acid) was added and mixed by shaking and kept away from light for 10 min. This was designated Solution B and its absorbance was measured soon after 10 min at 350nm. The blank for this measurement was made by mixing 5 mL of trimethylepentane and 1mL of p-anisidine solution (0.25% w/v in glacial acetic acid). The *p*-Anisidine value was calculated by Equation 2.4.

Equation 2.4

$$AV_i = 25\ X\ (1.2\ \text{absorbance of Solution A - absorbance of Solution B})/\text{weight of oil taken (g)}$$

2.3.10.5 Freezing Point

An oil sample was frozen using carbon dioxide in a test tube with an electronic thermometer probe. Test tube was then introduced to ambient conditions and the temperature of phase change (melting) was noted[31]. Tests were conducted in triplicate.

2.3.10.6 Refractive Index

Refractive indexes of the oils were measured using an Abbe style refractor meter (Mark III, Reichert Technologies, USA)[35]. A drop of oil was introduced to the optical surface and the value is noted as displayed. Tests were conducted in triplicate.

2.3.10.7 Viscosity

Viscosity was measured using a Bohlin viscometer (Visco-V88, UK). Samples of 3 g each were introduced to the cylinder with a gap of 0.7mm and the shear rate was set for 45.1 s^{-1}. The reading was taken at 200 seconds. Tests were conducted in triplicate and results were obtained from the software. (Bohlin, UK).

2.4 Statistical Analysis

All data were collected and enters to Microsoft Excel spreadsheets. General statistical analysis, graphs and calculations were performed using the Microsoft Excel 2007-2010. Further analysis presented in Chapter 3, Chapter 4 and Chapter 7 were conducted using SPSS 16.0 (SPSS, Chicago, USA). The percentage inhibition of prostaglandin E_2 and half-maximal inhibitory concentration (IC_{50}) values for cytotoxicity were calculated using GraphPad Prism® version 4 and 5(La Jolla, CA, USA).

All the data given in tables are presented as the mean ± standard deviation, graphs show the mean ± standard error. Statistical significance was analysed by t-test where p-values were less than 0.05 were considered significant. Correlations were demonstrated on a scatter graph with linear regression closer to 1. Comparative analysis between groups were analysed by Scheffe's test and the similar groups were marked with different superscript letters.

Chapter 3 Assessment of Seed Source for Western Australian Sandalwood Seed Oil

3.1 Introduction

Commercial operations involving sandalwood seed oil began in Western Australia from 2013[75]. A major limiting factor for any natural product industry is the supply of raw material containing the active constituents in suitable concentrations[76]. Ximenynic and oleic acids are the major fatty acids species found in the lipid fraction of the seed[45]. Western Australian sandalwood seed oil could be a plentiful supply of ximenynic acid; seeds which are rich in oil and ximenynic acid could be profitable for the planters and industry[70]. It is necessary to analyse seeds from different sources to determine their overall suitability for use as a raw material. Several field trials were established by forestry scientists in the Wheatbelt and coastal areas of Western Australia to study the factors affecting the heartwood essential oil quality in comparison to natural stands and plantations[11]. Sandalwood trees are affected by many factors influencing their growth and maturity, among them the selection of host trees and geographical conditions are identified as key factors[1, 6].

Most sandalwood plantations are in the Wheatbelt region of Western Australia where the soil is clay loam with an annual rainfall of 500-600mm and average annual temperature range of 9-27 ^{o}C[77-79]. Naturally found sandalwood is abundant in the Goldfields region of Western Australia with red loam soil where annual rainfall is 200-300 mm and average temperatures are 15-33 ^{o}C[77-79]. Some sandalwood is planted in the coastal areas mainly as field trials and has a possibility of plantations, the soil is sandy and the annual rainfall is 800mm with a temperature range of 12-27 ^{o}C[77-79]. Brand *et al*. has identified two distinct cultivars of *Santalum spicatum* in Western Australia. An arid variety found commonly in the Goldfields area and to some extent in the Pilbara and Gascoyne. A semi-arid variety found mostly in the Wheatbelt region. However, the natural stands would not be a suitable source of seeds because of the harvesting difficulties. Plantations trees would be the ideal

source of a continuous supply of seeds, hence this source needs to be studied in comparison with natural sources and research based field trial sources. A first step for this study was to analyse the seeds from these three regions for their chemical and physical properties.

An important factor affecting the growth of sandalwood is the effect of host trees. Sandalwood depends upon host trees throughout its life for nutrition through root hosturia made upon contact with a suitable host tree[1]. Most of the naturally found sandalwood hosts are legumes (Fabaceae) but other species are commonly seen[78, 80]. Some of the common hosts for sandalwood are *Acacia acuminata* (Jam), *Acacia burkittii* (Burkitt's Jam), *Acacia aneura* (Mulga), *Acacia saligna* (Golden wreath wattle), *Acacia resinimarginea* (Wodjil), *Acacia microbotrya* (Manna Wattle), *Acacia coriacea* (Wirewood), *Acacia jibberdingensis, Allocasuarina huegeliana* (Rock She Oak) and *Hakea recurva*[78, 80]. Any species without allelopathic effects could host sandalwood, but would not necessarily be providing a high level of nutrition[1]. Planters have used different host trees to support their plantations, which were recommended by the forestry researchers[11, 79, 80]. It was decided also to study the effect of different host trees on seed oil quality and quantity. Most of the sandalwood trees in commercial plantations or field trials have several hosts making it difficult to find a specific effect on seed oil. However, a sandalwood host trial established in 1997 by the Forest Products Commission in Narrogin, Western Australia has sandalwood trees dependent upon specific hosts. The effects of growth and heartwood formation based on these trials have already been reported[81]. This trial would provide an invaluable opportunity to analyse the seeds to compare with their host trees. Different host trees were trialled with sandalwood from a similar parentage. Sandalwood trees are hosted with single, double or multiple hosts of the same or different host species.

Objectives of this study were to evaluate the following;

- Effects of climate, location and seed size on seed characteristics of selected Western Australian Sandalwood
- Effects of the type of host trees on seed characteristics of Western Australian Sandalwood.

3.2 Methods

3.2.1 Sample Collection for Assessing Geographical and Size Effect

Six samples of seeds were collected in December 2009, which represented three regions of Western Australia where *S. spicatum* is currently found. Natural stand samples were collected from the Wiluna Goldfields area by Mr. Jonathan Brand and Mr. Benjamin Sawyer of the Forest Products Commission. Commercial plantation samples were from Quairading in the Wheatbelt region from a sandalwood farm harvested in a single season, but already graded according to the outer diameter as per the plantation requirements. Seeds from Wandering in the Wheatbelt region were collected from a private field trial. A sample was collected from the field trials area of Curtin University Bentley campus (Perth, Coastal) representing mature trees with multiple hosts. Except for the metropolitan area sample the remainder were collected on site and samples were withdrawn from each group. The outer husk (epicarp and mesocarp) was removed from the air dried fruit at the time of collection. When received at the laboratory the samples were stored in cloth bags under ambient conditions. Seeds (endocarp and kernel) were conveniently selected (n=100) from the cloth bags.

3.2.2 Sample Collection for Host Tree Effect

Seeds were collected from the sandalwood host tree trial established in 1997 on Agriculture College premises in Narrogin, Western Australia. Sandalwood trees selected for this study were of the same parentage and planted in 1998 with the host trees planted a year before. Seeds from four main host species planted as single host per sandalwood tree, one trial set of two hosts of the same species were selected. A total of 21 trees in five groups (Table 3.1) were selected from the middle of the trial area. The diameter at the base of the trees was recorded to compare the general growth. Collection was conducted in December 2011 once all of the mature seeds had fallen and dried on the ground. All the seeds found under the tree and near vicinity were collected and the soft pericarp was removed manually. Seeds with the shells were stored at ambient temperature and humidity in cloth bags for 15 days before size and weight were recorded. Physical parameters of seeds were measured on 20 conveniently selected seeds from each of 21 trees; average values for each tree

were used for statistical comparison. These samples were used for the chemical analysis as a combined kernel sample for each tree.

3.2.3 Physical Parameters of Seeds

The axial diameter was measured using Vernier callipers and the weight recorded before being de-shelled using a size adjustable rotary mill. Kernels were separated manually and stored in vacuum sealed bags at a controlled storage room (20-25^{o}C).

3.2.4 Determination of Oil Yield and Fatty Acid Composition

Seed kernels were size reduced and solvent extracted as described in Section 2.3.1 for determination of the oil yield. The oil was analysed for the fatty acid content as respective methyl esters by gas chromatography as described in the Section 2.4.

Table 3.1: Samples collected from different sources with geographical locations and seed size

Location	Region	Source	Seed size
Wiluna , WA	Goldfields	Natural	NA
Quairading, WA	Wheatbelt	Commercial plantation	Large (> 18mm)
Quaradine, WA	Wheatbelt	Commercial plantation	Medium (14 – 18 mm)
Quairading, WA	Wheatbelt	Commercial plantation	Small (< 14mm)
Wandering, WA	Wheatbelt	Field trial	NA
Perth Metropolitan	Coastal	Field trial	NA

Table 3.2: Seeds from sample groups representing different hosts from sandalwood-host trial in Narrogin, Western Australia on 2011

Host tree		Host per sandalwood tree	trees per group
Botanical name	Common Name		
Acacia acuminata	Jam tree	2	3
Acacia accuminata	Jam tree	1	5
Acacia microbotrya	Manna Wattle	1	5
Acacia aneura	Mulga	1	5
Allocasuarina huegeliana	Rock Sheoak	1	3

3.3 Results and Discussion

3.3.1 Effect of Geographical Location and Seed Size

The general characteristics of the seeds from the different sources and locations are shown in Table 3.1. Seeds from natural stands of the Goldfield area yielded the lowest kernel weight from whole seeds. Their outer diameter and weight were similar to many higher kernel yielding seeds from the Wheatbelt region; the only difference was their appearance which was rough and darker. Apart from the Goldfields sample most of the other seeds had a similar kernel yield (Table 3.3). A higher yield from the kernel was found from the Perth metropolitan sample which was grown in a coastal sandy soil (Table 3.3). Although this is not a natural habitat for *S. spicatum* these plants and their host trees are irrigated and fertilised. Seed size and weight data for a plantation of established *S. spicatum* corresponds with current values[1, 79]. Similar studies have reported the kernel weight and shell thickness of almond seeds compared with oil content and composition for industrial processing[82, 83]. Percentage kernel weight could be employed to predict raw material yield from whole seeds. Seed outer diameter and seed weight showed a weak correlation (R^2=0.4822), similarly observed for the kernel weight against seed weight (R^2=0.3283) as given in Figure 3.1. Using seed outer diameter as a parameter to estimate kernel yield would be inconsistent when sandalwood seeds are collected from different geographical locations.

Variation in oil yield was recorded within the seed lots (Table 3.4), mainly showing small differences between the groups. Larger seeds from the Wheatbelt region were found to contain more oil, whereas the lowest oil yield was recorded in the seed lot from the Goldfields (Table 3.4). The lowest kernel weight was also recorded for the seeds from the Goldfields and this correlated with the oil content. Coastal samples from Perth were found to have a low oil yield despite having the highest kernel yield. High oil content would render a higher commercial value. Seeds from plantations in the Wheatbelt region have given higher yields, while goldfields and coastal seeds lower. Considering the Perth and Quairading 2009 seed lots, oil yield was not constantly higher in larger nuts. Liu *et al.* have reported the lipid content of *S.*

spicatum seeds from different location in Western Australia, where both the highest and the lowest oil contents were reported from the Goldfields. In an industrial process variability of oil yield could be economically significant.

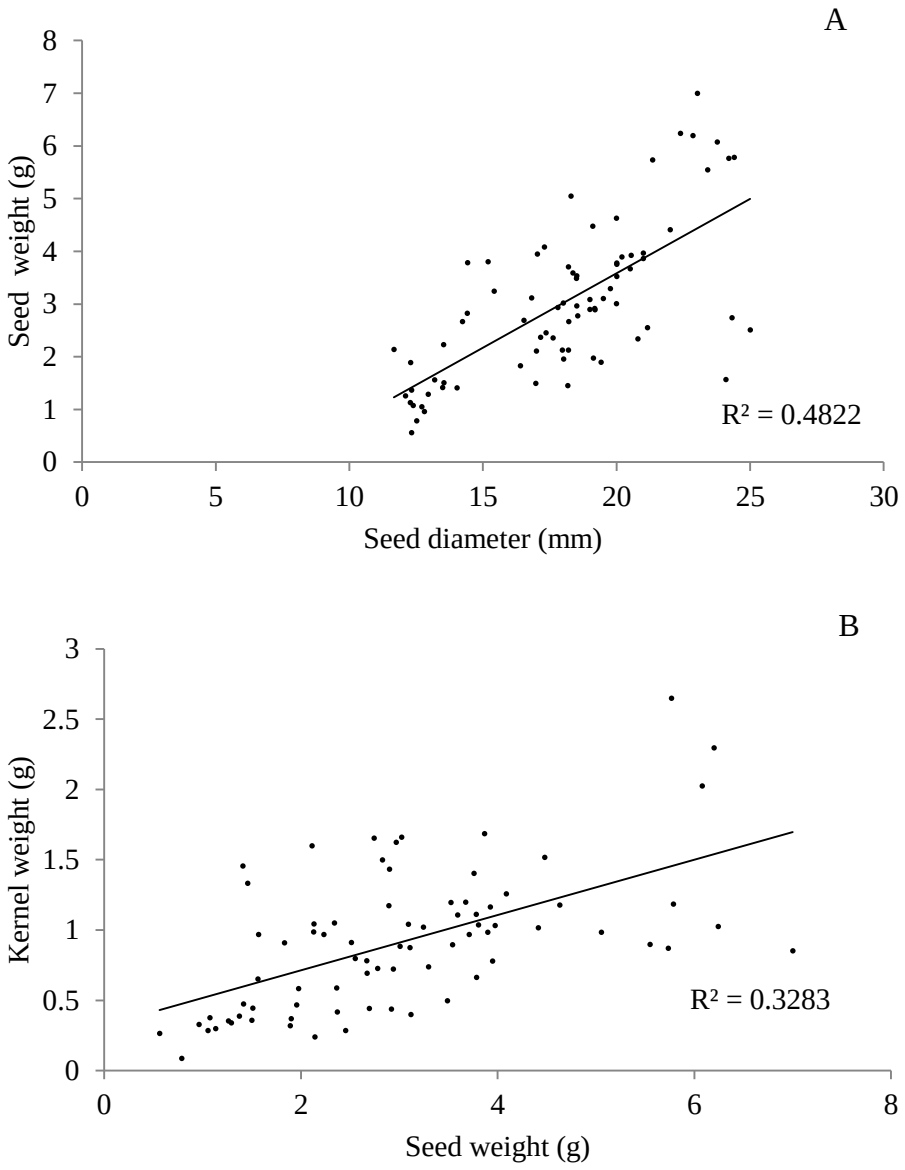

Figure 3.1: (A) Variation of average seed diameter against average seed weight for each tree; (B) variation of average seed weight against average kernel weight of each tree; correlation of dependence is given as R^2.

Table 3.3: Measurements of parameters of sandalwood seeds from different geographical sources and plantation size groups

	Goldfields (Wiluna)	Wheatbelt (Quairading large seeds)	Wheatbelt (Quairading medium seeds)	Wheatbelt (Quairading small seeds)	Wheatbelt (Wandering)	Coastal (Perth)
Seed Diameter (mm)	17.70 ±1.46^{a}	21.70 ±2.58^{a}	17.32 ±2.54^{a}	12.85 ±0.94^{b}	18.81 ±1.63^{a}	19.14 ±2.03^{a}
Seed Weight (g)	2.29 ±0.54^{a}	4.04 ±0.04^{b}	2.24 ±0.87^{a}	1.02 ±0.28^{c}	2.95 ±0.67^{a}	3.13 ±0.97^{ab}
Kernel Weight (g)	0.40 ±0.21^{a}	1.39 ±0.60^{b}	0.79 ±0.34^{ab}	0.32 ±0.14^{a}	0.92 ±0.26^{b}	1.16 ±0.35^{b}
Kernel % (w/w) of seed weight	16.62 ±5.40^{a}	33.81 ±2.46^{bc}	34.82 ±1.78^{cd}	30.36 ±5.60^{bc}	30.91 ±1.84^{bc}	37.13 ±0.34^{d}

Mean values with different superscript letters are significantly different (p<0.05); values are reported as mean values ± standard deviation (n = 100)

Table 3.4: Oil yield and fatty acid composition from seed kernel of sandalwood seed from different geographical sources and plantation size groups [84]

	Goldfields (Wiluna)	Wheatbelt (Quairading large seeds)	Wheatbelt (Quairading medium seeds)	Wheatbelt (Quairading small seeds)	Wheatbelt (Wandering)	Costal (Perth)
Oil yield (% *w/w of kernel weight)*	35.50±5.24^{a}	47.98±7.08^{b}	37.31±5.50^{a}	42.03±6.20^{ab}	40.28±5.94^{ab}	38.07±5.62^{a}
Fatty acids (% *w/w of oil weight*)						
Palmitic acid	3.74±0.10^{a}	3.98±0.15^{a}	3.43±0.10^{a}	3.67±0.08^{a}	3.51±0.07^{a}	3.33±0.11^{a}
Palmitolic acid	0.43±0.06^{a}	0.60±0.04^{a}	0.53±0.07^{a}	0.54±0.05^{a}	0.47±0.02^{a}	0.48±0.07^{a}
Stearic acid	1.79±0.05^{a}	1.78±0.08^{a}	1.81±0.05^{a}	2.05±0.06^{a}	1.57±0.03^{a}	2.09±0.07^{a}
Oleic acid	52.88±1.21^{a}	50.27±0.87^{ab}	50.13±0.74^{ab}	53.53±0.95^{a}	48.80±0.65^{b}	48.74±0.84^{b}
Linoleic acid	1.45±0.06^{a}	1.57±0.10^{a}	1.25±0.06^{a}	1.11±0.80^{a}	1.20±0.10^{a}	1.15±0.07^{a}
Linolenic acid	3.74±0.11^{a}	2.93±0.17^{b}	2.95±0.14^{b}	2.19±0.15^{a}	3.35±0.14^{b}	2.44±0.13^{a}
Sterolic acid	1.71±0.07^{a}	1.14±0.10^{a}	1.00±0.06^{a}	1.14±0.07^{a}	1.74±0.08^{a}	1.09±0.08^{a}
Ximenynic acid	32.72±0.29^{a}	36.19±0.27^{b}	37.24±0.08^{b}	34.46±0.15^{a}	37.79±0.09^{b}	39.14±0.01^{b}

Means with different superscript letters are significantly different (p<0.05), values are reported as mean values ± standard deviations (n = 3)

Fatty acid profiles of the seed oil (Table 3.4) from all the samples were comparable with previously reported data discussed in Chapter 1. A field study reported on proximate analysis of developing sandalwood seeds has reported that the maximum level of ximenynic acid could develop up to 35%[46]. Oleic acid is considerably lower in larger seeds from both the field trials in Wandering and Perth. High oleic acid was found both in natural stands of Goldfields and plantations in the Wheatbelt; providing no particular relationship between geography or seeds size. The current study has observed a significant difference between oleic and ximenynic acid composition. Similar versions are reported on fatty acid compositions between different cultivars of almonds and walnuts; however the differences in fatty acid composition among cultivars of olives are reported to be minimum[82, 85-87]. Considering the reports on other seed oils mentioned above, it would be useful to identify geographical locations or size grades to standardise the seed source for oil extraction. However, within the scope of this study we could not find a strong correlation between the seed source and the fatty acid composition and geographical location, even though the larger seeds from commercial plantations seem to be favourable in size and oil content.

3.3.2 Effect of Host Trees

The diameter at the base was recorded for every tree evaluated as a general marker of overall growth. *Acacia aneura* hosted sandalwood showed the maximum growth while *Acacia acuminata* hosted sandalwood also showed a good growth despite the number of host trees. Sandalwood hosted by *Allocasuarina huegeliana* reported the least growth (Table 3.5). In a recent study it was found that the diameter of the tree was proportional to the heartwood content[81]. Growth of sandalwood trees based on host tree effect has been studied previously and found that *A. aneura* was the best host for the Wheatbelt region[78, 80]. These trees are planted to be harvested for the heartwood after twenty to thirty years[17, 18]. The total number seeds collected from each tree reported in Table 3.5 needs to be considered approximate, as misplacement is inevitable in a natural environment.

Table 3.5: Diameter at the base (dbh) of sandalwood trees and total weight of seeds continently collected from each group hosted by different host trees.

	Host Species				
	Acacia acuminata 2 hosts (n=3)	*Acacia acuminata* 1 host (n=5)	*Acacia microbotrya* (n=5)	*Acacia aneura* (n=5)	*Allocasuarina huegeliana* (n=3)
Wt. of seeds with shell per tree (g)	550±14 [a]	554±16 [a]	448±11 [b]	591±15 [c]	276±16 [d]
Sandalwood tree diameter at the base (cm)	13.62±1.48[a]	13.75±1.34[a]	10.69±1.22[b]	14.00±1.81[a]	9.55±1.99[b]

Mean values with different superscript letters are significantly different ($p<0.05$), mean values ± standard deviation

Seed parameters were analysed on a conveniently selected 20 seeds per each tree used in this study as mentioned in Section 3.2.2. Data is given in Table 3.6.

Table 3.6: Measurements of parameters of sandalwood seeds from different host tree groups

	Host Species				
	Acacia acuminata 2 hosts (n=3×20)	*Acacia acuminata* 1 host (n=5×20)	*Acacia microbotrya* (n=5×20)	*Acacia aneura* (n=5×20)	*Allocasuarina huegeliana* (n=3×20)
Seed Diameter with shell (mm)	22.31±1.45[a]	21.18±1.20[ab]	20.38±1.12[ab]	20.76±1.04[ab]	19.38±0.98[b]
Seed Weight with shell (g)	4.74±0.88[a]	3.96±0.62[ab]	3.50±0.55[ab]	3.79±0.48[ab]	3.00±0.47[b]
Kernel Weight (g)	1.89±0.87[ab]	1.55±0.42[a]	1.35±0.35[a]	1.27±0.35[a]	0.81±0.2 [ac]
Kernel % (w/w of seed weight)	38.26±6.76[ab]	40.05±11.19[ab]	38.31±3.47[a]	33.47±6.28[ac]	28.38±5.18[ac]

Mean values with different superscript letters are significantly different ($p<0.05$), values are reported as means ± standard deviation

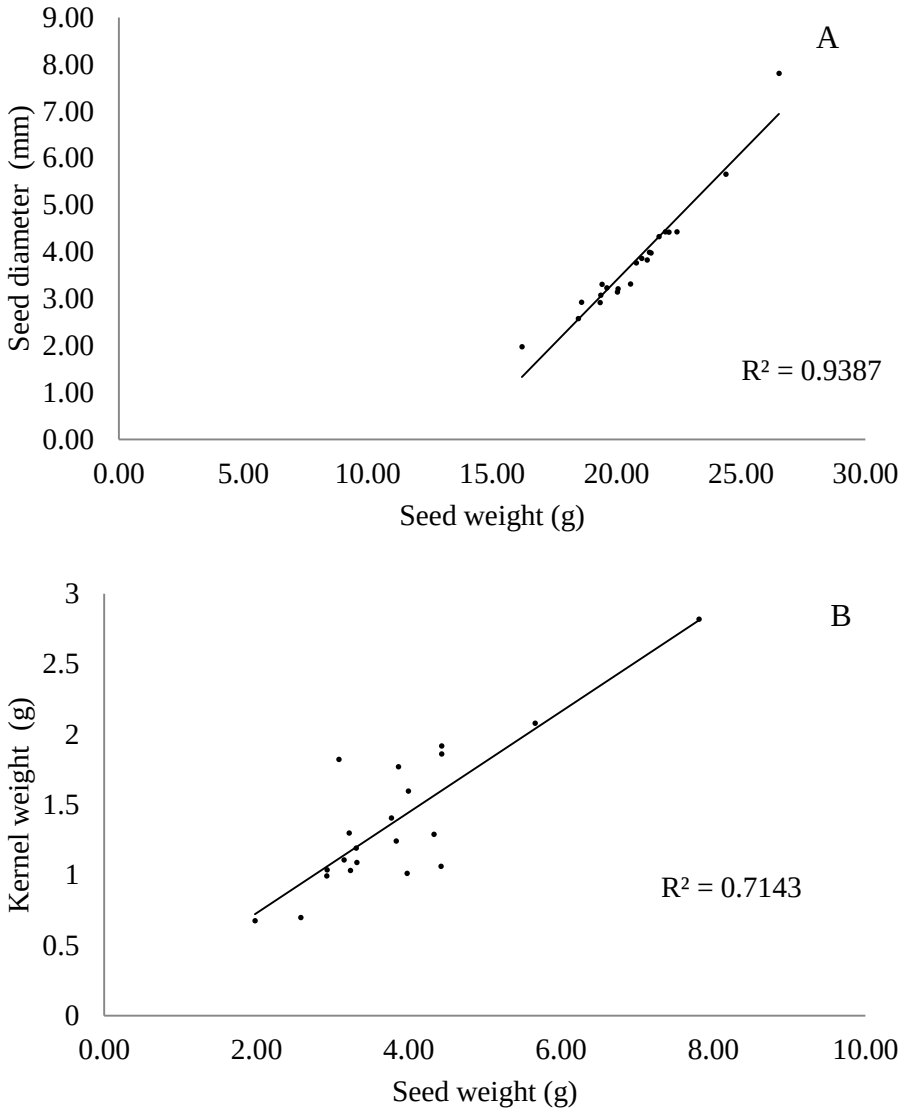

Figure 3.2: (A) Variation of average seed weigh against average seed diameter for each tree; (B) variation of average seed weight against average kernel weight of each tree; correlation of dependence is given as R^2.

Overall results on seed size and weight of sandalwood seeds do not show a clear difference among the Acacia host species (Table 3.6). It was apparent that *Allocasuarina huegeliana* is a poor host when compared to the Acacia species. When seed weights are compared with seed diameters there was a correlation observed (Figure 3.2 A), a correlation was also observed between seed weight to kernel weight (Figure 3.2 B). This would enable an estimate of the kernel yield based on seed diameter. This correlation is however limited to within the range evaluated, especially with respect to seed diameter.

In this study *A. aneura* produced the highest yield of oil, but the oil yields did not correlate well with seed sizes within the Acacia species. Sandalwood seeds from trees hosted by sheoak reported the smallest seeds size and least weight, also it had the lowest oil yield. Total sandalwood seed weight and yield were not affected by the number of *A. acuminata* host trees for the sandalwood tree. Other than the sandalwood hosted by *Allocasuarina huegeliana* all the others were found to contain oil contents reported on previous studies[71]. Fatty acid profiles between the trees showed no significant difference, and the values were similar to reported data[71]. When the averages are considered *Allocasuarina huegeliana* (Sheoak) hosted trees showed the highest ximenynic acid content while oleic acid content and oil yield were lowest for them (Table 3.7). *Acacia aneura* (Mulga) has shown the highest oil and oleic acid content while ximenynic acid was lowest. Fatty acid composition was found to be largely unaffected by the host trees. Use of host trees available in the geographical region would not affect the seed oil composition.

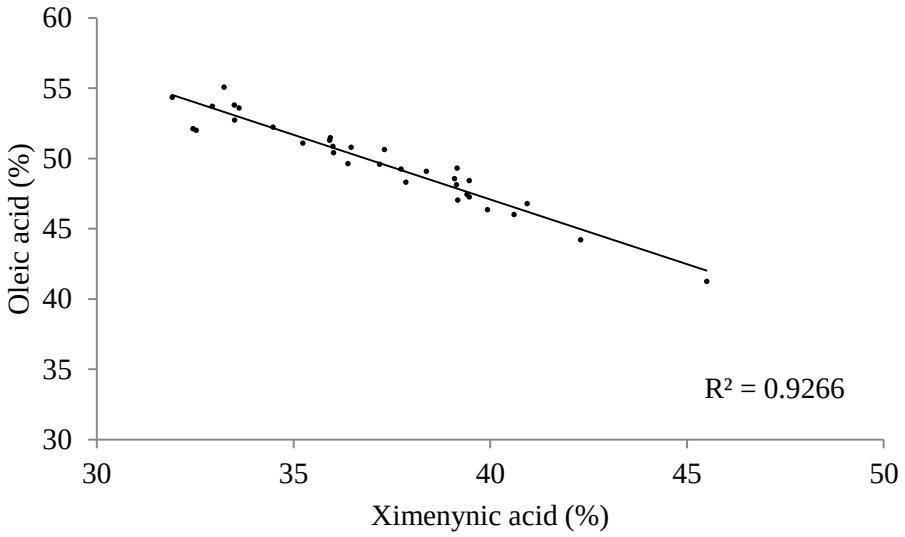

Figure 3.3: Correlation data reported as Sections 3.3.1 and 3.3.2 for average values of ximenynic acid content and oleic acid content.

Fatty acid data of from both the studies provided a correlation between oleic and ximenynic acid composition (Figure 3.3). A clear correlation has been observed between the oleic and ximenynic acids (Figure 3.3). A similar relationship was previously reported in a study conducted on seed samples from different Western

Australian locations[71]. Although it has been proposed that more acetylenic fatty acids are synthesised in stressed trees, there is not enough evidence from this study to confirm this hypothesis[21]. Fatty acids which originated from a secondary metabolic stage would require specific enzymes to desaturate particular carbon atoms[21]. A relationship between the mono-unsaturated and polyunsaturated fatty acids was seen in different cultivars of walnuts and almonds[87, 88]. Biosynthesis of ximenynic acid is reported to originate from oleic acid with stearolic acid as an intermediate[21]. This pathway was also suggested by Liu and Longmore based on a fatty acid analysis of developing *S. spicatum* seeds[46].

Table 3.7: Oil yield and fatty acid composition of samples from different host tree trials

	Acacia acuminata 2 hosts (n=3)	*Acacia acuminata* 1 host (n=5)	*Acacia microbotrya* (n=5)	*Acacia aneura* (n-5)	*Allocasuarina huegeliana* (n=3)
Oil yield (% w/w)	46.94±1.77[a]	46.47±4.48 [ab]	46.95±2.27 [a]	51.04±1.20 [b]	35.53±10.01 [c]
Fatty acids					
Palmitic acid	0.50±0.06 [a]	0.51±0.09 [a]	0.54±0.09 [a]	0.52±0.08 [a]	0.47±0.07 [b]
Palmitoleic acid	3.29±0.64 [a]	3.12±0.38 [a]	3.20±0.29 [a]	3.60±0.33 [a]	3.19±0.21 [a]
Stearic acid	1.14±0.26 [a]	1.31±0.26 [a]	1.30±0.09 [a]	1.07±0.13 [a]	1.42±0.18 [a]
Oleic acid	49.46±2.94 [a]	47.68±4.43 [a]	49.30±1.83 [a]	51.55±3.15 [a]	47.32±3.10 [a]
Linoleic acid	2.00±0.26 [a]	2.03±0.39 [a]	1.96±0.19 [a]	1.68±0.45 [a]	2.08±0.29 [a]
Linolenic acid	1.83±0.16 [a]	1.29±0.19 [a]	1.35±0.14 [a]	1.79±0.27 [a]	1.48±0.27 [a]
Sterolic acid	1.18±0.25 [a]	1.08±0.21 [a]	0.76±0.11 [a]	1.11±0.31 [a]	1.12±0.24 [a]
Ximenynic acid	37.25±3.25 [a]	38.77±4.93 [a]	38.23±2.08 [a]	35.03±3.00 [a]	39.25±3.15 [a]

Means with different superscript letters are significantly different (p<0.05), mean values ± standard deviation

3.4 Conclusion

Geographical locations of sandalwood seeds have a significant effect on percentage yield of seed kernel, but the oil yield and fatty acid profile showed no significant variation. Seeds collected from plantations of the Wheatbelt region of Western Australia could be used for sandalwood seed oil extraction. Seed grading may have an industrial benefit, but no particular advantage on oil content and composition was observed. Fatty acid content was found to be similar within these samples and also correlated well with the previously published data.

