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ENZYMATIC ESTERIFICATION OF CARBOHYDRATES WITH FATTY ACIDS DERIVED FROM VERNONIA GALAMENSIS

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DEDICATED TO:

My father Zelalem Walle

My mother Belaynesh Mitiku and

My sister Selome Zelalem

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LIST OF SYMBOLS AND ABBRIVIATIONS

V. galamensis	Vernonia galamensis
VO	Vernonia Oil
VOAC	Vernolic acid
VOME	Methyl Vernolate
SCFs	Supercritical fluids
DMSO	Dimethyl sulfoxide
DMF	Dimethyl formamide
$CHCl_3$	Chloroform
ILs	Ionic liquids
CAL-B	*Candida antarctica* lipase B
NMR	Nuclear Magnetic Resonance
ppm	Parts per million
δ(delta)	Symbol for chemical shift value
m	multiplet
RTILs	Room Temperature Ionic Liquids
SFAEs	Sugar Fatty Acid Esters
[Bmim][TfO]	1-butyl-3-methylimidazolium trifluoromethane sulfonate
[Bmim][dca]	1-butyl-3-methylimidazolium dicyanamide
$[Bmim][BF_4]$	1-butyl-3-methylimidazolium tetrafluoroborate
$[Bmim][PF_6]$	1-butyl-3-methylimidazolium hexafluoro phosphate
$CDCl_3$	Deuterated chloroform
Uv-Vis	Ultraviolet-visible
^0C	Degree centigrade
^1H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon-13 nuclear magnetic resonance
nm	nanometer
p-NPP	*para*-nitrophenyl palmitate
p-NP	*para*-nitrophenol

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ABSTRACT

Sugar fatty acid esters (SFAEs) are compounds produced from renewable and inexpensive substances such as natural oils and glucose or starch. SFAEs are normally non-ionic, non-toxic, non-irritants, odorless, tasteless and biodegradable. Due to these range of properties, these compounds have found applications as diverse as surfactants in the food and cosmetic industries, as insecticides and antimicrobial agents, in stabilizing and detergency or conditioning effects and even as non-caloric fat substitutes. Changing the nature of the starting materials such as using oils with special functional groups has the potential to provide with value-added products and highly versatile materials with interesting characteristics. *Vernonia galamensis* is a plant, endemic to Ethiopia, containing a very promising naturally epoxidized oil presenting also low toxicity and inherent biodegradability. The synthesis of sugar fatty acid esters based on vernolic fatty acids could provide with products with the potential to be used as a new type of epoxidized surfactants or as starting materials for a variety of value added products. The chemical synthesis of monosubstituted starch/glucose esters is very difficult to achieve due to the poor selectivity of this approach. Enzymatic processes are an excellent alternative due to the high selectivity of the reaction owing to their exquisite stereo-selectivity, regio-selectivity and functional group specificity. In this work, the synthesis of starch/glucose vernolates has been attempted from vernolic acid and methyl vernolate by enzymatic (*candida antarctica* lipase B) esterification in the ionic liquid [Bmim][TfO]. The use of ionic liquid avoids the use of environmentally unfriendly volatile organic liquids. The low vapour pressure exhibit by ionic liquids avoids the use of molecular sieves as the water produced in the reaction can be continuously extracted under vacuum. The synthetic procedure also includes the use of water mediated supersaturated solutions that allowed us to increase the concentration of glucose in the ionic liquid media. Moreover, the activity of the enzyme was found to be enhanced by the ionic liquid used. The products were characterized by melting point and ^{1}H and ^{13}C NMR spectroscopy and the degree of substitution of the glucose ester was investigated and found to be 2.17.

Key words: ionic liquids, [Bmim][TfO], vernonia oil, vernolic acid, methyl vernolate, candida antarctica lipase B, fatty acid esters, glucose esters, starch esters

1. BACKGROUND

Chemical industries provide us with variety of products. However, many of these products have some disadvantages from the environmental, economical and health point of view. Most of these chemicals come from fossil resources and this dependence results in rising of oil prices. Moreover, most of the chemical processes are environmentally damaging, produce toxic byproducts and non biodegradable wastes and products. Consequently, finding environmentally sound renewable natural resources and processes is needed in order to achieve sustainability [1].

The use of renewable raw materials can significantly contribute to a sustainable development, usually interpreted as ‘‘acting responsibly to meet the needs of the present without compromising the ability of future generations to meet their own needs’’. In ages of depleting fossil oil reserves and an increasing emission of green house gases it is obvious that the utilization of renewable raw materials wherever and whenever possible is one necessary step towards a sustainable development. In particular, this can perennially provide a raw material basis for daily life products and avoid further contribution to green house effects due to carbondioxide emission minimization [1].

Nowadays interest in the synthesis of compounds from renewable resources is growing due to advantages with regard to performance, commercial potential and environmental compatibility. Namely due to their potential of replacing petroleum derived standard products [21]. Furthermore, the utilization of renewable raw materials, taking advantage of the synthetic potential of nature, can (in some cases) meet other principles of green chemistry, such as a built-in design for degradation or an expected lower toxicity of the resulting products [1]. Products obtained from these renewable sources are as diverse as pharmaceuticals, coatings, packaging materials or fine chemicals [1].

Some of the most widely applied renewable raw materials in the chemical industry for non-fuel applications are plant oils and carbohydrates.

1.1. *Vernonia galamensis*

Vernonia galamensis (V. galamensis) is a plant in the sunflower family of the genus vernonia (*asteraceae*), known for its use as an oilseed. This species, often called ironweed, is the largest source of vernonia oil (VO). It includes more than 1000 species distributed widely in tropical and subtropical regions of Africa, Asia and America and has two major centers of origin, South America and tropical Africa. About 200 species ranging from annual herbs and shrubs to perennial trees are found in Africa of which about 50 species of vernonia have been recorded in Ethiopia. According to the species, *galamensis* is recognized to include six subspecies including *galamensis, mutomoensis, nairobensis, afromomntana, gibbosa*, and *lushotoensis* [2]. Subspecies *galamensis* grows in a wild form in Eritrea, Ethiopia, Malawi, Tanzania and Kenya [3, 4]. It is highly diverse and has four botanical varieties, namely variety *galamensis, petitiana, australis, ethiopica,* which are limited in distribution primarily to eastern Africa [2].

V. galamensis subspecies *galamensis* variety *ethiopica* was first identified by Perdue in 1964 in eastern Ethiopia along the Harar-Jijiga road at 9°14' N and 42°35' E, 1700 m above sea level. Later south and south-eastern Ethiopia was described as a natural habitat of this botanical variety. A number of studies demonstrated the presence of considerable variability in oil and vernolic acid (VOAC) contents in this botanical variety [2, 5, 9].

The subspecies *galamensis* is found in areas that receive as little as 200 mm rainfall per year [2]. A porous, well-drained and sandy soil is best for its growth. Vernonia plant does not dowel on heavy clay. It tolerates high temperature and full sun as long as the soil moisture is adequate. A superior species of vernonia in terms of variability in composition of fatty acid and oil content was originally found in Ethiopia [6]. List of geographical location and coordinates of *V. galamensis* subspecies *galamensis* varieties in Ethiopia are [2, 3]:

Gelemso (08° 49' N, 40° 31' E), Yirgalem (06° 42' N, 38° 21' E), Leku (06° 52' N, 38° 27' E), Melkabelo (09° 12' N, 41° 25' E), Awassa (06° 52' N, 38° 27' E), Harar Zuria (09° 19' N, 42° 07' E), Areka (06° 48' N, 37° 43' E), Metta (09° 25' N, 41° 34' E) and Arsi-Negele (07°00' N, 38° 35' E).

Figure 1: *Vernonia galamensis* plant [6].

An examination of the structure of VO (trivernolin shown in Figure 2 below) shows that there are three epoxy and double bond groups. Epoxy group, even the hindered ones such as those in VO, posses a relatively high degree of reactivity when compared with many other moieties [6].

1.1.1. Composition of vernonia seed oil

V. galamensis is a new potential industrial oil seed crop for semi-arid areas of the tropics and subtropics, with very high content of VOAC [6]. The oil and VOAC (18: 1 epoxy) contents were initially characterized over 30 years ago [7]. About 38% of the *Vernonia* seed is oil of which 72 80 % is VOAC, which is an enantiomerically pure unsaturated epoxy fatty acid [8]. VO also contains other fatty acids (shown Figure 3 below) such as linoleic acid (12 – 14 %), oleic acid (4 – 6 %), stearic acid (2 – 3 %), palmitic acid (2 – 3 %) and a trace amount of arachidic acid [2, 19]. Preliminary investigations also showed that the seed oil extraction is a valuable source of crude protein (43.75 %); it also consists of crude fiber (10.90 %), ash (9.50 %) and the carbohydrate fraction (6.57 %) with sucrose (2.36 %), fructose (1.90 %) and glucose (0.77 %). The major mineral elements, calcium (11.08 mg/g), potassium (14.18 mg/g), magnesium (6.90 mg/g) and high phosphorus (644 mg/g), which are higher than in most other oilseeds [9, 10].

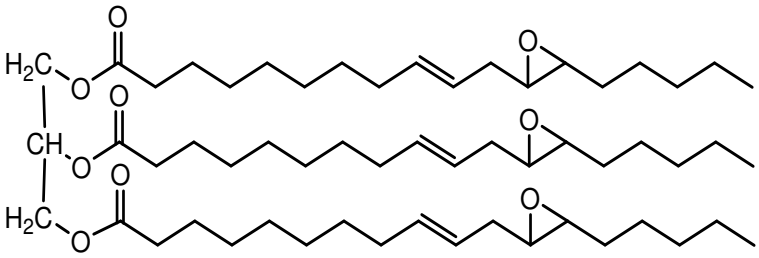


Figure 2: The structure of trivernolin [3, 6].

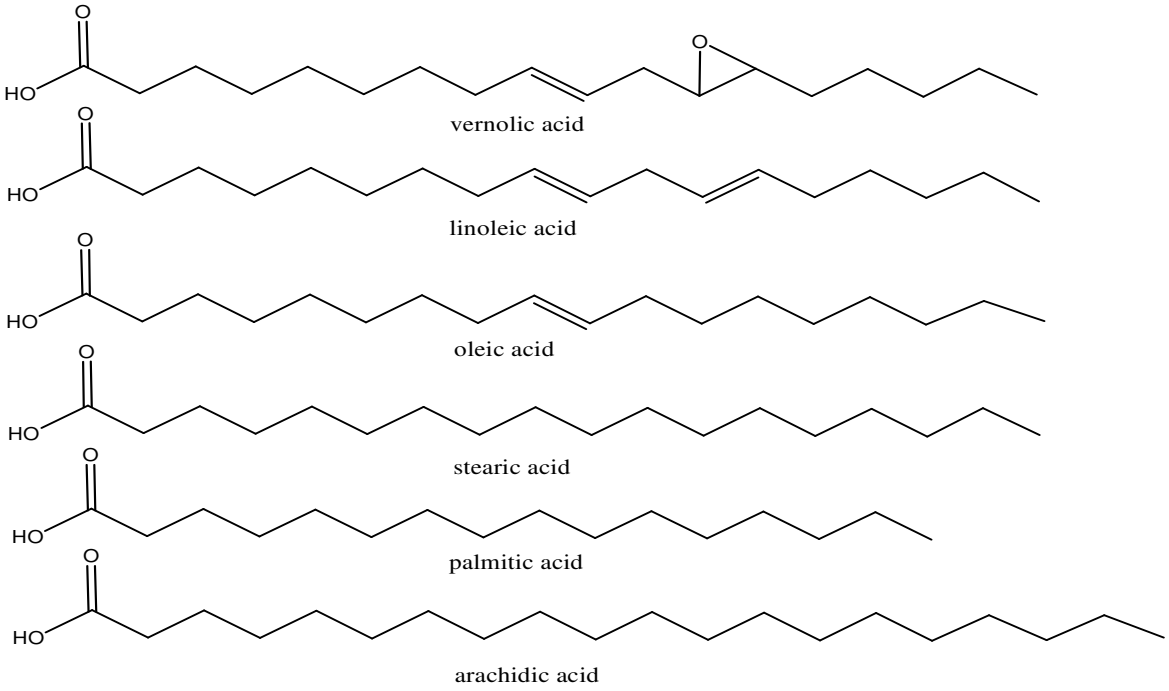


Figure 3: Structures of free fatty acids within of VO [3, 6].

1.1.2. Properties of VO

The natural, liquid epoxy oil from *V. galamensis* seed has properties, such as oxirane content (4.1 %), less viscous (110 cps) liquid compared to other artificial epoxy oils and molecular weight (926 g/mol), that relate to both commercial types. *V. galamensis* seed yields 40-42 % oil containing 72 - 78 % VOAC (cis-12,13-epoxy-cis-9-octadecenoic). Furthermore, oleic (4 %) and linoleic (13 %) acid levels in *V. galamensis* oil represent additional epoxidizable unsaturation (110 % monoene equivalent), so that fully epoxidized VO could have an oxirane value near 10 % [11].

1.1.3. Reactivity of VO

The unique structure of the fatty acid contained within the trivernoline triglyceride (VO) enables a wide variety of reactions. Functional group present within the structure include an ester group, a double bond and an epoxy group as shown in Figure 4 below [3, 6].

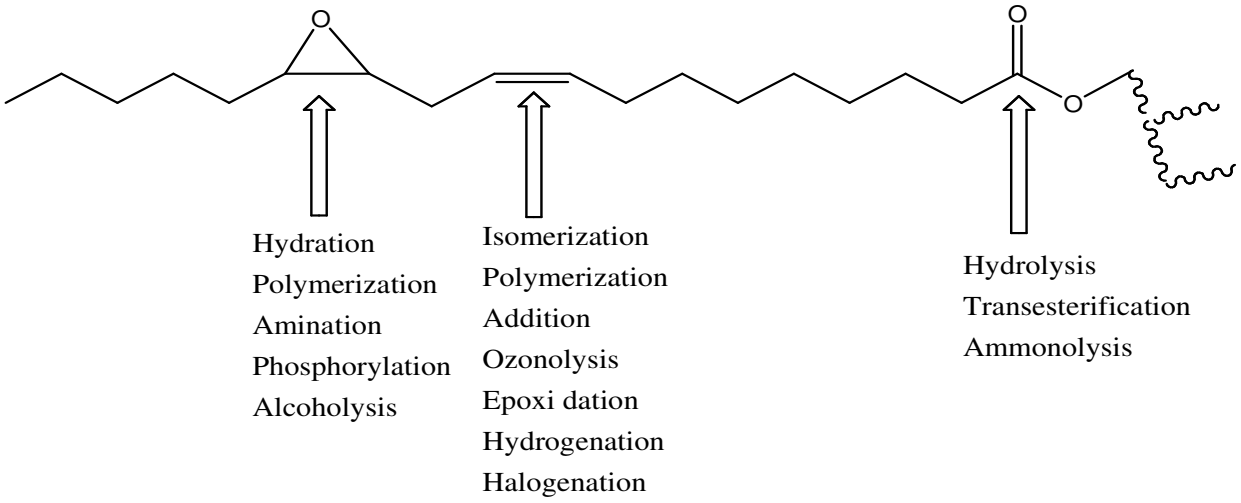


Figure 4: Reaction sites of VO (triglyceride) [3, 6].

1.1.4. Application of vernonia oil and vernolic acid

There has been a growing trend in utilizing epoxidized vegetable oils in various applications. In industry, epoxidized vegetable oil is currently used mainly as PVC stabilizers and plasticizers, in the painting and coating formulations [12], preparation of polyols for polyurethane use [8]. Moreover, epoxidized fatty acids are monomers suited for ring opening polymerizations [13] and polyetherpolyols derived from epoxidized fatty acid compounds may substitute the petrochemical compounds in various applications [8].

The main source for epoxidized oils is fossil fuels and synthetically epoxidized vegetable oil such as linseed or soya bean oils. However, the epoxidation process is expensive and has many environmental problems [6].

Vernonia galamensis is an enantiomerically unsaturated and naturally epoxidized plant oil with interesting applications as binder in coating and preferentially in photo curing coating [8]. This plant oil represents excellent renewable resources, has low toxicity and inherent biodegradability [3]. In addition, the oil used for the synthesis of the acrylate and methacrylate monomers by reacting VO with acrylic or methacrylic acid with high conversion of the epoxy group [14]; also it was used as solvent/reactive diluents in styrene–acrylate copolymerizations [15]. The oil can be used as a source of dibasic acid, these dibasic acids and their derivatives are used in the manufacture of polyurethanes, polyamides (nylons), alkyd resins, plasticizers, elastomers (synthetic rubber), lubricants and hydraulic fluids [16]. VO can also be used by its own in the reformulation of oil-based (alkyd-resin) paints to reduce emission of volatile organic compounds [9], in the animal feed industry and as a medicine to treat a variety of diseases [9]. Moreover, recent investigation shows that VO reacted with carbohydrates such as glucose and starch to give carbohydrate esters of fatty acids, which has more application on cosmetic and pharmaceutical industries [3].

The acid (VOAC) obtained from VO has been used for the synthesis of different important chemicals. For instance, bombykol (which is a sex pheromone of the silk worm Bombyx mori) is synthesized from VOAC. In addition, traumatic acid (which is active as wound hormone of plants) is synthesized from VOAC derived intermediate [3, 6].

1.2. Carbohydrates chemistry

Carbohydrates are naturally occurring organic compounds, containing carbon, hydrogen and oxygen in a typical ratio of 1:2:1 in their composition with highly oxidized organic molecules containing a large number of hydroxyl groups [17, 21]. They are usually defined as polyhydroxy aldehydes and ketones or substances that hydrolyze to yield polyhydroxy aldehydes and ketones, [18] which can be reduced to give sugar alcohols, oxidized to give sugar acids, substituted at one or more of the hydroxyl groups to give other compounds or derivatized at the hydroxyl groups [3]. The presence of hydroxyl groups allow carbohydrates to interact with the aqueous environment and to participate in hydrogen bonding, both within and between chains [17, 21].

Classification of carbohydrates is based on the size of the carbon chain, number of sugar units and location of the carbonyl groups. The simplest carbohydrates contain either an aldehyde moiety (aldose) or a ketone moiety (ketoses) [17, 21]. All carbohydrates can be classified as monosaccharide, oligosaccharides or polysaccharides. From two or ten monosaccharide units, linked by glycosidic bonds, make up oligosaccharides and polysaccharides are much larger, containing hundreds of monosaccharide units. Derivatives of the carbohydrates can contain nitrogens (amino), carboxyl phospho and sulfo groups imparting a net charge and further enhancing their hydrophilic nature [17]. Carbohydrates will form esters with both organic and inorganic acid derivatives. They have a defined stereochemistry, and may adopt a number of ring sizes and confrontations, their hydroxyl are easily protected or converted in to other functionalities often with great selectivity [19]. They are also a renewable natural resource which is widespread and inexpensive and from which a wealth of bulk and fine chemicals can be produced [3].

1.2.1. Glucose

Glucose is a simple monosaccharide ($C_6H_{12}O_6$). It contains six carbon atoms and an aldehyde group. Glucose can adopt several different structures; it can exist in an open-chain (Figure 5a) and ring (cyclic) form [3, 20]. The cyclic structure of glucose is the result of an intramolecular reaction between the aldehyde carbon atom and the C-5 hydroxyl group to form an intramolecular hemiacetal. When glucose is in its ring form, an additional asymmetric carbon, the anomeric carbon atom, is created at C-1. This leads to the formation of two ring structures, the anomers α-glucose (Figure 5b) and β-glucose (Figure 5c). In the case of α form, the hydroxyl group attached to C-1 is below the plane of the ring, in the β form it is above [20].

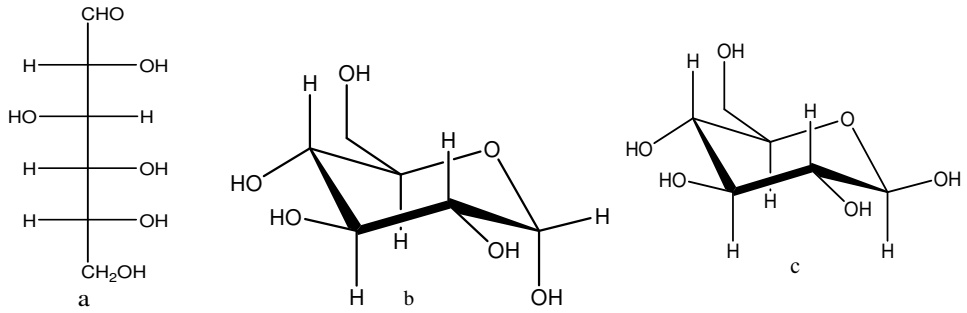


Figure 5: The structure of glucose a) open-chain D-glucose, b) α- glucose and c) β-glucose

1.2.2. Starch

Starch is the major form of stored carbohydrates in plants and a well known material for industrial purposes. This polysaccharide is composed of a mixture of two forms of α-D-glucose polymers, in which glucopyranose units are bonded by alpha-linkages. Most starches found in nature are composed of a mixture of amylose (15 - 20 %) and amylopectin (80 - 85 %). Amylose is essentially a linear polysaccharides linked by α-(1-4) linkages, where molecules coil into a helical structure and form a colloidal dispersion in hot water. Amylopectin is completely insoluble and a highly branched polysaccharide linked by α-(1-6) bonds, which provides starch with different properties [21].

