Characterization of Native Vernonia Oil and the Preparation of Derivatives for Application as Performance Materials

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This is to certify that the thesis prepared by Tegene Desalegn entitled: "***Characterization of Native Vernonia Oil and the Preparation of Derivatives for Application as Performance Materials"*** and submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy (Inorganic Chemistry) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Characterization of Native Vernonia Oil and the Preparation of Derivatives for Application as Performance Materials

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In recent years, the interest in finding environmentally friendly or green biodegradable materials derived from natural resources as alternatives to non-biodegradable petroleum-based polymers has increased dramatically. *Vernonia galamensis* is a native plant of Ethiopia whose seeds contain a significant concentration of a unique naturally epoxidised triglyceride oil. This type of moiety is particularly useful as a cross-linking agent and synthetic building blocks and therefore, vernonia oil offers great potential for numerous applications. This thesis explores the synthetic possibilities of vernonia oil in order to try to maximise the prospects for its industrialisation.

For this study, native *vernonia galamensis* seeds obtained from Haromaya University and Adet Agricultural Center were used. All the products obtained from vernonia oil are shown in Figure 1.

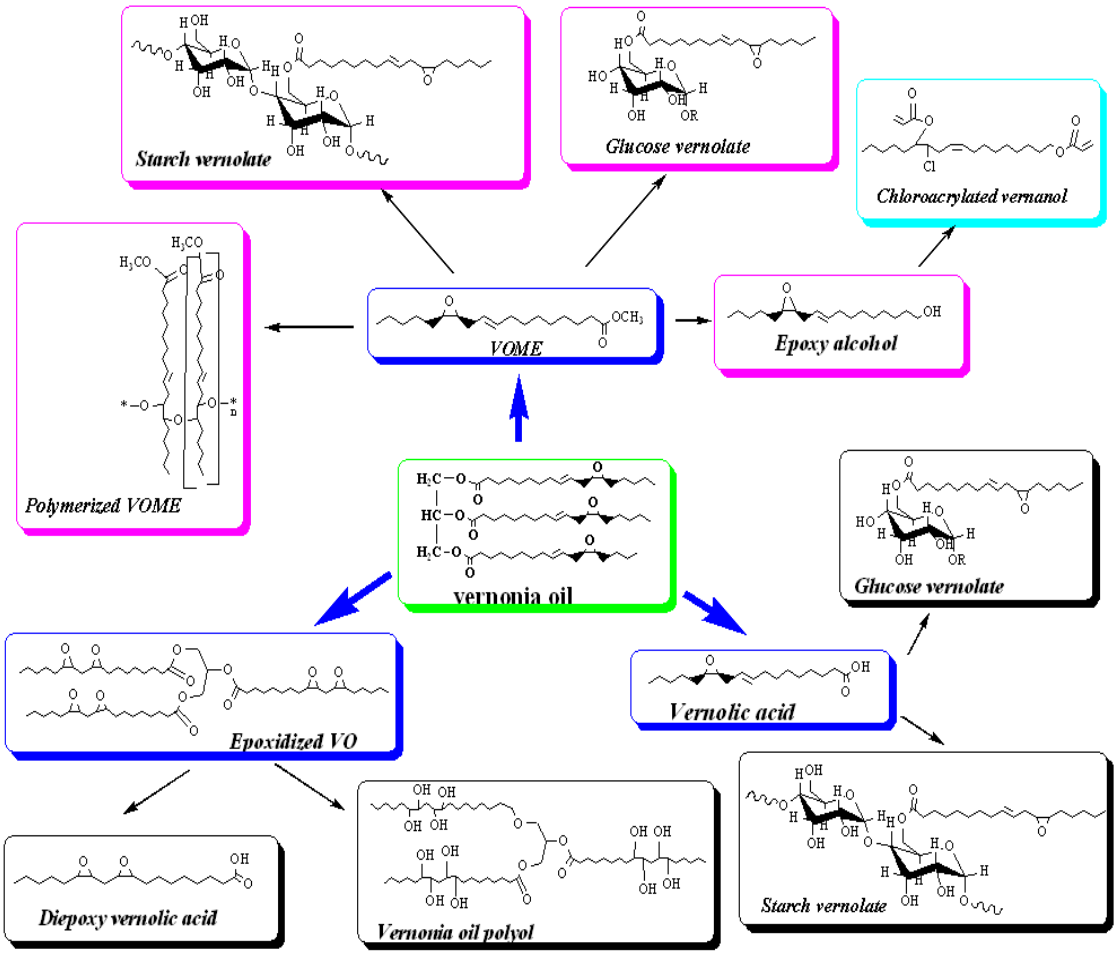

Figure 1: Schematic representations of derivatives of vernonia oil

The extracted vernonia oil, vernolic acid, vernonia oil methyl ester and vernanol were characterized using ^{1}H NMR, ^{13}C NMR, FTIR, MALDI-TOF MS, and TGA. The oil content of the plant and the concentration of the epoxy moiety were assessed. The vernonia oil derivatives such as vernolic acid, vernonia oil methyl ester, vernanol, were used as precursors in the synthesis of various value added products.

One of the major objectives of this thesis is to conduct preliminary studies for the synthesis of polymeric materials using sugar fatty esters containing an epoxy group as the

starting material. New epoxy starch and epoxy glucose fatty acid esters were prepared by esterification and transesterification reactions employing different reaction conditions, using both chemical and enzymatic synthesis and organic solvents and/or ionic liquids as solvents. Most of the products were found to be insoluble in NMR solvents, which complicated their characterisation. However, they were successfully characterised using a large array of techniques such as solid state NMR, FTIR, MS, DSC, TGA, powder XRD and SEM. All techniques confirmed the esterification of the sugars in a different degree of substitution, which were evaluated via titration methods. The stability of the epoxy group under the different experimental conditions was also evaluated. The degree of substitution and presence of epoxy group could also be related to properties such as the thermal stability or the morphology of the sample. The data collected helped to identify the most useful synthetic methods for further testing.