The selection of host trees has an effect on overall development of the tree which is manifested by the growth and amount of seeds produced. Among the commonly used hosts *Acacia* species were found to be better hosts than *Allocasuarina huegeliana*. Plantations could rely on different host trees without affecting the lipid composition to increase growth and oil yield.

Different hosts and climatic conditions have slightly increased the ximenynic acid content even though no significant change is observed. Oleic acid levels were observed high when the seeds were large and rich in oil, thus suggesting that certain factors might initiate acetylenic fatty acid synthesis. Identifying the factors and methods to increase ximenynic acid would be an important future study.

Chapter 4 Supercritical Fluid Carbon Dioxide Extraction and Characterisation of Sandalwood Seed Oil

4.1 Introduction

4.1.1 Lipid Extraction from Plant Material

Plant lipids are diverse in chemistry and found as a uniform mixture of triglycerides with other lipophilic components; Section 1.2.1 explained the different lipid classes and general composition of plant oils. Lipids are stored in plant cells as oil bodies surrounded by a phospholipid layer. Oil extraction methods separate the lipids from cellular debris[89]. The most commonly used method for cosmetic oil extraction is physical crushing or extruding the seed kernel, known as cold pressing[90]. The extruded material undergoes refining steps, which includes heating and some chemical treatment to coalesce the extract. In some instances an extrusion followed by a simple filtration or filter press provides clear oils which are known as virgin oils[90]. These oils also contain high free fatty acid and peroxide content dependent upon the raw material quality[20]. The main challenge in cold pressing is that most seeds do not expel oils upon physical extrusion [90]. High concentrations of phospholipids, lipid soluble proteins, glycolipids and sphringolipids hold the oil within the cell debris hindering extraction[90]. Upon shear pressure these compounds together with oil form a thick emulsion. There are some physical methods employed to overcome these difficulties. Use of heated rollers and a filter press are common methods employed for this purpose [90].

The majority of edible oils are solvent extracted and then refined before marketing. Solvent extraction with azeotopic mixtures of hexane and isopropyl alcohol and use of gum precipitants has been found to be an efficient and cost effective method[90]. Solvent extraction is commercially successful and considered safe under regulatory guidelines[91]. However cosmetic and nutraceutical industries prefer oils extracted without solvents to satisfy the growing demand for "greener" products. Methods used for oil extraction should not facilitate oxidation or hydrolysis of lipid components, this would require additional refining steps which are costly with loss of beneficial compounds. Hence scientists and industrial groups have worked on the

development of novel methods to extract oil with the least damage to the lipid composition. These methods include ultrasound treatment and enzyme treatment prior to extrusion, currently these methods are not used at an industrial scale [92]. Recovery of enzymes and removing the aqueous media could be possible difficulties in enzymatic treatment methods. However several successful operations have been reported[92]. Use of ultrasound treatment before extrusion is currently a laboratory scale process. This could be a useful pre-treatment method rather than a standalone extraction method[90]. These two methods could achieve considerable increase oil yield, but a multi-step process needs to be employed for refining and purification[90].

4.1.2 Supercritical Carbon Dioxide Extraction

Extraction using supercritical carbon dioxide is gaining popularity not only in natural products extraction; but also in the pharmaceutical and resources industries. This method is successfully used in large scale hops extraction in brewing, decaffeinating coffee beans, refining fish oil and spice extracts. Although known to be an expensive method with high capital investment, this method has advantages towards the final product quality and in the long term it is seen as commercially viable. In most roles it is a single step process which includes extraction and refining. This is considered "greener" technology with no solvent contamination of the product with minimum adverse environmental effects [76].

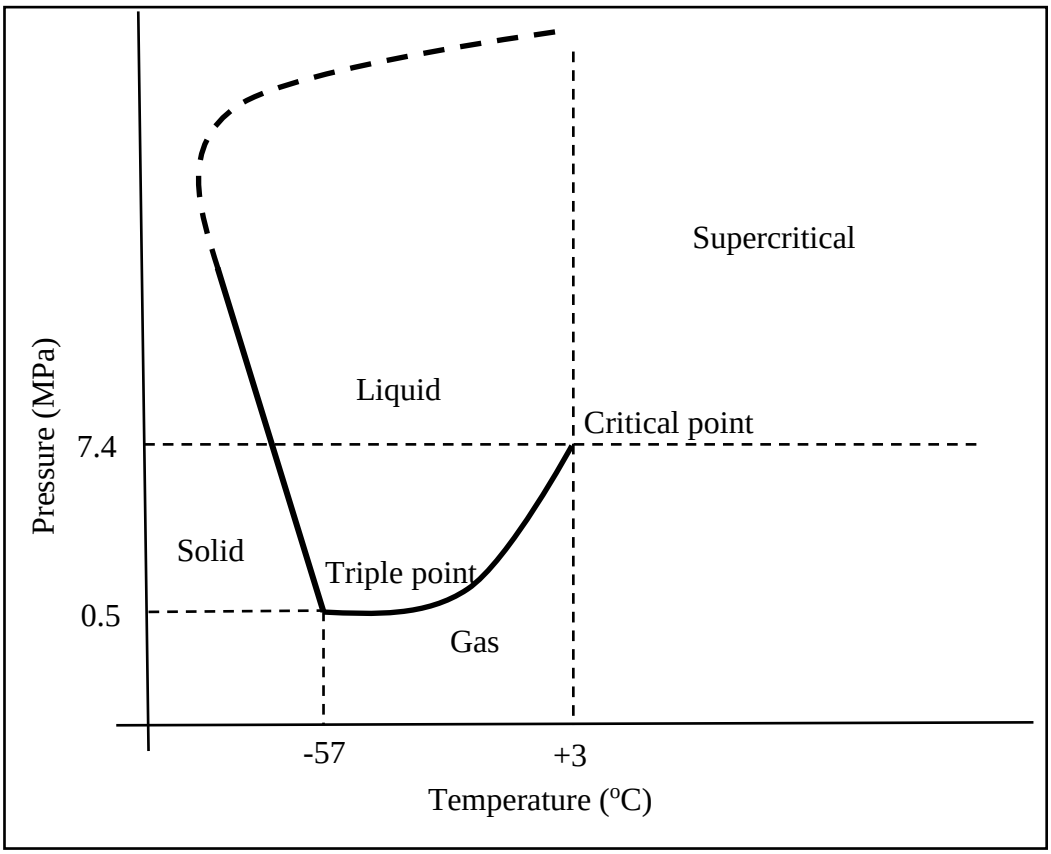

Figure 4.1: Phase diagram of a pure carbon dioxide [93]

Supercritical fluids are referred to a state of matter where a fluid exhibits characteristics of a liquid and a gas. Every pure gas has a particular temperature where beyond that would show liquid characteristics due to compression. This particular temperature is called as the critical temperature and the vapour pressure at this stage is known the critical pressure. In a phase diagram (Figure 4.1) this specific point is known as the critical point. When the pressure and temperature of a compound is above the critical point it is in a state of a supercritical fluid. Molecules in the supercritical state have lower volume, therefore the density and dielectric constant of the fluid would increase with pressure. Supercritical fluids will first diffuse to a substrate matrix and then dissolve the extractable substances depending upon their dielectric constant. Extracted substances and the supercritical fluid will move to the outer surface of the matrix by diffusion; then the fluid flow will carry the substance and the fluid away from the matrix[94]. Density, dielectric constant, flow rate of the fluid, extraction time and particle size of the matrix are the major factors governing extractability[95, 96]. Minor adjustments to the pressure and temperature

known as tuneability makes supercritical fluid extraction unique from other extraction methods used for natural product extraction[76, 93, 96].

Extraction is conducted by passing the fluid under defined pressures while maintaining the temperature of the extraction chamber. The extraction chamber is filled with the feed (Figure 4.2). Fluid with the dissolved extract is collected in a separation chamber where the pressure is reduced allowing the extract to precipitate, thus releasing the fluid to become a gas and be recycled. Design and the physical ability of each component of the plant are very important for an efficient extraction [97].

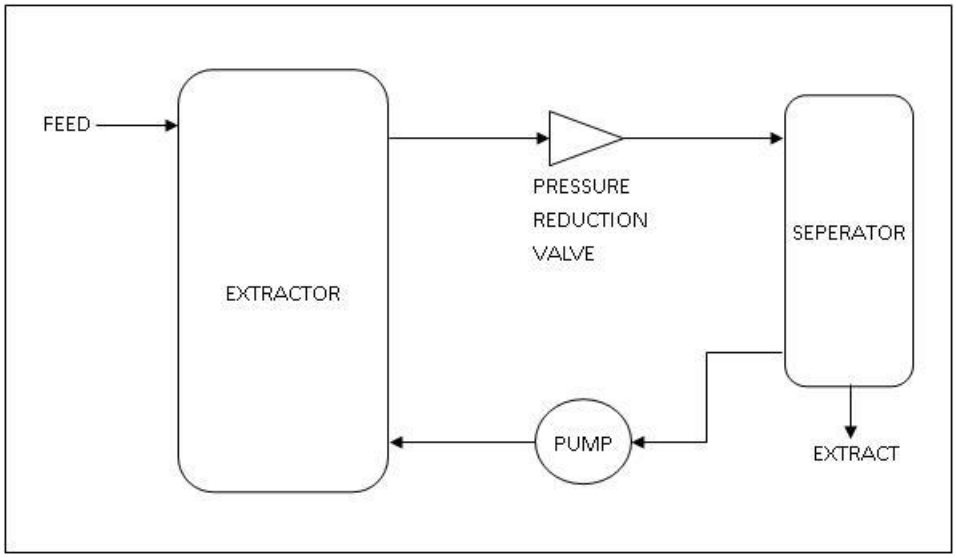

Figure 4.2: Schematic diagram of a typical supercritical extraction process [96]

The most commonly used supercritical fluid is carbon dioxide, since it operates at lower temperatures and pressure conditions, safety and availability. Carbon dioxide is mostly inert and its fluid characteristics are predictable. The dielectric constant of carbon dioxide increases with pressure and temperature. Increased density would facilitate carbon dioxide to reach the lipid containing structures and increase the leaching activity during the extraction [95, 97] .

4.1.3 Extraction of Plant Lipids Using Supercritical Carbon Dioxide

Currently supercritical carbon dioxide is used for extraction and purification of fixed oils. Isolating certain lipid classes such as squalenes and tocopherols from seed oils is another application of supercritical fluid extraction (SCFE)[95, 98]. It has been shown that the use of hydrophilic solvents as co-solvents or entrains can improve the extraction method and purity of the product. Lipid insolubility and inertness of carbon dioxide gives an added advantage. At a given pressure and temperature mass exchange between lipids containing seed material and carbon dioxide depends upon the particle size and flow rate[99, 100]. Fatty acids of triglycerides are different in molecular weights and nature. Increasing pressure has resulted in an increase in higher chain fatty acids in the total composition[101]. Differences in omega-3 and omega-6 fatty acid composition in supercritical and screw press extracted flaxseed oil have been reported[102].

Solubility characteristics of free fatty acids and triglycerides are similar during the extraction. However the extra free fatty acids could be removed during the separation process by fractionation[96, 98]. Entrainer solvents (co-solvent) such as ethanol with carbon dioxide are used to increase the efficacy at lower pressures[97]. This could have negative impact on yield as well as extracting more polar substances to the oil[103]. Little data are available on the extractability of oxidised species of fatty acids. It is considered that phospholipids, springolipids, glycolipids and proteins are only sparingly soluble at these pressures[96]. This would create an enormous advantage over many other methods where a refining step is needed to remove these co-extracted compounds[90]. Most of the lipids could be extracted using SCFE under lower temperatures, in less time without the use of any solvents[96]. Studies conducted on other oil bearing seed have reported to extract oils with similar or better properties to the solvent extracts or pressed oils[98, 104].

The possible advantages of SCFE over other methods and also the necessity to use a novel greener method has encouraged us to research this as a possible extraction method. A SCFE method was developed stepwise selecting suitable pressure, time of extraction, mass ratio, also considering using no entrained solvents and maintaining the lowest temperature. The extract was primarily assessed for the yield, particulate

matter and fatty acid balance between ximenynic and oleic acids. Several studies were conducted determining the optimal conditions for extracting lipids from seed sources[105-108].

4.1.4 Physicochemical Characterisation

Santalum spicatum seed oil has been identified as a potential cosmetic agent[70]. Fixed oils used in cosmetics and pharmaceutical products are first assessed for their physicochemical properties. These characteristics would be compared with other oils used in cosmetic and pharmaceutical products. Other than the initial chemical and biological studies by Liu *et al.* no other studies have been conducted on sandalwood seed oil[50]. The fatty acid composition has been published in several studies employing solvent extraction methods[44, 45, 52], therefore SCFE needs to be studied comparatively. Several studies have reported that the supercritical extract and pressed oils from oil bearing seeds show no difference in fatty acid content[96, 104, 109].

It is important to specify the presence of minor lipid components such as tocopherols and phytosterols. Tocopherols are important in topical applications due to antioxidant activity of oils as well as beneficial effects on the skin. Tocopherols can increase the shelf life of the oil significantly by their free radical scavenging activity, other conjugated compounds such as carotenoids and squalenes can exhibit similar activity. Studies have found that the fatty acid content show negligible differences between supercritical carbon dioxide extraction and other methods, however the tocopherol content was found to be higher on carbon dioxide extraction[104, 109]. Detailed work conducted by Mukhopadhyay *et al*. have reported that the tocopherol content in the oil seed extracts change with the pressure[96]. Seed oils have reported to have β-sitosterol, stigmasterol, campestrol and δ-5-avenasterol as major phytosterols[23, 110].

Physical properties of the oil would be used to give a comparative indication of the suitability in formulations for skin application[37]. Chemical properties of the oil depict the level of oxidation, free fatty acids increased non-fatty substances and degree of saturation of fatty acids in the oil[33]. These are important parameters to set standards and compare sandalwood seed oil and other vegetable oils. Oxidative stability of the supercritical extracted oil will be discussed in detail in Chapter Five.

Physicochemical properties are seen as important characteristics for oils; hence described in standard literature as quality parameters[31, 35].

The objectives of this part of the study were to develop an improved extraction method in a context of;

- Obtaining a high oil yield from sandalwood seed kernel free of undesired contaminants.
- Developing and characterising a supercritical fluid carbon dioxide extraction process and evaluate product quality and its feasibility.
- Evaluating the sandalwood seed oil from solvent and supercritical carbon dioxide extraction processes with respect to standards for vegetable oils used in cosmetics and pharmaceuticals.

4.2 Methods

4.2.1 Sandalwood Seeds

Western Australian sandalwood seeds were obtained from a commercial plantation situated in Quairading, Western Australia. These seeds were collected in December 2009 and de-husked prior to bagging. A total of 300 kg of seeds was stored in polyurethane bags and kept at room temperature before further processing.

4.2.2 Sample Preparation

Seed shells were removed using a modified portable macadamia nut de-sheller (MacQuip, Lismore, NSW, Australia). Seeds were graded into several groups according to diameter before feeding to the machine, rotors were adjusted accordingly. Kernels were separated from shells manually under visual inspection. Seed kernels were dried at 40^{o}C for 24 hours before pulverizing. A cutter mill (Mill Co. Ltd, Taiwan) was used with minimum attrition, particle sizes of 1 mm -3mm in diameter by sieve were collected. This product was stored in a dehumidifier at room temperature before packing into vacuum sealed bags.

4.2.3 Solvent Extraction

Solvent Extraction method was used as described in Section 2.3.1 was used.

4.2.4 Supercritical Extraction Analysis

A laboratory scale Helix Process Development Unit© extractor (Applied Separations, PA, USA) was used for initial studies. The extraction vessel volume was 1000 mL, temperature was controlled at 40^{o}C and flow of 0.6 kg hr^{-1} which was increased to 1.2 kg hr $^{-1}$ after 90 minutes. Two 500 mL cyclonic separator vessels were used, where pressure was lowered to 5 MPa in the first and then at ambient pressure in the second. Food grade carbon dioxide was used and the used gas was not recycled. Pulverised sandalwood seed samples (65g each, 1-3mm) were extracted with pressures of 30, 35, 40, 45, 50 and 55 MPa in the extraction vessel; in every experiment fractions were collected at 15, 30, 60, 150, 210, 270, 330 and 390 minutes.

Studies on fluid and mass exchange were conducted on a Profession Product Development Unit© (Natex Process technology, Ternitz, Austria). The extraction vessel of 5 L capacity was attached to two 2L separators with a carbon dioxide recycling unit. The first separator pressure was reduced to 6 MPa, second separator the fluid pressure was at 2 MPa. In a mass ratio experiment isobaric/isothermal conditions of 40 MPa/40 ^{o}C were maintained. Sandalwood seed kernel (4kg, 1-3mm particle diameter) was extracted for 7.6 hrs with a carbon dioxide flow of 30kg hr^{-1}. Extracts were withdrawn from the separators corresponding to the mass of carbon dioxide consumed.

4.2.5 Pilot Scale Production

Pilot scale production was conducted on two 80 L capacity extraction chambers attached to two 20 L separators with a carbon dioxide recycling unit. Conditions were 40 MPa at 40 ^{o}C with a carbon dioxide flow of 220 -320 kg hr^{-}. A sample of 55 kg (1-3 mm particle diameter) sandalwood seed kernel was extracted for 4 and 5.5 hr in two batches using the same extractor.

4.2.6 Chromatographic Analysis

Fatty acids were analysed as methyl esters following the methods described under Section 2.4 of General Methodology. Analysis of tocopherols, squalene and phytosterols were conducted as per the methods described in Section 2.5 of General Methodology using chromatography as described in Section 2.2.

4.2.7 Physicochemical Properties

Oil samples were analysed by the British Pharmacopoeia methods for their peroxide value, acid value, iodine value, saponification value and unsaponificable matter[31]. Physical properties such as specific gravity, freezing point and refractive index were also measured using standard methods. Viscosity was measured using a cup and bob type viscometer (Visco-V88, Bohlin, UK) commonly used in pharmaceutical pre-formulation studies.

4.3 Results and Discussion

4.3.1 Determination of Extraction Pressure

Using the laboratory scale apparatus described in the Section 4.2.4 pressures were altered in each experiment while temperature was kept constant at 40^{o}. Fractions were collected at time intervals up to 390 minutes from the start of the extraction cycle. There were no extracts found in the second separator for all the experiments. Yields were calculated as a cumulative percentage based on oil extracted to feed weights (Figure 4.3).

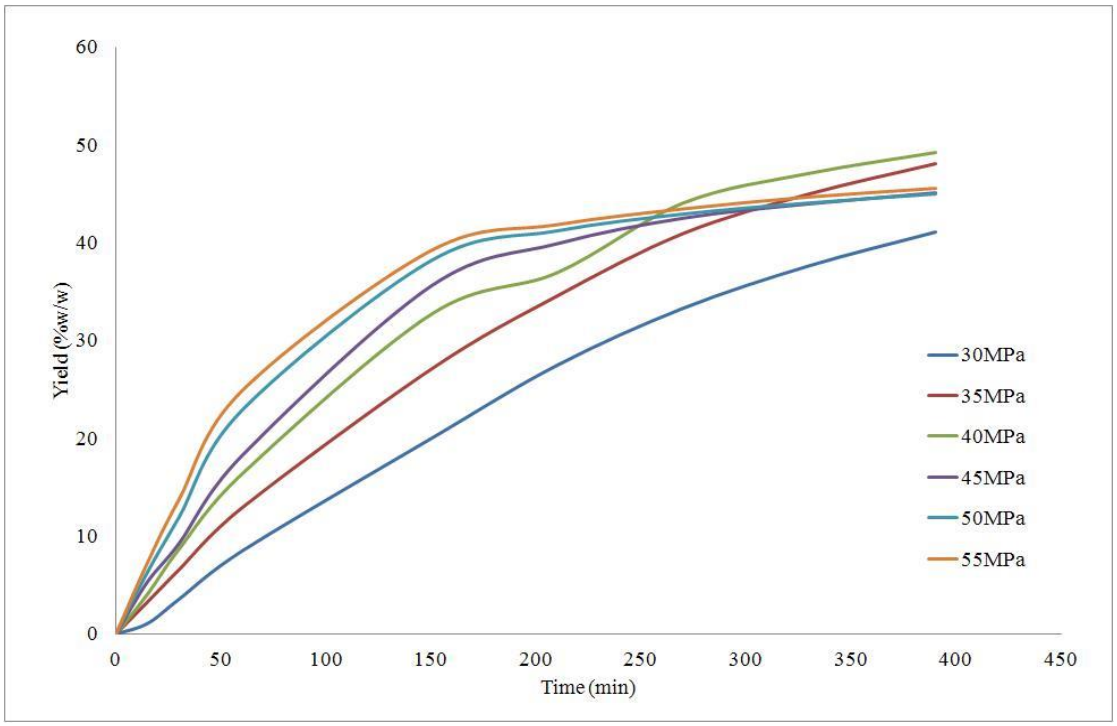

Figure 4.3: Oil yield obtained during time intervals at different pressures

The highest yield of 49.2% w/w was achieved at 40 MPa, at higher pressures total yield was reached in lesser time. It was identified that 40 MPa extraction pressure produced a comparatively a higher yield from 270 minutes onwards (Figure 4.3). This has several advantages, such as 90% of the extraction was completed at 270min; energy consumption is lower than the 45, 50 and 55 pressure systems. Upon visual observation the extracts obtained at 50 and 55MPa were opaque which precipitated as fine sediment upon refrigeration at 4^{o}C. The solvent extraction method with hexane and isopropyl alcohol resulted in 47.75%w/w yield. Liu *et al.* has reported that the total lipid content upon hot hexane extraction by Soxhlate apparatus was 56-60%w/w[46]. However the total lipid content included lipid soluble components which

are not desired in oil, such as phospholipids, glucolipids and lipoproteins[46]. Similar results were reported for sunflower seeds extracted by SCFE at 40^{o}C for different pressures[111]. Extracting at 40^{o}C and 40MPa was found to be the ideal conditions for palm kernel oil under different pressures and temperatures[101, 112].

Sandalwood seed oil yield was compared with respect to time and pressure to understand the best conditions based on these two factors. A surface graph was created to find the highest position in yield and then correlated with the best pressure and time values (Figure 4.4). Time showed a steady increase and the time factor needs to be studied in a future process development step. Surface graphs were used to correlate different factors in studies conducted on seed oil extraction of camphora, moringa, aquillaria and walnuts, [94, 105-108]. This study was not aimed to develop a detailed mathematical model, therefore the two major factors pressure and time were observed on the surface graph against the yield. Temperature was maintained at 40 ^{o}C as a requirement in this study to maintain the stability and reduce extraneous contamination, also the particle size and the flow rate were maintained constant during the current experiment. Extraction efficiency was considered based on the above discussed factors of time and pressure to obtain the highest yield. It was evident that 40 MPa pressure would be the most suitable pressure producing the maximum yield at a reasonable time scale of 270 mins.

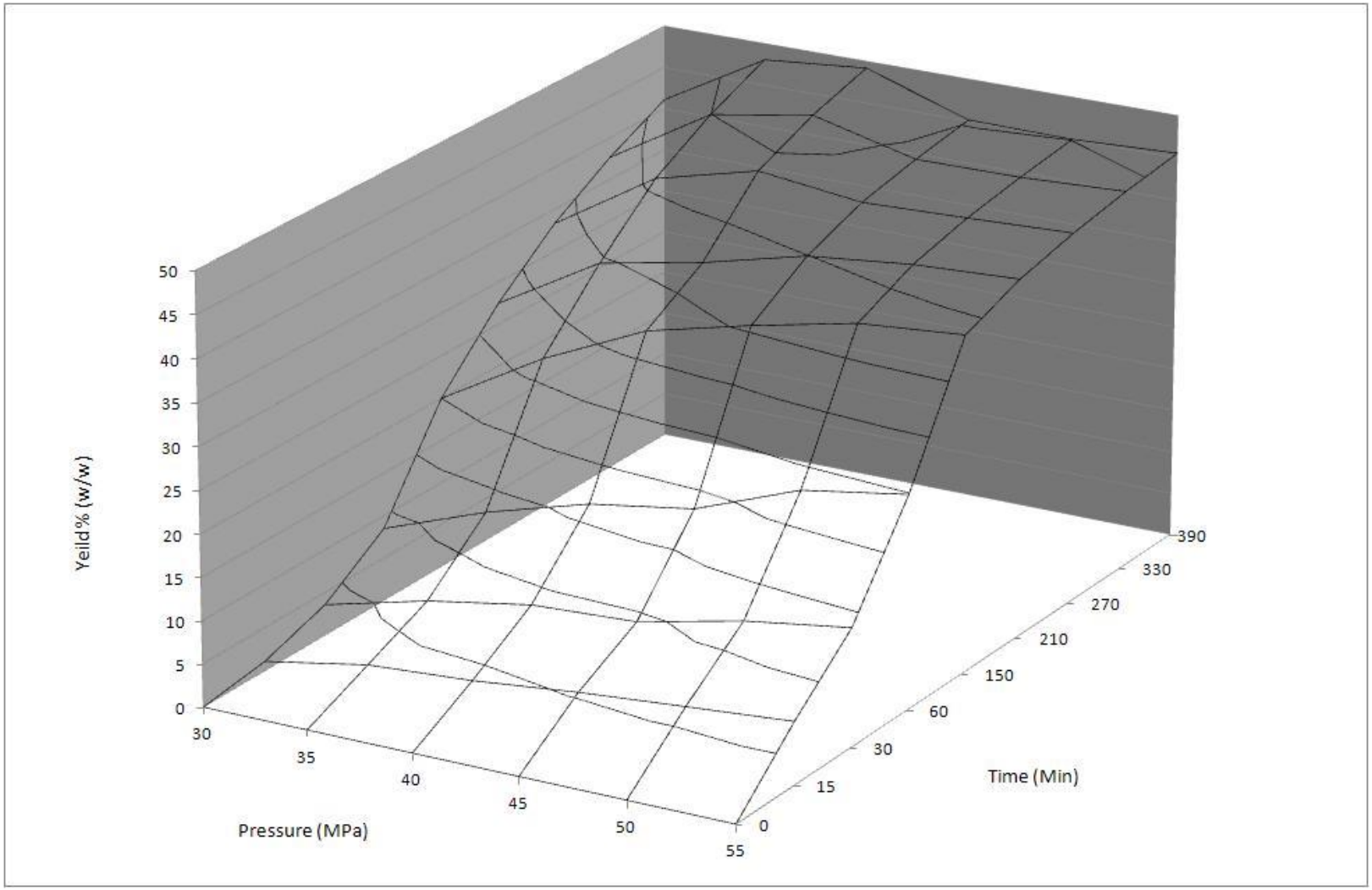

Figure 4.4: Surface diagram of percentage yield achieved at time intervals for different pressures

4.3.2 Mass exchange and carbon dioxide to feed ratio

The unit amount of carbon dioxide per unit amount of kernel feed against yields under different pressures is expressed in Figure 4.5. Yields were recorded to determine the efficiency, higher pressures were found to be more effective as higher yields were reached with less fluid consumption. An extraction pressure of 40 MPa was found to yield near the maximum for the amount of fluid utilised. Mass exchange is the unit amount of extract gained by a unit amount of carbon dioxide, which is an important parameter in the extraction process.

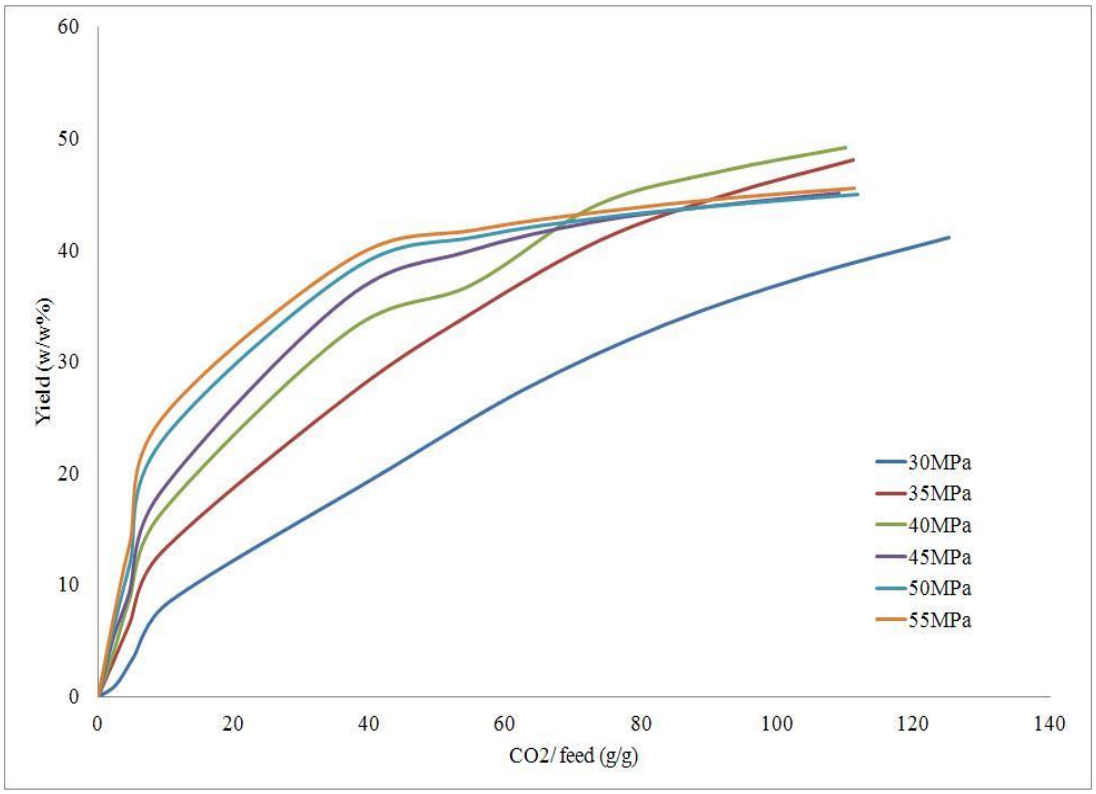

Figure 4.5: Mass exchange is expressed as percentage oil yield against weight ratio of carbon dioxide per feed for a standard time

Mass exchange with time was studied based on the data obtained from each fraction at different pressures (Figure 4.6). Higher pressures were found to give a better mass exchange reducing the extraction time. Lower pressure were weak in mass exchange capacity, this has been reported from a study on macadamia nut extraction[113]. The amount of carbon dioxide to feed would remain constant for a single extraction and the amount of extract achieved would reduce with time. This reduction of mass

exchange with time is also known as the extractability[96]. Fluid to feed ratio of 120 CO_2 kg/ Feed kg in this study, corresponded with results reported for oil extraction from walnut and aquilaria seeds under similar conditions[105].[104, 108]. A study of supercritical carbon dioxide extraction of sunflower oil used the same pressure and temperature as the current study with different flow rates; it was reported that the flow rate had no effect on the mass exchange but extraction time was significantly reduced[111].