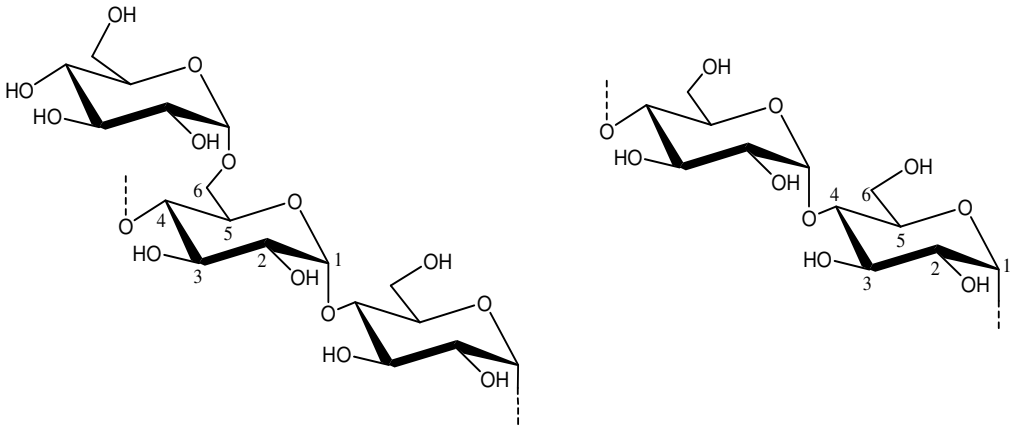


Figure 6: Structures of amylopectin (left) and amylose (right) [3]

1.2.3. Reactivity of Hydroxyl Groups in carbohydrates

The furanoid and pyranoid cyclic structures of monosaccharides generally may have four types of chemically distinguishable hydroxyl groups (shown below in Figure 7):

- The anomeric (hemiacetal) hydroxyl group

And the three types of alcoholic hydroxyl groups [22]:

- The primary hydroxyl group, which is always exocyclic with regard to the carbohydrate ring,
- The endocyclic secondary hydroxyl groups and
- The exocyclic secondary hydroxyl groups

Guidelines for the general order of reactivity of sugar hydroxyl groups:

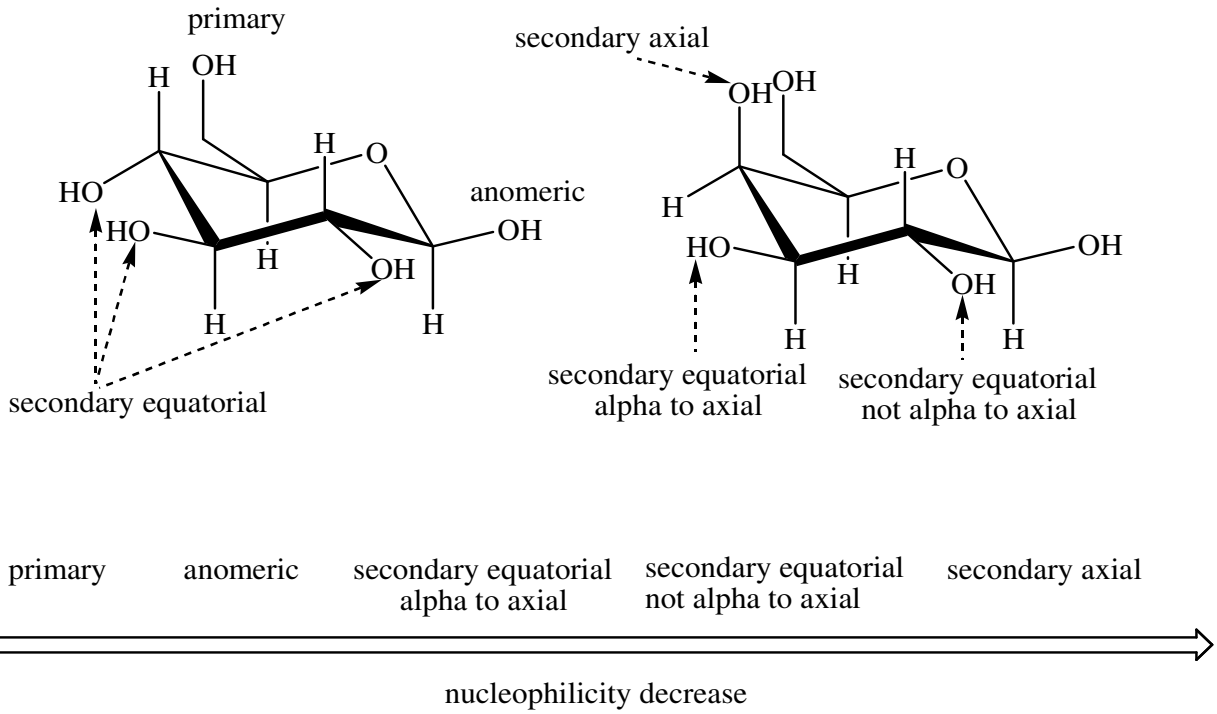


Figure 7: Order of reactivity of sugar hydroxyl groups [25].

The reactivity of hydroxyl groups, excluding the anomeric hydroxyl group, is controlled by several factors. First, it depends on whether the hydroxyl group is a primary or a secondary one. In general, the primary hydroxyl group is more reactive than the secondary one suggesting that the difference in reactivity is most likely due to steric control. The steric factor is probably also responsible for the exocyclic secondary hydroxyl groups being more reactive than endocyclic ones. The difference in the reactivity among the endocyclic secondary hydroxyl groups is most likely controlled by stereoelectronic factors and by the ability of individual hydroxyl groups to form the intramolecular hydrogen bonds with neighboring hydroxyl or alkoxy oxygen. This difference is evident from dependence of reactivity of a given hydroxyl group on its position in a furanoid and/or pyranoid ring. Finally, the reactivity of a hydroxyl group depends on its configuration, i.e., on whether it is in the axial or in the equatorial orientation on a pyranoid ring [22].

The relative reactivity of individual sugar hydroxyl groups depends also on the chemical nature of the acylating agent that is used for their transformation to sugar esters [22]. For instance, lower esters were best produced using combinations of lithium oleate with potassium or sodium oleate [20] and the highest conversion were obtained when vinyl esters (vinyl butyrate) and trifluoroethyl butyrate [23] irreversibly acylate the enol freed by transesterification is rapidly tautomerized into the corresponding volatile acetaldehyde, thus preventing the reverse reaction [24]. Moreover, selective acylation reactions of monosaccharides were obtained when acyl chlorides and acyl anhydrides are used as acylating agents [23].

2. SUGAR FATTY ACID ESTERS (SFAEs)

Sugar fatty acid esters (SFAEs), usually called sugar esters, are produced from renewable and inexpensive substances. SFAEs are non-ionic, non-toxic, non-irritants, odorless, tasteless, biodegradable surfactants that have very good emulsifying, stabilizing, detergency or conditioning effects [26].

Synthesis of carbohydrate esters from natural oils and glucose or starch has the potential to give value added products and highly versatile materials with interesting characteristics. These compounds, derived from natural oils and sugars, are used as surfactants in the food and cosmetic industries, as insecticides and antimicrobial agents and even as non-caloric fat substitutes [3].

2.1. Production of sugar fatty acid esters

SFAEs are synthesized by esterification of sugars with fatty acids, using either chemical or enzymatic processes. Methods can be selected in terms of the degree of substitution (mono-, di-, triesters, etc.), regioselectivity and also more feasible for an industrial scale or limited to the laboratory scale [21]. A multitude of different conditions have been used for the synthesis of sugar esters and include the variation of parameters such as the type of solvent, ratio of sugar to lipid, the specific sugar and lipid, temperature and type of immobilized lipase [27].

2.1.1. Chemical synthesis of sugar fatty acid esters

Currently, chemical production of SFAEs is carried out by reacting sugars with fatty acid vinyl esters (e.g. vinyl laurate, vinyl stearate) fatty acid chlorides or fatty acid methyl esters (e.g. methyl palmitate, methyl laurate) as reactants and basic salts such as potassium carbonate, sodium acetate, sodium dihydrogen phosphate and potassium methoxide as catalysts in organic solvents such as pyridine and dimethylsulfoxide (DMSO) [3].

Many acyl donors such as saturated fatty acids, unsaturated fatty acid, fat/oil and fatty acid derivatives, are used in sugar ester synthesis. In function of the effect of fatty acid chain length and distinct degrees of substitution on sugar ester synthesis was also investigated that the initial rates and conversion yield were decreased when the carbon chain length of fatty acid increase

from C-4 to C-12. The high conversion was obtained with saturated short chain fatty acids. Furthermore, saturated long chain fatty acids gives more yield than the unsaturated fatty acids. For instance, maximum conversion was obtained from stearic acid, while a monounsaturated fatty acid, oleic acid, gives low product [23].

The chemical methods of synthesis uses inexpensive basic catalyst [28], esterification in organic solvents offers inactivation of chemical catalysts and the addition of potassium carbonate as a catalyst allows for the formation of bicarbonate instead of water which will avoid reverse hydrolysis [20].

However, the chemical synthesis of carbohydrate fatty acid esters is hampered by less environmental friendly solutions, performed at high temperature and pressure in the presence of an alkaline catalyst, which is accompanied by high-energy consumption, formation of colored derivatives and unwanted side products due to low selectivity and the use of toxic solvents [21, 26, 29, 30]. Due to some of the drawbacks of chemical esterification process, another more appropriate method should be designed to minimize the above problems [21, 26].

2.1.2. Enzymatic synthesis of sugar fatty acid esters

Enzymes are complex protein molecules produced by living organisms for catalyzing specific reactions, such as breaking down a polymer or synthesizing a chemical. Enzymatic processes are likely to be an essential part of the production of chemicals from biomass due to their potential for high specificity. Although enzymes are formed within living cells, they can continue to function *in vitro* (in the test-tube) and their ability to perform very specific chemical transformations is making them increasingly useful in industrial processes [31].

The enzymatic synthesis of sugar esters has been investigated for over 20 years and is preferred to the chemical synthesis since it is more specific and conducted under milder conditions [27]. The process has often chiral (stereo-selectivity), positional (regio-selectivity) and functional group specificity (chemo-selectivity). Such high selectivity is very desirable in chemical synthesis as it offers several benefits such as reduced or no use of protecting groups, minimized side reactions, easier separation and fewer environmental problems. Other advantages like high catalytic efficiency are also very attractive in commercial applications [32]. Moreover, the enzymatic

synthesis can be performed in less toxic or non-toxic solvents compared to the chemical synthesis [26]. Although, enzymatic processes have more advantages over the chemical methods of SFAEs synthesis but have some drawbacks. For instance, the cost of biocatalyst is very high even so this can be overcome if the enzyme can be reused [33].

Historically, enzymes have been preferably used in aqueous media [32] to keep enzyme hydration because enzymes are inactive in a completely 'dry' system [34] and water helps enzyme to maintain its catalytically active conformation in the majority of noncovalent interactions increasing its flexibility [21]. However, the use of aqueous media negatively affects the synthesis of SFAEs [35]. As enzyme act towards the rapid achievement of reaction equilibrium, when water is used as a solvent, the concentration of one of the products of the esterification reaction is increased and the equilibrium is shifted towards the reactants and a low degree of conversion is achieved. Too much water will also facilitate enzyme aggregation, participates in most of the denaturation reactions and leads to a decrease in enzyme activity [21, 34].

Over the last two decades, studies have shown that enzymes can work in organic solvents. Progress has also been made in developing simple, scalable, and low-cost techniques to produce highly active biocatalyst preparations for use in organic solvents [32]. The use of enzymes in nonaqueous organic media for the modification of various substrates, including natural compounds, has been introduced as an advantageous new approach. Several research groups have reported the feasibility of the enzymatic modification of various polyhydroxylated compounds in both toxic and less toxic organic media using either lipases or proteases [38].

The use of an organic reaction medium can offer some interesting advantages [21]:

- Enhancement of the thermal stability of the enzyme
- Easy separation of the suspended enzyme from the reaction medium for recycling
- Increased solubility of the substrate
- Favorable equilibrium shift to synthesis over hydrolysis and
- Elimination of undesired reactions caused by water

In the enzymatic esterification of carbohydrates the choice of the solvent is a very important and difficult task due to the different chemical nature of the starting materials. Namely, one reactant

is polar (carbohydrate), the other has a nonpolar character (i.e. fatty acid or fatty acid ester) [21]. As the result, it is very difficult to find the appropriate solvents to dissolve both the hydrophilic carbohydrate and the hydrophobic fatty acid, while at the same time maintaining enzyme activity [36]. In fact, there is no solvent compatible with enzyme activity that is both nontoxic and allows the solubilization of both substrates [37].

The use of organic solvents as media for the biocatalytic modification of such polyhydroxylated natural compounds has often shown several disadvantages, such as slower reaction rates, decrease catalytic activities, harmful to the environment and limited productivity that arise from the reduced solubility of such compounds in hexane and chloroform [38, 39]. Unfortunately, polar solvents used in the enzymatic synthesis of sugar fatty acid esters that can dissolve both sugars and lipids include pyridine, dimethylsulfoxide (DMSO) and dimethylformamide (DMF) are often deleterious to most lipases, resulting in partial or complete inactivation [35], and they are not compatible with applications in the food and pharmaceutical industry [21]. Hydrophobic solvents including tertiary alcohols and ketones have been used to solvate both fatty acids and carbohydrate esters, but they are generally poor solvents for carbohydrates [35].

The search for a suitable solvent has driven some groups to research in the use of alternative non-conventional media for enzymatic reaction such as supercritical fluids, which have been used in a large number of studies. The main advantages are that they can be easily removed after the reaction by decreasing the pressure [34], are non-flammable, have low toxicity compared to organic solvents, are chemically inert in most conditions and they have excellent solvent properties for non-polar solutes. These characteristics make them suitable as a medium for a biocatalytic transformation in non-aqueous environments [40]. However, the poor stability exhibited by enzymes in SCFs, the high pressure needed (energy intensive) and the requirement of special equipment is probably the main drawback for using SCFs in biocatalytic processes [34, 41]. In spite of the advantages obtained with all these approaches, the best results for enzyme-catalyzed reactions were observed when the biocatalyst was applied in suspension or coated with another green solvent, such as an ILs [40].

2.1.3. Water removal in the synthesis of SFAEs

As mentioned above, the synthesis of SFAEs and enzyme activity or stability are negatively affected by higher concentration of water. With the aim of increasing the yield, the water generated during esterification has to be removed from the reaction mixture by vacuum line process or direct addition of molecular sieve [26]. However, direct addition of activated molecular sieves in the reaction system has some problems [35]:

- Molecular sieves occupy large space, leading to a low space-time yield.
- Mass transfer limitations can occur due to difficult stirring.
- Molecular sieves are broken due to the strong stirring which is necessary to keep mass transported in the reaction system.

The use of molecular sieves can be avoided by the use of vacuum line processes to extract the water. However, using vacuum line for the removal of water from organic solvents is difficult if the boiling point of organic solvent is lower than water. Therefore, it is necessary to restrict the range of solvents that can be used by this method [41]. To overcome this problem, reaction conditions in other non-conventional reaction media such as ionic liquids (ILs) has been investigated [26].

2.2. Enzymatic Esterification of Sugars in Ionic Liquids

2.2.1. Ionic liquids (ILs)

ILs have emerged as exceptionally interesting nonaqueous reaction media for enzymatic transformations, and research interest in this area has increased widely in recent years. ILs are simply salts and therefore, entirely composed of ions that are liquid below 100°C and usually also close to room temperature. Typical room temperature ILs are based on organic cations, for example, 1,3- dialkylimidazolium, N-alkylpyridinium, tetraalkylammonium and tetraalkylphosphonium, paired with a variety of anions that have a strongly delocalized negative charge (*e.g.* BF_4^-, PF_6^-, SbF_6^-, triflate, bistriflimide, etc), resulting in colorless, low viscosity and easily handled materials with very interesting properties as solvents [40].

2.2.2. Properties of Ionic liquids

Important properties of an IL in terms of its applications in biomass processing are its thermal stability, have negligible vapour pressure and are non-flammable [42]. Due to their non-volatility, they are considered to have a low impact on the environment and human health. They can also be designed to have high thermal stability, high conductivity and low toxicity. Many ILs have even been developed for specific synthetic problems [43]. For this reason, ILs have been termed "designer solvents", which means that the solvents can be customized with a particular end use in mind or to possess a particular set of properties [44]. Hydrophobicity or hydrophilicity is also an important property, which can be controlled by selecting an appropriate cation and anion combination [42].

2.2.3. Advantages of ionic liquid in sugar ester enzymatic synthesis

As mentioned in Section 2.1.2 sugar ester synthesis should be performed in non aqueous media in order to be able to drive the equilibrium towards the required products. Different organic solvents can be used but their application is hindered by different problems. Ionic liquids offer numerous advantages for the enzymatic synthesis of sugar fatty esters. In the first place, the use of ionic liquids avoids the use of atmospheric contaminants such as volatile organic solvents. Moreover, because of their specific physicochemical characteristics, ionic liquid have the ability to dissolve many kinds of compounds including polar (carbohydrates) or non-polar (fatty acids) organic compounds. In addition, ILs are non-volatile and therefore, the water generated during the reaction can be easily removed under vacuum [41]. Finally, some ionic liquids have been found to enhance the stability, activity and stereoselectivity of enzymes and inhibit side reactions [40, 43].

However, the choice of ionic liquid for a specific application is not simple as most of the ionic liquid that are able to dissolve sugars have been found to deactivate enzymes and enzyme friendly ionic liquids normally show low solubility for sugars. ILs containing $[Cl]^-$, $[Br]^-$ and dicyanamide ([dca]) anions have been reported to be good solvents for sugar dissolution but cannot be used in enzyme-catalyzed reaction due to the inactivation of most enzymes [45]. Similarly other scientific groups have reported that anhydrous ILs containing $[BF_4]^-$ and $[PF_6]^-$

anions were used as reaction media in the lipase-catalyzed transesterification of glucose with fatty acid vinyl ester, but the solubilities of sugars in these ILs are very low [45]. Moreover, the solubilities of glucose in ILs containing $[TfO]^-$ and $[BF_4]^-$ anions were greatly influenced by temperature. In these ILs the solubility of glucose increased by a factor of 2–5 when the temperature was increased [41] but resulted in losing the stability and activity of enzymes [21].

Table 1: Glucose solubility and enzyme activity in ILs.

ILs	Concentration (mM)	Activity of enzyme (µmol/min/g)
[Bmim][TfO]	30.6* [45]	1.31 [45]
[Bmim] $[BF_4]$	6.3* [45]	0.65 [45]
[Bmim] $[PF_6]$	0.6* [45]	0.67 [45]
[Bmim] [Cl]	High [45]	Very low [45]
[Bmim] [dca]	211* [42]	Very low [45]

* = at 40^0C

To overcome the above problems several research groups have reported methods to optimize the sugar solubility and enzyme activity and/or stability. For instance, decrease enzyme activity was obtained although much higher enzyme stability was achieved by mixing of two different ILs which show different physicochemical properties [45]. In 2007, Sang Lee prepared a high concentration of sugars in enzyme friendly ILs, by a procedure referred to as water mediated supersaturation. This procedure entails mixing of an aqueous sugar solution into ILs followed by removal of the water from the solution [21]. The increase of dissolved glucose concentration within an enzyme friendly ionic liquid that keeps the enzyme active and stable yielded better conversions because the equilibrium was forced toward synthesis [45]. Water mediated supersaturated glucose solution in ILs is more stable even in the presence of undissolved glucose than supersaturated glucose solution in general organic solvents. Low crystallization rate of a supersaturated glucose solution in ILs may be primarily caused by high viscosity (90 cp at 20^oC for [Bmim][TfO]) of ILs [45].

2.3. Enzyme activity

Lipases, triacylglycerol hydrolases that cleave triacylglycerols at the oil/water interface, have extensive applications in the food, paper, pharmaceutical, cosmetic, detergent, leather and textile industries. Widespread practical use of these enzymes requires fast and reliable analytical routines to assess their activity. Typical protocols for lipase assay include use of a PH- stat, back titrations and spectrophotometric determination of para-nitrophenyl palmitate (p-NPP) hydrolysis, etc [46].

Enzyme activity is a measure of the quantity of active enzyme present and is thus dependent on conditions, which should be specified. The SI unit is the katal, 1 katal = 1 mol s^{-1}, but this is an excessively large unit. A more practical and commonly used value is 1 enzyme unit (U) [47]. One unit of enzyme activity is defined as the quantity of enzyme required to liberate one micro mole of *para*-nitrophenol (p-NP) per minute under the assay conditions [48].