Ring opening polymerisations of vernonia oil and vernonia oil methyl ester were also successfully carried out. These studies contain useful information that will be used in the future work for the design of the polymerisation reactions of the epoxidized sugar esters.

Different modifications of vernonia oil and its derivatives were performed in order to increase the scope of applications of materials available from vernonia oil. These include epoxidation of vernonia oil, preparation of vernonia oil polyol and hydroxylation of VOME. In all cases, ^{1}H NMR, ^{13}C NMR, FTIR, ESI-MS results obtained confirmed the synthesis of the intended products.

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Table of Contents

List of Figures

List of Tables

List of Schemes

Abbreviations

2M2B……2-methyl-2-butanol

AGU……..Anhydroglucose unit

APGs …….Alkyl polyglycosides

BMIM…….1-butyl-3-methylimidazolium

CALB…….*Candida antarcitca* lipase B

DMF……Dimethylformamide

DMSO ……Dimethylsulfoxide

DSC…….**....**Differential Scanning Calorimetry

ESI-MS……Electron Spray Ionization Mass Spectrometry

FAME……. Fatty Acid Methyl Ester

IL ……Ionic liquid

MALDI-TOF MS …..Matrix Assisted Laser Desorption Ionization Mass Spectrometry

MEK……..Methyl ethyl ketone

PXRD…….X-ray powder diffraction

SEM……...Scanning Electron Microscopy

SFAE……..**.**Sugar Fatty Acid Esters

TGA……Thermogravimetric Analysis

VO …….Vernonia Oil

VOA……..Vernolic Acid

VOME ……Vernonia Oil Methyl Ester

CHAPTER ONE

1. INTRODUCTION

1.1 Renewable Resources

Over the last century our dependence on fossil fuels as energy source for transportation, heating and the provision of industrial feedstock for a multitude of products that we use in every aspect of our daily lives has dramatically increased. Owing to the finiteness of fossil fuel resources, oil prices are inevitably escalating to an even greater level at a faster rate [1]. The growing demand for petroleum-based products, depletion of fossil oil reserves and the negative impact of petroleum based products on environment are the driving force in searching for alternative, sustainable and renewable resources of energy and industrial feedstock. Sustainable development requirements, *i.e.*, to meet the needs of current and future generations can only be maintained by a significant contribution of renewable raw materials in the chemicals industry particularly for non-fuel applications [2].