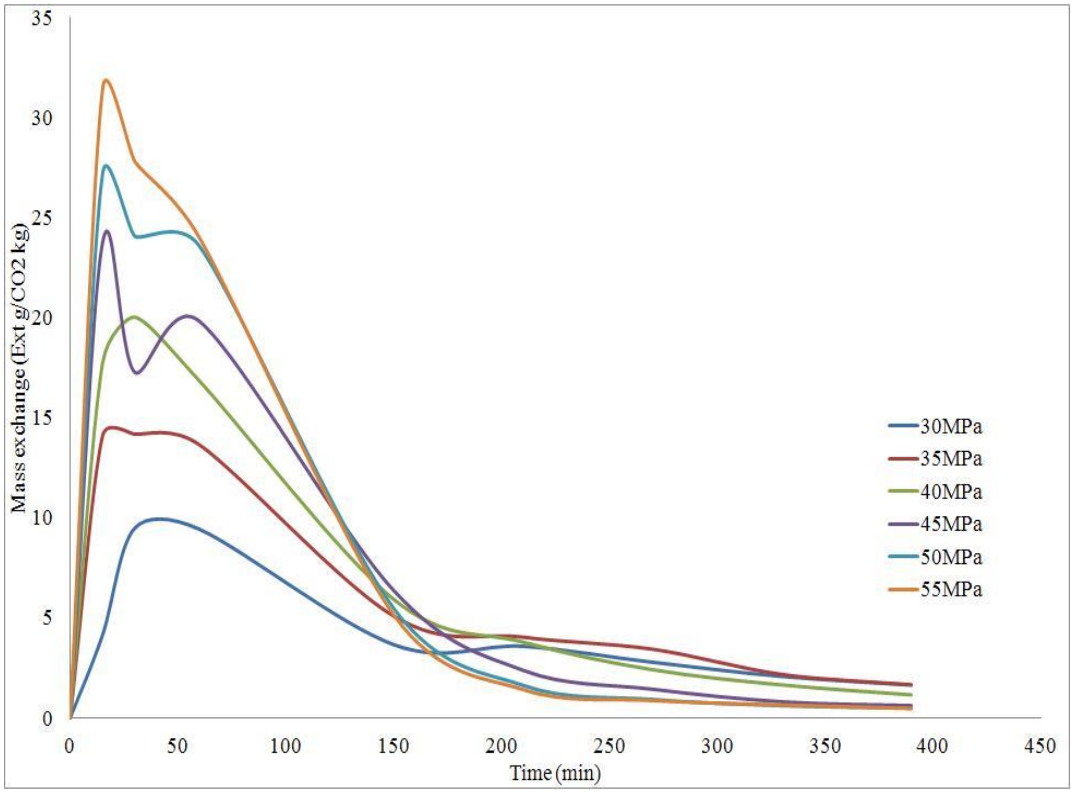

Figure 4.6: Total mass exchange of different extraction pressures with time

A larger sample of oil was extracted using a 5 L capacity process development unit. Unit mass of carbon dioxide per unit of feed was found to in a ratio of 55 CO_2 kg/ Feed kg. This experiment was conducted for up to 450 min and the total collective yield was 48% w/w. Mass ratio could be maintained without affecting other conditions such as pressure and temperature by using a larger extractor.

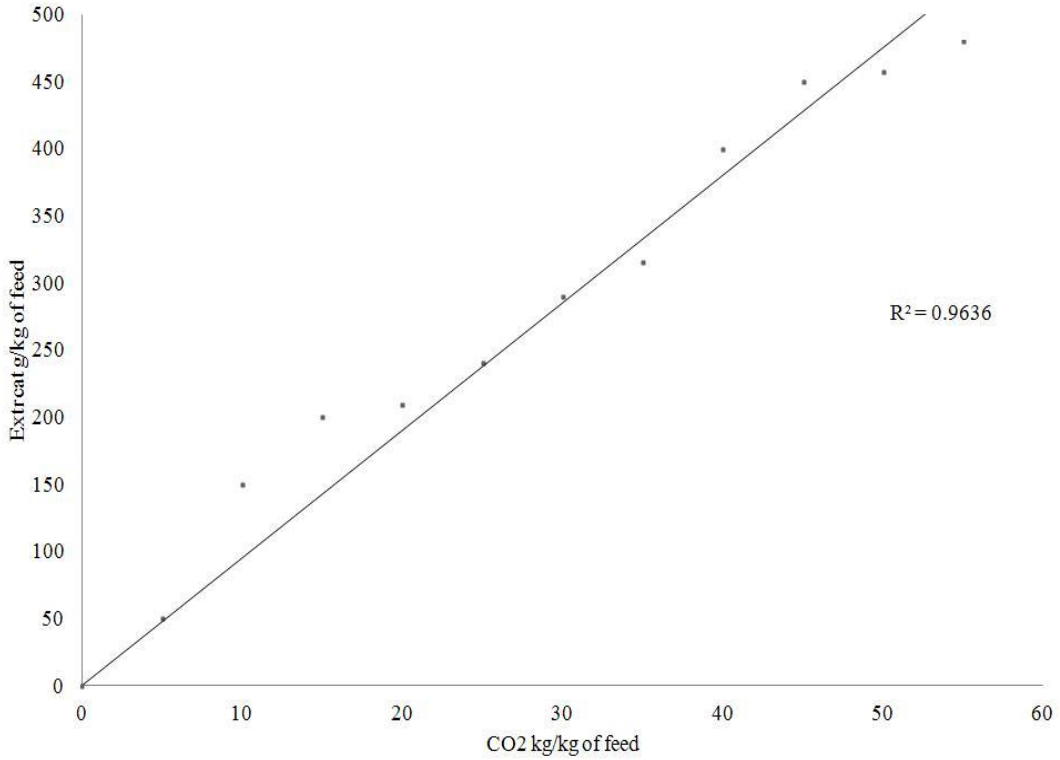

Figure 4.7: Cumulative extract mass (g) per kilogram unit of feed with respect to mass of carbon dioxide (kg) per kilogram unit of feed.

The relationship is linear and shows a positive correlation (r^2=0.9636) until it reaches the maximum extractable level (Figure 4.7). It is found that the mass of carbon dioxide could be increased under isothermal and isobaric conditions until the maximum extraction was obtained. Carbon dioxide flow was maintained at 30 kg hr^{-1} for a mass exchange at 55 kg of CO_2/ Feed kg. A linear relationship between carbon dioxide to feed ratio was reported by Oliveira *et al*. based on extractions conducted on walnut at 40^{o}C for 40 MPa extraction pressure[108]. Several method validation and process development studies have outlined the selection criteria for supercritical extraction of lipids. Major factors emphasised by Fiori are the adjustment of pressure and temperature for better mass exchange, carbon dioxide to solute ratio and end product quality[99, 100].

4.3.3 Pilot Processing

Processes at an industrial capacity have certain considerations which are different from the above experimental findings[97]. Pressure, temperature and particle size were maintained as in the earlier experiments. Carbon dioxide flow was changed to a

ranged between 220 – 330 Kg hr^{-1} to obtain necessary mass exchange ratio in an extraction chamber of 80L capacity.

The first experiment was conducted on 22.03 kg loaded in the extraction chamber. Extraction was carried out for 4 hr and carbon dioxide flow rate was 290 kg hr^{-1}. The first separator was kept at 40 ^{o}C and 6 MPa, while the second separator was maintained at ambient conditions, carbon dioxide to feed ratio was maintained at 52.65 CO_2 kg/ Feed kg. Separator one collected 10.90 kg (49.5% w/w) of extracts which appeared clear and yellowish free flowing oil. The sample was refrigerated at 4^{o}C for 18 hr, no sediment or opalescence was observed. The sample had not undergone considerable oxidation or hydrolysis as the peroxide value (2.4 meq O_2 kg^{-1}) and the acid value (2.4 mg KOH g^{-1}) were less than reported for virgin grade oils. This trial worked effectively in less extraction time and higher yields producing oil which complies with the desired chemical properties.

The above method was repeated with 27.51 kg of feed using the same unit. Temperature and pressure of the system were kept at 40 MPa and 40 ^{o}C. Flow of CO_2 was maintained at 220 kg hr^{-1} for 3 hrs and then increased to 330 kg hr^{-1} for 2.5 hrs, in the total extraction time 53.96 CO_2 kg/ Feed kg was maintained. First separator was maintained at 40^{o}C and 6MPa, second separator at ambient conditions. The first separator collected 14.377 kg of extract; yield was recorded as 52.2%. Extraction yields from sandalwood seeds seem to be promising with increased carbon dioxide mass. The above extraction method could be scaled up to a larger extraction system up to 1000kg feed amount. Sandalwood seed extract using this method was used for further studies reported in subsequent Sections and Chapters.

4.3.4 Physicochemical Characterisation

Oils extracted from the pilot plant and solvent extraction appeared clear pale yellow, free flowing liquids free from gums. Sandalwood seed oil samples obtained in this study were crude extractions; no refining step was included, other than reducing moisture and residual solvent content. Total lipid content reported by Liu *et al.* included gum and other hexane soluble material[50]. Physicochemical parameters of the sandalwood seed oil samples were compared with reported values of other oils

used in the cosmetic industry, such as virgin almond oil, virgin olive oil, cold press linseed oil and supercritical extracted grape seed oil (Table 4.1)[31, 35].

Acid values of the supercritical extract were in agreement with the standards and much lower than the free fatty acid content of solvent extract[31]. Peroxide value is an important parameter to determine the level oxidation in oils; results were similar to those of virgin grade oils as stated in the British Pharmacopeial standards (Table 4.1). These oils had higher unsaponifcable matter (Table 4.1), some unsaponificable matter such as phytosterols, tocopherols and squalenes in the oil could be beneficial in health maintenance[90]. Analysis of these compounds would find the composition of this unsaponificable matter. Specific gravity and freezing point are important parameters which would limit an oils capacity to be used as a formulating agent; values found were comparable with other oils used in formulation (Table 4.1)[31]. Viscosity of the oil was found to be lower than reported for Grape seed oil and generally was a lighter less viscous oil (Table 4.1)[31, 91]. Refractive index is mostly used as an identification of oils, fixed oils have a particular range different to other extracts or synthetic substances[31]. The refractive index of sandalwood seed oil extract does fall within the range of fixed oils (Table 4.1)[31]. Iodine value of the supercritical extract was lower than the solvent extract; decrease of iodine value is due to reduced unsaturation. Fatty acid profile could confirm whether the level of unsaturated fatty acids had reduced when compared to the solvent extract.

Physicochemical parameters of the supercritical extract have improved with the extraction method and feed material changes. Samples at the process development stage were likely to undergo oxidation were not acceptable at a peroxide value of 9.08 meq O_2 kg^{-1}. Hydrolysis of the seed oil was higher with an acid value of 6.33 mg KOH g^{-1}. This could be improved by altering particle size and moisture, which warrant further investigation. Some cosmetics use cold press virgin or crude grade oils in formulations, while refined oils are used in pharmaceuticals. If sandalwood seed supercritical oil is to be used in pharmaceuticals it needs to be refined further using physical methods such as heat or counter current supercritical extraction[76].

Table 4.1: Physicochemical comparison of sandalwood seed oil with reported data for other oils commonly used in cosmetics

	Sandalwood seed	Sandalwood seed	Grape seed[91]	Grape seed[91]	Almond[31]	Olive[31]	Linseed[31]
	SFE CO_2	Solvent	SFE CO2	Solvent	Cold press	Cold press	Cold press
Acid value (mg KOH g^{-1})	1.99 ±0.01[a]	7.22 ±0.24[b]	3.4	33.8	<2	<2	4.5
Peroxide value (meq O_2 Kg^{-1})	2.35 ±0.06[a]	6.57 ±0.19[b]	383	101	<15.0	<20	15
Saponification value (mg KOH g^{-1})	309.69 ±0.11[a]	296.74 ±0.16[b]	259	289	NA	NA	191 ±4
Iodine value (g I_2 $100g^{-1}$)	81.09±0.57[a]	89.73 ±0.19[b]	98	124	NA	NA	180 ±20
Unsaponificable matter (% *w/w*)	4.37 ±0.32[a]	3.08 ±0.21[b]	0.27	2.89	<0.9	<1.50	<1.50
Viscosity (Pa. s)	3.7 x 10^{-2} [a]	3.4 x 10^{-2} [a]	6.9 x 10^{-2}	6.6 x 10^{-2}	NA	NA	NA
Refractive index	1.477[a]	1.474[a]	1.475	1.474	NA	NA	1.480
Freeze point (^{o}C)	-11[a]	-11[a]	NA	NA	-18	0	-20
Specific gravity	0.9226 ± 0.0001[a]	0.9162 ±0.0008[b]	0.924	0.928	0.916	0.913	0.931

Mean values with different superscript letters are significantly different ($p<0.05$), values are reported as means ± standard deviations ($n = 5$)

4.3.5 Fatty acid content

Ximenynic acid from *S. spicatum* seeds has been previously identified and the acetylenic structure was confirmed several decades ago[43, 44, 52]. Results of the current study show no significant difference between the solvent extracts and supercritical extracted oil (Table 4.2), which also comply with previous findings[45, 46]. Previous studies on walnut and okra seeds have reported no difference between the fatty acid content of solvent extracts and supercritical extracts[96, 104, 108, 109].Mass spectra of the separated fatty acid methyl esters were matched with the previously reported data. Ximenynic acid comprised approximately 35% of total fatty acid content, oleic acid accounted for approximately 51% of the total. In a previous study it was observed that oleic and ximenynic acid concentrations increased as the seed matured and other saturated fatty acids and unsaturated fatty acids decreased. Stearolic acid which is another acetylenic acid was found in very low concentration in this oil. However saturated fatty acid contents were approximately 5% of the total, while long chain fatty acids of C20-C24 were not observed. Oleic acid and ximenynic acid make up 85% or more the total fatty acids. A geographical variation study found that in a single season ximenynic content varied between 27-38% within different geographical and climatic zones of Western Australia[84]; this could be due to several facts which are not thus far established.

Table 4.2: Fatty acid profile of supercritical and solvent extracts of sandalwood seed oil

Fatty acid	Notation	Percentage composition (%)	
		Supercritical CO_2	Solvent
Palmitic acid	16:0	3.64 ±0.03 [a]	3.58 ±0.01 [b]
Palmitoleic acid	16:1	0.53 ±0.05[a]	0.54 ±0.06 [a]
cis-9-Oleic acid	18:1	0.33 ±0.00 [a]	0.32 ±0.03 [a]
Stearic acid	18:0	1.79 ±0.03 [a]	1.78 ±0.07 [a]
Oleic acid	18:1	52.28 ±0.42 [a]	50.87 ±1.43 [a]
cis-9,12-Linoleic acid	18:2	1.26 ±0.01 [a]	1.27 ±0.03 [a]
cis-9,12,15-Linolenic acid	18:3	2.87 ±0.02 [a]	2.88 ±0.00 [a]
Sterolic acid	18:1(9a)	1.09 ±0.03 [a]	1.08 ±0.03 [a]
Ximenynic acid	18:2 (9a,11t)	34.96 ±0.03 [a]	35.13 ±0.83 [a]
Others (Unknown)		1.26 ±0.26	2.56 ±0.52

Mean values with different superscript letters are significantly different ($p<0.05$), values are reported as mean value ± standard deviations (n = 5)

4.3.6 Tocopherols

Tocopherols were found in a very low concentration (Table 4.3), where δ-tocopherol was higher than α-tocopherol. Supercritical carbon dioxide extracted more δ-tocopherol than solvent extraction, there was no significant difference between the α-tocopherol levels. Supercritical carbon dioxide extracts of walnut and okra seeds contained more tocopherols than the solvent extracts of the same[104, 109]. Huang *et al.* [114] has shown that α-tocopherol shows the maximum antioxidant activity at a minimum concentration of 10 mg/100g, where sandalwood seed oil has a much lower concentration. Seed oils of hazelnut, macadamia and almond have reported to have higher concentrations of tocopherols[115]. However the results for tocopherol content in walnuts, cashew, pistachio and pecan nuts were similar to the current findings on sandalwood seed oil, except that this study did not find γ-tocopherol [110, 115]. A study on walnuts (kernels) has also reported a reduction of tocopherol content by 30% even when stored at refrigerated conditions for less than three months [116]. The sandalwood seeds used in this study had been stored for more than a year in

room temperature conditions before the seeds were subjected to extraction. There is also a possibility that the extraction methods employed might have excluded all of the tocopherols. Oxidative stability would confirm whether the lack of tocopherols has a significant effect on the oil stability. Thus far the low peroxide value suggests that the oils are stable even with low tocopherol content.

Table 4.3: Tocopherol, squalene and phytosterol content of sandalwood seed oil

	Supercritical CO2 (mg/100g)	Solvent (mg/100g)
Campesterol	10.50 ±2.99[a]	12.09 ±3.15[a]
Stigmasterol	20.79 ±3.98[a]	20.73 ±6.54[a]
β-Sitosterol	85.35 ±2.94[a]	88.90 ±0.72[a]
δ-5-Avenasterol	31.53 ±1.28[a]	25.77 ±0.24[b]
Squalene	15.46 ±0.68[a]	12.72 ±0.19[b]
α-Tocopherol	1.22 ±0.17[a]	1.72 ±0.29[a]
δ-Tocopherol	5.48 ±0.34[a]	2.20 ±0.01[b]

Mean values with different superscript letters are significantly different ($p<0.05$), values are reported as mean value ± standard deviations ($n = 5$)

4.3.7 Sterols

Squalene which is a precursor in phytosterol synthesis is also a known antioxidant [117]. The squalene content in the sandalwood seed oils was similar to hazelnut, macadamia and almond oils (Table 4.3) [115]. It was higher than previously reported for the oils of, walnuts, cashew, pine and pecan nuts but lower than for brazil nut [110]. Four major phytosterols were identified from the oil, other minor sterol compounds were below the limit of quantification. The most abundant sterol was β-sitosterol, followed by δ-5-avenasterol, sigmasterol and campesterol. Other than campesterol which is a C28 4-desmethyl sterol the other three are C29 4-desmethyl sterols. Proportional distributions of the phytosterols are similar to that reported for other nut oils [23, 110, 115, 118]. Higher content of β-sitosterol (> 100mg/100g oil) has always been reported for nut oils, but sandalwood seed oil has approximately 87 mg/100g oil. However the campasterol and stigmasterol contents were higher than reported for other nut oils. There is a significant difference in the concentrations of δ-5-avenasterol. Concentrations of β-sitosterol were reported to be lower in the solvent

extraction than the supercritical fluid extractions[109]. Walnut oil extracted with solvents and supercritical carbon dioxide did not show a difference of β-sitosterol concentration [108].

4.4 Conclusion

Supercritical carbon dioxide fluid extraction is seen as a novel and "greener" method of extraction, where it is highly regarded to be used in cosmetics and nutritional supplements. In this study carbon dioxide extraction method was found to achieve quality sandalwood seed oil with a considerable yield by a technically feasible method. This method could be used in large scale extraction of sandalwood seed kernel.

Oil extracted by this method showed a similar fatty acid composition as that by solvent extraction. Physicochemical properties matched the currently used oils in the cosmetic industry. These findings would allow the introduction of a novel product which could support the sustainability of the Western Australian sandalwood industry.

Chapter 5 Stability of Sandalwood Seed Oil

5.1 Introduction

The stability of fixed oils is mainly based upon the degree of oxidation of fatty acids which results in changed chemical composition and physical properties[32, 119]. Hydrolysed triglycerides and oxidised fatty acids would affect the quality of the oil in many different ways[119]. Anticipated therapeutic, nutritional or topical effects would be diminished due to oxidation[37]. In certain cases free fatty acid and oxidised fatty acids could be toxic or irritant[120-122]. Oxidised and hydrolysed oils could also affect formulation stability, especially for emulsions. In the case of cosmetics it would be less appealing due to changed odour and texture. Hydrolysis of triglycerides gives the free fatty acids which are more susceptible to oxidation. Breakdown of triglycerides contributes to physical and chemical changes in oils, hence hydrolysis is considered a stability issue especially when oils are exposed to moisture as particularly occurs in creams[33, 90, 119, 123, 124].

5.1.1 Fatty Acid Oxidation

Fatty acids and certain other organic molecules react with atmospheric oxygen in the presence of different catalysts and increased temperatures. The major causes of oxidation are exposure to atmospheric oxygen, light, heat, metal ions and pH[123]. A free radical from the lipid components triggers oxidation, known as auto-oxidation. It is understood that unsaturated fatty acids undergo a free radical chain reaction due to the presence of one or more double bonds in their chain; when acetylenic bonds are present it is also considered an unsaturated fatty acid. Unsaturated bonds have an extra pi bond between the carbons apart from the strong sigma bond between them. These pi bonds are weaker as they are nucleophilic in nature and easily react with electrophilic species[125]. This reaction could be facilitated by thermal or metal ion catalysis of the free radical auto-oxidation of a lipid. This step of creating a free radical fatty acid is called the initiation step of oxidation[33, 125, 126]. This occurs by homolytic cleavage of an RH bond or by hydrogen atom abstraction from RH by an initiator free radical (Scheme 5.1 and Figure 5.1).

Scheme 5.1

Initiation

$$RH + I\bullet \rightarrow R\bullet + IH$$

Unsaturated lipid +initiator ion →alkyl radical + hydrogenated species

The next step is the reaction of free radical lipid with molecular oxygen species, oxygen in their molecular triplet format could not react with fatty acids until a free radical is formed (Scheme 5.2 and Figure 5.1). Hydrogen atom attached to the alkyl group with unsaturated bond will be abstracted. The radical formed on the fatty acid chain is stabilised by delocalisation to the unsaturated bond. This would allow the bi-radical oxygen to form a peroxyl radical [33, 125, 126].

Scheme 5.2

Propagation

$$R\bullet + O_2 \rightarrow ROO\bullet$$

Alkyl free radical + molecular oxygen → alkyl peroxide (peroxyl radical)

$$ROO\bullet + RH \rightarrow ROOH + R\bullet$$

Alkyl peroxide + unsaturated lipid → hydroperoxides + alkyl free radical

Highly reactive peroxyl (alkyl peroxide) would then react with an unsaturated fatty acid to form another free radical fatty acid. This in return would react with oxygen and react again continuing the cycle (Scheme 5.2). This step is known as the propagation phase of oxidation in lipids (Figure 5.1). The hydroperoxides formed would undergo dissociation in the presence of metal ion or temperature to provide initiator ions[125-127]. Antioxidants are active in this stage as they reversibly contribute a proton that would reduce the peroxyl radical to a hydroperoxide[127]. Fatty acid chains could break to form short chained hydroperoxides at the bonds where peroxyl radicals have occurred[33, 125-127].

However, the increase of alkyl free radical and hydroperoxides would limit the reaction itself (Figure 5.1). Alkyl radical can react with hydroxyl radical to form aldehydes (Scheme 5.3). Some of these carbonyl compounds are short chained fatty

acid derivatives of high volatility, and responsible to the rancid odour of decaying oil. Depending upon the positioning of the unsaturated bond there could be shorter chain carbonyls and medium chain carbonyl groups which are non volatile. Peroxides will react with each other to form dimers which are non-reactive[33, 125, 126].

Scheme 5.3

Termination

ROOH $\rightarrow$ RO• + •OH

hydroperoxides $\rightarrow$ alkoxyl radical + hydroxyl radical

R• + •OH$\rightarrow$ ROH $\leftrightarrow$RCOH

alkyl free radical + hydroxyl radical $\rightarrow$ alcohol $\leftrightarrow$ aldehyde

RO• $\rightarrow$ RCOH + RCOOH (β-scission)

alkoxyl radical$\rightarrow$ aldehyde + short chain fatty acid

R• + R• $\rightarrow$ R-R

alkyl free radical + alkyl free radical $\rightarrow$ stable dimers

Saturated fatty acids have no reactive pi bond to facilitate the reaction with oxygen by stabilising the free radical formed by abstraction of a H atom from adjacent alkyl group. Therefore thermal energy is required to dissociate saturated fatty acids before oxidation from a peroxyl or hydroxyperoxyl radical[123, 124]. Oxidation of oleic acid and polyunsaturated linoleate has been extensively studied and the mechanisms of these reactions have been established[128]. Each fatty acid has a specific oxidation pathway, understanding the positioning, number and strength of double or triple bonds provides a background to control and detection of oxidation[119]. The two major fatty acids in sandalwood seed oil are oleic acid and acetylenic ximenynic acid. Studies into the oxidation of acetylenic acids and stability of oils containing the above are not available in literature. Even though there have been previous studies on the isolation of ximenynic acid from plant seeds oxidation has not been addressed[33, 119, 129].

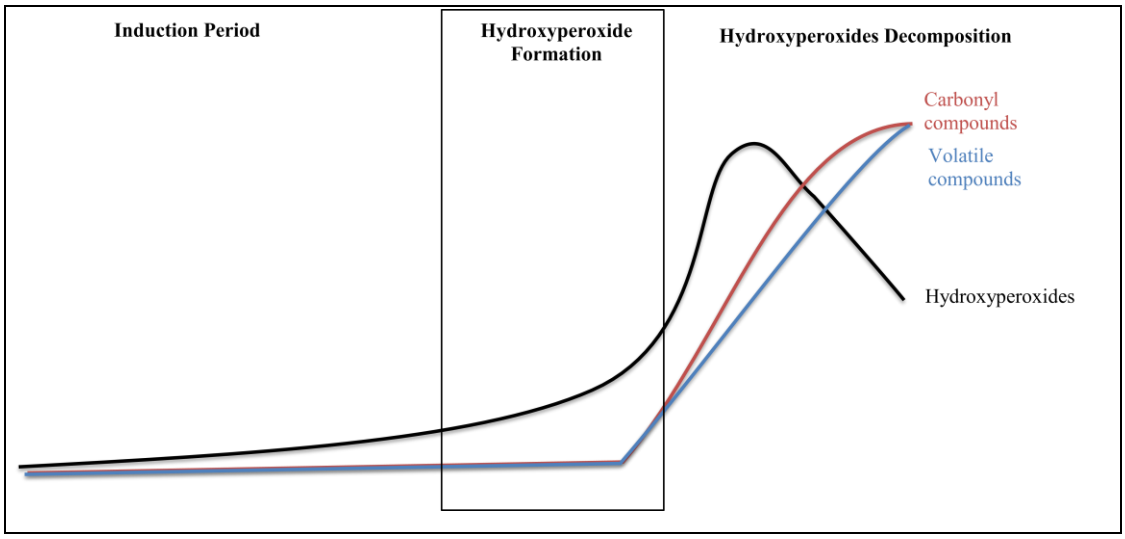

Figure 5.1: Oxidation of fatty acids observed as hydroperoxide, carbonyls and volatiles[130]

Photo-oxidation or the oxidation of lipids in the presence of light is a different process to auto-oxidation. Photoenergy in the presence of photosensitisers (carbonyl, conjugated alkenes, etc) can convert the inert triplet molecular oxygen ($^3O_2$) in to highly reactive singlet oxygen ($^1O_2$). Singlet oxygen reacts with π bonds sometimes changing the isomerism of the fatty acids. Photo-oxidation is more rapid than the auto-oxidation and continues as it depends only upon the oxygen concentration and light[33, 119, 123, 126].

5.1.2 Hydrolysis

Triglycerides are the main form of lipid molecules occurring in fixed oils. There are also free fatty acids, di and mono glycerides present as minor components. Glycerides are the esters of fatty acids and glycerol, they are otherwise known as triacylglycerols (TAGs) or acylglycerides because of the acyl bonds which forms the molecule. Living tissue and organisms continually synthesise and metabolise TAGs through a group of enzymes known as lipases. Outside living tissue acyl bonds could react with water molecules to breakdown as free fatty acid and glycerol catalysed by heat or other factors such as metal ions, pH or light. It has been identified that the TAGs show stability upon hydrolysis based on the fatty acids and their positioning in the acyl bond with glycerol. In the nomenclature of glycerol carbon atoms *sn*1 and *sn*3 are the external carbons while *sn*2 is the middle or internal carbon. Hydrolysis in *sn*1 and *sn* 3 would happen much earlier due to the spatial arrangement. The nature

of the fatty acids and its chain length plays an important role. In sandalwood seed oil the main triglycerides are comprised of oleic and ximenynic acids. Both have the same chain length of eighteen carbons, except for the presence of an extra acetylenic bond in ximenynic acid, and the positioning of the double bond[106, 131].

The formation of hydrolysed and oxidised lipids are seen as a negative development; total protection of lipids from hydrolysis and oxidation is not practical in the domestic and industrial situations. Oils can be protected to minimise decomposition and increase shelf life. Several standard methods have been established to determine stability by studying the levels of oxidation and hydrolysis[130].

5.1.3 Determination of Stability

Stability is a relative term used for a compound stating the time taken to degrade from its original form to chemically different forms. A range of parameters depicting the original nature of the compound is studied over a period of time and the onset of degradation and the time it remains in its original form would be measured or predicted[33].

The main stability determining factor for oils is oxidation, which is evaluated by measuring the level of hydroperoxides present in the oil. The most commonly used standard method is the peroxide value determination by iodometric titration. Potassium iodide is reacted with the hydroperoxides in oxidised lipids to liberate free iodine, which is titrated with sodium thiosulfate in the presence of starch which produces a blue colour complex with iodine (Scheme 5.4)[132, 133]. Disappearance of blue colour is considered the end point, milliequivalents of iodine measured is reported as the miliequivalent of peroxides per kilogram of oil. There are other methods to measure hydroperoxides such as conductivity and colorimetric assays; in the scope of the current study the above standard method was employed[32, 130].

Scheme 5.4

Iodometric reaction of hydroperoxides

$ROOH + 2H^+ + 2KI \rightarrow I_2 + ROH + H_2O + 2K^+$

hydroperoxide(acidity) + potassium iodide $\rightarrow$ iodine + aldehyde + potassium ion

$I_2 + 2\ NaS_2O_3 \rightarrow Na_2S_4O_6 + 2NaI$

Iodine + sodium thiosulfate $\rightarrow$ sodium tetrathionate + sodium iodide

In the case where an oil is oxidised over a long period or too rapidly due to the high levels of oxygen or heat peroxide value readings would reduce due to the lack of peroxides, thus producing a false interpretation. In order to overcome this, a different method is used to identify the secondary derivates such as carbonyl species. p-Anisidine (p-methoxyaniline) is reacted with the secondary carbonyl compounds such as alkenals to produce a complex which would increase in absorbance reading. This increase in absorbance reading is in proportion to the secondary compounds produced. The majority of the secondary oxidised products are 2-alkenals and 2,4-alkadienals, these could be readily reacted with p-anisidine in an acidic solution. The resulting dimers produce a yellowish solution with photo absorption at 350 nm. Anisidine value (AV) is an absorbance value ratio between the reacted and free anisidine[133]. Another method is to measure the volatile secondary products collected over the headspace of the oxidised oil and measured by gas chromatography or direct conductivity measurement. Weight gain, change of colour and reduction of iodine value are commonly noted as indicators of oxidation[130].