3. OBJECTIVES

The functionalization of glucose and starch is widely used to modify the physical and chemical characteristics of the sugars to obtain desirable properties for industrial applications. There is a strong interest in the development of new surfactants of glucose and starch derivatives from endemic natural resources that could be used as surfactants in the food and cosmetic industries, as insecticides and antimicrobial agents, as non-caloric fat substitutes, as biodegradable delivery systems, stabilizers and coatings. Sugar esters of vernolic acid could form new type of epoxidized surfactants with interesting properties. In addition, as these materials are naturally epoxidized they could be used as starting materials for the synthesis of various polymer materials.

As the chemical synthesis route is hindered with many drawbacks, the main objective of this project was to develop and optimize a mild and environmentally friendly enzymatic process for the synthesis and characterization of novel mono-substituted epoxy-sugar derivatives. As no proper organic solvent has been found to give good results for enzymatic esterification, we decided to use ILs as reaction media because they can dissolve the starting materials and additionally can also stabilize the enzyme to accomplish its task. A 'water mediated supersaturation' method will be used to prepare highly concentrated sugars in ILs. The ionic liquid used in the esterification reaction was [Bmim][TfO] because the enzymes are stable in it and the by-product water, generated during the reaction can be easily extracted from ILs under vacuum. The use of an ionic liquid also avoids the use of toxic and environmentally unfriendly organic solvents.

In this work, Lipase B from *C. antarctica* will be used. This enzyme is the most frequently used enzyme for sugar ester synthesis in organic solvents. It was also found to be active in solvent systems containing ILs.

4. EXPERIMENTAL PARTS

This part describes the chemicals, methods and instruments used in this thesis and procedural details of the synthesis and the NMR data of the substrates (starting materials) and sugar ester.

4.1. Materials and Methods

4.1.1. Enzymes and chemicals

Immobilized lipase Novozym 435 from *C. Antarctica* B, *Vernonia galamensis* seeds, n-hexane, sodium hydroxide (NaOH), sodium methoxide ($NaOCH_3$), 85 % potassium hydroxide (KOH), Methanol, D-glucose, Cassava Starch, glacial acetic acid, pyridine, dimethylsulfoxide (DMSO), activated charcoal, tetrahydrofuran (THF), acetone, [Bmim][Tfo], [Bmim][PF_6], [Bmim][BF_4], *para*-Nitrophenyl palmitate, Tris-base, HCl, iso-propanol, triton x-100, gum Arabic.

4.1.2. Materials

Materials that were used during synthesis are: Soxhlet extractor, TLC, measuring balance, litmus paper, rotator flask and rotavapour, vacuum line set-up, different types of flasks, measuring cylinders and thermometer with its set up.

4.1.3. Instruments

Instruments that were used for characterization of the oil and substrates (VOAC and VOME) and carbohydrate ester of fatty acid (glucose vernolate) are: Bruker 400 MHz spectrometer (^{1}H and ^{13}C NMR), melting point device (SMP3). Uv-Vis spectrophotometer was used for enzyme assay.

4.2. Extraction and Purification of Vernonia Oil

4.2.1. Extraction of Vernonia Oil

Dried *V. galamensis* seeds were heated in an oven for 1 hour at 90^0C for lipase deactivation and powdered seeds of *V. galamensis* were extracted with n-hexane as a solvent for three hours using Soxhlet extraction systems. Then the solvent was removed using rotary evaporator and the crude oil was subjected to refining process [3, 6].

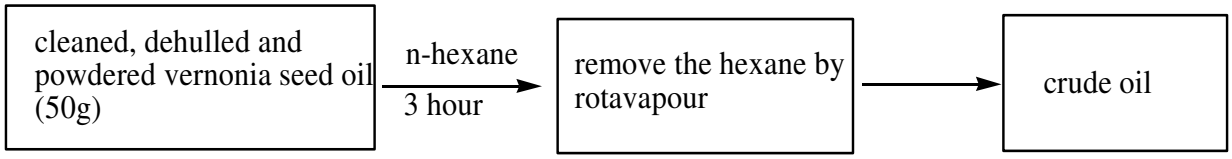


Figure 8: Extraction of the oil from *vernonia galamensis* seeds.

4.2.2. Refining of Vernonia Oil

In addition to triglycerides, the crude oil contains variable amounts of objectionable substances, which must be removed to produce pure VO. Some of these impurities are of the non-glyceride type such as the free fatty acids, which may be built up due to enzymatic processes (lipase) resulting from damage to the seed. While others are of the non-oil kind such as the mucilage volatile including moisture and solvent, pigment or coloring materials primary and secondary oxidation products, waxes and saponifiable and odoriferous materials [3, 6].

4.2.2.1. Bleaching

Crude VO was refined with 5 % to 8 % by weight of activated charcoal was mixed with the oil and heated at a temperature of 60^0C by a continuous stirring for one hour. The decolorized oil was isolated by hot filtration [3, 6].

4.2.2.2. Degumming

Almost all seed oils contain impurities in the colloidal state or dissolved in them. These substances must be removed from the oil. This purification process is known as degumming and is usually carried out immediately before neutralization or concurrently with it. Crude VO was degummed by stirring with 2.5 – 5 g by weight of distilled water, heated 60-70^0C for one hour followed by centrifugation at 5,000 rpm. Gum and oil were separated and the oil was dried at 60^0C on a rotary evaporator [3, 6].

4.2.2.3. Neutralization

Organic acids, which are always dissolved in the oil, are removed by saponification with sodium hydroxide solution. Separations occur easily because the resulting soaps are practically insoluble in the neutral oil under standard operation conditions. The degummed VO was mixed with sodium hydroxide solution and then heated to 40^0C followed by stirring for 30 minutes. From the mixer the oil-soap stock suspension passes through the centrifugal separator, which separates the soap stock from the neutral oil. Oil was dried for one hour on a rotary evaporator at 60^0C [3, 6].

4.2.2.4. Re-Refining of the Neutral Oil

The neutral oil obtained from the neutralization contains minute quantities of free fatty acids and other impurities. Such impurities were removed by treatment with a dilute solution of sodium hydroxide. The purpose of re-refining is to remove the last trace of fatty acids, phosphatides etc. from the neutralized oils [3, 6].

4.2.2.5. Washing of the Oil

To obtain soap free oil after the degumming, neutralization and re-refining steps, a vigorous washing by hot water was carried out. This is necessary because the soaps are always partially soluble in the neutral oil. Finally, NMR analysis of the purified oil has been obtained [3, 6].

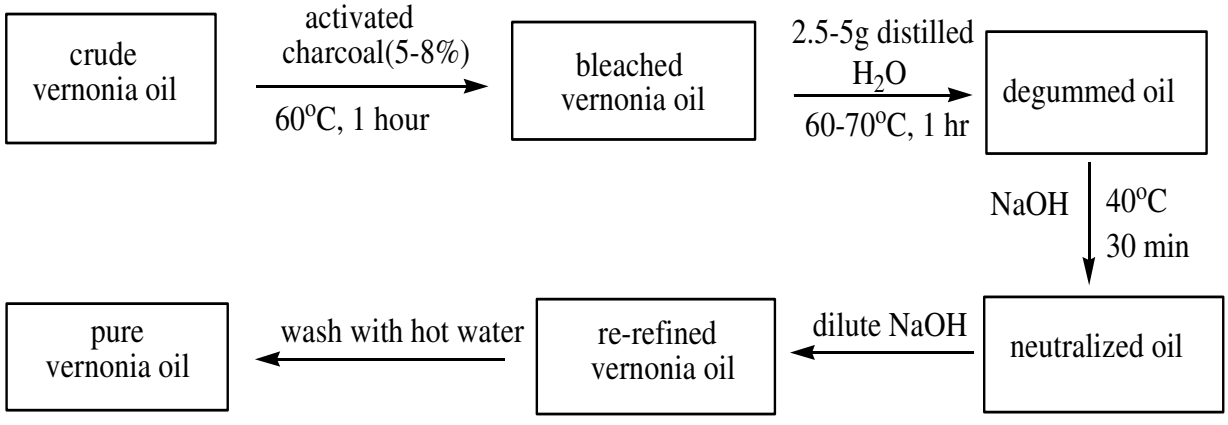


Figure 9: Summary of purification of crude *vernonia* oil.

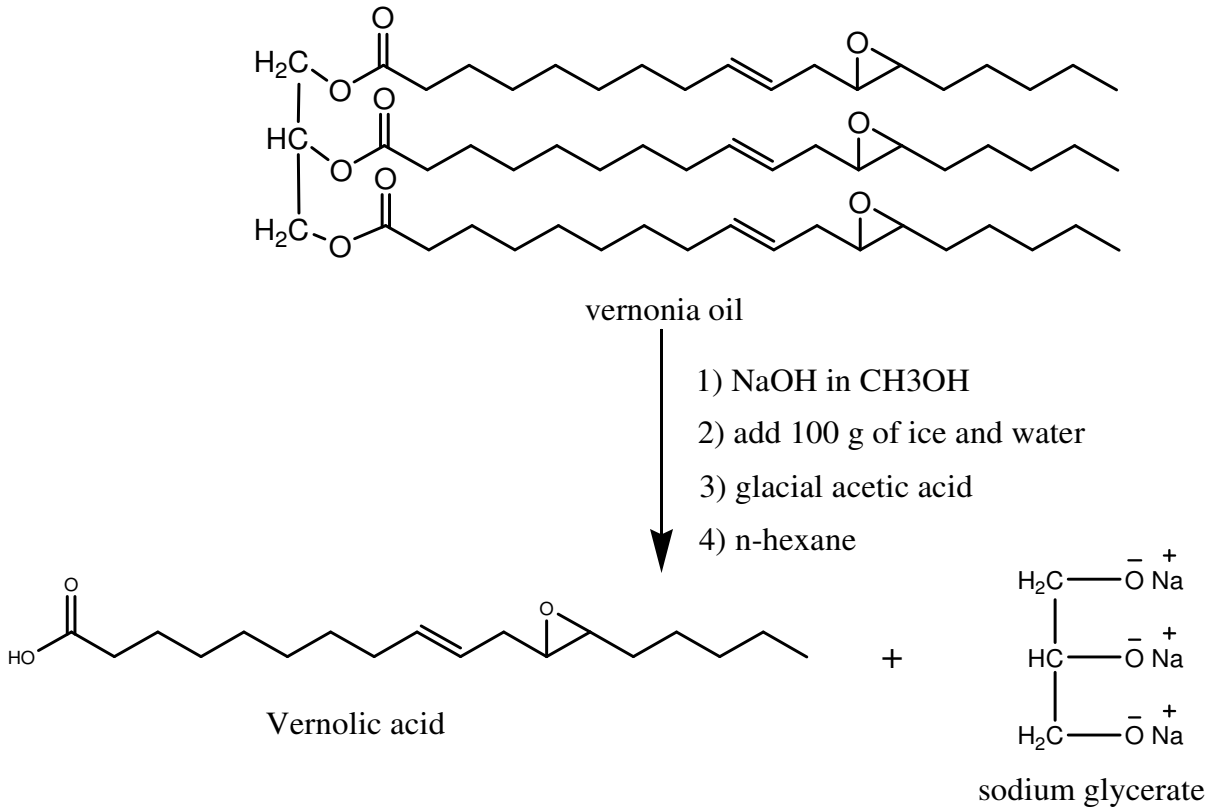
4.3. SYNTHESIS

4.3.1. Synthesis of Starting Materials

4.3.1.1. Synthesis of vernolic acid from vernonia oil

To a 250 mL distilling flask, equipped with magnetic stirrer bar, was transferred 50 mL methanol and 5 g (0.125 mol) sodium hydroxide. The flask was then fitted with a condenser and the mixture was heated to reflux until complete dissolution of the sodium hydroxide. To the hot alkaline solution 5.12 g (5.53 mmol) VO was added. The resulting brownish solution was refluxed with continuous stirring for 10 minutes, after which it was immediately transferred into a beaker and allowed to form a semisolid on cooling. About 100 g ice was added, mixed thoroughly, followed by addition of 100 mL water with mixing. The cold mixture was vacuum-filtered to afford an off-white solid soap in the filter bed. The soap was transferred into a beaker and mixed with 100 g ice and 100 mL water, then acidified with 4 mL glacial acetic acid. The acidified mixture was immediately vacuum filtered to afford a white solid acid. The cold white solid was transferred into a beaker, containing 100 mL hexane, with mixing to dissolve the acid and the resulting mixture was transferred in to a separatory funnel to allow separation of the organic and aqueous phases. The hexane layer was stripped to afford crude VOAC. Purification of the acid was accomplished by low-temperature recrystallizations. Hexane (50 mL) was added to a 150 mL beaker containing the crude VOAC and the beaker was placed in a freezer for 24 hour. The resulting solid was vacuum-filtered and rinsed with an additional 50 mL ice-cold hexane to give pure VOAC [3].

The schematic chemical reaction for vernolic acid which was obtained from vernonia oil is shown below:

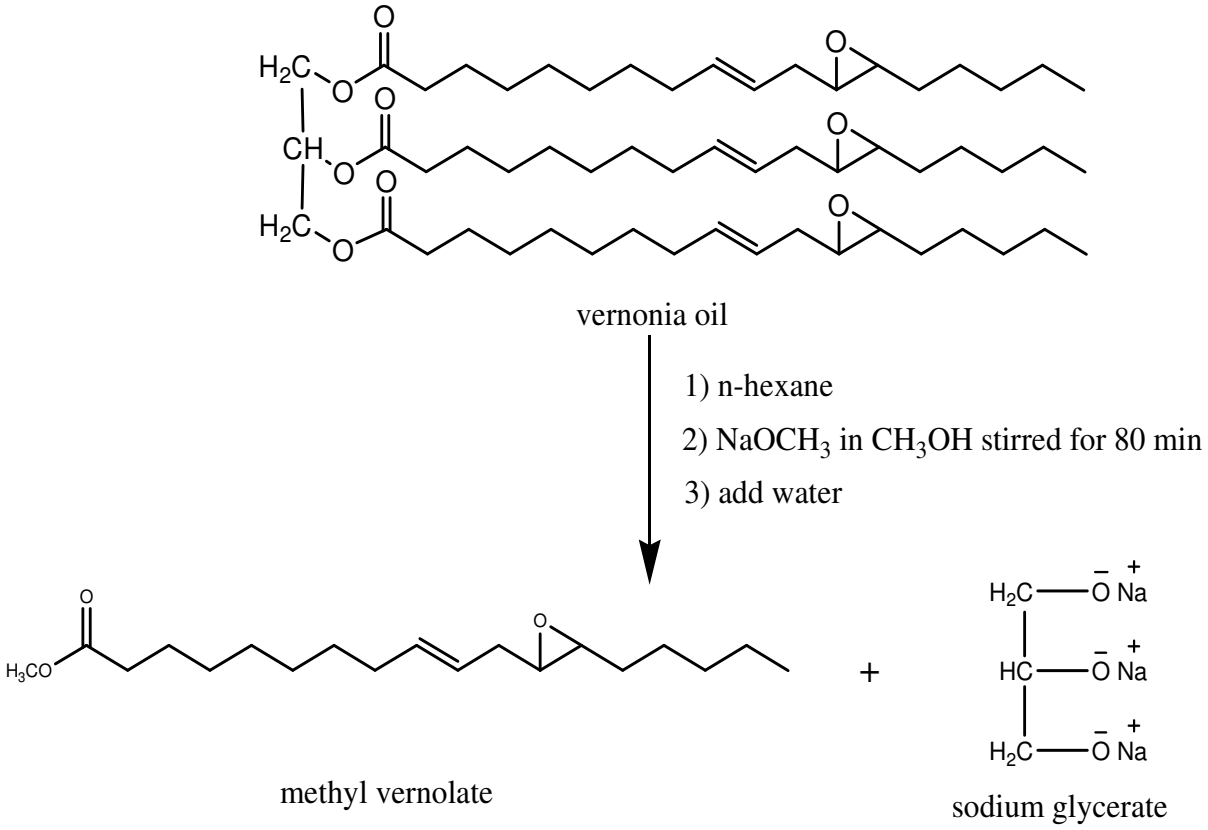


Scheme 1: Synthesis of VOAC from VO.

4.3.1.2. Synthesis of methyl vernolate from vernonia oil

VO (20 g, 21.6 mmol) was transferred into a 500 mL round-bottomed flask. Hexane (125 mL) was added, followed by 12.5 mL sodium methoxide in methanol (25 wt %). The flask was fitted onto a rotary evaporator, then allowed to rotate (approximately 240 revolutions/min) for about 80 min without heat or vacuum. The resulting mixture was transferred into a separatory funnel and 125 mL water was added. The flask was rinsed with approximately 20 mL water and the rinse was added to the separatory funnel. The hexane layer was drawn off and removed by rotary evaporator to result VOME [49].

The schematic chemical reaction for methyl vernolate which was obtained from vernonia oil is shown below:



Scheme 2: Synthesis of VOME from VO.

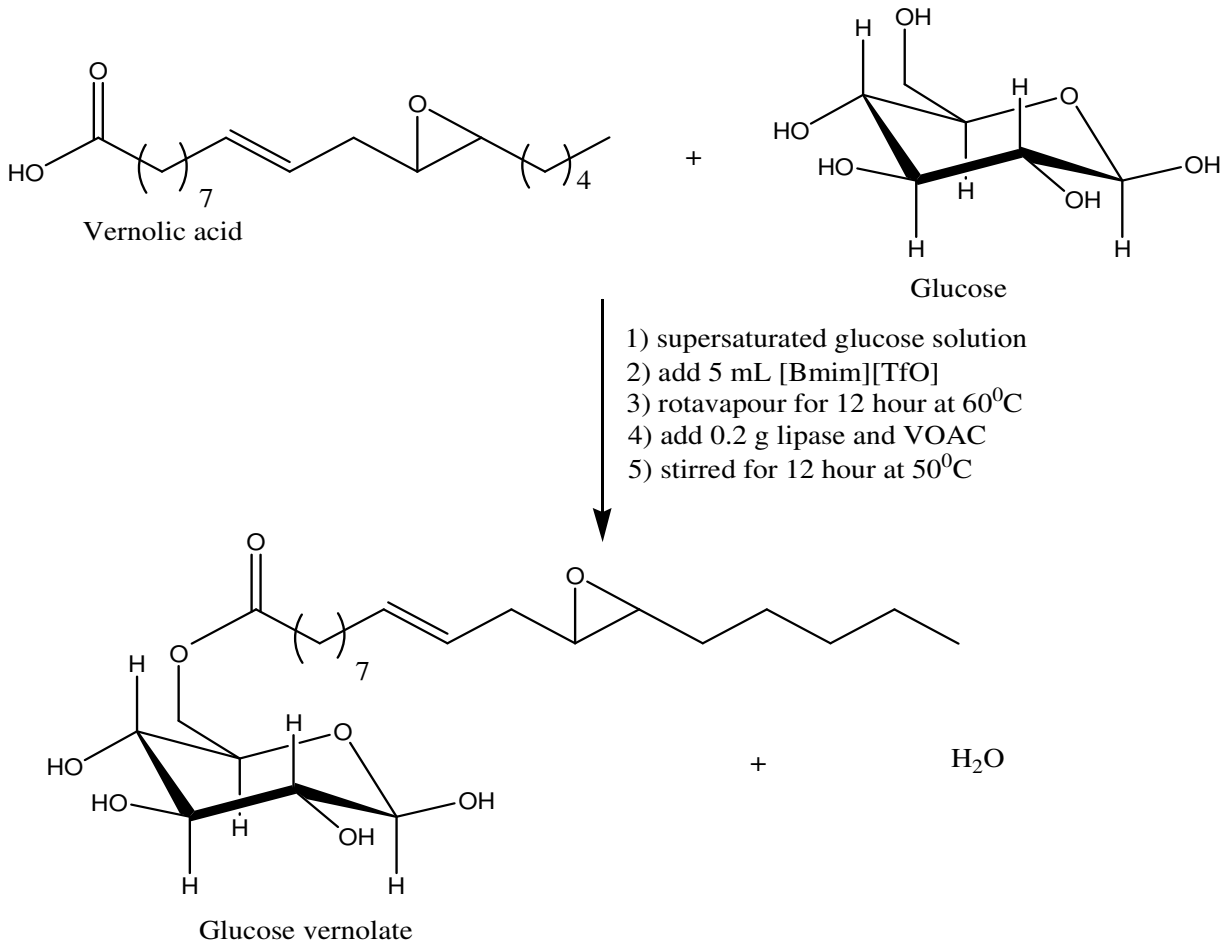
4.3.2. Synthesis of Sugar Esters

4.3.2.1. Synthesis of glucose ester from vernolic acid

Supersaturated glucose solution in ILs was obtained through water-mediated supersaturated method. 1.11 mmol (0.2 g) of glucose was dissolved in about 1.5mL of distilled water and to this solution 5mL of ILs ([Bmim][TfO]) was added at room temperature. After a white solution was obtained, the water in the mixture was removed by vacuum evaporation for 12 hour at 60^0C. Then 0.2 g of lipase enzyme (Novozym 435) was added to the supersaturated glucose solution. The reactions were started by adding 2.2 mmol (0.65 g) of VOAC to the supersaturated glucose solution and the mixture was stirred for 12 hours at 50^0C in a rotary evaporator or under vacuum to remove the water being produced in the course of reaction. To remove the ILs and unreacted

glucose 4mL of deionized water was added to the product. Then the product containing part was further washed with acetone or THF to separate the product from enzyme and unreacted VOAC. The product (glucose vernolate) which was extracted by acetone was cooled at -10^{0}C and the products were collected by filtration. Finally, the product was dried in vacuum oven for 48 hour at 35^{0}C and the NMR data for VOAC and glucose vernolate were characterized using Bruker 400 MHz spectrometer (both ^{1}H and ^{13}C).