Recently, the rate of desertification of agricultural lands is increasing dramatically, with over one-third of our planet's land now becoming arid. The fast growing human population is responsible for the currently noticeable scarcity of water that crops require for their growth. The development of industrial crops for semiarid zones is important in both developing and developed countries. A majority of the principal crop plants of the world are not yet well adapted to arid lands, consequently, the development of industrial crops for cultivation in semi-arid zones is important to developing and developed countries alike [3, 4].

A number of agricultural research institutions including United States Department of Agriculture, USDA were involved in plant screening to identify renewable resources, especially new and unique crops which essentially could grow in arid and semiarid areas, noncompetitive with the existing crops, and with a potential to provide new products for industrial applications [5]. Recent reports indicate that crops such as *vernonia galamensis* are receiving significant attention as they can grow under low rainfall and are found to be most suitable for dry land farming [5, 6].

The past decade witnessed that a considerable attention has been focused on the use of renewable and biodegradable resources such as plant oils, as raw materials in the production of useful chemicals and new materials. This is motivated by the potential advantages offered by the bio-based resources, in environment compatibility and economical feasibility, as compared to the traditional petrochemical derivatives. Furthermore, the utilization of renewable raw materials, taking advantage of the synthetic potential of nature, can (in some cases) meet other principles of "green"/ sustainable chemistry, such as a built-in design for degradation or an expected lower toxicity of the resulting products [7]. The most widely applied renewable raw materials in the chemical industry for non-fuel applications include plant oils, polysaccharides (mainly cellulose and starch), and sugars [7].

1.2 Vegetable oils

Plant oils, naturally occurring triglycerides of fatty acid are among the major sources of renewable feedstocks for oleochemical industries [7]. They have the potential to provide functionally equivalent, renewable, biodegradable, less toxic and environmentally

friendly replacements for these finite fossil-based raw materials, provided that their composition can be matched to end-use requirements, and that they can be produced on sufficient scale to meet current and growing industrial demands [8].

The seed oils of plants are structurally similar to long chain hydrocarbons derived from petroleum, and thus represent excellent renewable resources for oleochemical production. About 15-20 % of the plant oils produced (15-20 million metric tons) are currently used in the non-food applications. These oils are competitive alternatives to petroleum-based products for the production of a multitude of specific products such as detergents, paints, plastics and lubricants. The sustainable nature of these oils will also benefit the environment particularly with respect to their biodegradable nature and the fact that they will result in an overall reduction in CO_2 production compared to fossil fuel derived feedstocks [9].

Vegetable oils represent one of the cheapest and most abundant biological feedstocks available in large quantities, and their use as starting materials offers numerous advantages, such as low toxicity and inherent biodegradability. They are primarily water insoluble, hydrophobic substances that are made up of one mole of glycerin and three moles of fatty acids, so called *triglycerides* (fig.1.1). The triglyceride structures of vegetable oils such as soybean oil, canola oil, rapeseed oil, sunflower oil, peanut oil, castor oil, palm oil, linseed oil etc., have different functionalities, such as double bond, allylic carbons and ester groups which are potential sites for chemical modification [10].

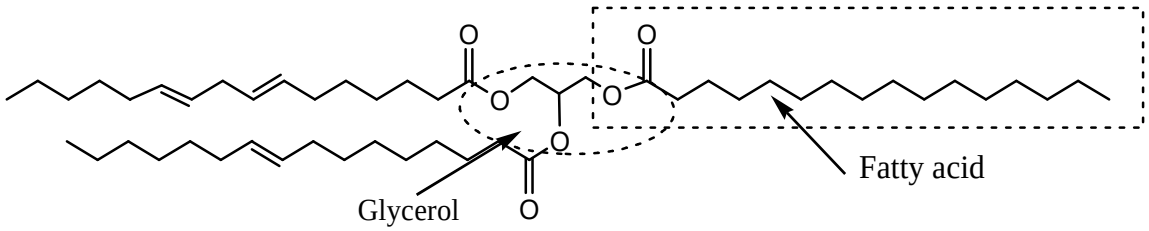

Figure 1.1*: Triglyceride structure of vegetable oils*

Approximately, 80 % of the world oil and fat production is obtained from vegetable oils while the remaining 20 % is of animal origin. Soybean oil makes about 25 % of this share followed by palm oil, rapeseed and sunflower oil [7, 92].