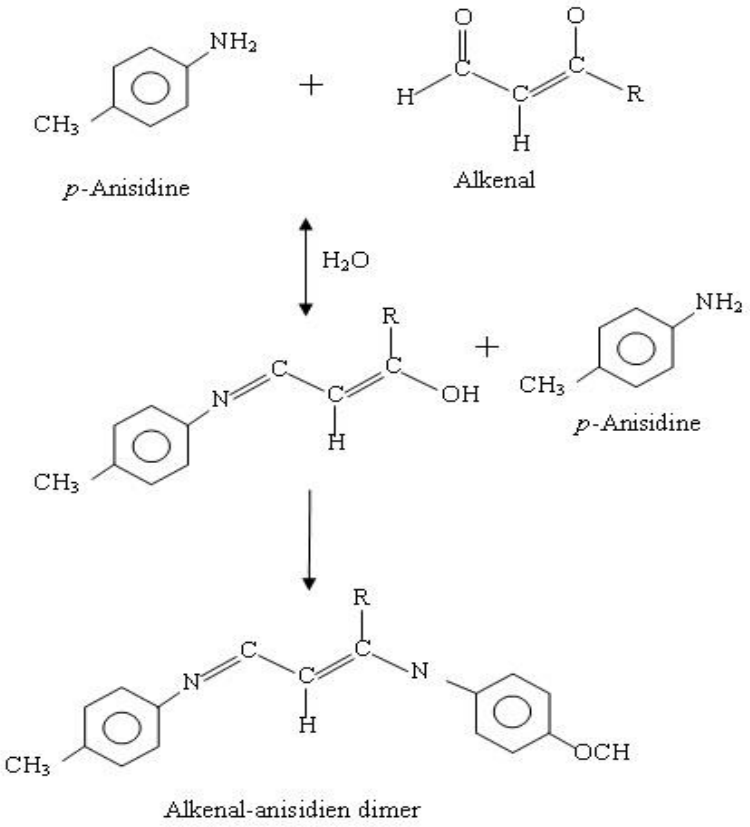

Figure 5.2: Reaction of alkenedienals with p-anisidine to form a photo absorbing dimer[133]

The above mentioned methods are to determine the by-products of oxidation, whereas determination of fatty acids and changes to the fatty acid composition are widely used methods. Gas chromatography analysis of fatty acid methyl esters is the best available method for this assay[33, 123]. This method could be used to identify the secondary products as well as quantify the reduction of certain fatty acids. However, the early stages of oxidation are not clearly depicted on chromatograms due to the low concentration of secondary products[123]. Iodine value is utilised to determine the unsaturation of fatty acids. Unsaturated bonds can uptake reactive halogens, this principle is used in the iodine value by reacting iodine monochloride with unsaturated fatty acids. Unreacted iodine monocholoride is then reacted with a saturated potassium iodide solution and the resulting iodine is titrated with a standardised solution of sodium thiosulfate. The endpoint is determined by the disappearance of the blue colour produced by the starch iodine complex, iodine value is determined as the gram amount of iodine taken by 100 grams of oil[31]. Lipid

peroxidation occurs in saturated bonds, a reduction of iodine value could be observed upon prolonged oxidation.

Hydrolysis is depicted in lipids by increased free fatty acids. These fatty acids could be easily measured by a weak acid strong base titration or precise conductivity measurement. The most commonly used method is the assay of acid value, increased acid value indicates the hydrolysis level[90]. Free fatty acids are a product of hydrolysis; these weak acids will readily react with a strong base to form a salt which is otherwise known as soap. The oil sample is reacted with a known volume of standardised potassium hydroxide and the excess base is back titrated with standardised hydrochloric acid in the presence of phenolphthalein as the indicator. A blank titration would determine the total potassium hydroxide used by the free fatty acids, thus expressing the value as milligrams of potassium hydroxide consumed by a gram of oil[31, 32].

The above parameters are the major factors for oil stability; however the oil is studied when subjected to certain conditions in order to record physicochemical changes. Under different conditions the above parameters are studied for longer or shorter periods of time with corresponding sampling frequency. The time period to exponential increase in oxidation rate is considered the induction time, which is the step where oxidation or hydrolysis has begun in an unrecoverable phase. As oxidation is continuing as a self catalysing process, the level of oxidation is beyond control once the onset has initiated. The main aim is to prolong the induction time[34, 119].

Some researchers have published conditions to accelerate short term studies to observe the degradation products. In these studies the oil would be subjected to heat and oxygen sources and samples are collected over a shorter period and measure the stability parameters[134, 135]. Commonly used active oxygen method is structured on bubbling of oxygen through an oil sample kept at different temperatures. Less aggressive methods use 40 - 60^{o}C while most of the rapid methods use higher temperatures of 80 - 120^{o}C. Light is considered as a trigger of oxidation; some researchers suggest light and dark controls to compare the effects of oxidation. There is a novel method for rapid oxidation at very high temperatures, where volatile

oxidative products are collected in a controlled headspace for assessment. Results obtained under different conditions would be used as variables in predicting the induction time for oxidation and hydrolysis[33, 34].

These methods are more suitable to evaluate the underlying potential for oxidation; fatty acids act differently under variable conditions. Oxidation and hydrolysis of lipids in natural conditions are very different to the elevated temperatures used in these studies. Long term studies under controlled conditions have been adopted for pharmaceuticals, food and cosmetic industries as the true representation of the degradation of products[136]. Samples are subjected to a condition which is naturally extreme. Samples would be analysed at longer intervals to determine the slow onset of oxidation or hydrolysis. This method would show actual induction times to predict shelf life. Under this assessment, samples could be treated in different ways representing package and light conditions[129].

The current study is designed to assess the stability of sandalwood seed oil based on oxidation and hydrolysis. The best method employed to conduct the study was determined to be a long term assessment method discussed above with both inclusion and exclusion of oxygen. Some samples would be packed exposed to air and light while others are packed with nitrogen headspace and kept in the dark. Oil samples would be studied for 360 days to observe parameters to determine its degradation. Samples would be analysed for peroxide, p-anisidine, acid and iodine values. The fatty acid profile would be analysed to study any specific depletion of a particular fatty acid as the oxidation progresses.

The objectives of this study were to evaluate the long term stability of the sandalwood seed oil by following parameters;

- Evaluating the oxidation by measuring hydroperoxides
- Evaluating secondary oxidation by measuring carbonyl compounds
- Evaluate the hydrolysis by measuring free fatty acid content
- Analyse the fatty acid profile with respect to oxidation and hydrolysis.

5.2 Methods

5.2.1 Sandalwood Seed Oil

Sandalwood seed oil was extracted using a supercritical fluid extraction process and was obtained from Wescorp Sandalwood Ltd (WS/SWSO/2012A1). A sample batch of 1 kg was received in the laboratory within 10 days after extraction on June 2012. The oil was packed in food grade aluminium containers with a high density polyethylene lining and stored at 15^{o}C with minimum headspace for transport.

5.2.2 Sample Allocation and Conditions

Samples were stored in 50 mL sealable clear glass vials (Standard Line Injection, Schott Aust Ltd, NSW); silicone lined 20mm aluminium crimp (Goldstand Seal, Alltech, USA) were used as the seal. Two sample groups were designated, First group (16 vials) was defined as the "protected sample", which was filled with 50 mL of oil and flushed with nitrogen and filled headspace (approximately 10 mL) with nitrogen; vials were covered with aluminium foil (Table 5.1). Two vials from this set was considered as the fresh sample representing the day 0 and stored at -20^{o}C immediately. Second group contained 14 vials with 25 mL each sample and were poured with aeration, this would be the "unprotected sample" with approximately 35 mL headspace exposed. Samples were stored at 40^{o}C ± 1^{o}C controlled chamber (Challenge 700, Angelantoni Test Technologies srl, Italy) with 12 hrs light per day and relative humidity of 40% ± 5% (Table 5.1).

At specific time intervals of 30, 60, 90, 120,180, 270 and 360 days samples were collected (Table 5.1). At each interval two vials from protected set and two vials from unprotected set were selected at random for analysis. The representative vials were labelled then immediately stored at -20^{o}C in the dark until all the samples were recovered from the controlled chamber.

Table 5.1: Sandalwood seed oil samples prepared for stability study with conditions and collection time

Group	Vial Number	Vial capacity	Oil Volume	Headspace	Sampling days
Protected	1	50 mL	50 mL	Nitrogen	0
	2	50 mL	50 mL	Nitrogen	0
	3	50 mL	50 mL	Nitrogen	30
	4	50 mL	50 mL	Nitrogen	30
	5	50 mL	50 mL	Nitrogen	60
	6	50 mL	50 mL	Nitrogen	60
	7	50 mL	50 mL	Nitrogen	90
	8	50 mL	50 mL	Nitrogen	90
	9	50 mL	50 mL	Nitrogen	120
	10	50 mL	50 mL	Nitrogen	120
	11	50 mL	50 mL	Nitrogen	180
	12	50 mL	50 mL	Nitrogen	180
	13	50 mL	50 mL	Nitrogen	270
	14	50 mL	50 mL	Nitrogen	270
	15	50 mL	50 mL	Nitrogen	360
	16	50 mL	50 mL	Nitrogen	360
Unprotected	17	50 mL	25 mL	Air	30
	18	50 mL	25 mL	Air	30
	19	50 mL	25 mL	Air	60
	20	50 mL	25 mL	Air	60
	21	50 mL	25 mL	Air	90
	22	50 mL	25 mL	Air	90
	23	50 mL	25 mL	Air	120
	24	50 mL	25 mL	Air	120
	25	50 mL	25 mL	Air	180
	26	50 mL	25 mL	Air	180
	27	50 mL	25 mL	Air	270
	28	50 mL	25 mL	Air	270
	29	50 mL	25 mL	Air	360
	30	50 mL	25 mL	Air	360

5.2.3 Analysis

Samples were brought to room temperature and analysed within a short period of time. Parameters analysed were the following based on the methods described in general methodology section;

- Peroxide value
- p-Anisidine value
- Iodine value
- Acid value

Fatty acid composition was analysed using a gas chromatography mass spectrometry as described in Section 2.4. Samples were analysed for physicochemical parameters as described on Section 2.7 following the British Pharmacopeial methods[31]. After exposing for set time intervals samples were frozen at -20^{o}C to terminate further oxidation and retain the chemical characteristics. This procedure has been practiced by researchers to avoid increasing variations and other errors[33, 130]. Values obtained were plotted against the time represented for further prediction of induction times.

5.3 Results and Discussion

5.3.1 Oxidation

A clear difference was observed between the protected and unprotected oils during the observation period. The unprotected sample had reached highest maximum peroxide value of 16.3 Meq O_2 kg^{-1} at 180 days against a 3.55 meq O_2 kg^{-1} reported for protected oil at 360 days (Table 5.2). This indicates that sandalwood seed oil would undergo a considerable level of oxidation when exposed to air in a high ambient temperature environment. Lipid peroxidation occurs as several different reactions in bulk oils[33]. The unprotected sample showed an immediate reaction without a detectable intermediate step (Figure 5.2). The current study would indicate that sandalwood seed oil is stable against oxidation for 360 days when stored in light protected containers under nitrogen at 40^{o}C.

Peroxide values observed during time intervals for the unprotected sandalwood seed oil sample correspond with the established pattern for lipid peroxidation [130]. Standard monographs for unrefined oils such as linseed, olive and almond have upper limits of peroxide values of 15-20 meq O_2 kg^{-1} [31], sandalwood seed oil is within these limits when unprotected for 360 days (Table 5.2). Several previous studies have identified the peroxide value of 10 meq O_2 kg^{-1} as the limit for reaching oxidation[137-139]. Vegetable oils containing polyunsaturated fatty acids such as linolenic acid have followed this limit of 10 meq O_2 kg^{-1} more frequently in both normal and accelerated stability testing[33]. A study conducted on Chia seed has found that the oil exposed to air at 4^{o}C had reached a peroxide value of 10 meq O_2 kg^{-1} within a 180 day period[138]. Further to this screw pressed almond oil reached the above value within 12 days in ambient conditions[139].

The effect of metal ions and other radicals could promote oxidation of the oil during storage[90]. The extraction method used for the sandalwood seeds has minimised any possibility of pro-oxidant molecules and ions from the oil as described on Chapter 4. Oxidation induction time for the protected oil was not determined as it was not reached at 360 days. Rapid oxidation of the unprotected oil was seen even at the first sampling at 30 days. Polyunsaturated fatty acids showed slow oxidation when stored without exposure to air below 30^{o}C in the dark [129]. Refined oils of walnut, almond,

macadamia and hazel nuts reached a maximum of 0.43 meq O_2 kg^{-1} upon air tight and light protective storage conditions. Observing these results, it is understood that sandalwood seed oil needs the same storage and packing conditions as many commonly used oils require.

A subsequent drop of peroxide values after 180 days for the unprotected sample suggests that the oxidative by-products from peroxyls would interfere the oxidation process (Figure 5.2). This has been commonly observed in accelerated stability testing of unsaturated fatty acids[32, 135, 140]. This reduction of peroxide value could be due to the increased conversion to carbonyl compounds. This observation on oxidised lipids can be misleading in the interpretation of oxidative stability. Because of this phenomenon it is always advised to assess the secondary oxidation products[33].

Conversion of peroxyls to carbonyls was determined by assessing the increase of carbonyl compounds in oil by AV[130]. Analysis of the secondary oxidative products showed a parallel increase to the peroxide value up to 180 days (Figure 5.3). The AV was monotonically increasing up to 360 days while the peroxide value had fallen. The unprotected oil demonstrated a marked increase of the AV within the first month (Figure 5.3). The subsequent phase was slow progression, there is a significant difference (P=0.0001) between the final AV of protected and unprotected oils (Figure 5.3). These values are reasonably high when compared to other oleic acid containing seed oils but less than reported for polyunsaturated fatty acids[31]. These ω-3 fatty acids are easily converted to carbonyl compounds by oxidative addition at the unsaturated bonds[32]. Formation of these secondary products could affect the quality of oil. A study on crude sunflower oil exposed to air at 30^{o}C protected from light reached AV of 10 units within 3 months [141]. Similarly long term stability of seed oils containing polyunsaturated fatty acids have shown that AV were independent of temperature and light[140, 141,142].

The protected sample showed a lower value with little change during the observation time of 360 days (Table 5.3). Several studies have been reported where researchers have studied both peroxide and AV to understand and establish the oxidation of fatty acids and different vegetable oils[141]. A detailed study on peroxide and AV were

measured for vegetable oils containing polyunsaturated fatty acids at accelerated conditions by Chander [140]. Findings of the above accelerated stability study and the current study on sandalwood seed oil both showed a complete peroxidation and a continuous increment of secondary carbonyl compounds[140]. However, the chemical nature of the fatty acids would affect the levels of these oxidation parameters. Some fatty acids would form hydroperoxides but would remain intact, other fatty acids would tend to breakdown from the peroxide bridge bond eventually forming two carbonyl compoundss. Oils consisting predominantly of oleic acid have shown higher peroxide value increase when compared to the AV[128]. Current findings of decreasing peroxide values and a steady increase of AV (Figures 5.2 and 5.3) suggest that the acetylenic fatty acid tends to breakdown and form carbonyl groups on oxidation.

There are other methods used to determine the oxidation of seed oils such as Rancimat® method, headspace analysis and DPPH colorimetric assay[130, 143]. Findings of these studies suggest that polyunsaturated fatty acids undergo oxidation faster than saturated or monosaturated oils[135].

Table 5.2: Peroxide value (meq O_2 kg^{-1} of oil) data for protected and unprotect samples during 360 days observation

Time (Days)	0	30	60	90	120	180	270	360
Protected	2.36±0.06	2.38±0.03	2.38±0.03	2.38±0.01	2.39±0.01	2.40±0.01	2.67±0.13	3.55±0.02
Unprotected	2.36±0.06	10.8±0.58	11.76±0.25	13.18±1.67	14.99±0.85	16.30±0.17	4.97±0.30	3.52±0.08

Values are reported as mean ± standard deviation (n=4)

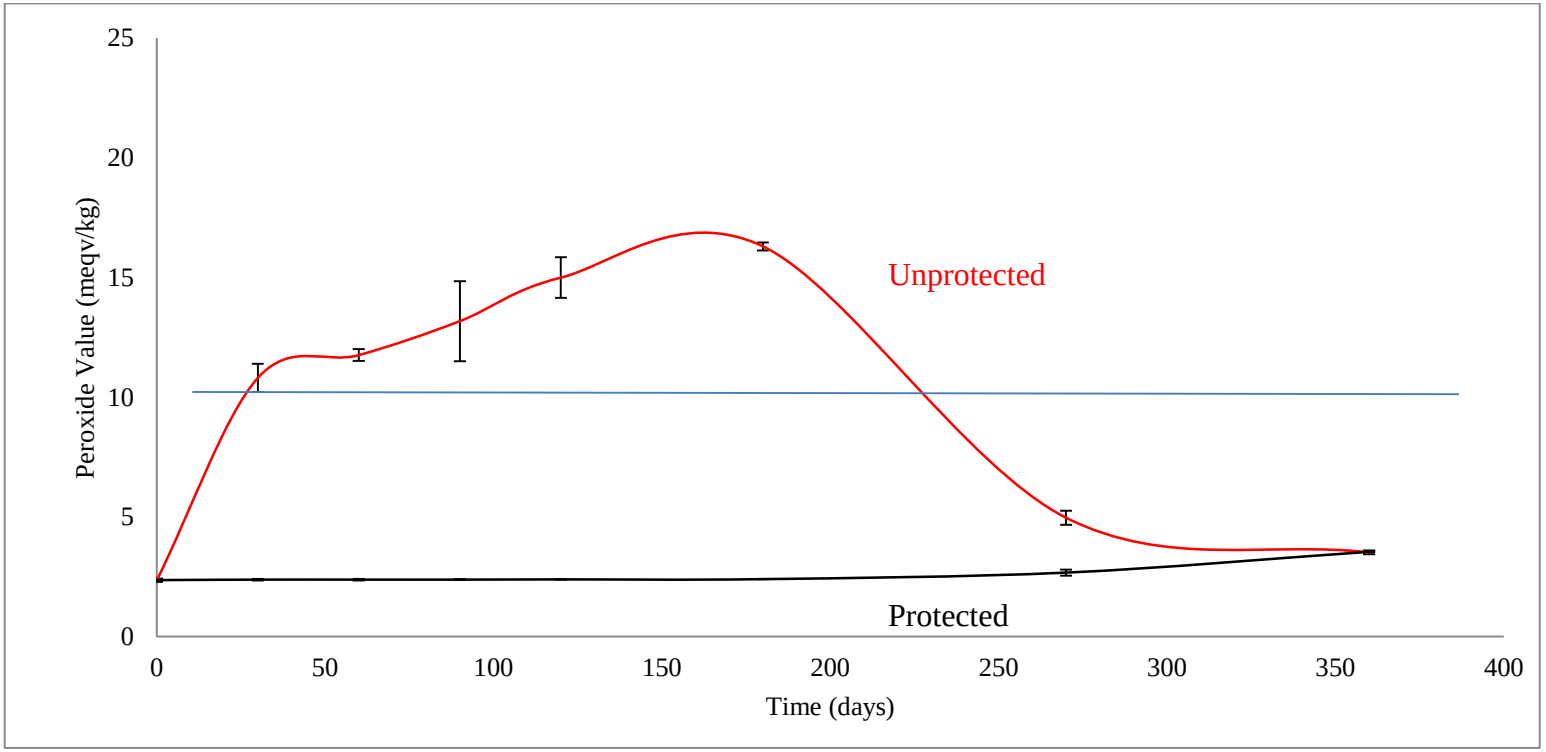

Figure 5.3: Peroxide value of protected and protected samples against time

Table 5.3: Anisidine value (AV) data for protected and unprotect samples during 360 days observation

Time (Days)	0	30	60	90	120	180	270	360
Protected	3.14±0.70	4.14±0.06	3.73±0.15	4.16±1.09	4.78±2.86	5.10±0.17	5.33±0.42	6.06±0.37
Unprotected	3.14±0.70	12.57±0.97	13.62±1.14	17.39±0.48	18.66±0.98	21.36±1.63	23.32±2.03	28.06±0.29

Values are reported as mean ± standard deviation (n=4)

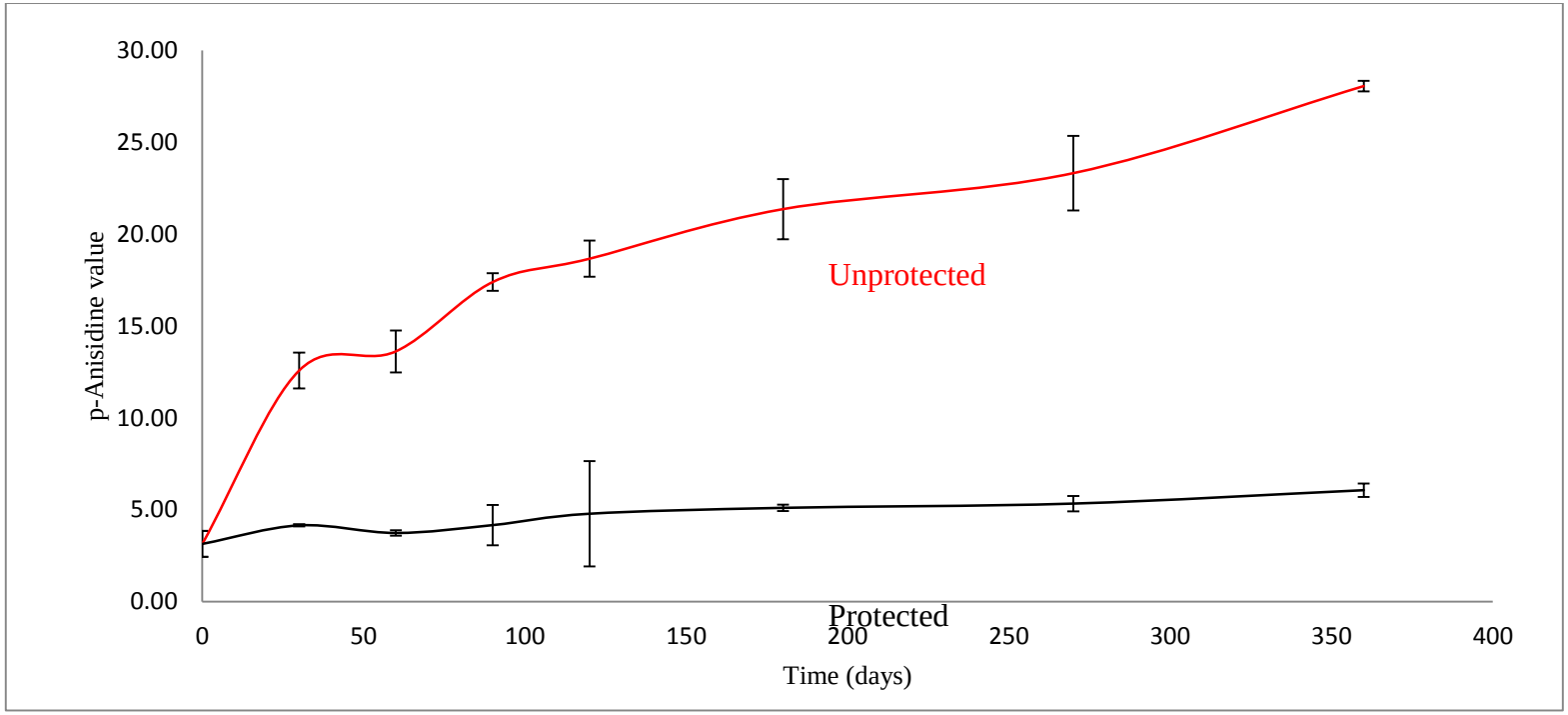

Figure 5.4: Anisidine value (AV) of protected and protected samples against time

5.3.2 Fatty acid profile

Sandalwood seed oil is of mainly C18 chain length fatty acids, with more than 50% of it comprised of monounsaturated oleic acid and 30-35% of acetylenic ximenynic acid[71]. This acetylenic acid has triple bond at C9 position and *E* (*trans*) double bond at the C11 position. These two fatty acids could undergo oxidation in different ways due to their chemical nature. PV and AV of this study (Section 5.3.1) suggest a considerable oxidation of the unprotected oil. However, there is no difference in fatty acid profile observed under the current study conditions within experimental error (Table 5.4), in contrast to the peroxide and AV. Results suggest that total composition of fatty acids of sandalwood seed oil were not changed (Table 5.4). Composition of polyunsaturated acetylenic ximenynic acid depicts no significant difference between the protected and unprotected oils (p =0.077). A similar observation was made for oleic acid where no statistical significance was observed (p =0.171). A minor change in their fatty acid composition would not be identified from a total fatty acid analysis. Polyunsaturated linolenic and linoleic acids are present in sandalwood seed oil but in very low concentrations. These fatty acids have been previously reported for change in chain length during oxidation[129]. Even the composition of these fatty acids have not been affected during the current oxidation study (Table 5.4). Oxidised fatty acids and secondary carbonyl products can be identified by mass spectrometry[130]. However, it is difficult to detect and quantify these carbonyl compounds and their fatty acid origin when present in low concentration. Oxidation of oleic acid in bulk oils and as free fatty acids have been reported previously and an oxidation pathway has been identified[119]. There are no previous studies reported on oxidation of acetylenic fatty acids.

Reduction of unsaturated bonds was measured by the iodine value as indicated by grams of iodine reacted with a 100 grams of oil. Reduction of iodine value with time or storage condition suggest that fatty acids have lost unsaturated bonds, therefore a change in their chemical nature possibly due to oxidation[130]. In this study iodine value has reduced only 4 g iodine per 100 g of oil in both protected and unprotected samples (Table 5.5). Changes of iodine value for both the samples show no statistical significance (p values for unprotected 0.22 and protected 0.09). The data in Figure 5-5 is enlarged to observe the change of values between 4 points, it shows the drop

near 100 days identically for both the samples (Figure 5.5). Iodine value is a unique value of oils which is commonly used to identify adulteration by inferior products[31]. Standard documents have given a considerable range in iodine value for oils containing unsaturated fatty acids[31, 35]. The current difference of four points in iodine value could be considered negligible. Peroxide formation and chain shortening is a result of reactions of pi bonds in unsaturated fatty acids[130]. The iodine value results did not show a depletion of unsaturated bonds in sandalwood oil in comparison to oxidation observed in the Section 5.3.1. Oxidised oils contain short chain fatty acids in the oil as a result of chain break from saturated bonds[119]. The fatty acid profile of oxidised sandalwood seed oil showed no traces of the short chain fatty acids.

Table 5.4: Fatty acid composition as %w/w of sandalwood seed oil for protected and unprotected oils in different time intervals (days)

Sample	Days	Fatty acid							
		Palmitic acid	Palmitoleic acid	Stearic acid	Oleic acid	Linoleic acid	Linolenic acid	Sterolic acid	Ximenynic acid
Protected	0	3.49±0.04	0.54±0.01	1.30±0.23	58.05±1.06	0.88±0.06	2.15±0.20	0.64±0.13	32.94±0.42
	30	3.53±0.01	0.55±0.01	1.05±0.09	56.71±0.08	0.98±0.02	2.28±0.03	0.82±0.02	34.08±0.10
	60	3.49±0.03	0.53±0.01	1.00±0.03	56.56±0.11	0.95±0.02	2.45±0.25	0.79±0.02	34.40±0.20
	90	3.54±0.01	0.55±0.02	1.04±0.08	56.69±0.52	1.00±0.03	2.32±0.08	0.81±0.05	34.06±0.26
	120	3.46±0.03	0.54±0.01	1.03±0.01	56.77±0.01	0.95±0.03	2.26±0.01	0.77±0.02	34.22±0.08
	180	3.41±0.02	0.52±0.01	1.01±0.04	57.27±0.23	0.95±0.02	2.27±0.02	0.78±0.06	33.84±0.11
	270	3.46±0.10	0.54±0.01	1.14±0.02	57.07±1.30	0.94±0.03	2.22±0.12	0.79±0.06	33.59±2.17
	360	3.43±0.07	0.54±0.03	1.11±0.07	56.90±0.59	0.93±0.02	2.19±0.08	0.79±0.05	34.44±0.21
Unprotected	0	3.49±0.04	0.54±0.01	1.30±0.23	58.05±1.06	0.88±0.06	2.15±0.20	0.64±0.13	32.94±0.42
	30	3.48±0.05	0.54±0.01	1.32±0.34	57.58±0.86	0.93±0.01	2.08±0.03	0.72±0.01	32.98±0.03
	60	3.51±0.06	0.53±0.02	1.16±0.13	57.62±0.37	0.91±0.01	2.09±0.01	0.70±0.03	33.47±0.11
	90	3.42±0.06	0.55±0.02	1.15±0.12	57.17±0.40	0.94±0.05	2.11±0.10	0.79±0.06	33.68±0.54
	120	3.49±0.01	0.55±0.01	1.03±0.15	56.75±0.17	0.94±0.03	2.16±0.04	0.80±0.02	34.05±0.53
	180	3.51±0.07	0.54±0.02	1.10±0.05	57.47±0.21	0.93±0.04	2.08±0.04	0.77±0.06	33.15±0.04
	270	3.56±0.20	0.55±0.02	1.09±0.02	57.42±0.84	0.94±0.02	2.31±0.03	0.72±0.04	33.23±1.60
	360	3.50±0.06	0.54±0.00	1.11±0.03	57.19±0.51	0.95±0.01	2.09±0.39	0.75±0.03	33.58±0.68

Values are reported as mean ± standard deviation (n=4)

Table 5.5: Iodine value (g I_2 $100g^{-1}$ of oil) data for protected and unprotect samples during 360 days observation

Time (Days)	0	30	90	120	180	360
Protected	81.09±0.57	80.39±1.81	79.04±1.75	79.93±2.08	78.25±3.69	76.87±1.42
Unprotected	81.09±0.57	80.69±0.63	80.68±0.43	80.37±1.97	78.12±0.88	76.93±6.21

Values are reported as mean ± standard deviation (n=4)

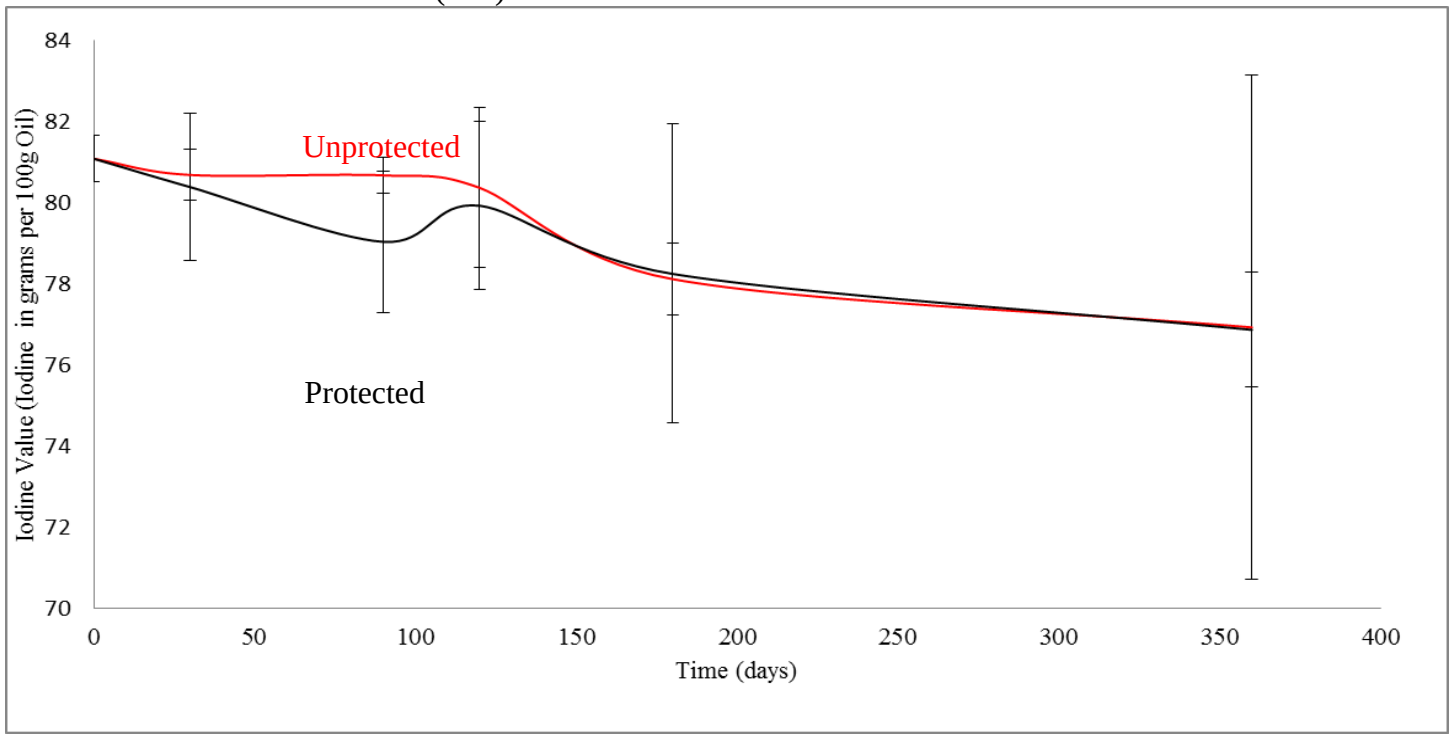

Figure 5.5: Change of iodine value (g I_2 $100g^{-1}$ of oil) of protected and unprotected samples against time

5.3.3 Hydrolysis

Hydrolysis of triglycerides would change the matrix of the oil and also release free fatty acids. Free fatty acids are more reactive than triglycerides, hence changing the chemical nature of oil and making the oil less desirable to be consumed or used in formulations[90]. However, the acid value has not been discussed as a major stability concern in the literature when compared to the emphasis given to oxidation. The basis of this study was to assess the total chemical stability which includes the hydrolysis of triglycerides. Hydrolysis of triglycerides was measured by titrating free fatty acids with a strong base[35]. The main factors governing hydrolysis in oil are moisture content and temperature, an effect of catalysts such as metal ions also been reported[123, 124, 131]. Sandalwood seed oil used in this study was extracted by supercritical fluid extraction and considered virgin or crude grade oil. The temperature of storage for both the samples was the same. Overall change of acid value for the unprotected sample was higher than for the protected sample (Table 5.6).There is a statistical significance in the change for unprotected oil (P = 0.003), whereas protected oil did not show a significant change (P=0.11).