The schematic chemical reaction for glucose vernolate which was obtained from vernolic acid is shown below:

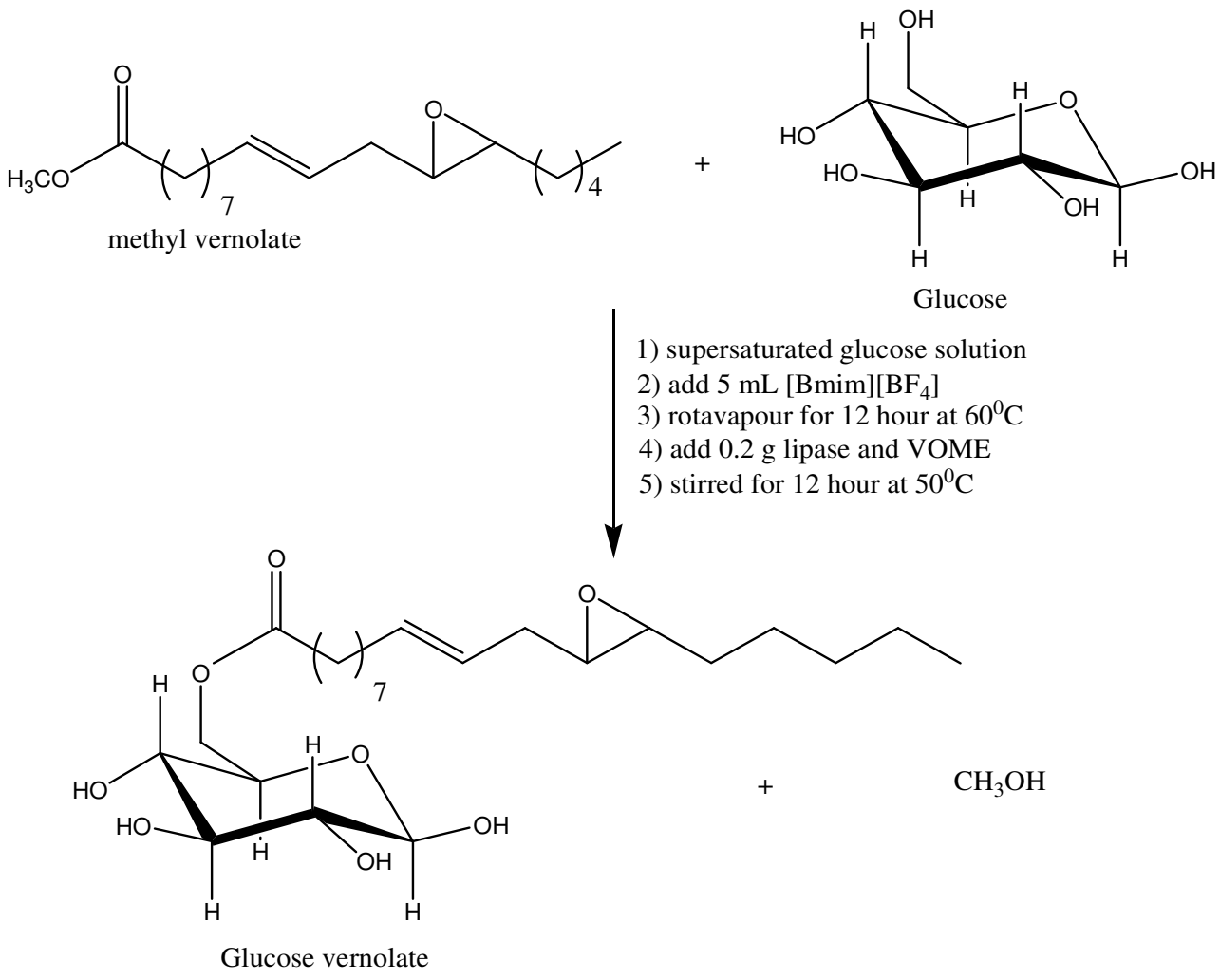


Scheme 3: Synthesis of glucose ester from VOAC.

4.3.2.2. Synthesis of glucose ester from methyl vernolate

After the supersaturated glucose solution was prepared, as mentioned in the above procedure, 0.2 g of lipase enzyme (Novozym 435) was added to the supersaturated glucose solution. The reaction was started by adding 2.2 mmol (0.68 g) of VOME to the supersaturated glucose solution and the mixture was stirred for 12 hours at 50^0C in a rotary evaporator or under vacuum to remove the methanol being produced in the course of reaction. To remove the ILs and unreacted glucose, 4 mL of deionized water was added to the product. Then the product containing part was further washed with acetone or THF to separate the product from enzyme and unreacted VOME. The product (glucose vernolate) which was extracted by acetone was cooled at -10^0C and the products were collected by filtration. Finally, the product was dried in vacuum oven for 48 hours at 35^oC.

The schematic chemical reaction for glucose vernolate which was obtained from methyl vernolate is shown below:



Scheme 4: Synthesis of glucose ester from VOME

4.3.2.3. Synthesis of starch ester from vernolic acid

Cassava starch (0.4 g, 2.47 mmol, anhydroglucose unit, AGU) was first gelatinized in DMSO (5 mL) at 70^0C. To the solution obtained, ionic liquid [Bmim][PF_6] (2 mL) and VOAC (1 g) was added. Then 0.2 g lipase enzyme (Novozyme 435) was added. To remove the water which is formed during the reaction vacuum line was used and the reaction was stirred at 40^0C for 72 hours. After cooling, the product was precipitated using methanol (100 mL) and separated from the liquid phase by decantation. The product was washed twice with methanol. Finally, the product was dried in a vacuum oven (70^0C) and a hard brown solid product was obtained.

4.3.2.4. Synthesis of starch ester from methyl vernolate

Cassava starch (0.4 g, 2.47 mmol, anhydroglucose unit, AGU) was first gelatinized in DMSO (5 mL) at 70^0C. To the solution obtained, ionic liquid [Bmim][TfO] (2 mL) and VOME (1 g) was added. Then 0.2 g lipase enzyme (Novozyme 435) was added. To remove the methanol which is formed during the reaction vacuum line was used and the reaction was stirred at 40^0C for 72 hours. After cooling, the product was precipitated using methanol (100 mL) and separated from the liquid phase by decantation. The product was washed twice with methanol. Finally, the product was dried in a vacuum oven (70^0C) and a hard brown solid product was obtained.

4.3.3. Reagent preparation for enzyme assay

Reagent A

50 mM tris base (MW = 121 g/mol, 0.0655 g) was prepared by dissolving solid tris base in water and the pH was maintained at 8 by adding HCl. Then 0.1 g gum Arabic and 0.4 mL triton x-100 was added.

Reagent B

0.03775 g of paranitrophenyl palmitate (p-NPP) was dissolved in 5 mL iso-propanol at 37-50^0C.

4.3.3.1. Lipase assay reagent

The iso-propanol p-NPP solution was heated to 37-50^0C until all crystals dissolve. Then in two 250 mL flasks, 19 mL reagent A and 1mL reagent B was added. To the first flask 0.25 g of lipase enzyme (Novozym 435) was added. The second flask was left in the absence of enzyme. The solution was stirred by magnetic stirrer and the absorbance was measured at different time intervals. The activity was assayed by detecting the product, *p*-nitrophenol, spectrophotometrically at 410 nm with a GENESYS UV-Vis spectrophotometer at 35^0C against an enzyme-free blank. The molar extinction coefficient of p-NP at pH 8 was calculated as 15.1 $M^{-1}cm^{-1}$ [48].

4.3.3.2. Lipase activity in ionic liquid media

The immobilized enzyme was mixed with [Bmim][PF_6] solution to check whether the ILs media affect the activity of lipase. CAL-B were exposed in [Bmim][PF_6] for 3 hour at 35^0C. Then the enzyme was filtered and its residual enzyme activity was measured by UV-Vis spectrophotometer using p- NPP as substrate under specified assay conditions.

4.3.4. NMR data for starting materials

The ^{13}C NMR spectra and the ^{1}H NMR spectra were recorded using Bruker 400 MHz spectrometer. Chemical shifts were reported in ppm downfield from TMS using $CDCl_3$ and as a solvent. Table 5 and 6 shows all the ^{1}H and ^{13}C NMR data for the oil (crude and pure), VOAC and VOME.

4.3.4.1. NMR data for crude vernonia oil

^{1}H NMR data (ppm) (400 MHz, $CDCl_3$): The ^{1}H NMR data of the crude VO (Appendix 1 or Table 5) δ 5.17 - 5.48 (m, 7H), 4.02 - 4.28 (m, 3H), 2.66 - 2.91 (d, 6H), 1.90 - 2.35 (m, H), 1.16 - 1.69 (m, 54H), 0.76 - 0.87 (m, 9H).

^{13}C NMR data (ppm) (400 MHz, $CDCl_3$): The ^{13}C NMR data of the crude VO (Appendix 2 or Table 6) δ 173.33, 123.82 - 132.40, 68.88, 62.00 - 64.86, 56.41 - 57.17, 22.50 - 33.90, 13.95.

4.3.4.2. NMR data for pure vernonia oil

^{1}H NMR data (ppm) (400 MHz, $CDCl_3$): The ^{1}H NMR data of the pure VO (Appendix 3 or Table 5) δ 5.22 - 5.56 (m, 7H), 4.02 - 4.34 (m, 4H), 2.71 - 2.98 (d, d, 6H), 1.94 - 2.42 (m, 18H), 1.18 - 1.68 (m, 54H), 0.81 - 0.95 (s, 9H).

^{13}C NMR data (ppm) (400 MHz, $CDCl_3$): The ^{13}C NMR data of the pure VO (Appendix 4 or Table 6) δ 173.27, 123.86 - 132.53, 68.88, 62.01 - 64.90, 56.41 - 57.16, 22.55 - 33.88, 13.96.

4.3.4.3. NMR data for vernolic acid

^{1}H NMR data (ppm) (400 MHz, $CDCl_3$): The ^{1}H NMR data of the VOAC (Appendix 5 or Table 5) δ 10.56 (s, 1H), 5.26 - 5.55 (m, 2H), 2.71 - 2.97 (dd, 2H), 1.95 - 2.40 (m, 6H), 1.18 - 1.68 (m, 18H), 0.80 - 0.94 (t, 3H).

^{13}C NMR data (ppm) (400 MHz, $CDCl_3$): The ^{13}C NMR data from the VOAC (Appendix 6 or Table 6) δ 179.78 (C-1), 123.84 - 132.49 (C-3), 56.63 - 57.30 (C-5), 22.55 - 34.25 (C-2, 4, 6), 13.93 (C-7).

4.3.4.4. NMR data for methyl vernolate

^{1}H NMR data (ppm) (400 MHz, $CDCl_3$): The ^{1}H NMR data of the VOME (Appendix 7 or Table 5) δ 5.29 - 5.57 (m, 2H), 3.62 - 3.74 (s, 3H), 2.74 - 2.98 (d, 2H), 1.99 - 2.42 (m, 6H), 1.21 - 1.67 (m, 18H), 0.84 - 0.95 (m, 3H).

^{13}C NMR data (ppm) (400 MHz, $CDCl_3$): The ^{13}C NMR data of the VOME (Appendix 8 or Table 6) δ 174.28 (C-2), 123.79 - 132.49 (C-4), 56.42 - 57.16 (C-6), 51.31 (C-1), 22.59 - 34.18 (C-3, 5, 7), 13.99 (C-8).

4.3.5. NMR data for sugar fatty acids

4.3.5.1. NMR data for glucose vernolate

^{1}H NMR data (ppm) (400 MHz, $CDCl_3$/TMS): The ^{1}H NMR data of the glucose ester (glucose vernolate) (Appendix 9) 0.83 - 0.92, 1.22 - 1.68, 2.06 - 2.40, 2.66 - 2.98, 3.31 - 3.63, 3.9, 4.12 - 4.19, 4.73 - 4.84, 5.26 - 5.11, 5.58 - 5.72.

^{13}CNMR data (ppm) (400 MHz, $CDCl_3$/TMS): The ^{13}C NMR data of the glucose ester (glucose vernolate) (Appendix 10) 13.92, 22.51 - 34.23, 56.67 - 57.34, 71.92, 72.2, 73.82, 74.05 75.74 99.98, 123.68 - 132.55 173.63.

NMR data for the rest of the products could not be obtained because of the insolubility of the sugar esters in common NMR solvents.

5. RESULTS AND DISCUSSION

5.1. Characterization of the Starting Materials

5.1.1. Analysis of crude vernonia oil

After extracting the oil and removing the solvent (hexane) by rotavapour the crude VO was analyzed by using Bruker 400 MHz spectrometer.

As seen in the experimental part characteristic peaks for the crude vernonia oil (Appendix 1 or Table 5) were found. On the spectra, the peaks at 5.17 - 5.48 ppm indicate the presence of olefin protons (C<u>H</u>=C<u>H</u>) and the glyceryl proton (C<u>H</u>), the peaks at 4.02 - 4.28 are glyceryl protons (C<u>H</u>$_2$), the peaks at 2.66 - 2.91 are epoxy protons(O-C-<u>H</u>), protons of methylene attached to olefinic group (-C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-) at 1.90 - 2.35, protons of methylene groups $(CH_2)_n$ at 1.16 - 1.69 and methyl protons (CH_3) at 0.76-0.87.

The ^{13}C NMR data also shows that the VO (Appendix 2 or Table 6) contains carbonyl carbon (O=<u>C</u>) at 173.33, olefinic carbons shows a peaks (<u>C</u>H=<u>C</u>H) at 123.82 - 132.40, glyceryl carbons (<u>C</u>H) at 68.88, and glyceryl (<u>C</u>H$_2$) at 62.00 - 64.86, epoxy carbons (O-<u>C</u>-H) at 56.41 - 57.17, methylene carbons $(\underline{C}H_2)_n$ at 22.50 - 33.90 and methyl carbon (<u>C</u>H$_3$) at 13.95.

The area of peaks (Table 2 shown below) of the methyl protons signal for crude VO is too high because of the presence of other saturated triglyceride oils of fatty acids such as stearic acid, palimitic acid and arachdic acids. This result was confirmed by the presence of a peak at 178.23 ppm near to the ester carbonyl carbon of VO on ^{13}C NMR spectra. Moreover, there are also other unsaturated fatty acids such as linoleic and oleic acids. This was confirmed by the presence of peaks at 127 and 130 ppm on the carbon spectra of crude VO. In addition, the peak area integration on the proton NMR confirms also the presence of saturated triglyceride oils. However, once the oil was purified the methyl protons ratio decrease due to the removal of those saturated fatty acids impurities.

The amount of integral peak area ratio of epoxide is low because of the high reactivity of epoxide groups on the triglyceride VO may undergo ring opening polymerization reactions and form triricinolein which has the same structure with trivernolein except that instead of the

epoxidized group, a nonconjugated hydroxyl group is found, this results in a minimum ratio of epoxide with respect to the double bond [54].

5.1.2. Analysis of pure vernonia oil

After the necessary purification steps, pure VO was analyzed by using Bruker 400 MHz spectrometer.

As seen in the experimental part characteristic peaks for the pure vernonia oil (Appendix 3 or Table 5) were found. On the spectra, the peaks for double bond (C<u>H</u>=C<u>H</u>) and the glyceryl proton (C<u>H</u>) at 5.22 - 5.56 ppm, the glyceryl proton (C<u>H</u>$_2$) at 4.02 - 4.34, the peaks for epoxy protons (O-C-<u>H</u>) at 2.71 - 2.98, protons of methylene attached to olefinic group (-C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-) at 1.94 - 2.42, protons of methylene groups $(CH_2)_n$ at 1.18 - 1.68, protons of methyl (CH_3) group at 0.81 - 0.95.

The ^{13}C NMR data also confirms that the pure VO (Appendix 4 or Table 6) contains the carbonyl carbon (O=<u>C</u>) at 173.27, olefinic carbons (<u>C</u>H=<u>C</u>H) at 123.86 - 132.53, glyceryl (<u>C</u>H) at 68.88, and glyceryl (<u>C</u>H$_2$) at 62.01 - 64.90, epoxy carbons (O-<u>C</u>-H) at 56.41 - 57.16, methylene carbons $(\underline{C}H_2)_n$ at 22.55 - 33.88 and methyl carbon (<u>C</u>H$_3$) at 13.96.

Table 2: The integral peak areas of the functional groups of crude and purified VO

Integral Peak area of different functional groups of VO			
Compounds	Double bond	Epoxy	Methyl
Crude VO	1	0.69	1.72
Purified VO	1	0.63	1.38
Theoretical value	1	1	1.5

The above integral peak area of the functional groups of the crude and purified VO (Table 2) indicates that after the purification of VO the epoxide protons ratio of pure VO was lower than the crude VO, the decrease in value is because of during neutralization and washing of the oil hydration may occur on one of the epoxy groups on the triglyceride structure of VO [6].

After the purification step, the methyl and epoxide protons ratio decreased by 22.6 % and 6 % with respect to the double bond, respectively. This value showed that most of the impurities were saturated fatty acids.

5.1.3. Analysis of vernolic acid

After the synthesis and purification of vernolic acid, it (light golden yellow color) was analyzed by NMR. NMR data of the vernolic acid indicated the presence of major functional groups namely the double bonds, the epoxy and the hydroxyl group of the VOAC.

As seen from the experimental part, ^{1}H NMR of the VOAC (Appendix 5 or Table 5) indicated the presence of hydroxylic (-OH) at 10.56, olefinic protons (C<u>H</u>=C<u>H</u>) at 5.26 - 5.55, epoxy protons (O-C-<u>H</u>) at 2.71 - 2.97, protons of methylene attached to olefinic group (-C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-) at 1.95 - 2.40, protons of methylene groups (C<u>H</u>$_2$)$_n$ at 1.18 - 1.68, protons of methyl (C<u>H</u>$_3$) group at 0.80 - 0.94.

The ^{13}C NMR also (Appendix 6 or Table 6) showed the presence of the carboxyl carbon (O=<u>C</u>) at 179.78, olefinic carbons (<u>C</u>H=<u>C</u>H) at 123.84 - 132.49, epoxy carbons (O-<u>C</u>-H) at 56.63 - 57.30, methylene carbons (<u>C</u>H$_2$)$_n$ at 22.55 - 34.25 and methyl carbon (<u>C</u>H$_3$) at 13.93.

Generally, in the synthesis of VOAC from VO, we can observe (Figure 10 top) clearly the appearance of new peaks on proton NMR at 10.56 ppm, is due to the hydroxyl groups of vernolic acid. On the other hand, the disappearance of glyceryl protons of vernonia oil (Figure 10 bottom) was observed on the proton NMR spectra of vernolic acid at 4.02 - 4.34 ppm.