Hydrolysis was initiated during the first 30 days for the unprotected oil and reached stable condition until 270 days where it showed an increase of acid value towards the end of the study (Figure 5.6). The protected oil showed reasonable stability and gradual increase up to 85 days which could be seen as an induction time (Figure 5.6). It reached 2.14 mg KOH g^{-1} within less than 30 days and remained relatively constant (Table 5.6). Even though the increase of acid value was not large when compared it could have affected the oxidation. A study conducted on by adding free acids to bulk oils have resulted 1-10% increase in oxidation[144]. Acid value increment during the latter part of the study could be explained with increased carbonyl compounds which were detected by the anisidine value. Secondary oxidation products such as carboxylic acids and β-diketones formed together with carbonyl compounds could react with potassium hydroxide resulting a higher acid value[130]. Acid values for both the oils are in the range of accepted values for the other virgin grade oils. Vegetable oils containing monounsaturated fatty acids such as olive and almond oils have less than 2 mg KOH g^{-1} while polyunsaturated fatty acid containing virgin linseed oil was given a upper limit of 4.5 mg KOH g^{-1} [31].

Table 5.6: Acid value (mg KOH g^{-1} of oil) data for protected and unprotect samples during 360 days observation

Time (days)	0	30	60	90	120	180	270	360
Protected	1.99±0.01	1.99±0.01	2.00±0.02	2.01±0.01	2.14±0.22	2.15±0.01	2.17±0.082	2.17±0.091
Unprotected	1.99±0.01	2.06±0.01	2.11±0.01	2.11±0.01	2.12±0.01	2.12±0.01	2.14±0.095	2.35±0.01

Values are reported as mean ± standard deviation (n=4)

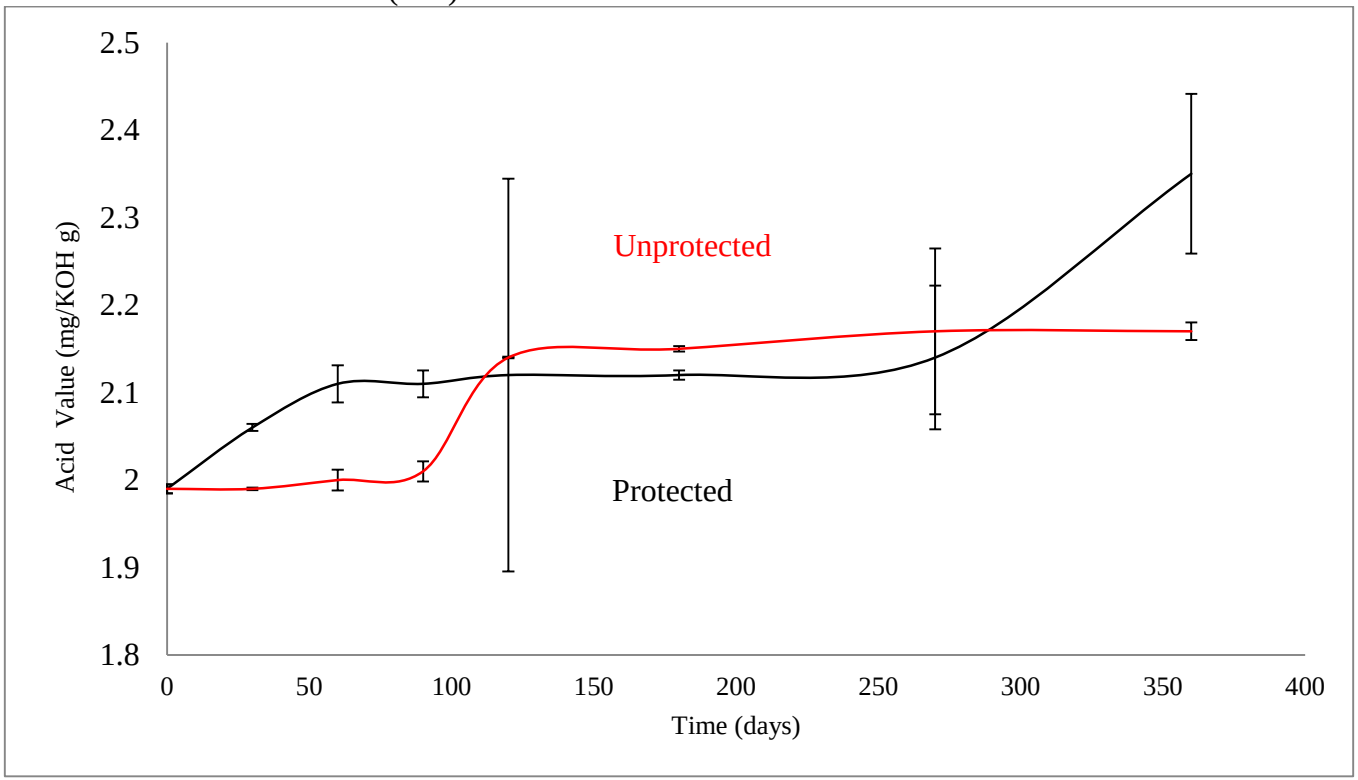

Figure 5.6: Acid value (mg KOH g^{-1} of oil) of protected and protected samples against time (days)

5.4 Conclusion

Current study used long term storage under high ambient conditions opposed to accelerated conditions where high temperatures where used. Sandalwood seed oil remains stable and maintained the original physiochemical characters with negligible oxidation in the absence of oxygen and light even at high ambient temperatures. However, under the same temperature conditions samples exposed to air and light have undergone considerable oxidation. Although the oxidation parameters of protected and unprotected samples showed notable differences, there were no differences observed in the fatty acid profile. Both the samples showed minimum hydrolysis; which is mainly due to lack of moisture in the oil obtained by supercritical carbon dioxide fluid extraction method. This study claims that when protected from oxygen and light sandalwood seed oil remained stable over 360 days. Proper care need to be taken soon after the supercritical carbon dioxide extraction of this oil by flushing with nitrogen and immediate storage. Sandalwood seed oil should be stored in airtight containers with headspace replaced by nitrogen gas and kept away from light. These findings on stability and storage conditions are useful for industrial production of sandalwood oil and its use in cosmetics and pharmaceuticals.

Chapter 6 Purification of Triximenynin from Western Australian Sandalwood Seed Oil

6.1 Introduction

Seed kernels of Western Australian sandalwood (*Santalum spicatum* R.Br.) contain three main triglycerides; one of the triglycerides (triximenynin) composed entirely of ximenynic acid, while the other two contain one and two ximenynic acid moieties together with oleic acid moieties[48]. Liu *et al*. have developed chromatographic methods to separate and identify triximenynin from *Santalum spicatum*[48]. A study on *Santalum album* has isolated triximenynin from seed kernel by thin layer chromatography[53]. Triximenynin together with the other two triglycerides have been characterised for their fatty acid composition and their structural arrangement[48, 53]. The major triglyceride species in *S. spicatum* and *S. acuminatum* were found to be diximenynin-monoolein while diolein-monoximenynin was found to be greater than the triximenynin in both the species[48, 145]. The proportion of ximenynic acid is highest in *S.album*, hence the presence of triximenynin as the main triglyceride compound [53].

Triglycerides are synthesised in the endoplasmic reticulum in plant cells. The glyceryl backbone of triglycerides would be initiated as a glyceryl-3 monophosphate moiety which will undergo acylation reactions with fatty acid CoA groups forming mono, di and tri glycerides subsequently[20, 21] . Positioning of the fatty acid where it has formed an acetyl bond is known denominated as *sn* position; where *sn*-1 and *sn*-3 reactions from α-acyl bonds and *sn*-2 forms β-acyl bonds. These bonds can be acetylated or hydrolysed by lipase group enzymes[131]. These enzymes play a valuable role in the biochemistry of triglycerides. When seeds or other lipid sources are consumed lipases in the gastrointestinal tract would hydrolyse the triglycerides, upon absorption they are then metabolised in hepatic tissue with lipases to triglycerides which will be stored in adipose tissue. Not all the fatty acids would be rearranged as triglycerides as essential fatty acids would convert into other physiologically important molecules[146].

In vitro studies conducted on acetylenic ximenynic acid and *in vivo* studies on seed oils containing this fatty acid were discussed in Section 1.3.3. However, triximenynin has never been studied for its pharmacological properties, unlike other naturally available triglycerides containing beneficial fatty acids.

Plant oil contains triglycerides made up with different fatty acids, whereas only a few of these fatty acids are therapeutically important. Oil fractions rich in particular triglycerides are becoming pharmaceutically important as nutritional supplements and therapeutic agents[147]. Scientists have developed methods to synthesise desired triglycerides from necessary free fatty acids[147]. Pharmacologically important triglycerides such as conjugated linoleic acid (CLA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are currently used as nutritional supplements[148]. A particular interest was taken in studies where the authors have separated all γ-linolenic triglycerides from evening primrose oil to be used as a nutritional supplement without synthesis[28, 149].

Separation of triglycerides for analytical and isolation purposes have been previously reported[26, 27]. A common method of separating uses normal phase silica and a gradient of solvents of increasing polarity for lipid class separation[72]. However, this method is mainly depending upon the polarity of the whole molecule rather than the chemical dissimilarity between the fatty acids. Another method is using arginated silica; silver ions increase the affinity of double bonds (*pi* bonds) towards the stationary phase, the number of double bonds and spatial arrangement can be exploited by this method[29]. This method has shown excellent separation for triglycerides, as well as free fatty acids such as CLA, EPA and DHA[29, 150]. The main disadvantage of this method is the cost as the silver ions leach from the column with eluent, which also requires removal from the fractions. Use of reverse phase silica has been reported mainly for HPLC methods[151, 152]. Some studies have utilised this method in large scale purification of lipids[28]. Separation of critical pair triglycerides has been reported using a column packed with 5µm octadecyl reverse phase silica. In the above study El-Hamdy and Perkins found a relationship between carbon number and capacity factor for the separation triglycerides[151]. Though this is not a specific method, lipophilic affinity towards the stationary phase can be altered with mobile

phase. Reverse-phase silica is highly stable and the columns are reusable numerous times with solvents of varying polarity under high pressure[27, 151, 152].

Liu *et al.* has reported a HPLC method to separate and identify the triglycerides of sandalwood seed oil with reverse phase silica and a refractive index detector, however this detector was seen as less sensitive [48]. A routine method utilizing a reverse-phase columns and UV detection needs to be developed for future chemical and biological assays. Large scale purification of triximenynin using suitable stationary and mobile phases is seen as essential at the beginning of this study. This triglyceride compound could be subjected to a range of biological studies and to understand kinetic characteristics. A large scale column chromatographic method could be ideal to produce this compound industrially.

The objectives intended to be achieved form this current study were as follows;

- Develop a HPLC method to separate and identify the triglycerides of sandalwood seed oil.
- Develop a chromatographic method to isolate triximenynin in high purity, which could be extended to a large scale.

6.2 Materials and Methods

6.2.1 Sandalwood Seed Oil

Sandalwood seed oil was received as an authentic sample Wescorp Sandalwood Pty Ltd, WA, Australia (batch number WS/SWSO/2010A2).

6.2.2 Solid Phase Extraction

Oil was dissolved at a concentration of 50mg/mL for the initial experiments using chloroform and methanol (1:1 v/v) as solvent. Mobile phase experimentation was on solid phase extraction cartridges of normal phase silica (Discovery DSC-Si 1g/6mL, Sigma Aldrich, USA), reverse phase silica (Discovery DSC-18 1g/6mL, Sigma Aldrich, USA) and silver ion silica (Discovery Ag-Ion 1g/6mL, Sigma Aldrich, USA) was undertaken. Cartridges were wetted with mobile phase and 100µL of

solution was introduced and solvents were passed through and 500µL fractions were collected. Mobile phase trialled were n-hexane (100%), acetone (100%), acetone and *n*-hexane (60:40), acetone and propan-2-ol (65:35) and propan-2-ol (100%).

6.2.3 Chromatographic Separation

Preparative and analytical TLC methods were according to the Sections 2.2.3 and 2.6.1. A mobile phase of n-hexane, diethyl ether and glacial acetic acid (70:30:1) was used for analytical and isolation studies. Plates were developed and then dried under a hot air flow, then observed under 254 nm and the compounds were identified as quenched spots on a fluorescent green background.

Analysis was carried out using a HPLC system described in Section 2.2.2. Column chromatography methods followed those described in Section 2.6.2. Samples were dissolved as a 200 mg/mL solution in n-hexane. The solvent mixture was fed to the top of the column and a positive pressure was created using nitrogen gas at 50 kPa producing a flow rate of 5 mL/min. A 20mLsample was collected for each fraction. Fatty acids were analysed and quantified as the fatty acid methyl esters by GC-MS and the fatty acid analysis methods described in Section 2.4.2.

6.2.4 Characterisation

Triglyceride fractions were characterised by Dr. Lindsay Byrne (School of Biomedical, Bimolecular and Chemical Sciences, University of Western Australia) using NMR system as mentioned in Section 2.2.6. Mass spectra of the triglycerides were obtained by the LC-MS system mentioned in Section 2.2.5 and methods in Section 2.6.4.

6.3 Results and Discussion

Three main bands for the TAGs were clearly visible on preparative TLC upon observation under UV light at 254 nm in a chamber. Content of these bands were isolated extracted and analysed by GC-MS for their fatty acid composition. Fatty acids corresponding to TLC fractions confirmed the following distribution; band 1 contained two oleic acid and one ximenynic acid esters, band 2 two ximenynic acid and one oleic acid esters and band 3 with all three ximenynic acid esters. Positioning of the fatty acids could not be characterised based on this data. This step was repeated several times and band R_f values were observed to be reproducible and complying with results of Liu *et al*[48]. Sufficient amounts of triglycerides were isolated using eight preparative TLC plates. Highest absorbance (λ max) was found to be 236nm for the all three triglycerides and sandalwood seed oil in chloroform and methanol (1:1).

6.3.1 HPLC Method

Triglycerides were successfully separated using reversed phase HPLC and UV absorption at 236 nm. The mobile phase used was acetonitrile propan-2-ol (65:35) which was identified as a suitable mobile phase for a reverse phase silica stationary phase to separate triglycerides based on the above studies. The triglycerides formed clear and sharp peaks with considerable retention time differences when subjected to HPLC analysis. Triximenynin showed the shortest retention time of 11.13 min followed by diximenynin-mono olein at 19.21 min, diolein-monoximenynin required 35.41 min to elute from the system. When sandalwood seed oil was introduced to the system it showed the three major peaks and several minor peaks on a clear base line and separated from each other with suitable retention times. Three main peaks showed similar retention times to the isolated triglycerides. Further analyses were conducted on the same HPLC system for sandalwood three major peaks which were collected and the fatty acid composition was analysed as FAMEs by GC-MS. The fatty acid compositions of the three peaks were found to correspond with the triglycerides isolated by TLC.

The current method showed good separation with considerable retention time differences, and symmetrical peaks. The current system with 1.5ml/min flow consumes more time and solvents, which could be rectified by increasing the flow rate. As the flow rate increases back pressure developed significantly due to the relatively high viscosity of isopropyl alcohol and the oils. Isopropyl alcohol was replaced by ethanol and methanol in two trials but the separation of peaks was not satisfactory. In order to obtain a good separation, flow rate was kept at maximum of 2.5mL/min while total run time was extended to 20min. This has given considerable retention time difference between the three major triglycerides (Figure 6.1). Peak compositions were confirmed by analysing the eluents corresponding to the peaks by GC-MS (Table 6.1). The peaks showed some contamination but the proportion of major fatty acids corresponded to the ratios expected for the three triglycerides of sandalwood seed oil.

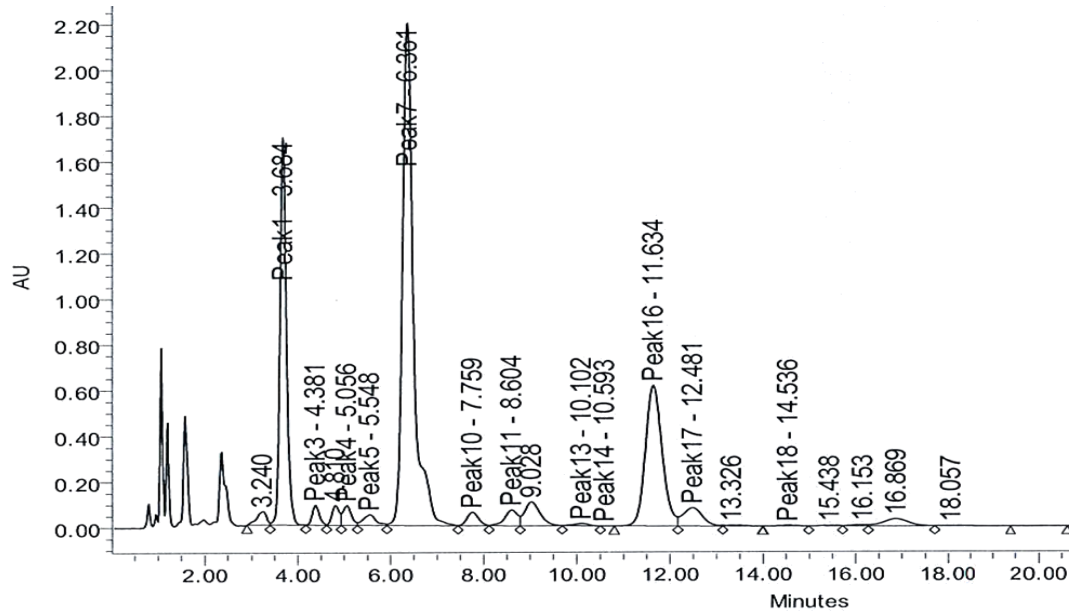

Figure 6.1: HPLC trace of the sandalwood seed oil with major triglycerides separated; (Peak1) triximenynin, (Peak 7) diximenynin-monoolein, (Peak 16) diolein-monoximenynin

Reverse-phase HPLC systems described by Liu *et al.* on sandalwood seed oil and Redden *et al.* on evening primrose oil have similarities to the current method[48, 74]. Rivett *et al.* has reported that diximenynin-monoolein is the major triglyceride species in *S. acuminata* seed oil[48, 145]. This current study has reported a higher

relative percentage of triximenynin than reported by Liu *et al*[48]. In addition this method could be used with no special dimension columns or refractive index detectors.

Table 6.1: Triglyceride retention times by HPLC with fatty acid composition

Triglyceride	Relative Area % by HPLC	Retention time	Fatty acid composition by GC-MS		
			Oleic acid	Ximenynic acid	others
Triximenyin	19.93±1.09	3.63±0.05	1.91±0.64	77.28±2.00	20.81±1.13
Di-ximenyin, mono-olein	44.16±2.30	6.24±0.12	20.69±1.89	68.27±1.68	11.04±0.95
Di-olein, mono-ximenyin	30.03±1.56	11.45±0.19	57.15±2.24	33.02±2.04	9.82±1.35

6.3.2 Triximenynin Purification

Solid phase extraction was considered as a step to determine a suitable stationary phase for large scale purification of triximenynin. This was assessed by using three silica based solid phase micro cartridges. Mobile phases were examined using all three stationary phase cartridges, each with five different mobile phases. Each test was carried out by collecting five fractions of 500µL aliquots. Every fraction was tested using the TLC method and comparing the distribution of spots corresponding to the triglycerides. Normal phase silica and arginated silica did not separate the triglycerides with any of the mobile phase, the three major triglycerides eluted without separation. Reverse phase silica showed considerable separation with the acetone: propan-2-ol (60:40) mobile phase. This stationary and mobile phase was introduced to column chromatography system without further analysis as it appeared more suitable for larger scale.

A sandalwood seed oil sample was separated on 100g reverse phase silica using the acetone propan-2-ol (65:35) mobile phase at 5ml/min , 25 fractions were collected of 20 mL each (Table 2.2). These were first analysed using the TLC method and simultaneously using HPLC.

Table 6.2: Percentage purity as total area percentage for triximenynin in fractions 4 to 15 of column chromatograph as analysed by HPLC

fraction number	Triximenynin (Total peak area %)
4	7.86
5	38.38
6	72.80
7	81.06
8	91.38
9	91.75
10	84.77
11	76.31
12	48.62
13-15	0.00

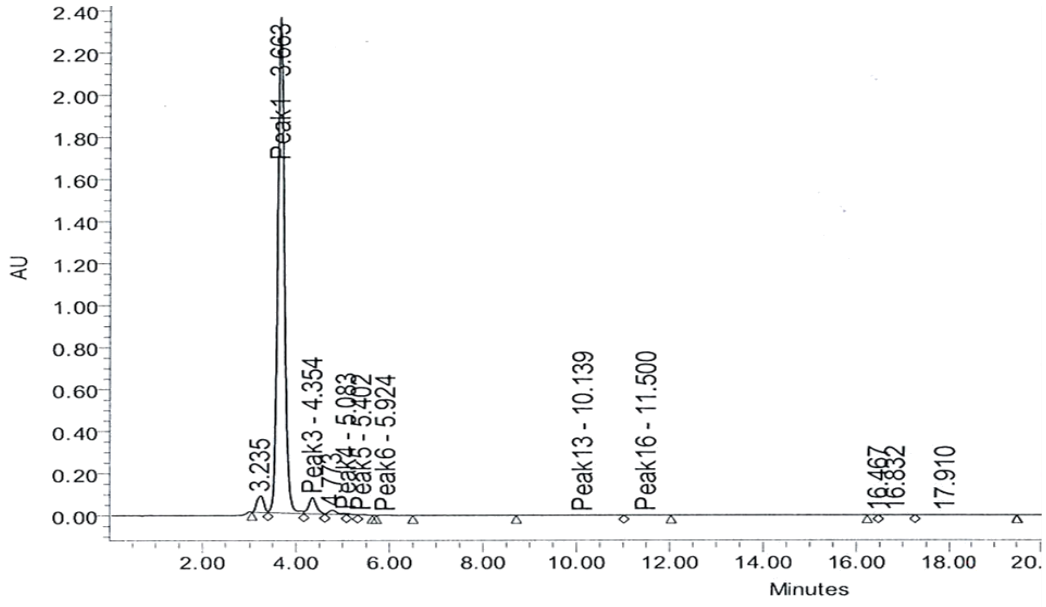

Figure 6.2: HPLC chromatogram of fraction 9 of column chromatograph showing Peak 1 for triximnenynin.

The HPLC chromatograms Fraction 9 showed a total area percentage of 91.75% for triximenynin indicating its purity (Figure 6.2). Triximenynin separated by this method was found to be satisfactory as the maximum purity achieved was 91.75% of total area (Table 6.2). The ximenynic acid was found to be 95.2% of total fatty acids of the fractions 8 and 9 (over 90% area of triximenynin) when analysed by the GC-MS. Fractions were combined and solvent was evaporated to get a total weight of 0.1476 mg, which was 2.95 $\%_{w/w}$ from the 5.008g of sandalwood seed oil.

Conditions of this experiment were later trialled with 200g reverse phase silica in the same column to separate sample sizes of 10g of oil. This was repeated using the same column and chromatographic method three times with 10 min n-hexane wash between the samples have yielded 0.8293g of triximenynin from total of 30.024g sandalwood seed oil (2.76% w/w). Purity was assessed fraction wise by the HPLC and the total area for triximenynin was found to be above 90% for these fractions. The GC-MS analysis found that the ximenynic acid composition above 95% of the total fatty acids.

This method was found to be versatile and economical as reverse-phase octadecyl silica could be used repeatedly as a separation medium; it also could be easily cleaned with non-polar solvents. Solvents used in this method are considered non-toxic, thus used commonly in plant drug extractions[90]. Using a non-gradient system would make the operation simple and these solvents could be easily recovered and reused as the mobile phase. Similar separation studies on tri-γ-linolenin triglycerides have obtained purity up to 59.7% using a silver-ion normal phase chromatography with the same mobile phase[28, 149].

6.3.3 Characterisation

Proton NMR showed shifts at δ5.460 and 6.039 which correspond to single hydrogen at C-11 and C-12 double bond (Figure 6.3). Multiple signals at δ1.2 to 1.5 were from the acyl chain, which could be assigned to hydrogen atoms at C-3 to C-7 and again C-14 to C17. Shifts observed at δ2.261 and 2.314 as triplet signals were produced from C-8 next to the acetyl bond and the C-2 next to a carbonyl group (Table 6.3). Shifts observed at δ4.1 to 4.4 were produced from hydrogen attached to *sn*-1 and *sn*-3 carbons of the glyceryl backbone. The methyl group at C-18 produced a triplet signal at 0.888 (Figure 6.3).

The major shifts on ^{13}C NMR spectra were observed for all the carbon atoms corresponding to ximenynic acid (Figure 6.4). The chemical shifts at δ88.623 for C-9 and δ 79.435 for C-10 were due to the presence of triple bonds. Double bond at the C-11 and C-12 are represented by the shifts at δ109.840 and δ143.584 (Table 6.3). Shift for the double and triple bonds confirm the presence of *trans*-ximenynic acid.

Three carbon atoms of the glyceryl backbone have produced chemical shifts between δ60-70 (Figure 6.4). Acyl chain carbons at C-2 to C-8 and C-13 to C17 could be seen producing shifts between δ19- δ34 (Figure 6.4), whereas the C-18 methyl group produced a shift at δ 14.220 (Table 6.3). The first carbon of the fatty acid is the carbonyl group which showed a shift at δ172.968 (Table 6.3). Contamination from other fatty acids with a C9-C10 double bond could be seen as minor shifts at 129-131ppm (Figure 6.4).

Table 6.3: NMR spectral data for triximenynin

Carbon	δC (ppm)	Protons number	δH (ppm)	Signal	Coupling constant J (Hz)
glyceryl	60 - 70		NA	-	
1	172.968		NA	-	
2	34.14	2-H	2.314	t	9.025
3	24.942	3-H	1.231-1.394	m	72.072
4	28.939	4-H			
5	29.205	5-H			
6	29.085	6-H			
7	29.126	7-H	1.496-1.585	m	2.702
8	19.471	8-H	2.261	t	9.025
9	88.623	9-H	NA	-	
10	79.435	10-H	NA	-	
11	109.89	11-H	5.46	q	1.040
12	143.584	12-H	6.039	d	1.000
13	33.12	13-H	2.049	t	2.629
14	29.205	14-H	1.496-1.585	m	2.702
15	29.085	15-H	1.231-1.394	m	72.072
16	31.816	16-H			
17	22.738	17-H			
18	14.225	18-H	0.888	t	15.005

Chemicals shifts of the isolated triglyceride matched with the ^{13}C NMR data reported by Li Ken Ji *et al*[53]. There were no proton NMR chemical shifts published for the triximenynin, although there are published proton NMR data for ximenynic acid. The above study has found the *trans* isomer of ximenynic acid as showing a chemical shift at 88.547 in 13 C NMR studies; while the synthesised *cis* isomer produced a shift at 94.277. It confirmed that ximenynic acid moieties in triximenynin were in all *trans* isomers.

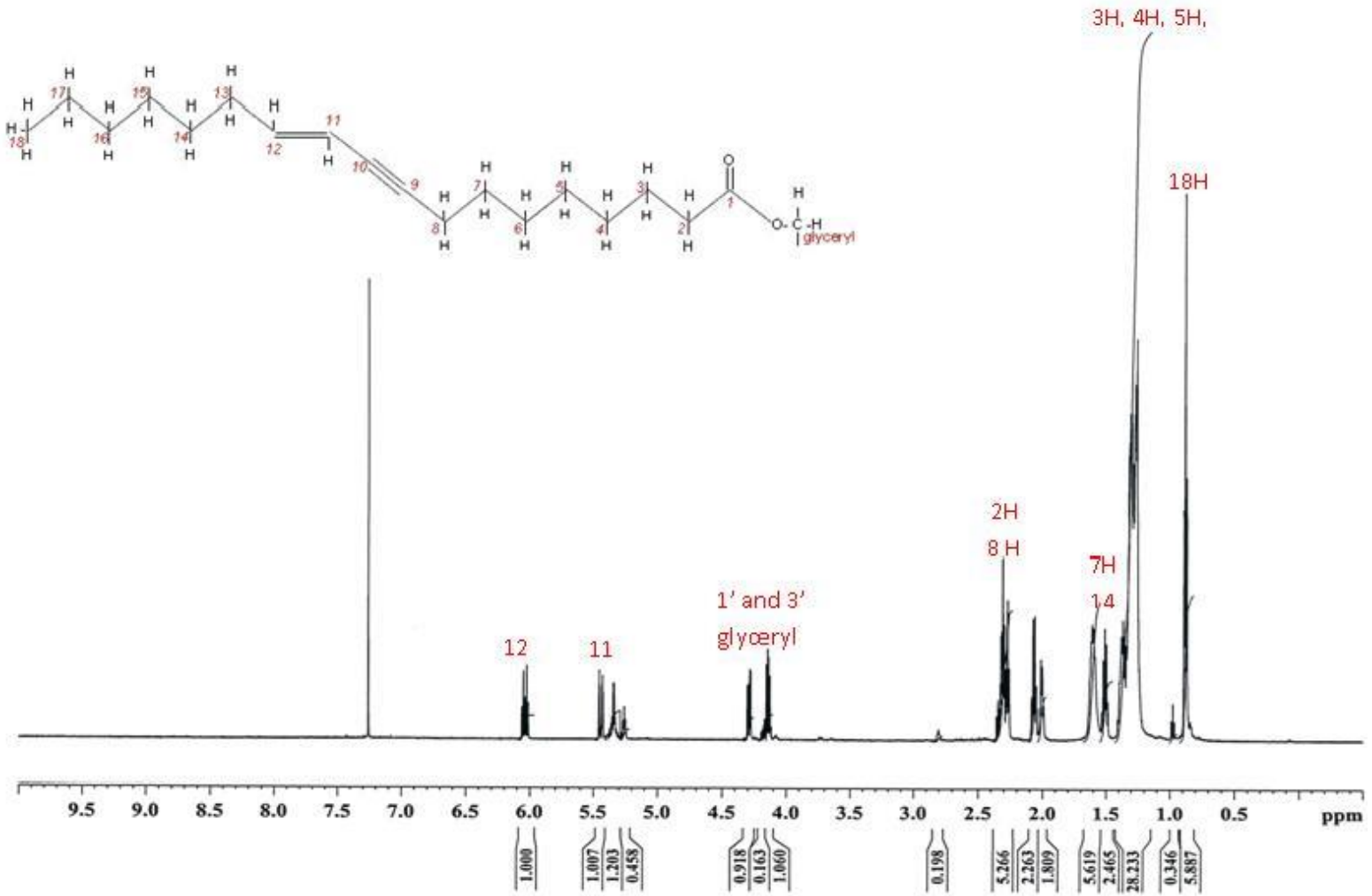

Figure 6.3:Proton NMR of triximenynin with identified chemical shifts and ximenynic acid with protons numbered based on carbon

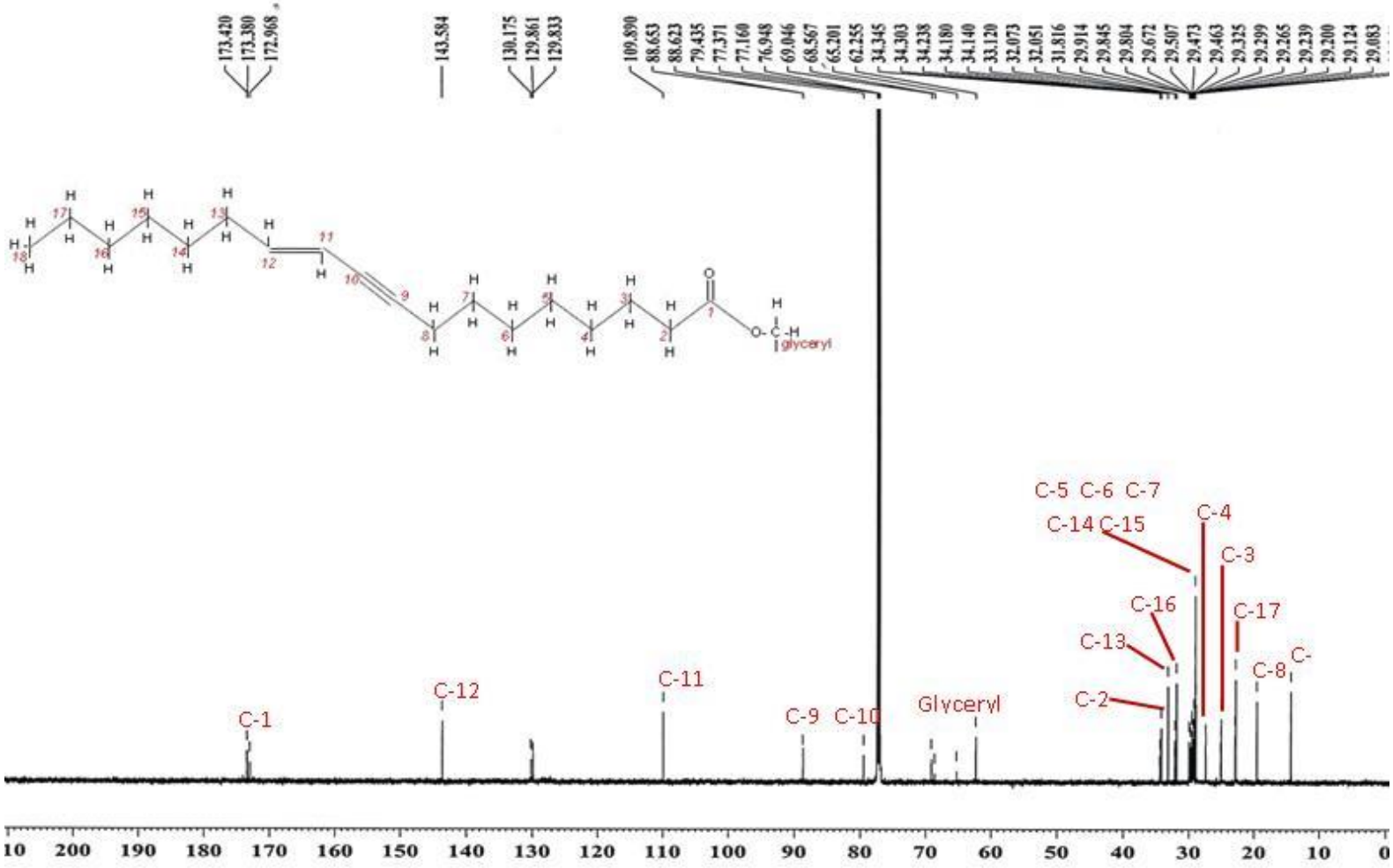

Figure 6.4: ^{13}C NMR of triximenynin with identified chemical shifts and ximenynic acid structure with carbon number.

Mass spectrometry analysis data corresponds to a triglyceride with a possible molecular weight of $C_{57}H_{92}O_6$ (Figure 6.5). The molecular peak is expected at 872m/z, however there are peaks observed at 896 *m/z* and 916 *m/z* could be quasimolecular ions with or without the solvent molecules (Figure 6.6). There are peaks observed at 595 *m/z* for the diglyceride and the 305 *m/z* for the monoglyceride (Figure 6.6). However, the most stable ion was found to be at 507 *m/z* which corresponded to a diglyceride with a C_7H_{14} fraction fragmented from the C11 double bond rather than the C9 triple bond (Figure 6.5). A peak observed at 727 *m/z* matches with a triglyceride with fragmentation at the C9 triple bond (Figure 6.5). The abundance at 551 *m/z* is higher which could be correlated to a triglyceride structure where all the C_7H_{14} fragments for fatty acids have been lost at the C11 double bond (Figure 6.6).

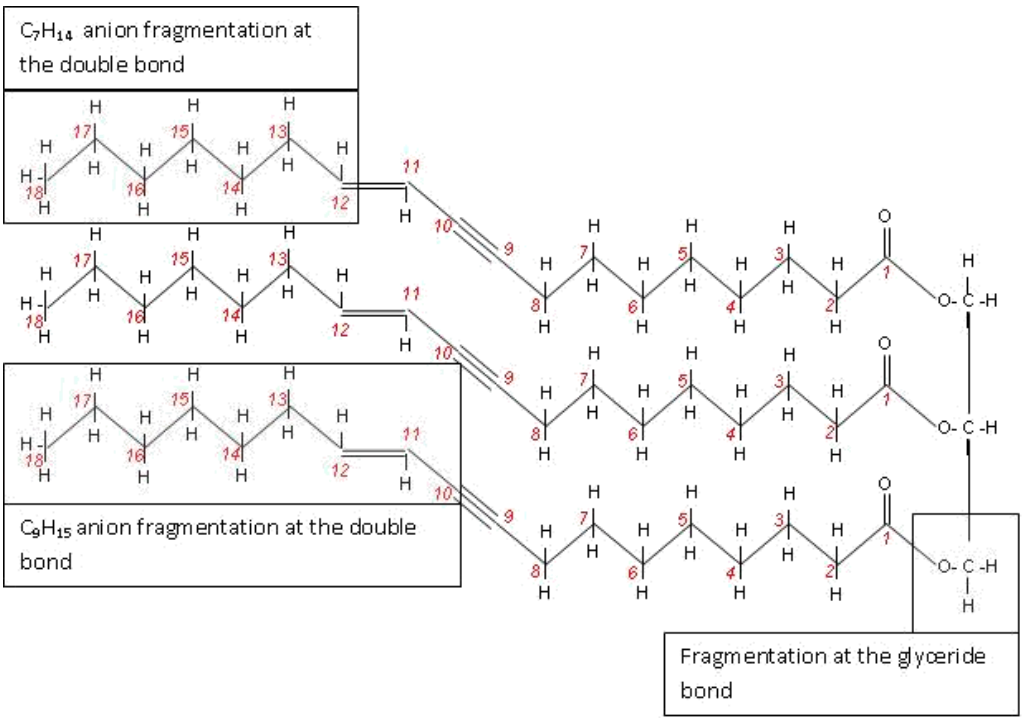

Figure 6.5: Triximenynin structure with identified major fragmentation points

These major fragmentations could be seen on a triglyceride structure for triximenynin (Figure 6.5). Potential early fragmentations are seen at the saturated bonds and the carbonyl bonds. Fragments with lesser mass to charge ratios correspond with molecular fragments and fatty acid ions. The peak at 261 *m/z* corresponds with ximenynic acid cation (Figure 6.6).

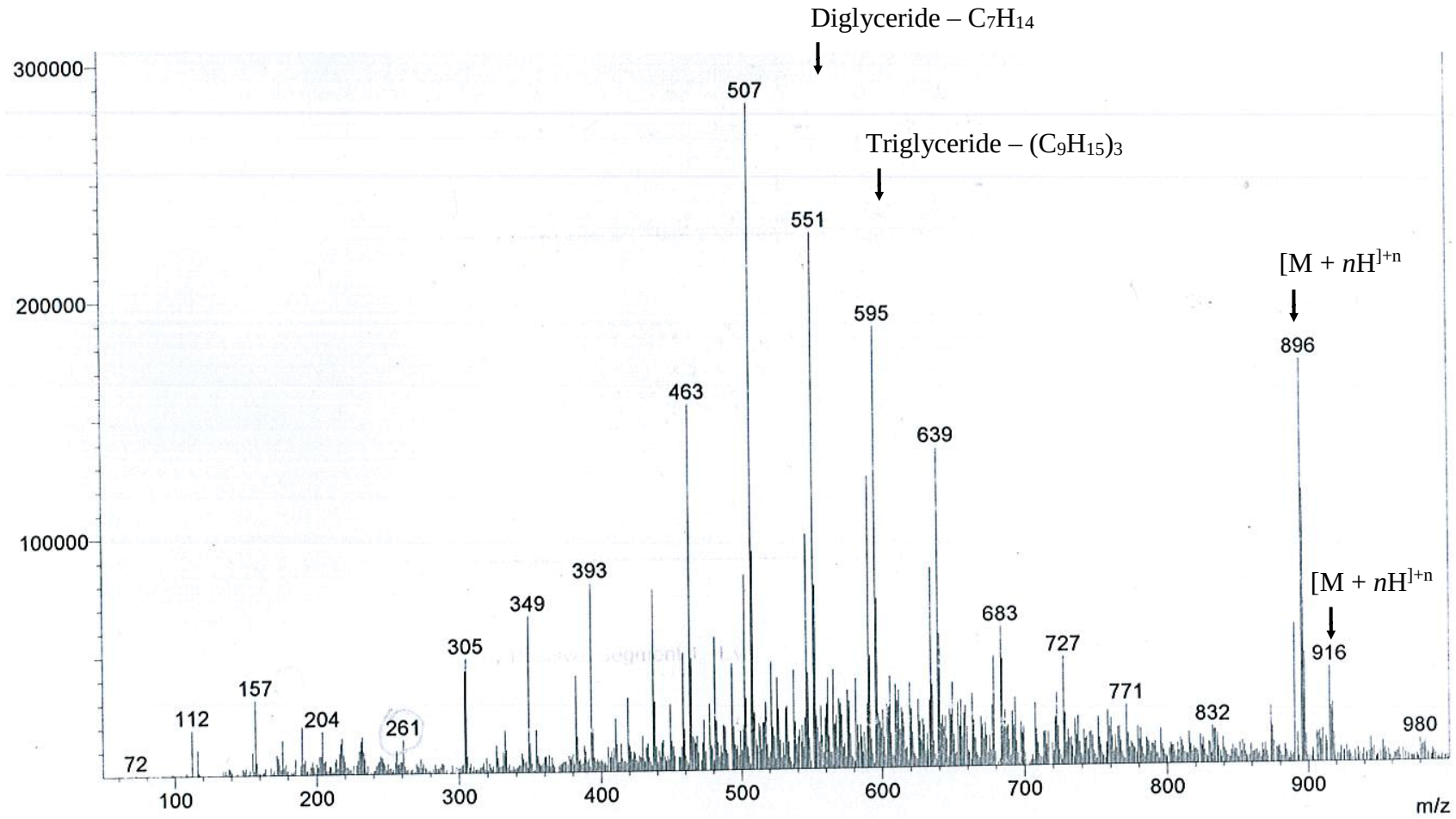

Figure 6.6: Mass spectrum of triximenynin obtained by ASI/LC-MS marked with identified fragments

6.4 Conclusion

A new HPLC system based on a routine reverse-phase silica octadecyl column with ultraviolet detection was developed. This method is suitable for the routine analysis of triglycerides during purification, assessment of seed oil, detection of stability and biological studies. Effectively, this method could replace the previously discussed HPLC and GC methods which are more complicated to practise. Method validation and quantification could be developed when more pure triglyceride is obtained. It was found that diximenynin-monoolein is the major triglyceride species in *S.spicatum* seed oil.

A suitable liquid chromatography method was developed with reverse-phase silica and mobile phase where purifications up to 91.75% were achieved. This method could be routinely used, as the stationary phase and the solvents used are recyclable. Under current conditions, sandalwood seed oil is separated to gain 2.95% w/w of triglyceride at over 90% purity. This method is currently providing triximenynin for the biological assays and would facilitate more studies to understand this rare acetylenic triglyceride.

Chapter 7 Biological Characterisation of Sandalwood Seed Oil as a Novel Excipient

7.1 Introduction

Sandalwood seed oil has a potential for use as an excipient in cosmetic and topical pharmaceutical preparations[70]. Bioactivity and toxicity are major concerns of any novel agent used as an excipient[120]. A majority of the seed oils in cosmetic and therapeutic preparations have been used since ancient times and also consumed as food. Toxicity or other biological effects are negligible in these oils except for a few oils containing allergens[120, 121, 153, 154]. However, fixed oils isolated from novel seed sources lack this lengthy time of use and thus require rigorous safety testing. Plant lipids consist of triglycerides formed from different fatty acids and other lipophilic compounds. The effects of fatty acids on human physiological functions have been studied and well established [121, 155-157]. The topical effects of fatty acids and their triglycerides on human skin have been reported [121, 122]. Beneficial effects on the skin such as the humectant properties of coconut oil and jojoba oil are well established while polyunsaturated fatty acids are known to act as antioxidants[121]. However, most of the benefits or adverse effects on the skin are produced by minor lipophilic components in fixed oils. These could range from tocopherols and carotenoids producing antioxidant activity to anaphylactic reactions by lipophilic proteins[37, 120-122, 158]. Fixed oils extracted from plant seeds contain certain toxic compounds which could produce adverse effects on human skin. Ricin is a commonly known toxic protein present in castor oil; this is generally inactivated by denaturation[153]. Jatropa seeds contain the oil soluble diterpenoid phrobol derivatives which are irritant in nature, these compounds are known to cause discomfort to people working in the biodiesel industry using Jatropha oil[154, 156]. The well known insecticide azadirachtin is a triterpenoid present in neem oil, which is found to cause reproductive toxicity[159]. Some oil seeds such as peanuts have specific hypersensitivity reactions for certain individuals[155, 160-163]. Refined oil of peanut is found to produce no hypersensitivity due to the absence of these allergens[160, 162].

Some seed oils have been reported to exert anti-inflammatory effects and it is an important biological characteristic for a natural product. New seed oil sources such as pomegranate seeds, celery seeds and caesalpinia seeds have been reported to have anti-inflammatory activity[164-166]. Several pharmacological studies have been conducted on ximenynic acid including the biological effects of the seed kernels and seed oils. These *in vivo* and *in vitro* studies are discussed in Chapter 1. Studies conducted by Liu *et al.* by feeding mice sandalwood seed oil found lipid lowering effects and serum aspartate aminotransferase enzyme levels increased, signalling a possible xenobiotic effect[57]. A recent study found an increased n3:n6 ratio on sandalwood seed oil fed rats[58]. Studies conducted on *Santalum accuminatum* seed oil and isolated ximenynic acids have shown positive results for anti-inflammatory activity in rats; hepatic P450 enzyme levels increased but no histopathological damage to vital organs was observed[59]. It is understood that acetylenic fatty acids interrupt the arachidonic acid pathway (Figure 7.1) to produce an anti-inflammatory effect by inhibiting prostaglandin, thromboxane and leukotriene synthesis[61, 66]. Naturally occurring acetylenic fatty acids, crepenynic and ximenynic have been reported to show anti-inflammatory activity by inhibiting leukotriene B_4, thromboxane B_2 and 6-ketoprostaglandin–$F_{1\alpha}$. It is reported that crepenynic acid containing plants were found to be poisonous to ruminants[61]. The ethyl ester of ximenynic acid was found to have a micro-vasoconstrictor effect[62].

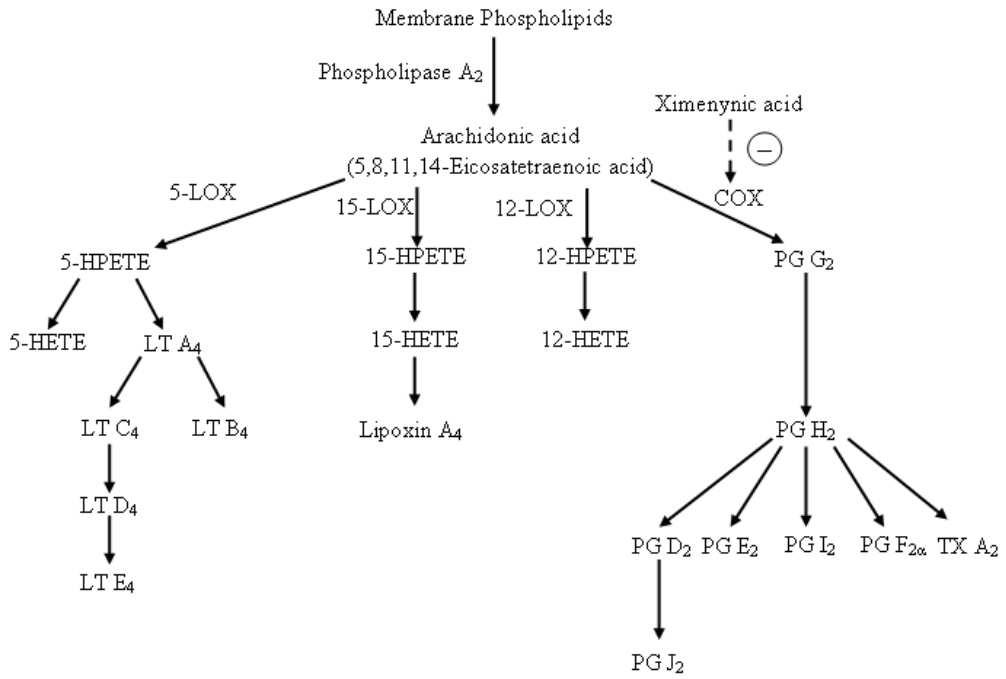

Figure 7.1: Proposed mechanism of action of ximenynic acid; interruption of arachidonic acid pathway thus inhibition of lipid derived inflammatory mediators[61, 66, 167]. COX, Cyclooxygenase; PG, Prostaglandins; TX, Thromboxane; LOX, Lipoxygenase; HPETE, Hydroperoxy eicosatetraenoic acid; HETE, Hydroxyeicosatetraenoic acid; LT, Leukotrienes

In this study sandalwood seed oil will be compared with free ximenynic acid, triximenynin and other oils for their anti-inflammatory action. Initially this involves studying the previously suggested disruption to the arachidonic acid pathway by ximenynic acid to synthesise eicosanoids by determining the prostaglandin E_2 levels and ability to inhibit its synthesis (Figure 7.1). Other commonly studied cellular inflammatory mediators are the cytokines and nitric oxide. It is important to investigate the ability of ximenynic acid or any other lipid components to inhibit these inflammatory mediators. This study also assayed the inhibition of nitric oxide and tumour necrosis factor-α. Measurement of the antioxidant capacity of sandalwood seed oil would be an added advantage. Most of the commonly used vegetable oils contain tocopherols, retinols and squalenes which are known antioxidants; however, sandalwood seed oil appears to contain minimum levels of tocopherol and average amounts of squalenes[168].

The toxicity of sandalwood seed oil, its components and other oils were determined initially as cytotoxicity on macrophages[169]. Pure seed oil was further assayed more specifically for acute toxicity on fibroblasts using neutral red[170]. This regulatory approved method would categorise the level of toxicity thus enabling a safety assessment. Dermal irritation is known as the reversible damage to skin caused by substances in contact with the epidermis. This effect could be measured on animal models or reconstructed human epidermis, for this study reconstructed human skin was used [171]. Cell viability was tested by a standard MTT assay which would be interpreted as relative cell viability. It is common to assess the ocular corrosiveness as most topical agents could affect the eye. Non-animal methods approved by regulatory bodies involved studying the corrosion of bovine cornea[172]. This is studied by measuring the corneal opacity and permeability after treatment with sandalwood seed oil .These tests would also provide safety data for sandalwood seed oil.

The objectives of the current study are to evaluate the following using in-vitro models;

- Anti-inflammatory, anti-oxidant and cytotoxicity effects of sandalwood oil, ximenynic acid and triximenynin.
- Acute toxicity, dermal irritation and ocular toxicity of sandalwood seed oil.

7.2 Methodology

The studies reported in this chapter on bioactivity studies (sections 4.2.2-4.2.5) were carried out by personnel in the Southern Cross Plant Sciences, Southern Cross University, Australia under supervision of Dr. Hans Wohlmuth during the month of April 2012. Toxicological analysis reported here (4.2.6 -4.2.8) were conducted in CiToxLAB, France by Dr. Mylène Valin and Dr. Cedric Gerbeix during the months of June and July 2012. Samples were prepared by me at the Curtin University. The raw data obtained from the above institutes were analysed and reported by me with the assistance of my supervisory team.

7.2.1 Materials

Sandalwood seed oil was an authentic sample Wescorp Sandalwood, WA, Australia (batch number WS/SWSO/2012A1). Triximenynin was isolated by the methods described in Chapter 6. Ximenynic acid was isolated using the method described under general methodology in Chapter 2. Oils of almond, olive and fish were obtained as refined grade from Sigma-Aldrich fine chemicals (MO, USA). Solvents used were analytical grade (Sigma-Aldrich, MO, USA).

7.2.2 Prostaglandin E_2 Assay

3T3 Swiss albino mouse embryonic fibroblast cells (ATCC, Manassas, VA, USA) were grown at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) containing 5% FBS (Interpath, Heidelberg, VIC, Australia), 5% newborn calf serum, L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (50 U/mL) and streptomycin (50 μg/mL) (all except FBS from Invitrogen, Mulgrave, VIC, Australia). For the assay, cells were seeded into 96-well cell culture plates (Interpath, Heidelberg, VIC, Australia) at a concentration of 27×10^3 cells/well in media as for the maintenance media but without phenol red. Cells were allowed to attach overnight. Test samples dissolved in DMSO were added to the cells and incubated for 3 hours at 37 °C, 5% CO_2. Final DMSO concentration was 0.5% in all wells. Calcium ionophore A23187 (0.5 mM, 10 μL/well) (Sigma-Aldrich, MO, USA) was added to the wells to stimulate PGE_2 production and the cells were incubated for a further 15 min. Culture plates were centrifuged (1500 g, 3 min) and the supernatant

was removed and stored at -80°C until assayed using a Prostaglandin E_2 EIA Kit-Monoclonal (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. The cell culture supernatant was diluted 81-fold in kit assay buffer before determination of PGE_2 content. A standard curve (%B/B_0 v $\log_{10}$ PGE_2 concentration) was plotted and the curve was fitted to a 4-parameter logistic equation using Graph Pad Prism® version 4 software. The percentage inhibition of PGE_2 production by each sample (assayed in triplicate) was calculated relative to the DMSO control[169].

7.2.3 Nitric Oxide and TNF-α Assay

RAW264 cells were cultivated as described in Section 7.2.2. Cell suspension (120 μL/well, 10^6 cells/mL) was added to the wells of a 96-well microplate and incubated for 20 hours (37ºC, 5% CO_2). Samples dissolved in DMSO at six concentrations (71.4, 23.8, 7.94, 2.65, 0.88, and 0.29 μg/mL), diluted in media, 10 μL were added to the cell suspension in wells. Following incubation for one hour, 10 μL of Lipoploysaccharide (LPS) solution (10 μg/mL) was added and the plate incubated for a further 20 h. Following this incubation, the plate was centrifuged (1500 g, 3min) and 90 μL of the supernatant transferred to a clear flat-bottom assay plate (PerkinElmer, Glen Waverley, VIC, Australia) and assayed immediately for nitrite. Nitrite standards (0–100 μM) were prepared in media, 90 μL of each standard and cell supernatant were transferred to a flat-bottom micro plate (Greiner Bio-One, Frickenhausen, Germany) with 90 μL of Griess Reagent (0.1% N-1-napthylethylenediamine dihydrochloride, 1% sulphanilic acid in 5% phosphoric acid) added to each well, followed by incubation (23°C, 20 min) on an orbital plate shaker. Following incubation the absorbance was read at 550 nm in a Wallac Victor 2 plate reader (Wallac, Turku, Finland). Samples and controls were assayed in triplicate. The nitric oxide (as measured by nitrite) production in sample wells was calculated as a percentage of the production in solvent control wells. Inhibition of tumour necrosis factor-alpha (TNF-α) was performed on the same cell supernatants as the nitric oxide assay (see above) using a Quantikine Mouse TNF-α immunoassay kit (R&D Systems, Minneapolis, MN, USA) The manufactures guidelines for the assay were followed and luminescence measured on a Wallac 1450 Microbeta luminescence counter (Wallac, Turku, Finland)[169].

7.2.4 Oxygen Radical Absorbance Capacity Assay (ORAC)

This assay was carried out in black 96-well fluorescence assay plates (Interpath, Heidelberg, Vic, Australia). All samples were diluted by combining 5 μL sample in DMSO (20 mg/mL) with 120 μL phosphate buffer (75 mM, pH 7.4) to give a concentration of 0.8 mg/mL. Further two-fold serial dilutions were performed in 75 mM phosphate buffer - 2% DMSO. Each sample was tested in two concentrations that gave AUC values within the range of the Trolox standard curve (12.5–100 μM). Epicatechin (Sigma-Aldrich, MO, USA) was used as a positive control. Into each well was added fluorescein solution (10 μL, 5 μM) and 20 μL sample, Trolox standard, epicatechin or solvent control solution, and lastly 170 μL AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) solution (20 mM). Immediately after the addition of the AAPH solution, the assay plates were placed in a Wallac Victor 2 Plate Reader (Wallac, Turku, Finland) and the fluorescence recorded at 37ºC every minute for 35 minutes. The fluorescence readings were referenced to solvent blank wells. Final ORAC values were determined using a regression equation for Trolox concentration versus net area under the fluorescein decay curve. Antioxidant activity was based on the mean value for two sample concentrations and expressed in micromol of Trolox equivalents per g sample. All assays were performed in triplicate[173].

7.2.5 Cytotoxicity Assay

Cytotoxicity in RAW264 murine leukemic monocyte-macrophages (ATCC, Manassas, VA, USA) was assayed in 96-well plates using the ATPlite™ Assay kit (PerkinElmer, Glen Waverley, Australia) with chlorambucil (C0253,Sigma-Aldrich, MO, USA) as a positive control. Cells were grown in clear 96-well plates. The growth medium consisted of colour free Dulbecco's Modified Eagle's medium containing 10% (v/v) foetal bovine serum (FBS; Interpath, Heidelberg, Australia), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (200 U/mL) and streptomycin (200 μg/mL) (all from Invitrogen, Mulgrave, Australia). Cells were plated out at a concentration of 30,000 cells/well (90 μL cell suspension/well), test and control compounds dissolved in DMSO at eight concentrations(100, 33.33, 11.11, 3.70, 1.23, 0.41, 0.14 and 0.05 μg/mL) and further diluted 20-fold in media were added to the cell suspension at 10 μL/well, and the plates were incubated at 37

ºC with 5% CO_2 for 24 hours. Following incubation, cell lysates were assayed for ATP with the ATPlite™ assay kit as per the manufacturer's instructions. Briefly, all kit components were equilibrated to room temperature. Mammalian cell lysis solution (50 µL) was added to each well of the cell culture microplate, the plate was shaken on an orbital microplate shaker (500 rpm, 5 min), then substrate solution (50 µL/well) was added and the plate further shaken (500 rpm, 5 min). The plate was dark adapted for 10 min and the luminescence measured on a Wallac 1450 Microbeta luminescence counter (Wallac, Turku, Finland). Value obtained for DMEM was considered the 100% cell viability and a dose-dependent inhibition was calculated as a percentage of test value corrected for DMEM media. Half-maximal inhibitory concentration (IC_{50}) values were calculated using Graph Pad Prism® version 4 (La Jolla, CA, USA)[169].

7.2.6 Acute Toxicity 3T3 NRU Test

The assay compares the cytotoxicity of chemicals applied to mouse fibroblasts (Balb/c 3T3, clone A 31; LGC standards, UK). Cytotoxicity is measured as the inhibition of the capacity to take up the vital dye, Neutral Red (NR). Solubility of the test substances were studied on 200-20mg/mL dilution in DMSO and then 100 fold dilution in DMEM for precipitation. Sodium lauryl sulfate was used as the positive control dissolved in DMEM. Test solutions were dissolved in DMEM 1% DMSO in a range from $1x10^{-5}$ to 100µg/mL. For each study, three 96-well plates were prepared (one plate for the positive control and two plates for the test item prepared independently). Peripheral wells of each plate received 100 µL of PBS whilst the remaining wells received 2 x 10^4 3T3 cells in culture media. Two plates were treated with the test item (6 replicates of the test item dilution series with 8 concentrations) and one with the positive control (6 replicates of the test item dilution series with 8 concentrations). The vehicle control was applied to the left and right sides of the test item dilution series (12 replicates in total). The cells were incubated at 37ºC, 5% CO_2 for 24 hours until they formed a confluent monolayer. After incubation, culture media was removed and 50 µL of fresh pre-warmed culture media added. Fifty microliter of culture media containing the appropriate amount of test chemical (2 x desired concentration) was added to the wells. The cells were incubated for 48 hours (± 30 minutes) at 37ºC, 5% CO_2. After at least 46 hours of treatment, each

plate was examined under an inverse phase contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells. Any changes in morphology of the cells due to the cytotoxic effects of the test substance were recorded in the study files. Visual observation codes were given for the cell damage ranging from 1-4 and a letter "P" was added if any precipitation was observed.