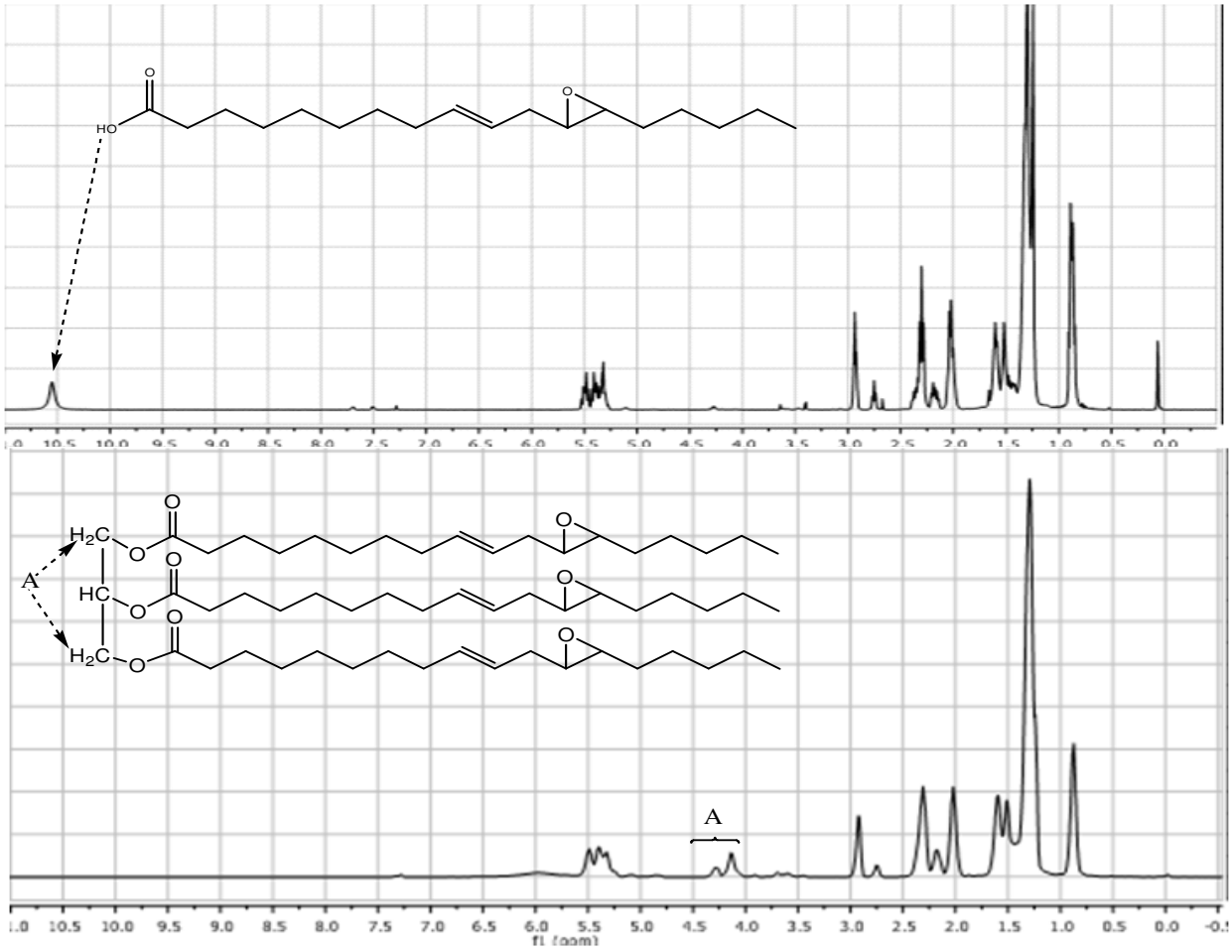


Figure 10: Comparison of the ^{1}H NMR spectra of VOAC (top) and VO (bottom)

Besides the proton NMR, the carbon NMR showed the appearance of a new peak (Figure 11 top) at 179.78 ppm is for the carboxyl carbon of vernolic acid. On the other hand, the disappearance of peaks at 173 ppm is due to carbonyl carbons of triglyceride structure of VO and a peak between 62 - 69 ppm also disappears on the spectra of VOAC is due to the glyceryl carbons of VO (Figure 11 bottom).

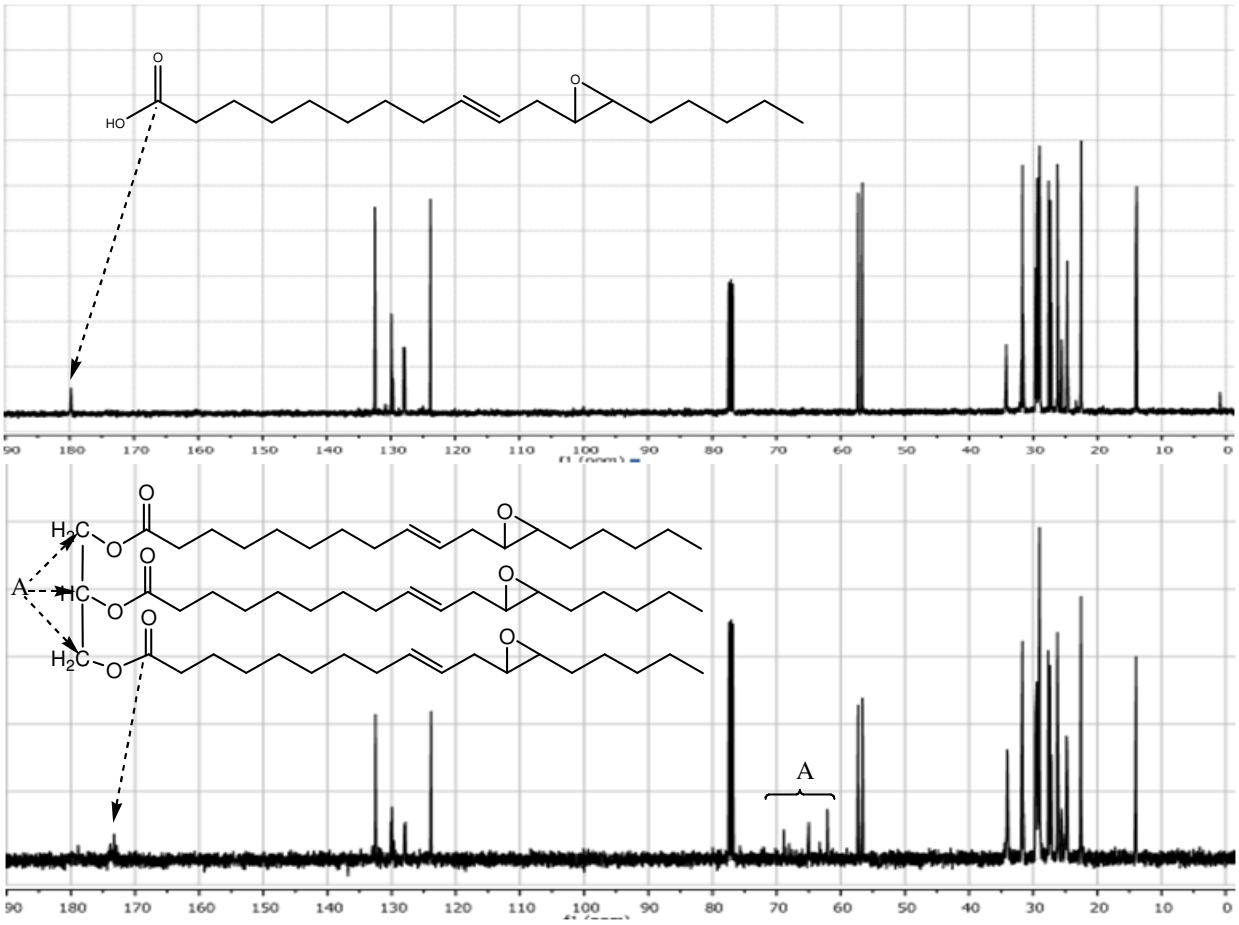


Figure 11: Comparison of the ^{13}C NMR spectra of VOAC (top) and VO (bottom)

Table 3: The integral peak areas of the functional groups of VOAC.

Integral Peak areas of different functional groups of VOAC				
Compounds	Double bond	Epoxy	Methyl	Hydroxyl
Vernolic acid	1	0.71	1.89	0.47
Theoretical value	1	1	1.5	0.5

The integral peak areas of the functional groups of VOAC containing the double bond, the epoxy, hydroxyl and methyl protons are shown in the above Table 3. The peak area integrations of the hydroxyl group approximately match with the theoretical value.

After the hydrolysis of VO to VOAC, the amount of epoxide was low, because the acid obtained may undergo ring opening reaction or other forms of reaction on the epoxide group of vernolic acid. In addition the presence of non-epoxidized saturated fatty acids impurities in VO will definitely increase the methyl protons ratio.

From the above integral peak areas, the methyl signal for VOAC is too high because of the presence of non-epoxidized saturated fatty acids and other free fatty acids from the starting material, VO.

5.1.4. Analysis of methyl vernolate

After esterification of VO with sodium methoxide the product obtained was analyzed by NMR. The NMR data for methyl vernolate (light yellowish color) indicated the presence of major functional groups namely the ester, the double bond, the epoxy and the methoxy.

As seen from the experimental part the ^{1}H NMR of VOME (Appendix 7 or Table 5) indicated the presence of olefinic protons (C<u>H</u>=C<u>H</u>) at 5.29 - 5.57, methoxy protons (O-C<u>H</u>$_3$) at 3.62 - 3.74, epoxy protons (O-C-<u>H</u>) at 2.74 - 2.98, protons of methylene attached to olefinic group (-C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-) at 1.99 - 2.42, protons of methylene groups (C<u>H</u>$_2$)$_n$ at 1.21 - 1.67, protons of methyl (C<u>H</u>$_3$) group at 0.84 - 0.95.

The ^{13}C NMR also confirms that VOME (Appendix 8 or Table 6) spectra showed the presence of the carbonyl carbon (O=<u>C</u>) at 174.28, olefinic carbons (<u>C</u>H=<u>C</u>H) at 123.79 - 132.49, epoxy carbons (O-<u>C</u>-H) at 56.42 - 57.16, methoxy carbon (O-<u>C</u>H$_3$) at 51.31, methylene carbons (<u>C</u>H$_2$)$_n$ at 22.59 - 34.18, and methyl carbon (<u>C</u>H$_3$) at 13.99.

In the synthesis of methyl vernolate (VOME) from VO, we can observe (Figure 12 top) clearly the appearance of new peaks on proton NMR spectra at 3.68 ppm, is the methoxy protons of VOME. On the other hand the disappearance of glyceryl protons of vernonia oil (Figure 12 bottom) was observed on the proton NMR spectra of VOME at 4.02 – 4.34 ppm.

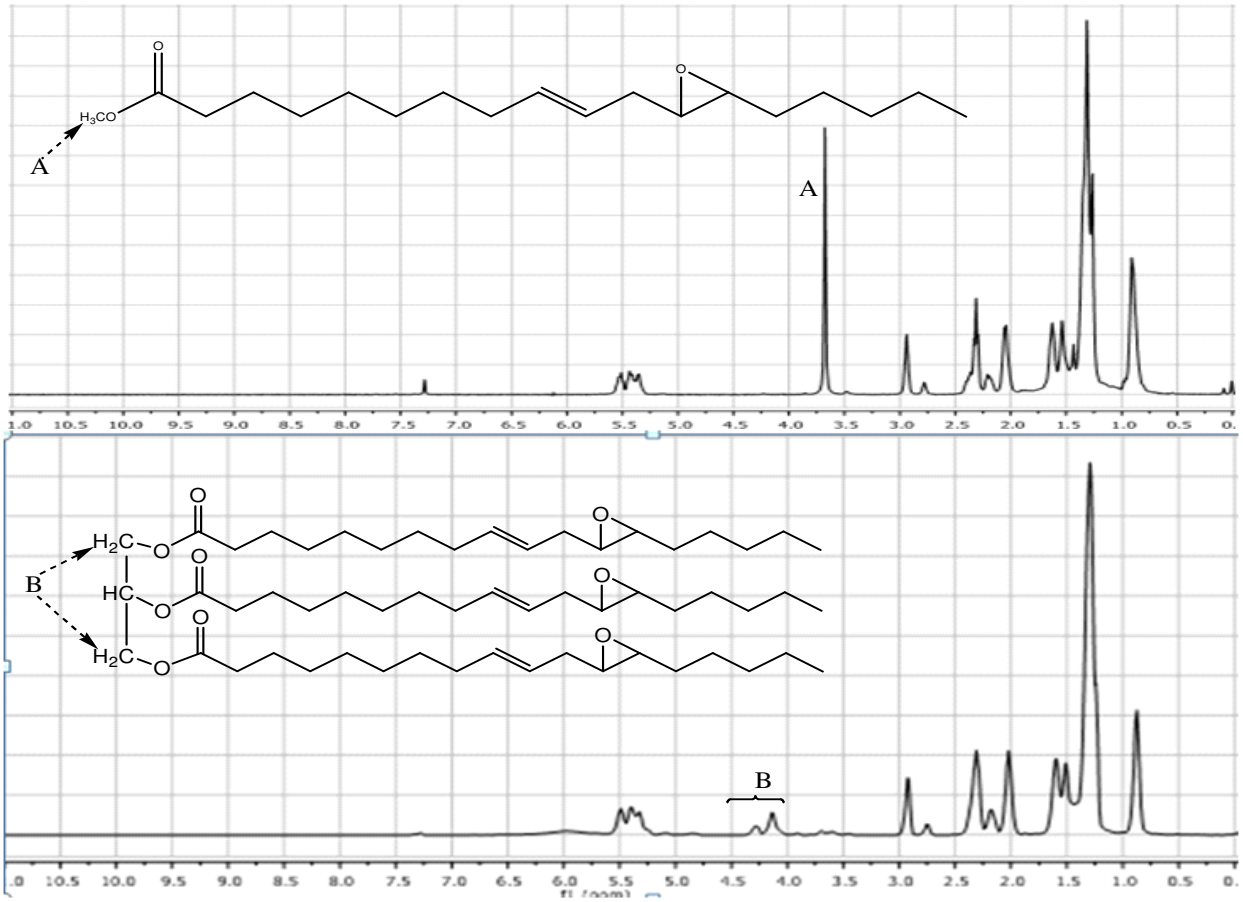


Figure 12: Comparison of the ^{1}H NMR spectra of VOME (top) and VO (bottom)

The ^{13}C NMR of VOME showed the appearance of a new peak (Figure 13 top) at 51.31 ppm is the methoxy carbon of VOME. Another new peak also observed at 174.28 ppm due to the ester carbonyl carbon of VOME. On the other hand the disappearance of the glyceryl carbons peaks between 62 - 69 ppm and carbonyl carbons of the triglyceride VO were observed on the spectra of methyl vernolate. The glyceryl and carbonyl carbon peaks were shown on the spectra of vernonia oil (Figure 13 bottom).

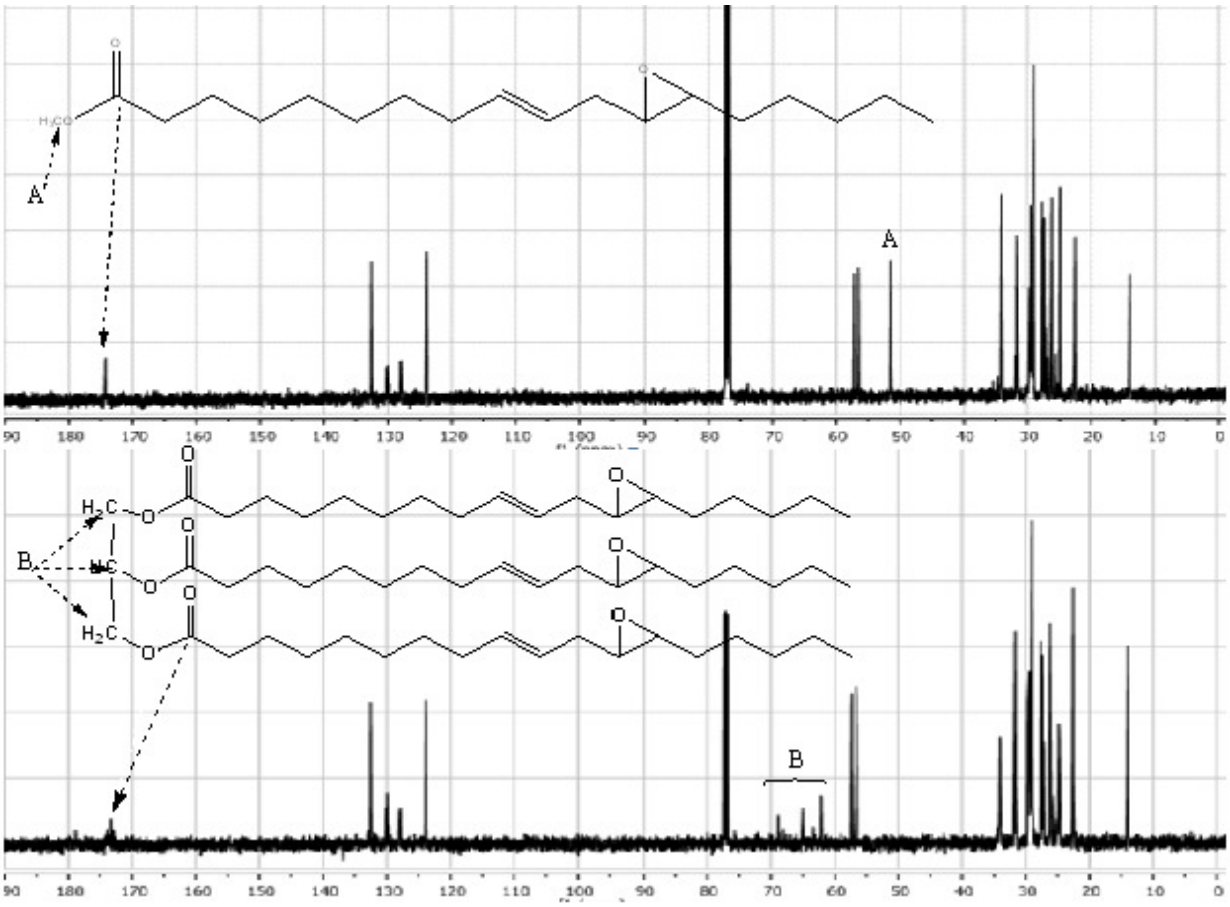


Figure 13: Comparison of the ^{13}C NMR spectra of VOME (top) and VO (bottom)

The integral peak areas (Table 4) of methoxy group with respect to the double bond showed slight decrease in value. This is because of the presence of non-esterified unsaturated fatty acids. The increase value of methoxy as compared to epoxy is due to the presence of other non-epoxidized saturated/unsaturated fatty acid esters of sodium methoxide. In addition, the methyl group ratio is too high because of the presence of saturated fatty acid esters.

Table 4: The integral peak areas of the functional groups of VOME.

Integral Peak area of different functional groups in VOME				
Compounds	Double bond	Epoxy	Methyl	Methoxy
VOME	1	0.74	2.2	1.40
Theoretical value	1	1	1.5	1.5

Generally, the ^{1}H and ^{13}C NMR chemical shift value for the starting materials are shown below in Table 5 and 6.

Table 5: ^{1}H NMR Chemical Shifts of crude VO, pure VO, VOME, and VOAC.

Types of protons	^{1}H NMR Chemical Shift data δ (ppm)			
	Crude VO	Pure VO	VOME	VOAC
Hydroxylic proton (-O<u>H</u>)	-	-	-	10.56
Olefinic protons (C<u>H</u>=C<u>H</u>)	5.17 - 5.48	5.22 - 5.56	5.29 - 5.57	5.26 - 5.55
Glyceryl proton (C<u>H</u>)	5.17 - 5.48	5.22 - 5.56	-	-
Glyceryl protons (C<u>H</u>$_2$)	4.02 - 4.28	4.02 - 4.34	-	-
Methoxy protons (O-C<u>H</u>$_3$)	-	-	3.62 - 3.74	-
Epoxy protons (-O-C-<u>H</u>)	2.66 - 2.91	2.71 - 2.98	2.74 - 2.98	2.71 - 2.97
methylene (-C<u>H</u>$_2$-CH=CH-C<u>H</u>$_2$-)	1.90 - 2.35	1.94 - 2.42	1.99 - 2.42	1.95 - 2.40
Methylene groups (C<u>H</u>$_2$)$_n$	1.16 - 1.69	1.18 - 1.68	1.21 - 1.67	1.18 - 1.68
Methyl (C<u>H</u>$_3$)	0.76 - 0.87	0.81 - 0.95	0.84 - 0.95	0.80 - 0.94

Table 6: ^{13}C NMR Chemical Shifts of crude VO, pure VO, VOME, and VOAC.

Types of carbon	^{13}CNMR Chemical Shift data δ (ppm)			
	Crude VO	Pure VO	VOME	VOAC
Carbonyl carbon (O=<u>C</u>)	173.33	173.27	174.28	179.78
Olefinic carbon (C<u>H</u>=C<u>H</u>)	123.82 - 132.40	123.86 - 132.53	123.79 - 132.49	123.84 - 132.49
Glyceryl carbon (<u>C</u>H)	68.88	68.88	-	-
Glyceryl carbon (<u>C</u>H$_2$)	62.00 - 64.86	62.01 - 64.90	-	-
Epoxy carbon (O-<u>C</u>-H)	56.41 - 57.17	56.41 - 57.16	56.42 - 57.16	56.63 - 57.30
Methoxy carbon (O-C<u>H</u>$_3$)	-	-	51. 31	-
Methylene carbons (<u>C</u>H$_2$)$_n$	22.50 - 33.90	22.55 - 33.88	22.59 - 34.18	22.55 - 34.25
Methyl Carbon (<u>C</u>H$_3$)	13.95	13.96	13.99	13.93

5.2. Enzymatic Synthesis of Sugar Fatty Acid Esters

In this research, SFAEs were produced by vacuum line. The experimental set-up is shown in Figure 14 below.

Figure 14: vacuum line reaction set-up

As can be seen the reaction flask was connected to the vacuum line so the water can be removed efficiently and continuously. The increase of dissolved glucose concentration and the continuous removal under vacuum of the water formed during the reaction time resulted in better conversion of the starting materials because the equilibrium was forced towards synthesis.

5.2.1. Physical characteristics

Some of the important physical characteristics of the substrates (starting materials) and esterified products and their solubility are listed in Table 7 and 8.