The cells were washed with 150 µL pre-warmed PBS which was then removed by pipetting. One hundred microliter of Neutral Red (0.4% w/v aqueous; Sigma-Aldrich, MO, USA) culture media was added and the plates incubated at 37ºC, 5% CO2 for 3 hours. After incubation, the NR media was removed and the cells washed with 150 µL PBS. To ensure complete removal of the PBS, the plate was blotted dry. One hundred and fifty microliter of desorption solution (ethanol/acetic acid) was added to each well and shaken rapidly on a plate shaker until NR has been extracted and formed a homogenous solution. Absorption of the resulting coloured solution was measured at 540 nm. Positive control (SLS) and test item data were analysed with the GraphPad Prism® software (V4.0). A bottom constraint was set to 0 for curve fitting[170]. Following determination of the IC_{50} for the test item, the LD_{50} value for mice was estimated using Equation 7.1.

Equation 7.1

$$\text{Log } LD_{50} \text{ (mg/kg)} = 0.372 \log IC_{50} \text{ (µg/mL)} + 2.024$$

7.2.7 Skin Irritation on Reconstituted Human Epidermis

Cell viability of human reconstructed epidermis (Episkin™, SinEthic Laboratory, Lyon, France) was studied using the reduction and conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT; Sigma-Aldrich, MO, USA) into a blue coloured formazan salt. To identify any test substance interference with the MTT endpoint 10 µL or 10 mg of the test item was added to 2 mL of a 0.3 mg/mL freshly prepared MTT solution. The mixture was incubated in darkness at 37°C for 3 hours. The intrinsic colour or the ability of the test item to become coloured in contact with water simulating a tissue humid environment was evaluated by adding 10 µL or 10 mg of test item to 90 µL of water for injection in a transparent recipient. After 15 minutes of mixing, the colouration was checked. If the MTT solution containing the test item (or dosage form) turns blue/purple when compared

to the negative control, the test item is presumed to reduce directly MTT. In this case additional controls are performed on water-killed tissues in parallel to the main test. Otherwise, the test item is considered as a common chemical and no additional controls are performed in the main test. Firstly, 2 mL of pre-warmed maintenance medium were added to the first column of 3 wells of 12-well plates (one plate per item). The plates were labelled with details of the test substance or control substance to be applied and the study code. Then, each tissue was transferred into the maintenance medium pre-filled wells (3 tissues per plate). Plates were incubated at 37°C, 5% CO_2 in a humidified incubator for 24 hours. Test solutions were used without dilution, positive control was sodium dodecyl sulfate (SDS; Sigma-Aldrich, MO, USA) as a 5% (w/v) aqueous solution, negative control was Dulbecco's phosphate buffered saline (D-PBS; Sigma-Aldrich, MO, USA). These were applied in triplicate for 15 minutes. At the end of the treatment period, each tissue was removed from the well of the treatment plate, and rinsed with D-PBS. Excess D-PBS was removed by blotting the bottom of the tissue culture insert with absorbent paper. If necessary the epidermal surface was gently swept with a cotton-bud to remove excess D-PBS. If the test material was not removed, this was noted in the study file. The rinsed tissues were transferred to the second column of 3 wells containing 2 mL of maintenance medium in each well and the plates were incubated at 37°C, 5% CO_2 in a humidified incubator for 42 (± 1) hours. Following the 42-hour incubation period, each 12-well plate was placed for 15 (± 2) minutes on a plate shaker to homogenize the released inflammatory mediators in the maintenance medium. Two millilitres of a freshly prepared MTT solution (0.3 mg/mL) were added into the third column of 3 wells of the 12-well plates. Then, the tissues were transferred to the MTT filled wells and incubated for 3 hours at 37°C, 5% CO_2 in a humidified incubator. At the end of the MTT incubation period, the underside of each tissue was blotted on absorbent paper to dry. A total biopsy of the epidermis was made by using the Episkin™ biopsy punch. Each tissue was examined with the naked eye and the degree of MTT staining was evaluated. The epidermis was separated from the collagen matrix using forceps and both parts (epidermis and collagen matrix) were placed into micro tubes and incubated with 500 µL of acidic isopropanol. After vortexing, each tube was stored refrigerated at +5°C protected from light until day 6 of the experiment to extract the formazan (reduced MTT) out of the MTT-loaded tissues. At the end of the formazan extraction period, each tube was mixed

thoroughly on a vortex mixer until the solution colour becomes homogenous. Each tube was used to fill two wells of a 96-well plate with 200 µL of extract per well. One 96-well plate was used for the negative and positive controls (placed at opposite ends of the plate), another one was used for the test item. For each 96-well plate, the average optical density value (OD) of six wells containing 200 µL of acidified isopropanol only was used as the blank. The OD was measured at a wavelength between 540 and 595 nm using a plate reader (Mutiskan Ascent, Labsystems, Helsinki, Finland)[171]. Data were analysed using Equation 7.2.

Equation 7.2

$$cOD_{\text{Untreated Killed tissues}} = OD_{UK} - \text{mean } OD_{blank}$$
$$cOD_{\text{Test Item treated Killed tissues}} = OD_{TIK} - \text{mean } OD_{blank}$$
$$TOD_{TI} = \text{mean } cOD_{TI} - (\text{mean } cOD_{TIK} - \text{mean } cOD_{UK})$$
$$\text{Relative mean viability} = (TOD_{TI} / \text{mean } cOD_{\text{Negative Control}}) \times 100$$

cOD=Corrected Optical Density; NSMTT= Non specific MTT reduction; TOD_{TI} =True MTT metabolic conversion

If relative mean viability is >50% it is considered as non-irritant.

7.2.8 Bovine Corneal Opacity and Permeability Test for Identifying Ocular Corrosives and Severe Irritants

Corneas obtained from freshly slaughtered calves (SOCAVIA, Beuvillers, France) were mounted in corneal holders (OPKIT, ED4004 SB; MC-2, Clermond-Ferrand, France). The corneas were used immediately after been carefully examined macroscopically before their assembly in the holders, to detect the presence of any defects. Any corneas with defects were discarded. The corneas were then mounted in the corneal holders with the endothelial side against the O-ring of the posterior chamber. For pre-incubation, both chambers of the corneal holder were filled to overflowing with MEM culture media supplemented with 1% fetal bovine serum plus penicillin/streptomycin in Modified Eagle's Medium (MEM) at room temperature (all from Sigma-Aldrich, MO, USA). The holders were pre-incubated for 1 hour and 5 minutes ± 5 minutes at 32 ± 1°C. Then, the opacity of the cornea was measured to obtain first reading (OPT0) using an opacitometer (OPKIT, MC-2, Clermond-Ferrand, France). Corneas that showed defects or if the OPT0 over 7 were discarded. Samples of 750uL applied on cornea, test samples were applied without dilution, negative control was 0.9% sodium chloride and positive control was 10% sodium hydroxide. After application of the dosage form, the holders were incubated

vertically in a water bath at 32 ± 1°C, for 10 mins. After treatment corneas were rinsed at least three times with pre-warmed MEM containing phenol red. Then, the corneas were finally rinsed with pre-warmed cMEM without phenol red. Following a 10-minute treatment, the holders were incubated horizontally (corneas placed vertically) for 2 hours ± 10 minutes in a water bath at 32 ± 1°C. On completion of the 2-hour incubation period, the medium of both anterior and posterior chambers was renewed with pre-warmed cMEM (32°C), then the second opacity measurement (OPT2) was performed. After the second opacity measurement, the medium of the anterior chamber was removed and the chamber received 1 mL of a 4mg/mL fluoresceine solution. Before use, the fluoresceine solution was tested: the solution of fluoresceine was diluted in cMEM in order to obtain a 5 µg/mL solution and the optical density at a wavelength of 490 nm (OD490 nm) of this dilution was measured. To be valid, the OD490 nm value of the fluoresceine solution between 0.850 and 0.940.The mean corrected opacity value of each series of three corneas was calculated from the individual corrected opacity values. The corrected OD490 nm (cOD490 nm) value (i.e. permeability) for each cornea treated by the test item or positive control was calculated by subtracting the average negative control cornea value from the original permeability value of each cornea[172]. The *in vitro* irritancy score (IVIS) was determined as per the equation 7.3.

Equation 7.3

$$IVIS = \text{corrected opacity} + (15 \times cOD_{490\ nm})$$

7.3 Results and Discussion

7.3.1 Inhibition of Prostaglandin E_2

Test samples described in Section 4.2.1 were tested for their capacity to inhibit the production of PGE_2 in 3T3 Swiss albino mouse embryonic fibroblast cells stimulated with calcium ionophore A23187. Only the ximenynic acid showed a dose-response; the other samples were not active at concentrations up to 100 µg/mL. Ximenynic acid was subsequently tested at six concentrations (500, 166.7, 56, 18.5, 6.2, 2.1 µg/mL) to determine the IC_{50}. The inhibition pattern of ximenynic acid seems to be similar to the positive control indomethacin, which is a known cycloxygenase inhibitor. The dose-response curve for ximenynic is shown below (Figure 7.2) indomethacin was used as the positive control. The IC_{50} for ximenynic acid was calculated to be 39.9 µg/mL (95% CI 17.9 to 89.0 µg/mL).

This value converts to an IC_{50} of 143.3 µM , which is higher than previously reported IC_{50} values. Croft *et al.* have reported the inhibition of leukotriene B_4 in rat peritoneal leucocytes to have an IC_{50} of 60µM, while the thromboxane B_2 was inhibited at 10µM concentrations[61]. Nugteren and Chris-Hazelhof have reported the inhibition of PGE_2 similar to this study but on sheep vascular gland microsomes to be an IC_{50} of 39 µM[66]. This and previous studies show different level of inhibition, the actual values could be influenced by different methods. Intact oils or their triglycerides have not shown a measurable activity; this finding suggests that only the free fatty acid is active as an anti-inflammatory agent. Studies conducted by Li *et al.* have reported considerably lower levels of inflammatory mediators in Sandalwood seed oil fed mice when compared to soyabean, safflower and olive oils fed controls. Reduced levels of PGE_2, thormoboxane B_2, leukotrien B_4 were observed in liver and plasma [58]. It is understood that sandalwood seed oil would not act as an anti-inflammatory agent upon application to the skin without hydrolysing to free ximenynic acid. Fixed oils and fatty acids are known to produce anti-inflammatory effects; γ-linolenic acid is a known to inhibit both interleukins and eicosanoids[174]. Several animal studies conducted on nigella and pomegranate seed oils have shown that inflammation on animal models have reduced significantly[166,

[175, 176]. A non-fatty compound in olive oil was reported to produce ibuprofen like activity[177].

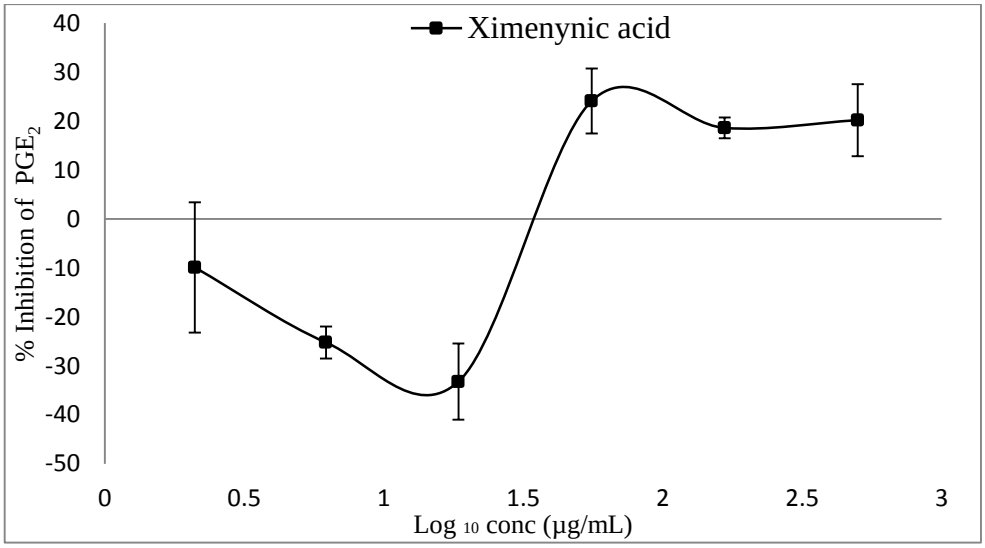

Figure 7.2: Dose-dependent percentage inhibition of PGE_2 in 3T3 Swiss albino mouse embryonic fibroblast cells stimulated with calcium ionophore by ximenynic acid.

7.3.2 Inhibition of Nitric oxide (NO) and Tumour Necrosis Factor Alpha (TNF-α)

This assay determines the capacity to inhibit the production of nitric oxide (NO) and cytokine TNF-α in RAW264 cells stimulated with bacterial lipopolysaccharide (LPS) when cells were treated with test lipids. Each sample was tested at six concentrations against positive control dexamethasone. No inhibition of nitric oxide was observed. The assay was performed on the same cell supernatants as the nitric oxide assay using a Quantikine Mouse TNF-α immunoassay kit. Dexamethasone showed a 41±12% inhibition of TNF-α by RAW264 cells at a concentration of 0.29µg/mL. Test compounds showed a clear dose dependent inhibition as showed on Figure 7.3. Based on the above results none of the test compounds including sandalwood oil and its components could inhibit the inflammatory response by TNF-α or NO. It is understood that ximenynic acid mainly inhibits inflammatory mediators generated from the arachidonic acid pathway[66]. However Li *et al.* has reported that TNF-α levels were reduced in the plasma of sandalwood seed oil fed mice[58]. This study was conducted on cell cultures and does not support the previous findings. It also

indicated that fish oil also lacked this activity in this system, whereas it has been reported that diet rich of omega-3 fatty acids have reduced the TNFα and IL1B levels in humans[178]. This might indicate a lack of adequate validity of the test. Although the oils were initially dissolved it is unclear whether this was achieved in the plates. Feeding seed oils of nigella, celery and pomegranate have been reported to reduce the TNFα and NO levels in animal models with induced inflammation[164, 175, 176, 179].

7.3.3 Oxygen radical absorbance capacity (ORAC)

The samples were tested for antioxidant activity using the ORAC assay, which measures the protection afforded by an antioxidant to a target molecule (fluorescein) being oxidized by peroxyl radicals. The assay employs Trolox (a water soluble synthetic vitamin E analogue) as a standard, and results are expressed in micromoles of Trolox equivalents per gram of sample. Epicatechin was included as a positive control. Each sample was tested at two concentrations (100 and 200 µg/mL) each in triplicate. The entire assay was repeated. All samples showed a remarkable lack of antioxidant activity when compared to the positive control (Table 7.1). Results of the two assays (on consecutive days) appear in Table 7.1.

Table 7.1: Antioxidant activity as Trolox equivalents per gram sample of test substances

Sample	Trolox equivalents per gram sample	
	Day 1	Day 2
Sandalwood seed oil	No Activity	No Activity
Fish Oil	No Activity	No Activity
Almond Oil	No Activity	No Activity
Olive oil	12.5±0.3	12.8±0.8
Triximenynin	8.6±0.9	9.3±0.1
Ximenynic acid	29.2±9.2	30.2±8.8
Epicatechin (positive)	31967±4457	32491±4985

Even though the antioxidant activity of olive oil is well established this study has shown negligible or minimum activity when compared to the control, however the

antioxidant activity of ximenynic acid was found to be higher than olive oil[180]. Miraliakbari *et al*. has reported a detailed account of antioxidant capacity of number of nut oils, which have produced promising results using DPPH (1,1-diphenyl-2-picrylhydrazyl) and the ORAC assays with some dissimilarities reported[143]. Antioxidant activity of hazelnut, peanut, pistachio, walnut and almond nut oils has been reported using DPPH assay[181]. Sandalwood seed oil has shown a considerable stability (Chapter 5) but lacked tocopherols when compared to other seed oils (Chapter 4). A fresh extract of sandalwood seed oil needs to be assayed using the DPPH assay to confirming the findings of this study. It is possible that poor oil solubility influenced these data.

7.3.4 Cytotoxicity

Samples of sandalwood seed oil, triximenynin and ximenynic acid were tested for cytotoxic activity together with almond, olive and fish oils. Percentage inhibitions of cell viability for the six samples are shown in Figure 7.4. Only the isolated ximenynic acid showed dose-responsive acute toxicity; the other samples were not cytotoxic at the concentrations tested. The IC_{50} for ximenynic acid was calculated to be 47.7 μg/mL (95% CI 12.1 – 188.3 μg/mL). This concentration is higher than the ximenynic acid in the oil if it was fully hydrolysed.

This study has compared the toxicity of the sandalwood seed oil, its components and other oils. The current study is focused on sandalwood seed oil and the more specific toxicity and irritability assays need to be performed. Safety of using sandalwood seed oil in topical preparations would need to be confirmed based on the regulatory approved assay methods discussed henceforth.

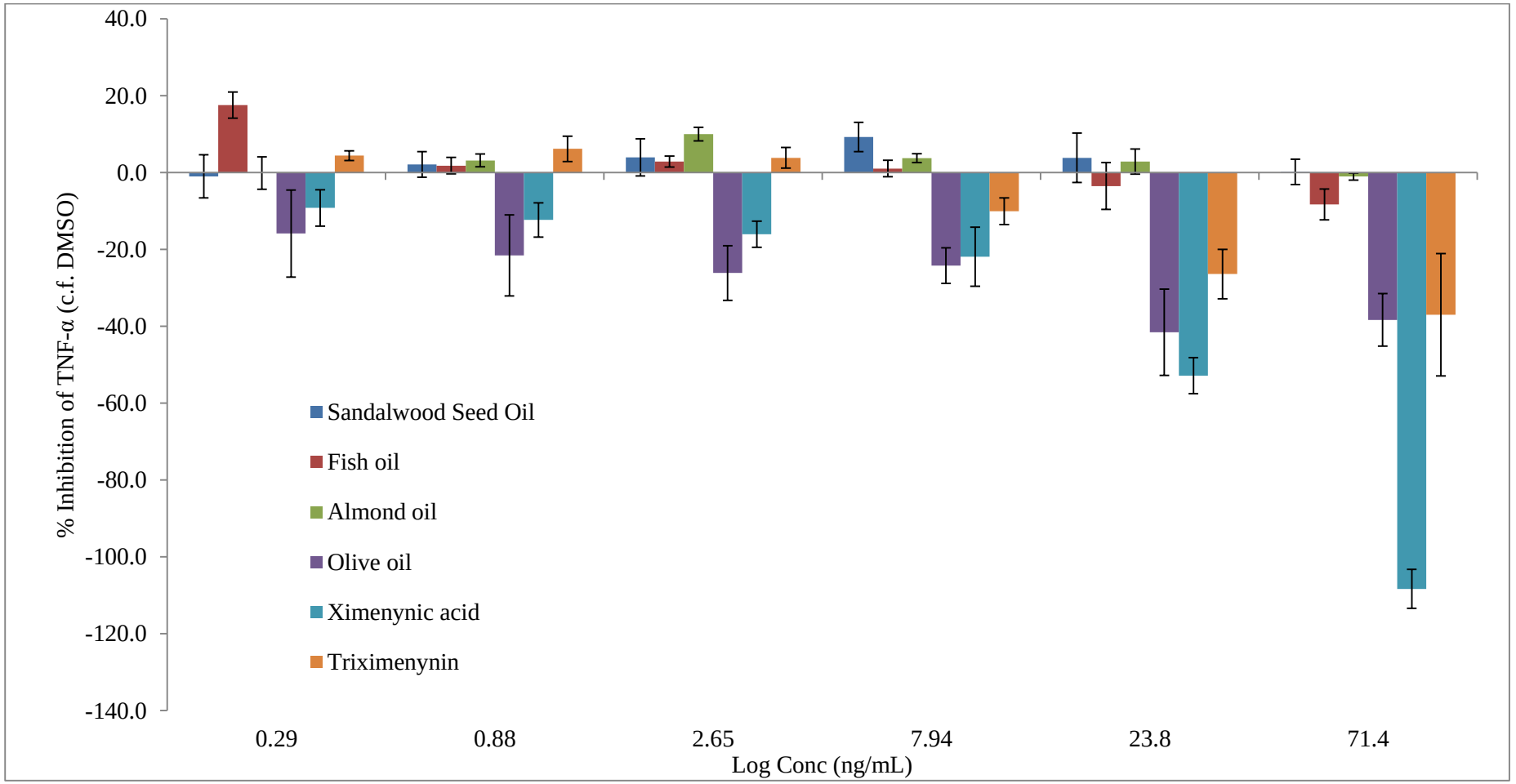

Figure 7.3: Dose-dependent percentage inhibition of TNF-α for test samples with vehicle correction for DMSO (n=6)

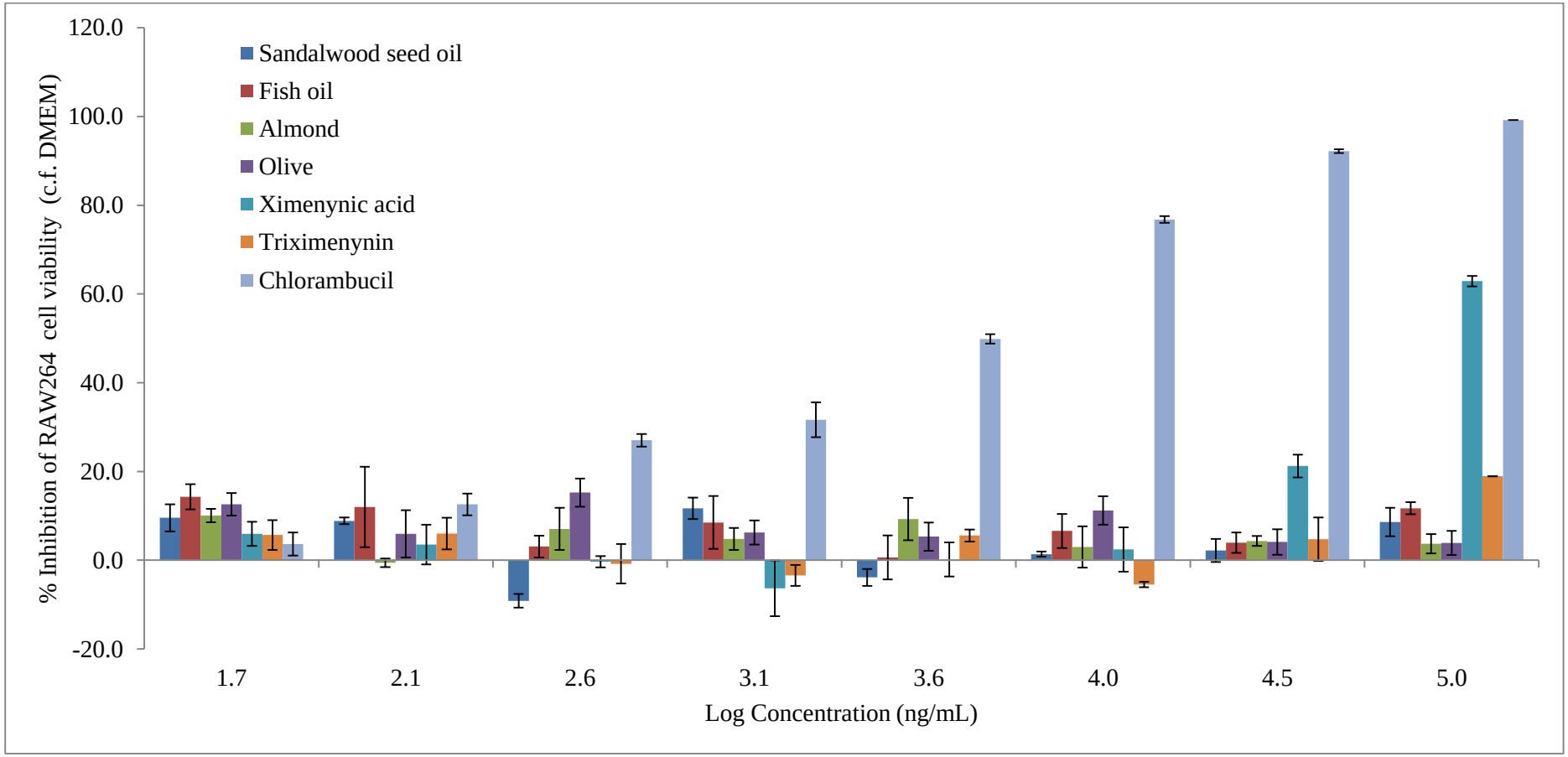

Figure 7.4: Dose-dependent percentage inhibition of RAW264 cell viability for test samples and chlorambucil (positive control) corrected for DMEM as 100% cell viability (n=6)

7.3.5 Acute Toxicity 3T3 NRU Test

The acute toxicity of sandalwood seed oil was studied in detailed using cell cultures of mouse fibroblast cells found in the connective tissues[169]. This study has confirmed that the sandalwood seed oil would not produce any acute toxicity on cells of dermal region. The highest soluble concentration to dissolve in the media was found to be 200 µg/mL following the test on sandalwood seed oil solubility. A preliminary test was performed using 8 tested concentrations of sandalwood seed oil dissolved in media from $1x10^{-5}$ to 100 µg/mL with a geometric dilution factor of 10. Upon microscopic analysis of cells no damage was observed caused by the sandalwood seed oil (Table 7.2).

Table 7.2: Numerical scoring for damage observed by microscopy with increasing dose of sandalwood seed oil on 3T3 NRU cells

Tested dose	Dose 1	Dose 2	Dose 3	Dose 4	Dose 5	Dose 6	Dose 7	Dose 8
Conc. (µg/mL)	$1x10^{-5}$	$1x10^{-4}$	$1x10^{-3}$	$1x10^{-2}$	$1x10^{-1}$	1	10	100
Score*	1	1	1	1	1	1	1	1

*Scale of 1-4 to grade the level of cellular damage observed by microscopy (1 none to 4 severe).

No cytotoxicity was noted following the preliminary test, an exploratory solubility test was performed between 200 mg/mL and 20 mg/mL in DMSO with 100 fold dilution in $DMEM_0$ and observed for precipitation. The highest soluble concentration for sandalwood seed oil was found to be 1500 µg/mL in media, 8 tested concentrations of sandalwood seed oil dissolved in media were used in the main test from 3.5 µg/mL to 750 µg/mL with a dilution factor of 2.15. Optical densities were measured for the plates for positive control (SLS) with no DMSO in $DMEM_0$ medium, percentage differences were found to be 0.8% (OD_{540}). Two plates were made for sandalwood seed oil with 1% DMSO in the $DMEM_0$ media, in which the percentage differences were found to be 10.3% (OD_{540}) for the plate 1 and 6.1% (OD_{540}) for plate 2. Guidelines have specified not to use a solvent if the percentage difference is more than 15% (OD_{540}).

The positive control study with SLS has shown a clear IC_{50} value of 159.8 µg/mL, this was within the limit range given by guidelines 20.34 -189.5 µg/mL. However, the increased concentration made sandalwood seed oil insoluble in the media. This insularity of 1% DMSO has made the determination of OD_{540} difficult. Upon incubation high viability was recorded for the cells (Figure 7.5), but a workable IC_{50} could not be achieved. Percentage inhibition of cells was around 100%, which signalled no cytotoxicity (Figure 7.5). Considering the current situation a LD_{50} on mice was determined based on the formula specified on guidelines. Using Equation 7.1, it was calculated the LD_{50} to be > 1240 mg/kg body weight of mouse. This level of LD_{50} is considered as safe and sandalwood seed oil could be considered as a non-toxic compound.

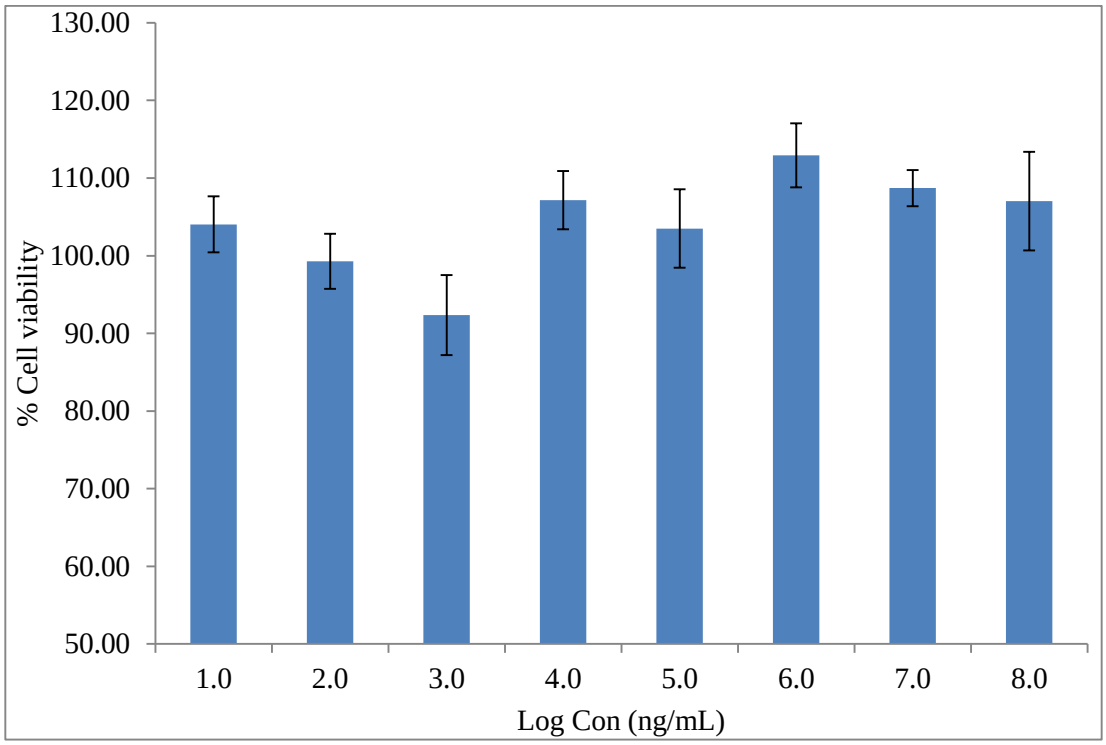

Figure 7.5: Dose-dependent percentage viability of 3T3 cell relative to vehicle control (DMSO 1% DMEM) for both plates with sandalwood seed oil (n=12)

Animal experiments conducted on sandalwood seed by Liu *et al.* and Li *et al.* have not reported any toxicity or pathological damage[57, 58]. The current test is typically designed for systemic toxicity however it is applicable to dermal tissues as the fibroblasts are used as the targeted cells[170].

The acetylenic fatty acid, crepnynic acid is linked to sheep motilities, however poisoning by fatty acids is rare but some lipophilic compounds in the seeds could be toxic[61]. Moreover the extraction process could produce more toxic by products and residual levels of solvents and enzymes in the oil could be toxic[182]. This study has confirmed that sandalwood seed oil a safe compound and would not cause any acute toxicity on the skin. Most of the seed oils used as cosmetics ingredients are non-toxic with reported LD_{50} values above 5000mg/kg body weight[121, 122]. Novel oils have always been assayed for acute toxicity due to the known activity of certain lipid soluble compounds present in seed oils[121]. The protein ricin in castor oil and the diterpene phorbol in jatropha oil are known examples of these toxic compounds found in seed oils[153, 154, 156, 183]. Studies conducted *in-vivo* and *in-vitro* on novel oils from nigella and pomegranate seeds have reported very high LD_{50} values[157]. These results are similar to the above reports on novel and established seed oils.