Table 7: Color, appearance and melting point value for products.

Compounds	Color	Appearance	Melting point (^{0}C)
Crude VO	Yellowish	Oil	-
Pure VO	Yellowish	Oil	-
Vernolic acid (VOAC)	Yellowish	Viscous Oil	-
Methyl vernolate (VOME)	Yellowish	Viscous Oil	-
D-Glucose	White	Powder	153-156 [50]
Starch	White	Powder	256-258 [50]
Glucose vernolate (VOAC) in [Bmim][TfO]	White	Solid	283-286
Glucose vernolate (VOME) in [Bmim][BF_4]	White	Solid	286-288
Starch vernolate (VOAC) in DMSO and [Bmim][PF_6]	Brown	Hard Solid	290-293
Starch vernolate (VOME)) in DMSO and [Bmim][TfO]	Brown	Hard solid	225-227

Table 8: Substrate and product solubility in different organic solvents

solvents	Compounds							
	Crude VO	Pure VO	VOAC	VOME	Glucose vernolate (VOAC)	Glucose vernolate (VOME)	Starch vernolate (VOAC)	Starch vernolate (VOME)
DMSO	+	+	+	+	-	-	-	-
Acetonitrile	-	-	-	-	-	-	-	-
Acetone	-	-	-	-	+/-	-	-	-
THF	+	+	-	-	+/-	-	-	-
t-Butanol	+	+	+/-	+/-	-	-	-	-
Pyridine	+	+	+	+	-	-	-	-
Hexane	+	+	+	+	-	-	-	-
Benzene	+	+	+	+	-	-	-	-
Chloroform	+	+	+	+	+/-	-	-	-
Water	-	-	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-
2-methyl-2-butanol	+	+	+	+/-	-	-	-	-

Soluble = +, insoluble = - , partially soluble = +/-

The melting points and solubilities of the products obtained after esterification reactions were measured (Tables 7 and 8) and showed a clear deviation from the values exhibited by their starting materials. This indicates that the identity of the product is different from that of its starting materials. The glucose vernolate from VOAC/VOME showed increase in melting point from the starting material, glucose. Their value also confirms that the glucose vernolate obtained from the two starting materials have a similar melting point value. However, starch esters from

VOAC showed high melting point while the starch ester from VOME were obtained with low melting points from their starting material, starch.

Comparisons were made between the solubility of the starch and glucose used as the starting material and the products obtained. The starting materials are soluble in most organic solvents tested. However, the ester products of glucose and starch were completely insoluble in all organic solvents. This indicates again that the identity of the product is different from that of the starting materials. However, as samples are not soluble in any of the available NMR solvents, in general, samples could not be analyzed by NMR. We have also tried using mass spectrometry to characterize glucose vernolate from VOAC however; due to the solubility problem the sample could not be analyzed effectively.

As the product was expected to have ampiphilic nature, it should have dissolved in both polar and non-polar solvents. This indicates that the products may undergo a ring opening reaction at the epoxide site and may form a crosslinked polymer. In general, products were not soluble when totally washed and purified. The limiting factors in the synthesis may be due to unwanted side reaction caused by the presence of epoxy and double bond on vernolic acid which has a maximum probability for polymerization reaction. As fatty acids contain naturally occurring functional groups, such as hydroxyl and epoxide, which make them candidates for ring opening polymerization to form polymer networks by direct crosslinking [54].

Only one product, the glucose vernolate obtained after reaction with VOAC, was found to be partially soluble in the NMR solvent used, $CDCl_3$. The reason for this was, the presence of unwashed ionic liquid in this solution might acting as a co-solvent and favor the dissolution of the product.

Table 9: Solubility of the starting materials in most organic solvents

Solvents	Glucose (mg/mL)	Starch
Hexane	insoluble [35]	insoluble
DMSO	540** [51]	Practically insoluble [51] but gelatinized at 70^0C
$CHCl_3$	insoluble	insoluble
Water	1,207 mg/mL ** [41]	insoluble
CH_3OH	7.2**[52]	insoluble
Acetone	0.348* [35]	insoluble

*= at 60^0C **= at 30^0C

5.2.2. NMR Analysis of Glucose vernolate from vernolic acid

As seen from the experimental part, the ^{1}H NMR data of the glucose ester (glucose vernolate) (Appendix 9) indicated the presence of protons of methyl group (H-14) at 0.83 - 0.92, protons of methylene groups (H-8 and H-13) at 1.22 - 1.68, protons of methylene attached to carbonyl carbon and olefinic carbons (H-7, H-9 and H-11) at 2.06-2.40, epoxy protons (H-12) at 2.66 - 2.98, glucose protons H-4, H-3 and H-2 at 3.37, 3.42 and3.60 respectively, (H-5) at 3.9, (H-6a and H-6b) at 4.78 - 4.84, olefinic protons (H-10) at 5.26 - 5.51 and the anomeric carbon proton (H-1) at 5.72 ppm.

The ^{13}C NMR data of glucose vernolate (Appendix 10) also indicates the presence of the methyl carbon (C-13) at 13.92, methylene carbons (C-8, C-10 and C-12) at 24.79 - 34.23, epoxy carbons (C-11) at 56.67 - 57.34, glucose carbons (C-6, C-4, C-5, C-3 and C-2) at 71.92, 72.2, 73.82, 74.05 and 75.74, respectively. The anomeric carbon shows a peak (C-1) at 99.98, olefinic carbons (C-9) at 123.68 - 132.55 and the carbonyl carbon (C-7) at 173.63. This result was in accordance to those in the literature.

The first eye catching feature in this NMR is the presence of peaks (Figure 15 bottom) at a chemical shift value of 9, 7.39, 7.37, 4.14, 3.90 (overlap with glucose proton), 1.8 ppm and 0.9 ppm (overlap with methyl protons). These peaks suggests the presence of the ionic liquid ([Bmim][TfO]), which was used as solvent in the reaction, within the sample. As commented in

the previous section the ionic liquid present in the sample could act as a co-solvent and this could be the reason of being able to dissolve this product and not the others.

Comparisons have been made between the proton NMR of the starting material and the product to confirm whether vernolic acid was converted into the vernolate. As seen from the two spectra (Figure 15), the hydroxyl protons of VOAC peak (Figure 15 top) at 10.56 ppm disappears in the spectra of glucose vernolate, which indicates that the acid is totally converted into ester form. The disappearance of anomeric hydroxyl of glucose on the spectra of glucose vernolate at 6.4 ppm indicates that it undergo esterification. Moreover, new peaks at around 4.75 appear confirming the presence of ester proton peaks. On the other hand, the appearance of new peaks from 3 - 5 ppm and from 5.5 - 6 ppm corroborates the presence of glucose in the sample. The combination of these two observations suggests the formation of glucose esters.

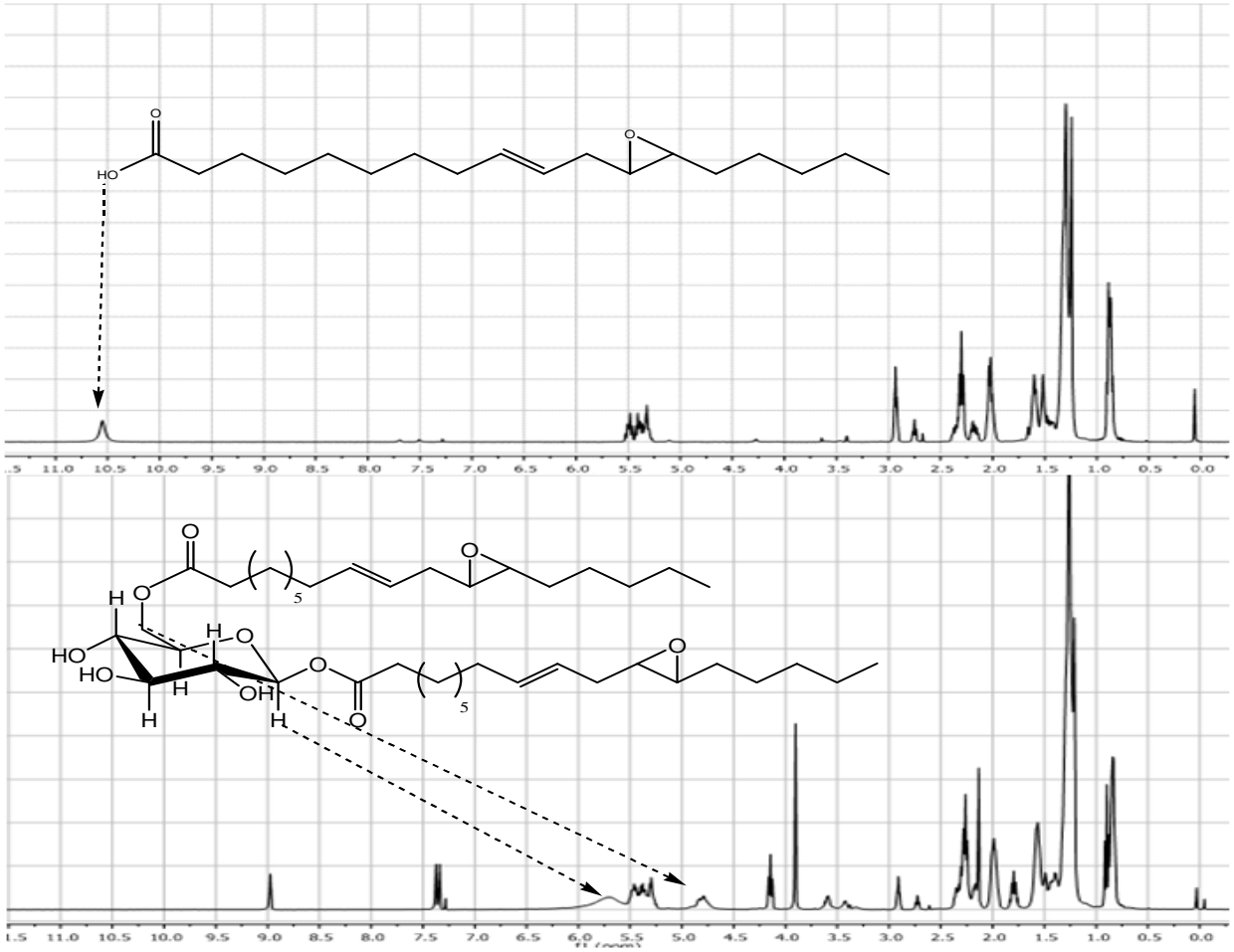


Figure 15: Comparison of the ^{1}H NMR spectra of VOAC (top) and glucose vernolate (bottom)

Comparisons have been made between the carbon NMR of the starting material and the product to confirm whether vernolic acid was converted into the vernolate form. The disappearance of carboxyl carbon at 179.78 ppm confirms that the acid is totally converted into glucose vernolate. The carbon NMR of the product also showed the appearance of a new peak (Figure 16 bottom) from 70-77 ppm and at 99 ppm. These are characteristic peaks of glucose carbons in glucose vernolate. Another two new peaks were observed at 174.32 and 178.3 ppm. This suggests the formation of two different types of vernolate esters, which suggests that the glucose molecule might have been di-esterified. The peaks around 13, 19, 36, 50, 119, 122 and 136 ppm are peaks of the carbons of the ionic liquids ([Bmim][TfO]).

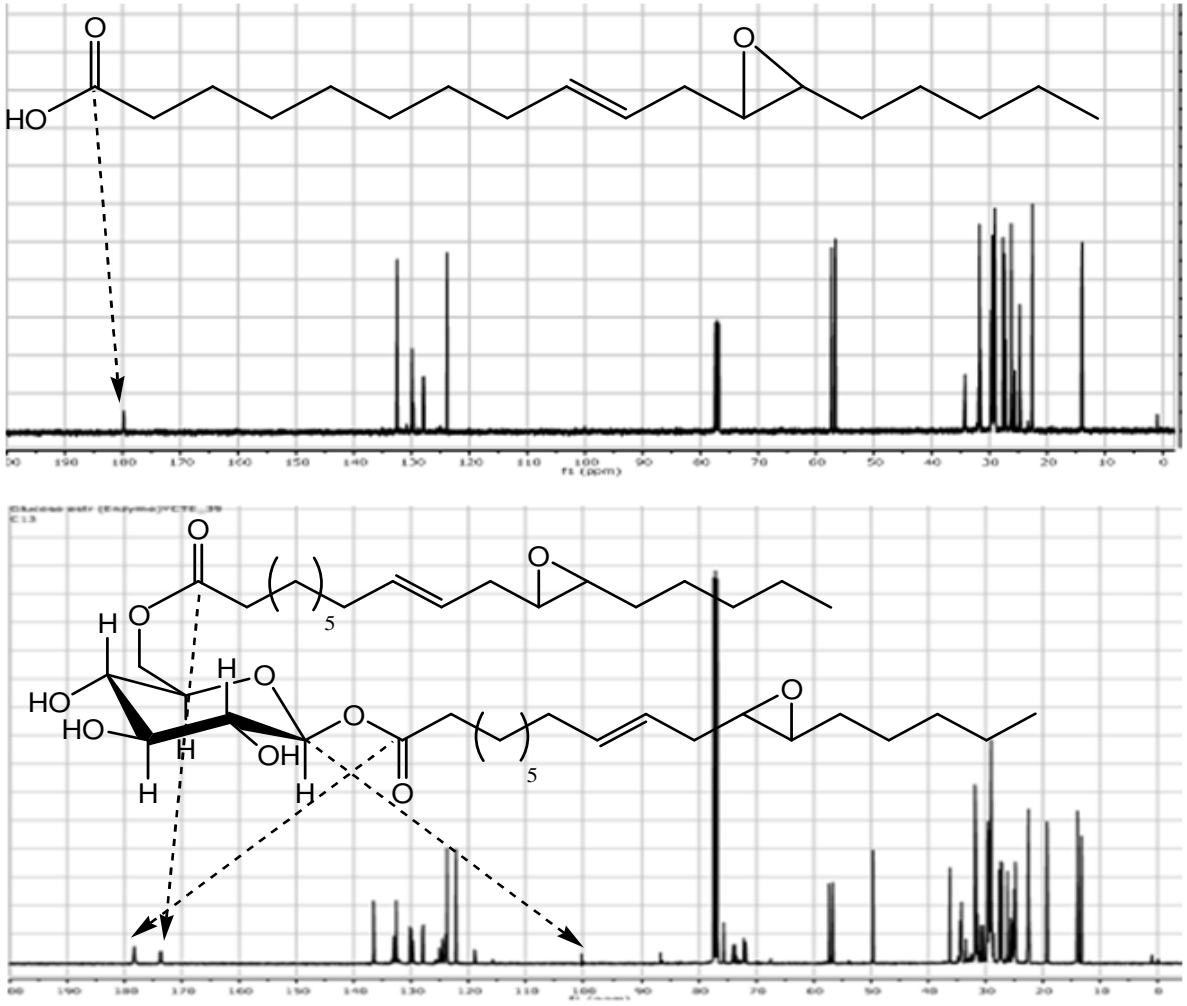


Figure 16: Comparison of the ^{13}C NMR spectra of VOAC (top) and glucose vernolate (bottom)

Table 10: peak area integration of the functional groups on the starting material and the product

Compounds	Double bond	Epoxy	Methyl ($-CH_3$)	acid-OH	Glucose-protons			
					H-1	H-2,3,4	H-5	H-6
VOAC	1	0.71	1.89	0.47	-	-	-	-
Glucose Ester	1	0.30	1.69	0.00	0.33	0.69	0.31	0.36
Theoretical (1:1)	2	2	3	0.00	1	3	1	2
Theoretical (1:2)	4	4	6	0.00	1	3	1	2
Theoretical (1:3)	6	6	9	0.00	1	3	1	2
Theoretical (1:4)	8	8	12	0.00	1	3	1	2
Theoretical (1:5)	10	10	15	0.00	1	3	1	2

Analysis of the areas underneath the characteristic signals from the proton NMR spectra of the starting materials (VOAC) and its product shows that the epoxy ratio on glucose vernolate decreases with respect to the double bond as compared to the vernolic acid. If there will be full conversion the peak area integration for epoxy protons of the product as well as the starting material should be the same, but only 42.25 % of the epoxy group in vernolic acid is converted to glucose vernolate. The rest of the epoxide groups of the acid may undergo reactions such as ring opening polymerization or epoxides on the disubstituted glucose ester may undergo direct-cross linking with each other or intra and intermolecular cross linking with free hydroxyl groups of one chain with another and results hydrolysis.

5.2.2.1. Degree of substitution (DS)

The DS is expressed as moles of vernolic acid residues per mole glucose units. The degree of substitution of glucose vernolate can be determined by the integration of ^{1}H NMR spectra using the area of vernolate olefinic protons (2 protons) as the reference for the fatty acid chain (using the peak at 5.4 ppm attributed to the double bond from the glucose vernolate) and the area of the protons characteristic of the glucose (7 protons) but one proton (H-5) overlap with the IL peak, so for the sake of accuracy only 6 protons were considered. Therefore, the degree of substitution was obtained by using the equation given below [53]:

$$DS = \frac{\text{moles of vernolic acid}}{\text{moles of glucose}} = \frac{6^* \text{ Ipa of vernolate olefinic protons}}{2^* \text{ Ipa of Glucose protons}} \quad \text{..... 4.1}$$

Where, Ipa is the integrated peak area of vernolate olefinic protons and glucose characteristic peaks in glucose vernolate.

As explained in section 5.2.2 glucose vernolate gives multiplet for two H-atoms of the olefinic group at 5.26-5.51 ppm in ^{1}H NMR spectra. We take that area as the reference for the NMR spectrum; therefore the area is equal to 1. The peak area integration for glucose protons in the glucose vernolate is 0.33 for the protons at 5.54-5.89 ppm, 0.36 for the protons at 4.7-4.9 ppm and 0.69 for protons at 3.26-3.6 ppm. The sum of the integral peak area of glucose protons in the glucose vernolate is 1.38.

$$DS = \frac{\text{Ipa of vernolate olefinic protons}}{\text{Ipa of Glucose protons}} = \frac{6^*1}{2^*1.38} = 2.17$$

The average degree of substitution was calculated by the above equation (4.1) and found to be 2.17. This indicates that in average, each glucose molecules at least di-substituted as the multiple peaks in the carbon spectra: 174.32 and 178.3 ppm were suggesting.

The reasons for this high degree of substitution may be due to the inefficiency of the enzyme to selectively mono-catalyze the esterification reaction. If the glucose is already monosubstituted and there is more fatty acid available this could result in another esterification taking place. Most probably as explained in section 1.2.3. (Figure 7) the anomeric hydroxyl proton shows more reactivity than the other secondary hydroxyl groups. Therefore the di-substitution of the substrate is on the anomeric hydroxyl group.

5.3. Lipase Activity

5.3.1. Lipase assay in the absence of ionic liquid

Enzyme activities were determined spectrophotometrically (GENESYS- UV/VIS Spectrometer) without exposing the enzyme in ILs ([Bmim][PF6]) at 410 nm by monitoring the liberation of p-NP against time. The absorbance and concentration are related by the Beer-Lamberts equation. The molar absorptivity coefficient value for para-nitrophenol is 15.1 M/cm at pH 8 [48].

$$A = \varepsilon lc$$

Where, A = absorbance, ε = molar absorptivity coefficient,

l = length of the cuvvet (1 cm), c= concentration

The concentration against different time interval was plotted.

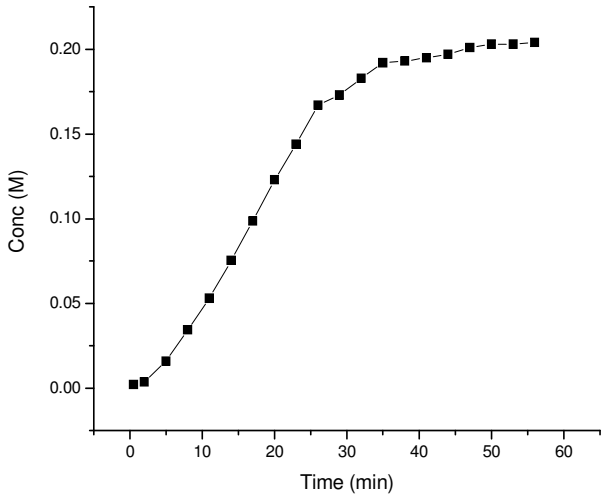


Figure 17: Plot of concentration vs time for enzyme activity test in the absence of IL at 35^0C

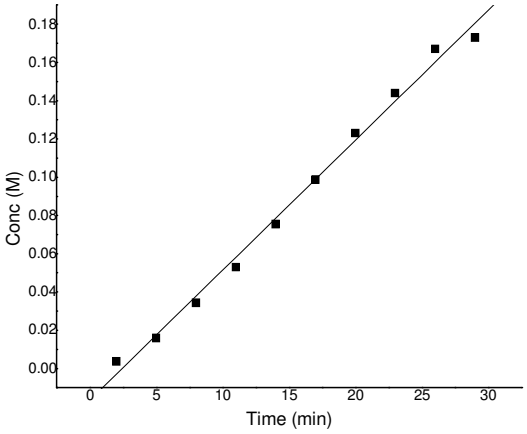


Figure 18: Slope for concentration vs time of enzyme activity test in the absence of IL at 35^0C

The slope of the initially linear portion of the plot indirectly represents the maximum rate of substrate hydrolysis as a function of time. The time in which the concentration showed sharp increase was taken and its slope was calculated and found to be 0.00679. Therefore, the activity which is the amount of para-notrophenol liberated from para-nitrophenyl palmitate was calculated and the amount is 0.00679 M/min, which is the same as 6.79×10^{-6} mol/min/mL = 6.79 U/mL.

5.3.2. Lipase assay in the presence of ionic liquid

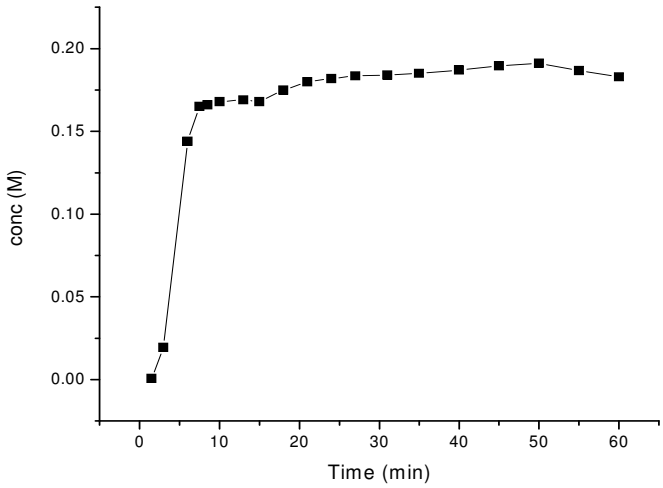


Figure 19: plot of concentration vs time for enzyme activity test in the presence of IL at 35^0C

However, after the enzyme was exposed in [Bmim][PF6] IL then the concentration showed sharp increase at the 3rd minute, but after the 10th minute the reaction rate become slower and kept constant throughout the experiment. This result shows that the presence of ILs as a reaction media will increase the activity of the enzymes.

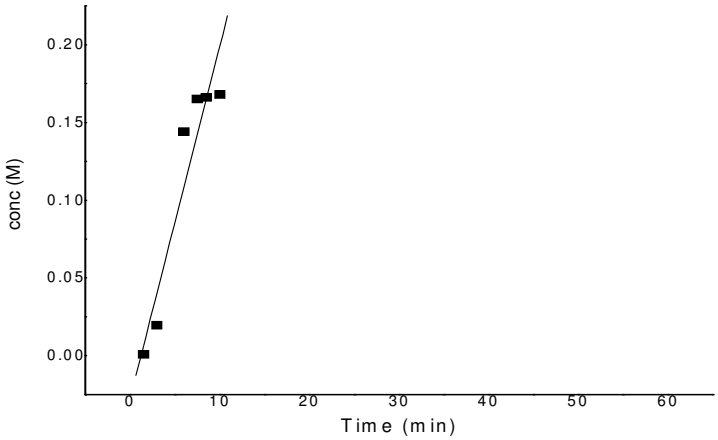


Figure 20: slope for concentration vs time of enzyme activity test in the presence of IL at 35^0C

From the above plot the time in which the concentration showed sharp increase was taken and its slope was calculated and found to be 0.02267. Therefore, the activity was calculated and found to be 0.02267 M/min, which is the same as 22.67 U/mL (enzyme unit).

Table 11: Comparison of lipase assay in organic and ILs media

Lipase assay type	Activity (U/mL)
Lipase in IL ([Bmim][PF6])	6.79
Lipase in the absence of IL	22.67

Therefore, we concluded that using ILs as media in enzymatic esterification reaction will increase the activity of the enzyme.

6. CONCLUSION AND FUTURE WORK

The synthesis of glucose/sugar esters from vernonia oil starting materials has been attempted via enzymatic reactions using an ionic liquid as a solvent and performing the reaction under vacuum to eliminate the water formed during the reaction.

Initial steps in the research work include the characterization of the Vernonia oil extracted by NMR. The crude oil contains a lot of saturated fatty acid impurities however, after the purification step the amount of impurities in the oil decrease as confirmed from the NMR data. The purified oil was derivatized into other compounds such as vernolic acid (VOAC) and methyl vernolate (VOME) and their successful synthesis was confirmed via NMR. VOAC and VOME were then used as the starting materials for the esterification of carbohydrates.

Esters of glucose were synthesized using enzymatic methods of esterification process. The increase of dissolved glucose concentration and the continuous removal of the by-product water under vacuum resulted in better conversion of the starting materials because the equilibrium was forced towards synthesis.

The esterification products of glucose and starch were completely insoluble in all organic solvents. Only in one case glucose vernolate from VOAC was found partially soluble in $CHCl_3$. This was probably due to the presence of ionic liquid ([Bmim][TfO]) which acts as a co-solvent and favor the dissolution of the product in the system as confirmed by NMR. For this reason, only glucose vernolate obtained from VOAC was analyzed by NMR.

NMR analysis confirmed the formation of glucose esters and the average degree of substitution was calculated and suggested the formation of disubstituted product (DS = 2.17). The insolubility of the product could be due to unwanted side reaction caused by the presence of epoxide and double bond groups on vernolic acid/methyl vernolate which could undergo polymerization reactions via ring opening. The formation of polymers would also explain the low solubility of the products. Ring opening polymerization could result in the formation of polymer networks by direct crosslinking of the fatty acid chains.

For the synthesis of starch esters the starting material starch was gelatinized in DMSO at 70^0C. As the product expected to have ampiphilic nature it should have dissolved in both polar and

non-polar solvents. However, the products obtained from starch were not soluble in any NMR solvents. This indicates that the products undergo a ring opening reaction at the epoxide site of VOAC/VOME and may form a crosslinked polymer due to secondary reactions of the reactive epoxide groups, like intra and intermolecular cross linking with free hydroxyl groups of polysaccharide chains and hydrolysis.

Enzyme activities were determined spectrophotometrically and those enzymes exposed in ILs ([Bmim][PF_6]) media showed higher activity than enzymes in the absence of ILs.

Future work should include the analysis of insoluble products obtained from the reaction of glucose/cassava starch and methyl vernolate by different techniques such as solid NMR, IR and x-ray crystallography. These analysis techniques should help in elucidating of the structure of the products. Moreover, the reactants should be taken in 1:1 proportion in order to have mono-substituted product.

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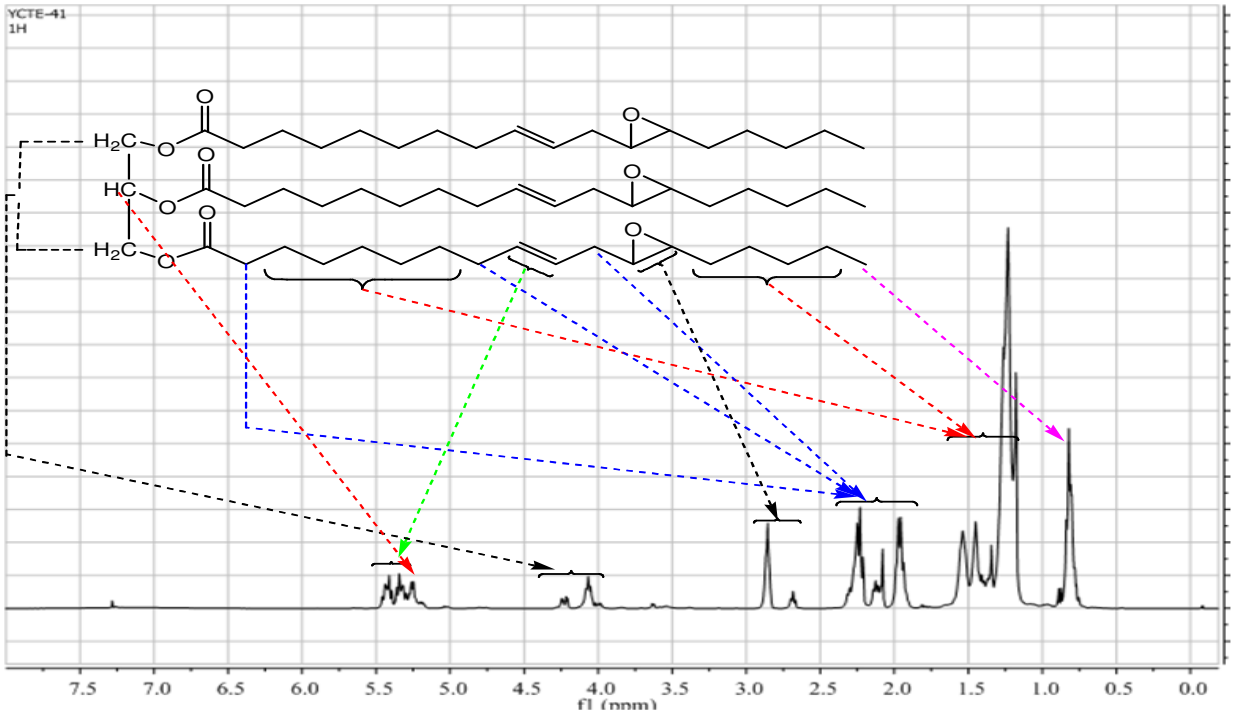
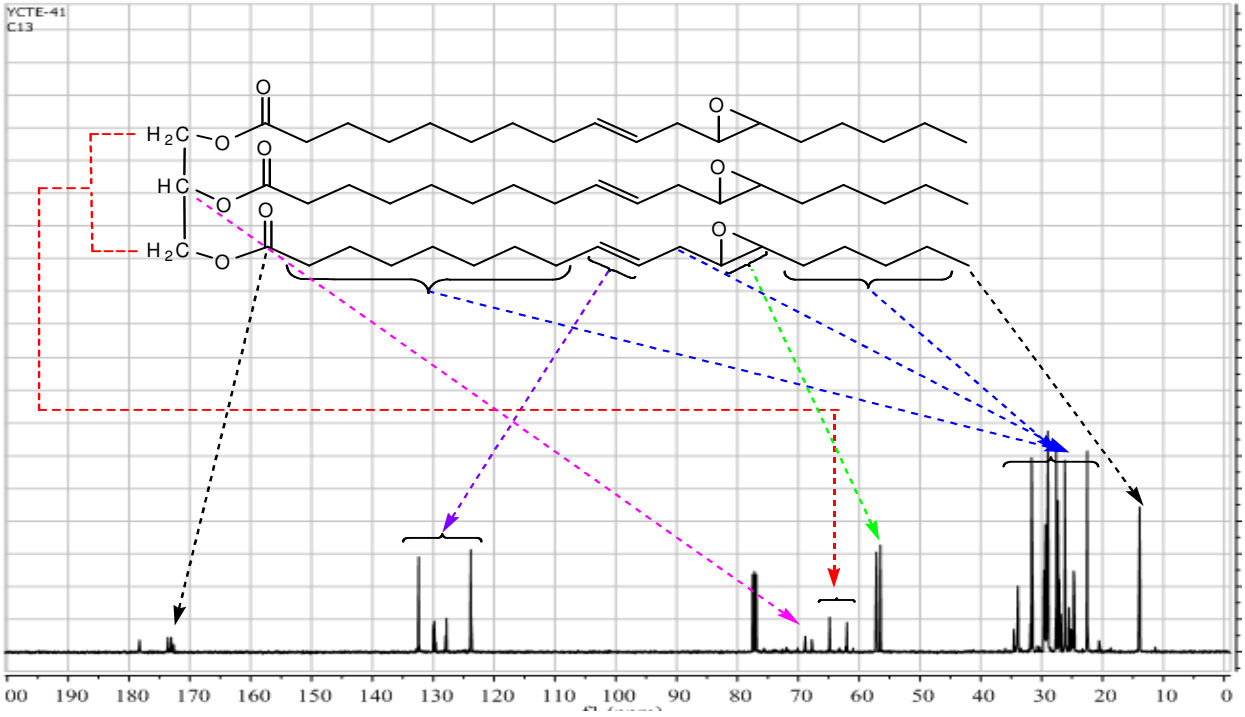
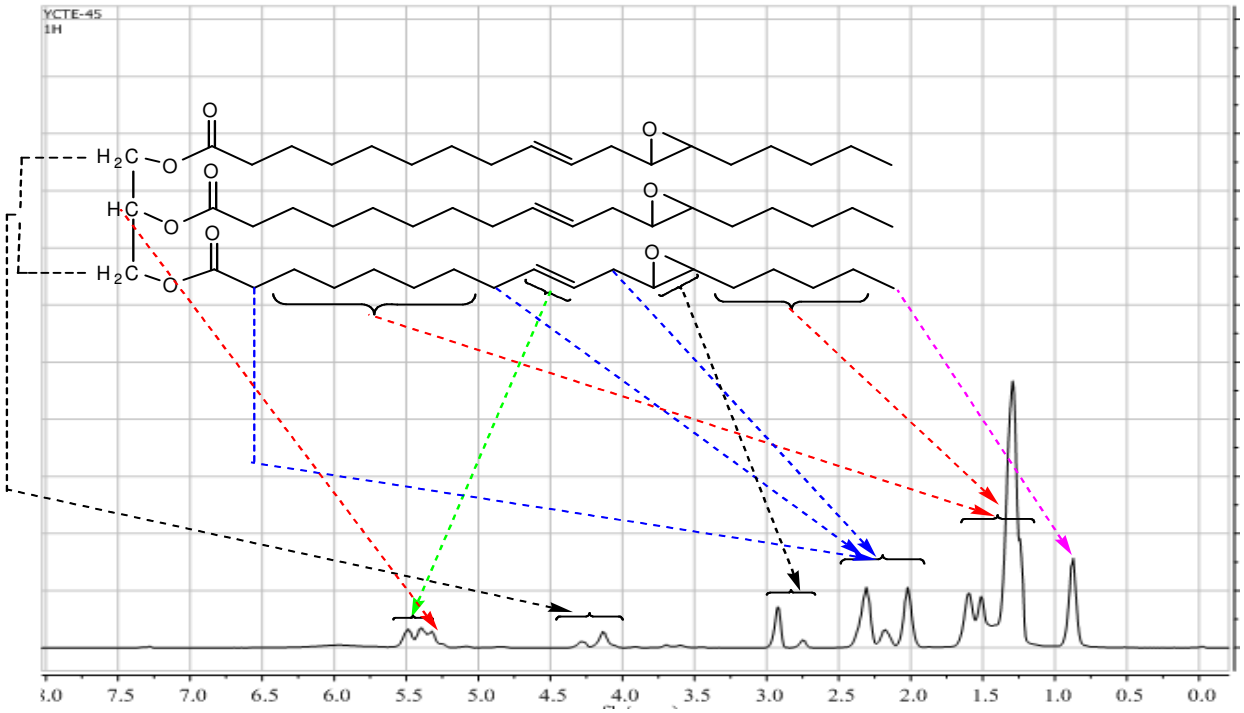
APPENDICES



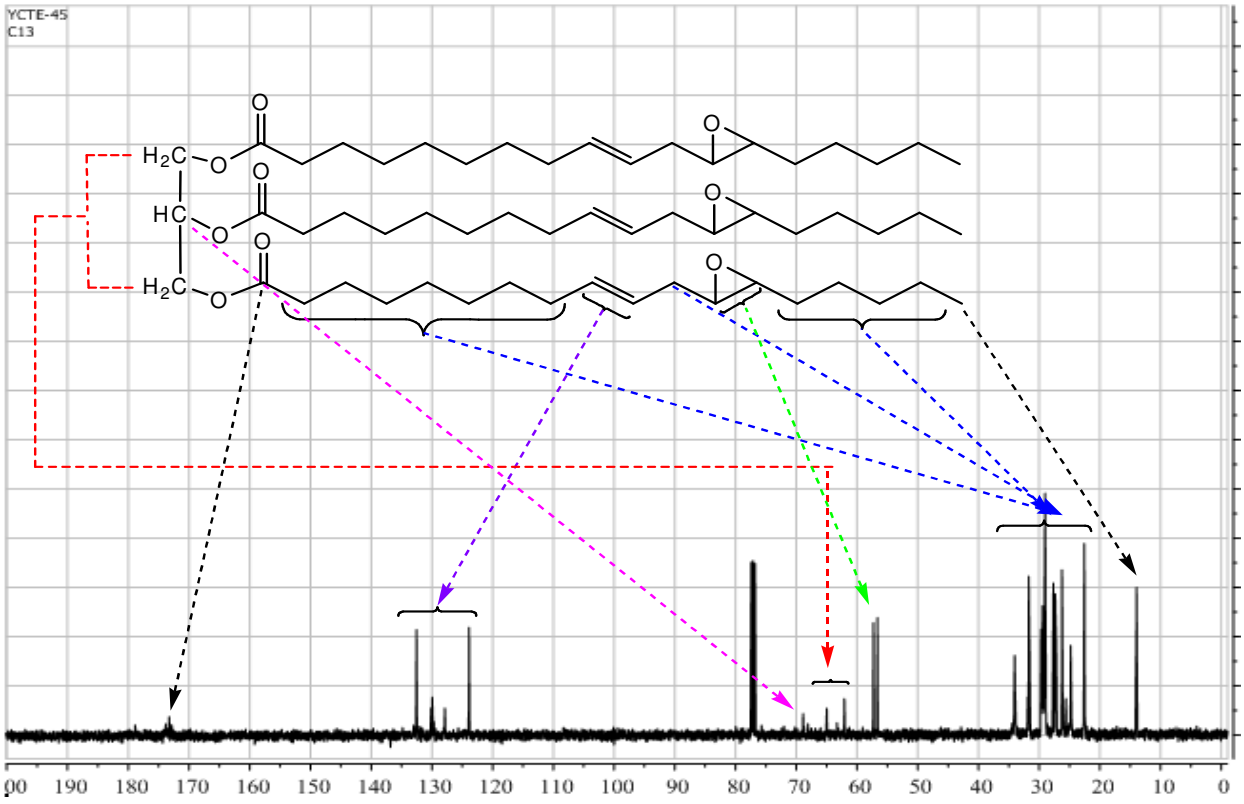
Appendix 1: ^{1} H NMR spectrum of crude VO in $CDCl_3$.



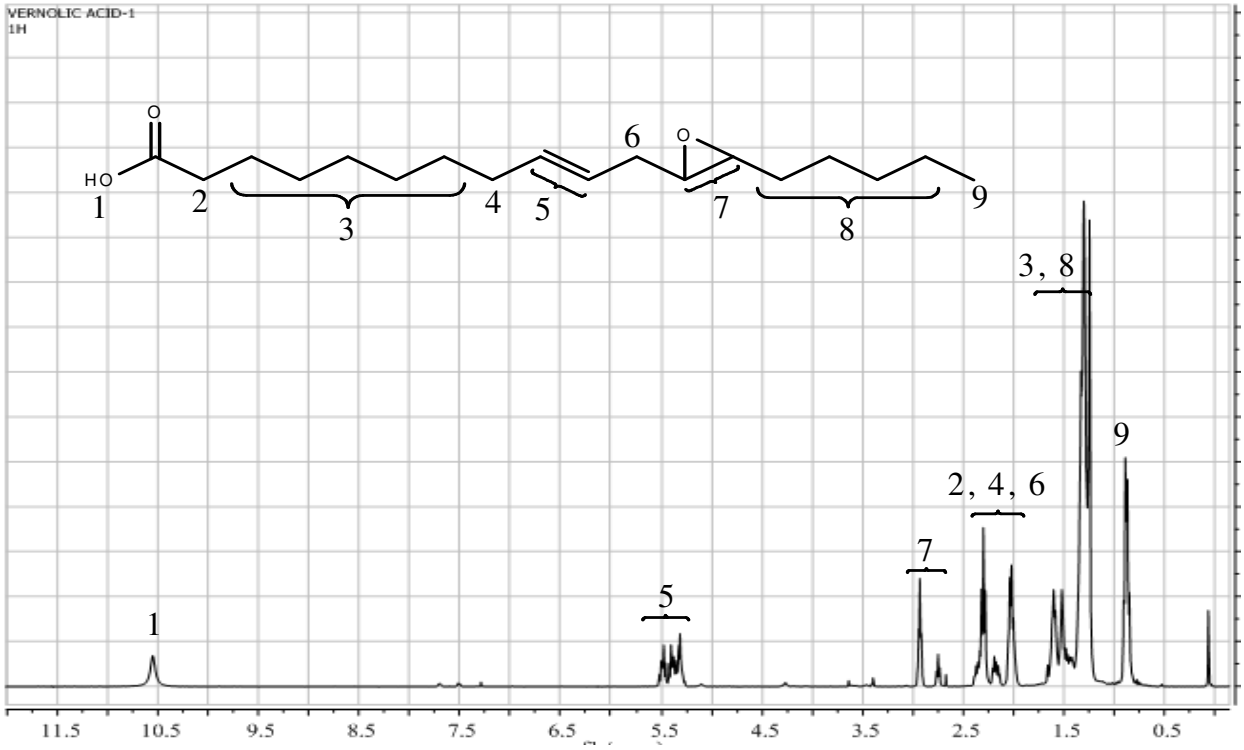
Appendix 2: ^{13}CNMR spectrum of crude VO in $CDCl_3$.