7.3.6 Skin Irritation on Reconstituted Human Epidermis

Although no acute toxicity was found certain compounds could still produce mild to severe irritation on the skin[121]. It is important that testing for irritancy by in-vivo or in-vitro methods is essential for an oil to be used in cosmetics[122]. Reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide) by treated reconstituted human epidermal tissue is used to measure any irritation produced[171]. The MTT solution containing the test item turned blackish when compared with the negative control. The test item was therefore considered to have direct MTT reducing properties. As a result, additional controls were performed on water-killed tissues in parallel to the main test. During this test, since the water solution containing the test item did not change colour, therefore, the test item was found not to have a colouring potential. As a result, no additional controls were used in parallel to the main test. The individual and mean optical density (OD) values, standard deviations and tissue viabilities for the test item, vehicle and positive controls are presented in Table 7.3.

Table 7.3: Viability measured as true metabolic conversion by Equation 7.2 for reconstituted human epidermis cells treated with sandalwood seed oil.

Group	cOD (Equation 7.2)	Viability	Non-specific MTT reduction
Negative control (D-PBS)	0.872±0.085	100±10	NA
Positive control (SDS)	0.043±0.004	5±0	NA
Sandalwood seed oil	1.011±0.017	NA	-2
Sandalwood seed oil treated killed tissue	0.100±0.035	NA	NA
Untreated killed tissue	0.114±0.031	NA	NA
True metabolic conversion of test item	1.011±0.017	116±2	NA

Mean values with ± standard deviation (n=3)

The qualitative evaluation of the tissue viability is given in Table 7.4. Following the treatment with the test item, all treated tissues appeared blue which was considered indicative for viable tissues.

Table 7.4: Qualitative assessment of tissue viability of sandalwood seed oil treated cells.

Treatment	Tissue 1	Tissue 2	Tissue 3
Negative Control	B	B	B
Positive Control	W	W	W
SWSO	B	B	B
SWSO -treated killed tissues	B/W	B/W	B/W
Untreated killed tissues	W	AB	W

B : blue discolouration of the tissue
B/W : blue/white discolouration of the tissue
W : white discolouration of the tissue
AB : absence of tissue

Since the test item was found to have direct MTT reducing properties in the preliminary test, three water- killed tissues and three untreated water-killed tissues were run as additional controls in the main test (for the evaluation of the non specific MTT reduction (NSMTT%). These control tissues followed the same treatment steps as the other tissues. One of the three untreated water killed tissue was lost during the rinsing. Based on the OD obtained with these control tissues, the NSMTT% was calculated as described in the methods section and the value obtained was -2%. Since this value was negative, the corrected OD values of the killed tissues were not subtracted from the OD values of the viable test item-treated tissues. Therefore the true relative mean viability was calculated using the Equation 7.2.

Following a 15 minute exposure and a 42 hour recovery period, the relative mean viability of the tissues treated with the test item was 116 ±2% as assessed by the MTT assay (Table 7.4). Under the experimental conditions of this study sandalwood seed oil is considered to be non-irritant to skin. Irritation to the skin is a major reason many botanical products are rejected for use in personal care products, most of the oils are selected to be non-irritant[120]. Removal of lipids from the stratum corneum was found to be a major cause of skin irritation[184]. Oils commonly used in cosmetics have been found to reduce irritation produced by surfactants[185]; sandalwood seed oil as a unsaturated fat would be ideal to treat such conditions. This finding renders sandalwood seed oil suitable to be used on the skin; however the specific sensitisation reactions need to be studied in future. Thus this study is not aimed at dermal sensitisation, and a separate study needs to be conducted[120, 186].

7.3.7 Ocular Corrosives and Severe Irritants

Ocular irritation is an important assessment for plant derived oils used on the skin, also the ocular tissues represent the other mucosal membranes in the body[121]. There are several *in-vitro* methods used to evaluate ocular corrosion and irritancy. A bovine corneal opacity and permeability test was reported to be useful in assessing cosmetic ingredients, thus used to identify the ocular corrosiveness and irritation of sandalwood seed oil[187,172]. No notable opaque spots or irregularities were observed on test item-treated corneas and on negative control corneas following the treatment (Table 7.5).

Table 7.5: IVIS score calculated as Equation 7.3 with opacity and permeability data for sandalwood seed oil treated bovine cornea

	Opacity				Permeability		IVIS Score
	OPT0	OPT2	OPT2-OPT0	cOPT	OD $_{490nm}$	cOD $_{490nm}$	
Negative (0.9% NaCl)	1.33 ±0.66	1.66 ±1.33	0.30 ±1.50	NA	0.005 ±0.01	NA	NA
Sandalwood seed oil	1.33 ±0.66	2.33 ±0.66	1.00 ±0.00	0.70 ±0.00	0.02 ±0.01	0.02 ±0.01	0.90 ±0.10
Positive (10% NaOH)	1.33 ±0.66	149.66 ±61.33	148.33 ±61.66	148.00 ±53.40	0.97 ±0.02	0.97 ±0.02	162.50 ±53.70

Mean values with ± standard deviation (n=3)

The individual and mean opacity and permeability values for the test item, positive control and negative controls are given in Table 7-5. The *in vitro* Irritancy Score (IVIS) was: 0.9, according to the regulatory requirements IVIS < 55.1 is not identified as being ocular corrosive or a severe irritant[172]. Under the experimental conditions of this study sandalwood seed oil was considered to be non corrosive to the eye. Eye irritation is an important assessment for a novel compound expected to be a cosmetic ingredient[120, 158].The value obtained for sandalwood seed oil is less than fifty fold below the expected value. Commonly available seed oils are reported to be non-corrosive and non-irritant on the eye[37, 121], these results would qualify sandalwood seed to be used as a safe cosmetic ingredient.

7.4 Conclusion

This study has characterised the toxicity and anti-inflammatory effects of sandalwood seed oil with additional testing of ximenynic acid and triximenynin. Fish oil, olive oil and almond oil were also used to compare the results. Free ximenynic acid was found to inhibit prostaglandin E_2 (IC_{50} 143.3 µM) and act as an anti-inflammatory agent with increased activity with dose. None of the samples inhibited nitric oxide and cytokine production as an inflammatory response; this was contrary to the reported *in-vivo* findings of sandalwood seed oil and the other reference oils. Ximenynic acid and other acetylenic fatty acids were found to inhibit the arachidonic acid pathway, but would not affect the other inflammatory mediators. None of the samples were found to be antioxidant when assayed by the ORAC method. Reference oils used such as olive were known antioxidants and this study needs to be repeated using more specific assay methods recommended in the literature. A lack of solubility of oils in the system used may have given rise to these results. Free ximenynic acid showed dose dependent cyctotoxicity with an IC_{50} of 47.7 µg/mL, while none of the oils and triximenynin have shown any cytotoxicity. However, the triglyceride of ximenynic acid, triximenynin, sandalwood seed oil and other reference oils did not depict any anti-inflammatory or cytotoxic effect. This study has found that sandalwood seed oil without much hydrolysed free ximenynic acid would not produce therapeutic or cytotoxic activity. More specific *in-vitro* studies were conducted only on sandalwood seed oil following guidelines specified for cosmetic ingredients to be used on the skin. Acute toxicity studies conducted on 3T3 NRU mice fibroblast has found an LD_{50} value over 1240mg/kg body weight, this is a high value which indicates negligible toxicity. Skin irritation was assessed on reconstituted human epidermal cells with the MTT assay and resulted in a 116% cell viability suggesting no irritation. Ocular irritation and corrosiveness was measured using bovine cornea, in-vitro irritation score was <55.1 which indicated no irritation or corrosion on ocular tissue. These findings would suggest that sandalwood seed oil does not cause any acute toxicity or irritation to skin or ocular tissue. It may not be biologically active unless hydrolysed to the free fatty acid form. Based on these data could lead to setting a limit of free fatty acids in sandalwood seed oil as increased levels could cause cytotoxicity and anti-inflammatory actions in tissues.

Chapter 8 General Discussion and Recommendations

8.1 Discussion

Western Australian Sandalwood (*Santalum spicatum* R.Br.) is cultivated for its valuable heartwood. During the long crop cycle seeds are seen as a possible annual product from these plantations. Seeds are high in lipid content and contain a rare acetylenic ximenynic acid. Sandalwood seeds are a potential source of fixed oil which could be used in cosmetics, pharmaceutical and food industries.

8.1.1 Seed Source

Sandalwood seeds were selected from three different geographical locations in Western Australia; Goldfields, Wheatbelt and field trials in the Perth metropolitan area. The selected seed groups showed a variation in seed size, seed weight, kernel yield, oil content and the fatty acid composition. Seeds from the Goldfields were the smallest while those from the Wheatbelt showed the highest diameter and weight. The seeds from field trials produced the highest yield of kernel per whole seed weight. On average plantation seeds produced 33% kernel per whole seeds. This is an important industrial parameter as the handling costs would relate to the yield of kernel from whole seed. Seed oil content was highest in the large seeds from the Wheatbelt region, whereas the Goldfields seeds had the least oil content. Geographical variation has a negligible effect on the fatty acid composition of sandalwood seed kernel; while the seed physical dimensions and oil content varied considerably. Sandalwood trees in the Goldfields region and Perth metropolitan area are economically not a feasible seed source. Seeds from the commercial plantations in the Wheatbelt region have a considerable kernel yield and high oil content with desired fatty acid profile. Seeds from this region were used as the source for studies on oil extraction as this would be a consistent source of raw material. Moreover a steady and reliable supply of seeds for an industry to be established could only be expected from the Wheatbelt region as the commercial plantations are located there.

A previous study has reported a variation of fatty acid composition between sandalwood seeds collected from single different trees in various locations [71]. However, the current study represented populations of trees from certain geographical regions. The intention of this study was to select regions where seeds

could be collected as a raw material for future oil extraction. Similarly studies have been reported on the almond and olive oil industries to find out the best cultivars and the geographical location providing higher oil yield and unsaturated fatty acids [82, 85, 86, 88].

Seeds produced by sandalwood trees hosted by Acacia species were found to yield larger seeds with more oil content. Whereas those hosted by *Allocasuarina huegeliana* produced smaller seeds with low oil content. Although variations were observed for different seed parameters between the three Acacia species, no particular species was found to be clearly superior. The number of *Acacia acuminata* trees used to host sandalwood did not affect the seed properties. There is a difference in ratio observed between oleic and ximenynic acid based on the host trees. Trees hosted by Acacia species were found have less ximenynic acid when compared to the *Allocasuarina huegeliana* hosted trees. A majority of the host trees used in sandalwood plantations are Acacia species which were selected for their suitability to the geographical and topographical conditions. Host tree effects on the development of sandalwood have also been reported in studies conducted on trees in the same controlled field trial area with different host species; findings from these studies have reported that *Acacia acumunata* is a superior host for sandalwood trees planted in the Wheatbelt region [78, 80].

Based on the findings of this study on geographical and host tree effect on sandalwood seeds a protocol has been developed (Table 8.1) for selecting seeds most suitable for oil extraction industrially. As the only collection criteria is seed diameter then from those harvested the seed size range will be easily obtained by grading.

Table 8.1: Guideline on limits and ranges for sandalwood industry to select seeds with shells of *Santalum spicatum* for seed processing and oil extraction

Seed size (Diameter)	14 mm – 22 mm
Kernel yield from seeds (% w/w)	Minimum of 30% w/w
Oil yield of kernel	Minimum of 40% w/w
Ximenynic acid (% of total fatty acids)	32 % - 40%

8.1.2 Extraction and characterisation of sandalwood seed oil

The traditional and most widely practiced method of fixed oil extraction is pressing; however oils produced by this means usually undergo refining steps to remove moisture, gums and phospholipids. Previous studies have used laboratory scale solvent extraction. Because of concern in the cosmetic industry of the effects of residual solvents in oils extracted by this method it was not considered for further development. As sandalwood seed oil would only be available in relatively small quantities commercially, to be viable it would need to find a niche as a boutique oil or oil with special qualities. Therefore this study has developed a supercritical fluid extraction process using carbon dioxide. Although requiring expenditure on equipment and use of carbon dioxide, this method produces oil acceptable to the boutique cosmetic market. Supercritical extraction is used in the pharmaceutical and food industries for the extraction and purification of botanical products[76, 95, 96, 188]. Lipid extraction by supercritical carbon dioxide has been reported at an industrial scale where the final product holds a high value and conventional methods are unable to produce the required quality or yield[98, 100, 189].

An aim was to identify the optimum extraction pressure, and mass ratio of carbon dioxide to extract higher yields of oil from sandalwood seed kernel. The oil extracted should fall in to a category where its physicochemical properties can be matched with other oils used in cosmetic and pharmaceutical formulations. Extraction

conditions should not be favourable to certain fatty acids and their triglycerides as the aim is to gain a representative lipid profile of the sandalwood seed kernel.

Laboratory scale extractions have resulted in yields of 40-50% w/w relative to the seed kernel for a pressure range of 30-55 MPa. The optimum extraction pressure was found to be 40 MPa to yield the maximum oil content with no turbidity or precipitation. The extraction temperature was kept unchanged at 40^{o}C, which is the minimum temperature for supercritical carbon dioxide extraction. The mass exchange and carbon dioxide to feed ratios were further studied at extraction conditions of 40 MPa and 40^{o}C in larger quantities. A linear increase in cumulative yield was observed with the increased ratio of carbon dioxide to feed. Maximum yields of oil were 48% w/w with respect to kernel weight at 55 kg of CO_2/ feed kg. This ratio could be achieved in an industrial scale plant making it feasible to yield high amounts of oil content with a considerable amount of carbon dioxide input. The pilot scale studies have yielded 52% w/w oil from kernel weight for larger quantities of feed material replicating a future industrial extraction. This is the first time a supercritical fluid extraction method has been used for sandalwood seed oil. It was decided from the commencement of the study that this process would be evaluated, because it is proposed to market it as specialised oil.

A comparison of supercritical extracted oil with solvent extracted oil found that both the solvent extract and the supercritical extract were comparable with the specifications for other oils used in cosmetic and pharmaceutical formulations. Sandalwood seed oil has not been physicochemically characterised previously. Development of the physicochemical parameters and other lipid components were based on parameters for other oils used in the cosmetic and pharmaceutical industry. The chemical parameters for both extracts were within the range of many unrefined oils[31]. The supercritical carbon dioxide extract had lower peroxide and acid values, indicating that the extraction process did not contribute to oxidation or hydrolysis of the lipids. Unsaponifiable matter and the specific gravity of the supercritical extract was higher indicating possibly more non-fatty acid composition in the oil.

The fatty acid composition was unchanged between the solvent and the supercritical extract, and also was consistent with the previous reports on sandalwood seed oil extracted using the same solvent extraction method[44, 45]. The compositions of the

sterols were slightly different to other seed oils as the β-sitosterol content was lower while campasterol and stigmasterol were higher. Squalene present in the seed kernel could be an intermediate product of sterol synthesis or acting as an antioxidant for the seed[30]. However the tocopherol content was considerably lower, δ-tocopherol was higher than α-tocopherol and supercritical extracts contained more δ-tocopherol. Other seeds such as walnuts are reported to contain less tocopherols and are depleted upon storage[104].

Sandalwood seeds used in this study were collected a few weeks after falling to the ground and stored in ambient conditions before extracting. The effects of seed source and storage time and conditions on tocopherol content are a subject for a future study. This study has conducted the first physicochemical characterisation of sandalwood seed oil. Based on these observations the following parameters were established as a basis of a monograph for sandalwood seed oil (Table 8.2) to enable its commercial standardisation and identification.

Table 8.2: Draft monograph developed for sandalwood (*Santalum spicatum*) seed oil extracted by supercritical carbon dioxide.

Definition	Sandalwood seed oil is the oil obtained by super critical fluid extraction using carbon dioxide from the kernel of *Santalum spicatum* R.Br. (Western Australian Sandalwood).
Appearance	A clear yellow, to golden-yellow transparent liquid.
Solubility	Sparingly soluble in ethanol (96 per cent); miscible with n-hexane, acetone and ether.
Relative density	0.910 to 0.930
Viscosity	3.65 X 10^{-2} to 3.75 X 10^{-2} Pa s
Freeze Point	-10.8 to -11.2 ^{o}C
Acid value	maximum of 3 mg KOH/ g
Peroxide value	maximum of 6 meq O_2 /kg
Unsaponifiable matter	maximum 4.5 % (w/w)
Iodine Value	minimum of 87 g I_2 /100 g
Specific absorbance (E 1% 1 cm)	Maximum 2.60 measured at 270 nm in cyclohexane
Residual solvents (headspace gas chromatography)	Not detectable
Fatty acid composition (percentage of total fatty acids)	
saturated fatty acids of chain length less than C_{16}	less than 0.1 %
palmitic acid 16:0	3.0 to 3.5 %
palmitoleic acid 16:1 (n-7)	0.2 to 1.0 %
stearic acid 18:0	2.0 to 3.5 %
oleic acid 18:1 (n-9)	50.0 to 53.0 %
linoleic acid 18:2 (n-6)	1.7 to 2.0 %
α-linoleic acid 18:3 (n-3)	2.5 to 3.5 %
stearolic acid 18:1 (9a)	1.0 to 2.0 %
ximenynic acid 18:2 (9a, 11t)	28.5 to 36.5 %
Tocopherol (Vitamin E) composition (mg in 100g oil)	
α-tocopherol	1.05 to 1.93 mg
δ-tocopherol	5.14 to 5.82 mg
Phytosterol content (mg in100g oil)	
Campesterol	7.55 to 13.50 mg
Stigmasterol	16.77 to 21.77 mg
β-sitosterol	82.41to 88.29 mg
δ-5-avenasterol	32.81 to 30.25 mg
Squalene	14.81 to 16.14 mg

8.1.3 Stability of sandalwood seed oil

Adequate stability is paramount for any ingredient to be used in pharmaceutical or cosmetic formulations. The stability of plant oils is determined based on oxidation, hydrolysis and changes to the fatty acid composition. This is the first study of the stability of an acetylenic fatty acid containing oil to be reported.

The evaluation of the stability of oils is complex owing to the wide range of compounds which interconnect with oxidative and hydrolytic degradation. Some studies of the stability of olive oil have been questioned because the oxidative stability has been based upon high temperature Rancimat© or oxidative stability index (OSI) methodology[128]. In addition at high temperatures the solubility of molecular oxygen decreases and there is rapid degradation of the antioxidant tocopherols[128]. As this study was primarily designed to evaluate long term storage of sandalwood seed oil it was therefore decided to use the accepted standards of peroxide value, free acid formation and p-anisidine values. There are standards established for these values in the pharmaceutical and cosmetic industries.

Oxidation was observed as an autoxidation reaction and evaluated by determining the peroxide value and then the secondary oxidative products by the anisidine value. Supercritical carbon dioxide extracted sandalwood seed oil was subjected to stability assessment both exposed to and excluded from air at high ambient temperature (40^{o}C) for 360 days. This study was designed based on the storage conditions given in ICH guidelines for pharmaceutical products[136]. A study on the stability of virgin olive oil involved long term stability assessment at 25^{o}C and 40^{o}C opposed to accelerated and antiradical assays[190]. The supercritical extract was found to be stable when not exposed to air up to 360 days, while rapid oxidation was observed when exposed to air at this temperature. Exposed samples degraded reaching an oxidation level in excess of 10 meq O_2 kg^{-1} of oil within the first 30 days and a rapid increase of secondary products was also observed. Towards the end of the observation period oxidation of the unprotected samples had reached the termination stage as PV have reached a minimum, complying with the theoretical oxidation process. Secondary reaction products of oxidation increased for the unprotected oil where 28 AV was recorded compared to the 6 AV for the protected oil. Fatty acids

with unsaturated bonds undergo peroxidation. The main fatty acids in the sandalwood seed oil are mono-unsaturated oleic acid and polyunsaturated acetylenic ximenynic acid. Hydrolysis of triglycerides would increase the free fatty acid contents which is also a sign of oil degradation. Hydrolysis observed by the formation of free fatty acids was low in both the protected and unprotected samples. Acid values of the unprotected oil only increased towards the final stages of the observation period.

It has been noted that for olive oil that it is more stable when stored in containers impervious to oxygen[191]. Another study has shown that olive oil stored in impervious containers outdoors with temperatures varying from 0 to 38^{o}C showed markedly lower peroxide values when the headspace was replaced with nitrogen[192]. These data supported replacement of the headspace of sandalwood seed oil with nitrogen and the selection of 40^{o}C as a high ambient temperature[190].

Fatty acid composition and the level of saturation have not been altered within experimental error for both protected and unprotected oils although considerable oxidation was observed for the unprotected oil. The measurement of oxidation has a higher degree of sensitivity when compared to total fatty acid composition. Continuity of secondary oxidation could ultimately depict a change of fatty acid composition with the depletion of the C18 chain acids and production of shorter chain length fatty acids and hydrocarbons.

There are no previous reports on the oxidation of ximenynic acid or other acetylenic fatty acids. However, oxidation of oleic acid which is the major fatty acid of sandalwood seed oil has been extensively studied[124,114,135]. It was reported that oleic acid undergoes rapid peroxidation but shows a slow onset of secondary oxidation to produce carbonyl compounds [123]. However, the results for the unprotected oil have shown a similar increment of secondary oxidation as in peroxidation, which may have resulted from oxidation of ximenynic acid. Secondary oxidation of ximenynic acid may follow a similar pathway as polyunsaturated linoleic acid; which was reported to rapidly produce carbonyl compounds from the peroxyls due to its polyunsaturated nature. The stability of the sandalwood seed oil can be maintained if stored in airtight containers with the headspace air replaced with nitrogen even at

elevated temperatures. Parameters listed in the monograph could be used as limits of oxidation and hydrolysis for different storage and formulation conditions.

8.1.4 Toxicity and Irritancy Assessment

Cytototoxicity of sandalwood seed oil, triximenynin and ximenynic acid was studied in comparison to other oils used in pharmaceutical formulations. Sandalwood seed oil and triximenynin showed no toxicity on cell lines; similar results were obtained for the other oils (almond, olive and fish) tested at the same time. However the free ximenynic acid showed cytotoxicity. It was evident that ximenynic acid produces cytotoxic effects in its free acid form but not in triglyceride or the bulk oil form.

Sandalwood seed oil was further studied in detail to confirm its toxicity by *in-vitro* methods for regulatory approval. The acute toxicity of the seed oil was found to be low as the $LD_{50\ mouse}$ was >1240mg/kg, this value thus considered as safe with a 70kg adult able to safely consume up to 80g of oil. Dermal irritancy was evaluated using reconstituted human epidermal cells. It was found that the viability was 116% which shows it as non-irritant. Ocular irritancy was tested on isolated bovine corneas where an ocular in-vitro irritancy score was given based on the opaqueness and permeability. A total score was obtained for sandalwood seed oil which was 0.9 where a value less than 55 was considered as non-irritant on eyes. This is the first time a biological safety evaluation has been conducted on sandalwood seed oil. These studies were conducted using OECD approved methods in an accredited cosmetic testing laboratory. These results would make the oil eligible to be used as a potential ingredient in the cosmetic industry. These in-vitro methods are novel but a more efficient path to assess the toxicity than conventional whole animal studies.

Major challenges for a novel excipient product would be its physiochemical characteristics, stability and safety. A suitable raw material source and guidelines to select seeds for oil extraction have been established as the first step. Supercritical carbon dioxide extraction was optimised and it was developed up to a larger scale extraction of the sandalwood seed kernel. The oil obtained was characterised and further compared with other oils used in cosmetic and pharmaceutical formulations. Stability of this oil was established, where sandalwood seed oil remained almost unaffected when protected from air. Sandalwood seed oil was found to be non-toxic

and non-irritant. Overall, this current study has established sandalwood seed oil as a feasible, safe, stable ingredient which is comparable with other oils currently in use.

8.1.5 Pharmacological Studies of Sandalwood Seed Oil

Sandalwood seed oil, ximenynic acid and triximenynin were first studied for anti-inflammatory and antioxidant properties with comparison to almond, olive and fish oils. Ximenynic acid was found to produce PGE_2 inhibition; the dose response curve of the ximenynic acid had a similar pattern to the non steroidal anti-inflammatory drug indomethacin. Sandalwood seed oil samples and triximenynin showed no inhibition for the above study. None of the samples inhibited nitric oxide or cytokine production. It is seen that only the free fatty acid of acetylenic fatty acid produced an anti-inflammatory effect by inhibiting PGE_2. Inhibition of leukotirene and thromboxane by ximenynic acid was reported in *in-vitro* studies[61]. However, the current finding shows that the triximenynin was inactive unless it was hydrolysed to the free ximenynic acid. Anti-inflammatory effects have been reported from *in-vivo* mice and rat experiments where sandalwood seed oil was fed together with food; this oil would have been hydrolysed by lipases and the free ximenynic acid might have given rise to the anti-inflammatory markers in animal plasma [57, 58]. Results of the antioxidant study give rise to concerns since no activity was recorded for olive and fish oils which are known to have antioxidant properties. This maybe a lack of response in the *in-vitro* study methodology employed. The most tenable explanation for this would be inadequate aqueous solubility of these components in the cell-line systems employed.

8.1.6 Novel Analytical and Isolation Methods for Triximenynin

Sandalwood seed oil was reported to contain three major triglycerides, triximenynin contains all ximenynic acid[48]. For future biological studies it would be essential to study the pharmacological properties of triximenynin to understand any unique behaviour of the acetylenic fatty acids and triglycerides. An HPLC method was developed to separate and identify triximenynin using photo diode array detection based on a previously reported reversed phase method[48]. The current method is more sensitive and shows a greater separation. A single step column chromatographic

method was developed to isolate triximenynin which could be scaled-up to a larger process. The isolated triximenynin was found to be over 90% pure based on the total area, by HPLC. Similar methods have been developed to isolate gamma linolenic acid from edible oils and triglycerides containing gamma linolenic acid[28, 149]. These studies have reported the purity based on the FAME composition, which is different to the current study. Triglycerides and fatty acids are separated and purified by supercritical separation methods; however, it is necessary to have specialised instrumentation and facilities to conduct these processes[193]. Current findings on chromatographic separation could be used to develop a supercritical separation method. Due to the unavailability of resources this supercritical separation could not be trialled during the present study. This would be a suitable method to isolate triximenynin as a specialty supplement to be used as cosmetic or pharmaceutical agent in future.

8.2 Conclusions

The development of novel oils for the cosmetic and pharmaceutical industries is challenging. A wide range of data needs to be obtained demonstrating suitability and lack of toxicity. This study has provided a range of data which provides much of the evidence for its future use. Important data identified in this study includes a suitable raw material source, an extraction method, physicochemical characteristics, long term stability and the *in-vitro* dermal toxicity of sandalwood seed oil. Developing sandalwood seed oil for future industry needs all the above assessments to be examined as part of establishment of the process.

Though sandalwood is found in many natural habitats, only the plantations trees in the Wheatbelt region of Western Australia were found to be a viable source for seed for an oil extraction process. Seed from plantations were used in developing the supercritical carbon dioxide extraction method to obtain seed oil. Supercritical extraction was found to be the best suited method for extracting ingredients for cosmetic and pharmaceutical industry. A systematic method identified suitable pressure and solvent ratios for optimal extraction conditions; particle size and temperature was maintained the least possible levels. Process developed on laboratory scale was developed to larger pilot scale operations to replicate the industrial extraction.

Physiochemical parameters of novel oils have to meet the standards of other oils with similar end use. Sandalwood seed oil contains a rare acetylenic ximenynic acid, and seed oils containing ximenynic acid have not been studied or reported for their properties or stability. Based on current studies it was found that sandalwood seed oil demonstrated physiochemical parameters suitable for cosmetic and pharmaceutical preparations. Although the phytosterol content was found to be similar to other oils, the tocopherols were far below that reported for other oils with an exception of walnut oil. However, a high squalene level was observed which could be the main antioxidant compound in this oil.

Long term stability studies of the oil showed excellent stability when the headspace was replaced with nitrogen with no marked change in oxidation and hydrolysis parameters and fatty acid composition. In contrast an unprotected sample underwent rapid oxidation. Polyunsaturated acetylenic structure of ximenynic acid could be susceptible for oxidation, however if preventive measures are taken this is avoided and the oil remains unchanged over one year even at relatively high ambient temperatures.

Dermal toxicity and irritation together with ocular irritation confirmed that sandalwood seed would not produce toxicity or irritation on human skin. The supercritical carbon dioxide extraction process does not leave any residual solvents which could cause toxicity and irritation.

Pharmacological findings of the current study has confirmed that only the free ximenynic acid produced cytotoxic and anti-inflammatory activity by PGE_2 inhibition properties, while the triglyceride form and the bulk oil remained inactive. However, the antioxidant and anti-inflammatory assays did not produce any effects. A novel analysis and isolation method for triximenynin was developed during the course of this study, which could be highly beneficial for a future study on human nutrition.

Sandalwood seeds are seen as a plentiful source form the plantations; development of a sandalwood seed oil as a novel product would support a new rural industry and sustain Western Australia's sandalwood industry.

8.3 Recommendations

Further studies need to be conducted for wider acceptance of this oil in cosmetic and pharmaceutical industries.

- Collaborative research with forestry scientists could investigate different factors or cultivars triggering the ximenynic acid synthesis in sandalwood seeds based on the established acetylenic fatty acid biosynthesis. These findings could be used to increase the rare fatty acid content in sandalwood seeds in future.
- A supercritical separation method to purify triximenynin from sandalwood seed oil should be investigated.
- Formulation methods and stability of semisolid dosage forms using sandalwood seed oil should be developed as a future study. Pharmaceutical studies need to be conducted on rheological and dispersion characteristics of sandalwood seed oil and oxidative stability during formulation and storage. A clinical based study on dermal application of sandalwood seed oil need to be conducted. Absorption of acetylenic fatty acids through skin has not been studied thus far; this would be an interesting addition to a clinical investigation in future.
- The nutritional properties of ximenynic acid, triximenynin and sandalwood seed oil are currently under study as a doctoral program in Zhejiang University, China in collaboration with the current study. Both the animal experiments and cell culture studies are been employed to investigate the nutritional properties. In future a clinical investigation needs to be conducted to establish the findings. This could provide evidence for sandalwood seed oil as a nutritional supplement. The next step would be to carry out a study in animals such as pigs to further investigate the pharmacological properties.

Chapter 9 References

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Appendix

Appendix 1 –Publications and Conference Proceedings on Related Subjects

Hettiarachchi DS, Coakley TD. Snakes Encirling Sandalwood: A Chemical Investigation. Proceedings of International Seminar on Sandalwood: Current Trends and Future Prospects. February, 2014. Bangalore, India. Institute of Wood Science and Technology, Indian Council for Forestry Research and Education. (*in press*)

Subasinghe U, Gamage M, Hettiarachchi DS. Essential oil content and composition of Indian sandalwood (*Santalum album*) in Sri Lanka. Journal of Forestry Research. 2013; 24 (1): 127-130.*

Subasinghe SMCUP, Rathnamalala E, Hettiarachchi DS**.** Agarwood-type Resin from *Gyrinops walla* Gaertn: A New Discovery. Journal of Tropical Forestry and Environment. 2012; 2(2):43-48.*

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* Part of collaborative research between Sri Jayewardenepura University and Curtin University which was awarded the National Research Council of Sri Lanka Grants for years 2012 and 2013.

Appendix 2 - Copyright Consent by Wescorp Holdings Pty Ltd

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To whom it may concern

The Wescorp Group (Wescorp Holdings Pty Ltd and its subsidiaries) has consented for Dhanushka Sugeeshwara Hettiarachchi to use some photographs published on our website (www.wescorp.com.au) in his doctoral thesis titled "Pharmaceutical Evaluation of Western Australian Sandalwood Seed Oil" from Curtin University. Our organisation reserves the rights for these photographs and anyone who wishes to reproduce them for printed or digital publication requires prior permission from the Wescorp Group.

Yours sincerely

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