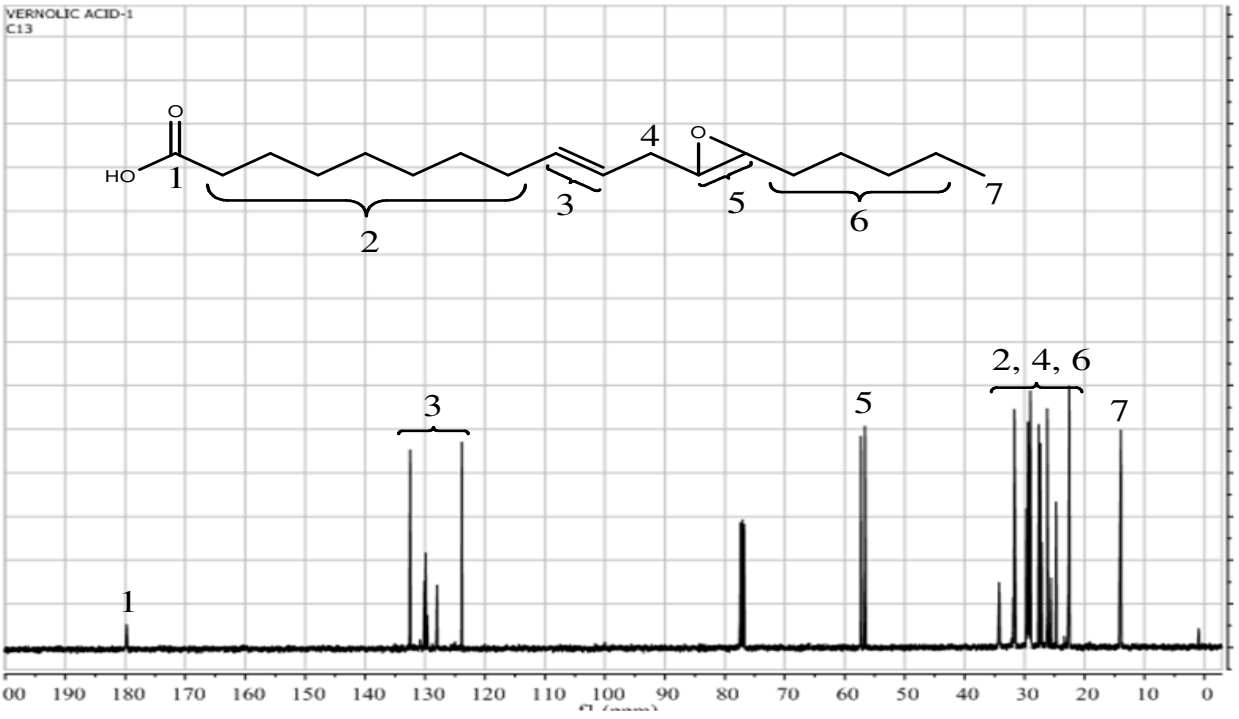
Appendix 3: ^{1} H NMR spectrum of pure VO in $CDCl_3$.



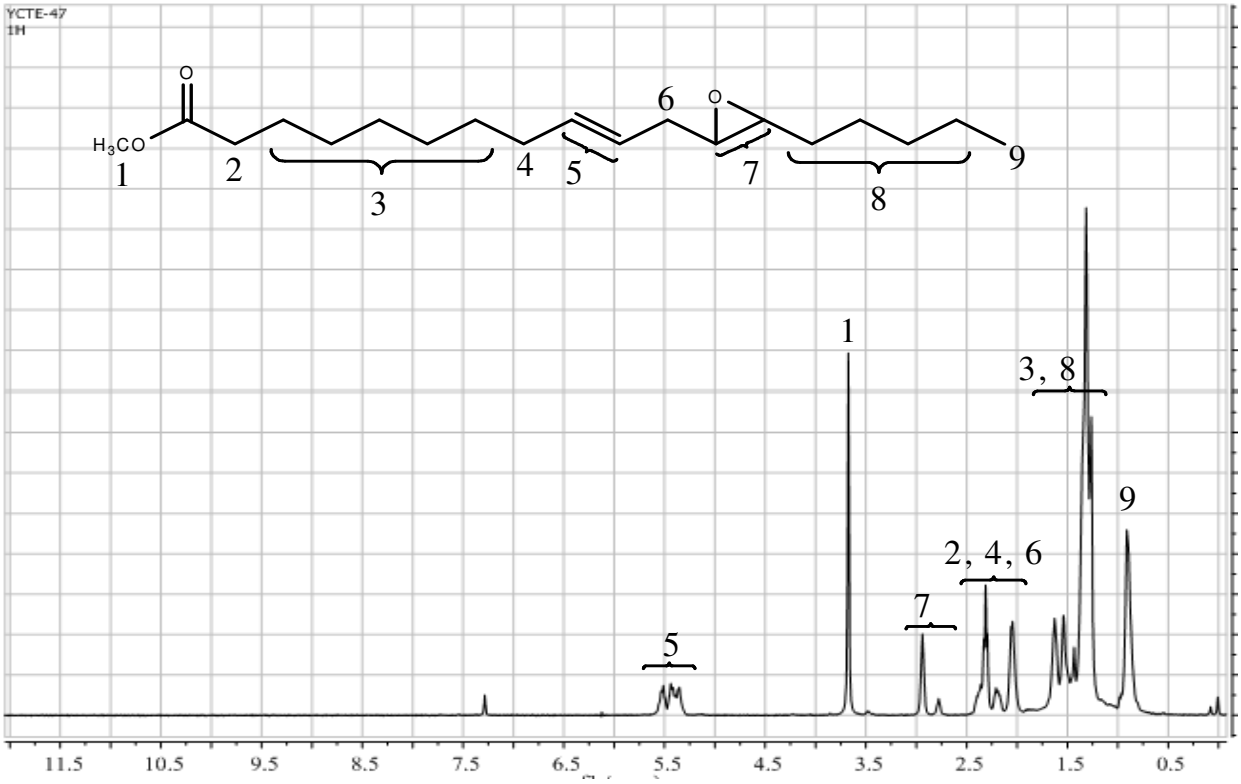
Appendix 4: ^{13}C NMR spectrum of pure VO in $CDCl_3$.



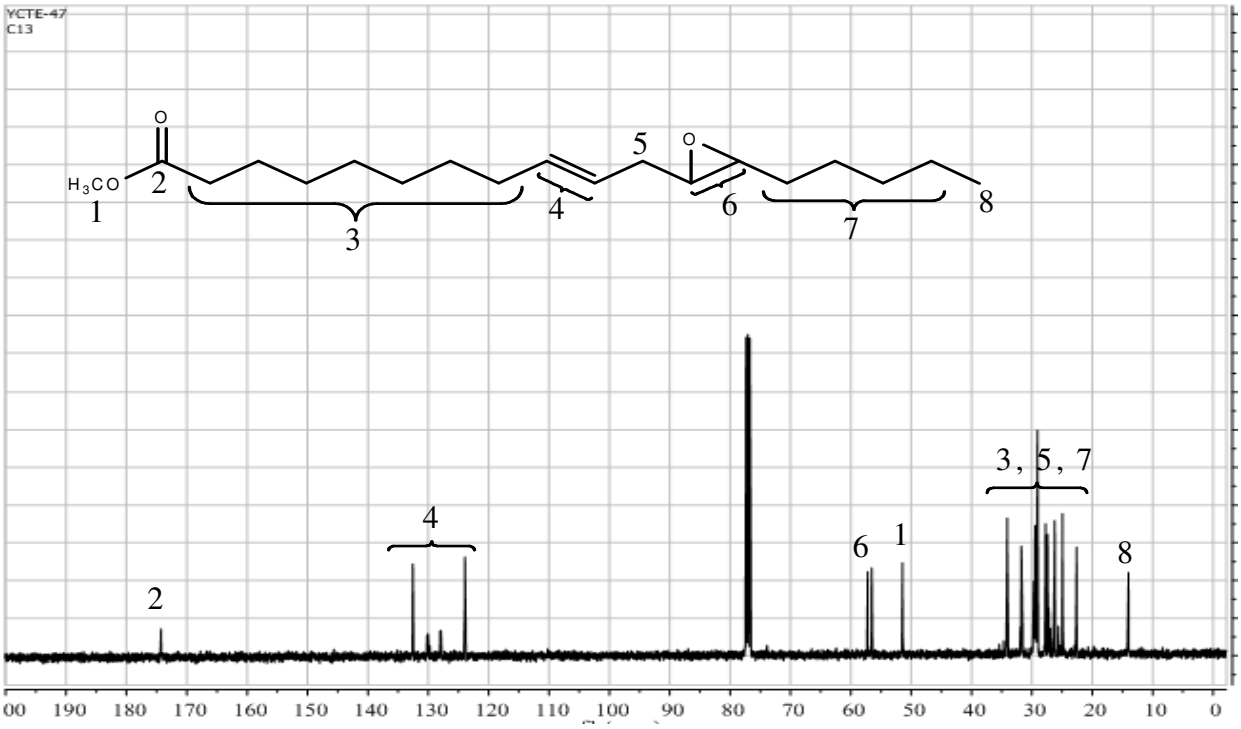
Appendix 5: ^{1}H NMR spectrum of VOAC in $CDCl_3$.



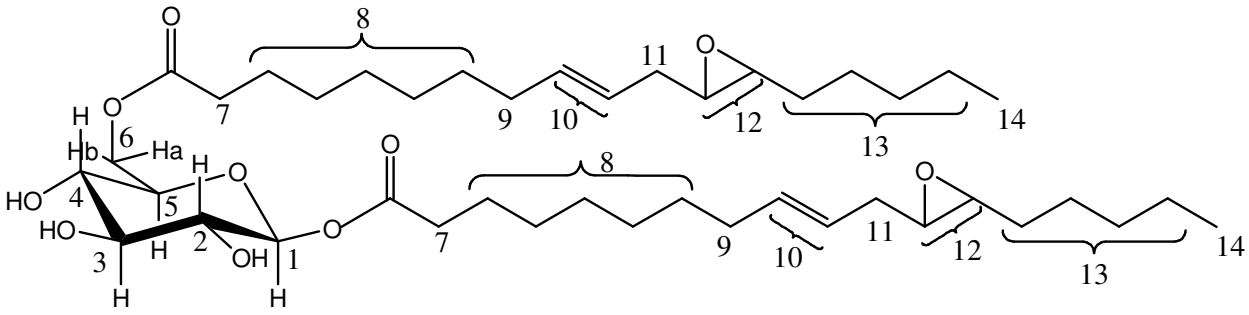
Appendix 6: ^{13}CNMR spectrum of VOAC in $CDCl_3$.

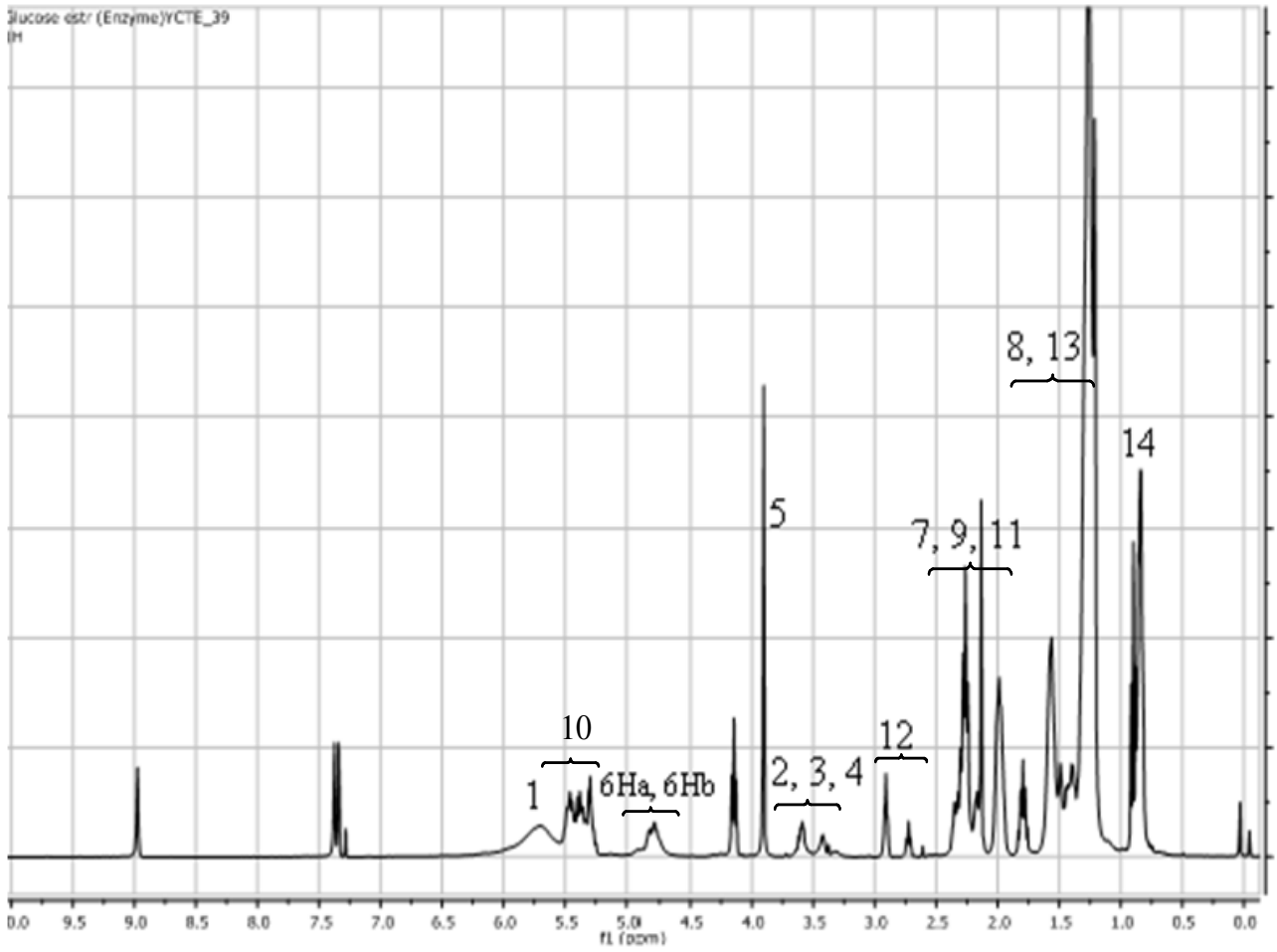


Appendix 7: ^{1}H NMR spectrum of VOME in $CDCl_3$.

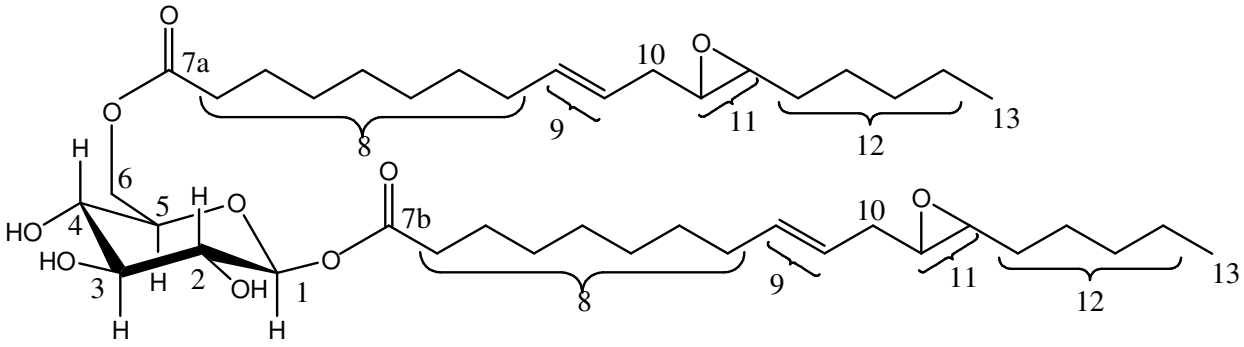


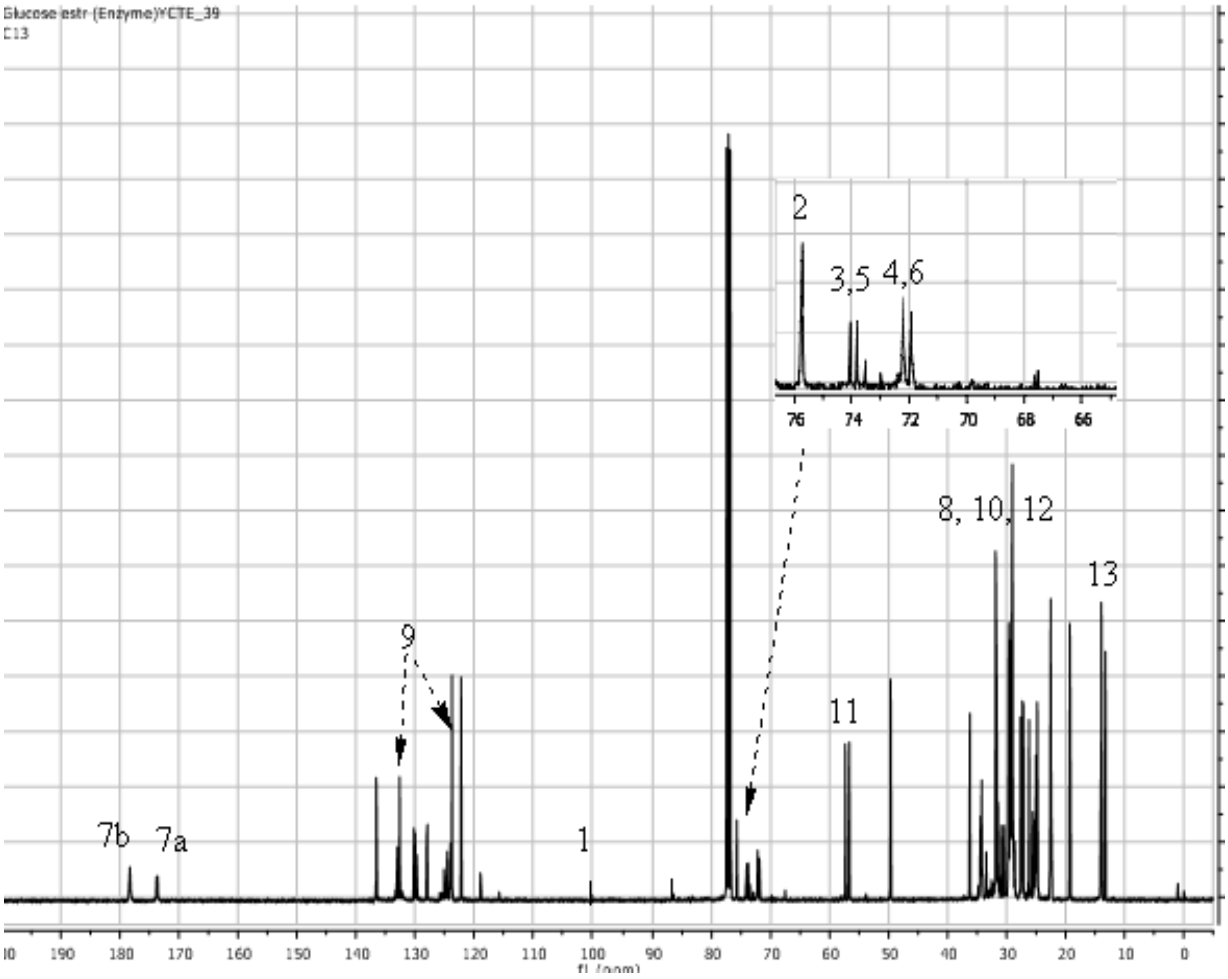
Appendix 8: ^{13}C NMR spectrum of VOME in $CDCl_3$.





Appendix 9: ^{1} H NMR spectrum of glucose vernolate in $CDCl_3$.





Appendix 10: ^{13}C NMR spectrum of glucose vernolate in $CDCl_3$.

DECLARATION

I the undersigned confirm that the results reported in this work were obtained by research carried out by me under the supervision of my advisor in the faculty of science, department of chemistry, Addis Ababa University in the academic year 2010-2011. No part of this work shall be published in scientific journals without the knowledge and consent of my advisor, who is the principal scientist responsible for any publication.

Name: Kumlachew Zelalem

Signature: _____

Date: _____

This MSc. Thesis has been submitted for examination with my approval as a university advisor.

Name: Dr. Ignacio J. Villar Garcia

Signature: _____

Date: _____

Place and date of submission:

School of Graduate Studies

Department of Chemistry

Addis Ababa University

June 3, 2011