More than 95 % of the total weight of triglycerides is due to fatty acids, therefore, the properties and reactivity of triglycerides strongly depends on their composition [11]. Fatty acids contain carbon atoms ranging from C_8 to C_{24} in a linear chain, with the C_{16} and C_{18} being the most common. A broad variety of structurally different and 'unusual' fatty acids occur in the seed oils of plant species, with outstanding potential as feedstocks for industry. They include unusual monounsaturated fatty acids, medium, short, or very-long-chain fatty acids, fatty acids with additional functional groups such as epoxy and hydroxy groups, or fatty acids with conjugated double bonds [12].

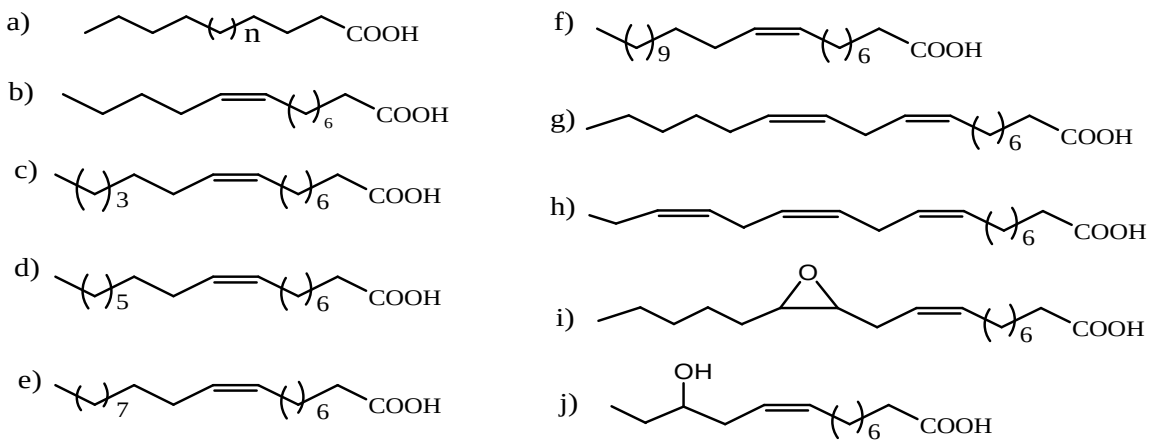

Figure 1.2*: Structures of the most common fatty acids:*

Saturated fatty acids (a), myristoleic acid (b), palmitoleic acid (c), oleic acid (d), gadoleic acid (e) erucic acid (f), linoleic acid (g), linolenic acid (h), vernolic acid (i), and ricinoleic acid (j)

Table 1.1 Names and descriptions of some of the common fatty acids [98]

Common Name	**IUPAC Name**	**Number of double bonds**	**Carbon number and symbol**
Lauric acid	Dodecanoic acid	0	C12:0
Myristoleic acid	Tetradecanoic acid	0	C14:0
Palmitoleic acid	9-Hexadecenoic acid	1	C16:1 n-7
Oleic acid	9-Octadecenoic acid	1	C18:1 n-9
Erucic acid	13-Docosonoic acid	1	C22:1 n-9
Linoleic acid	9,12-Octadecadienoic acid	2	C18:2 n-6
Linolenic acid	9,12,15-Octadecatrienoic acid	3	C18:3 n-3
Vernolic acid	12,13-epoxy-9-octadecenoic acid	1	C18:1 n-9

*n referes to the position where the first double bond appears in the structure of the fatty acid

Table 1.1A: Fatty acid composition (wt %) of some of the major oil crops [11, 13]

	Fatty acids									
Vegetable oil	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:1
Palm oil				5	36	2	50	8		
Soybean oil					11	4	23	54	8	
Canola oil					4	2	60	21	10	1
Sunflower oil					7	5	19	68		
Linseed oil					6	2	19	24	47	
Coconut oil	7	7	48	18	9	3	6	2		
Palm kernel	3	4	48	16	8	2	15	2		

The derivatization of oil-based fatty compounds has recently made significant progress. Chemical modification of fatty compounds is a key route to obtaining useful products from renewable resources [14]. Oleochemical industries have mainly focused on the carboxylic functionality of fatty acids; producing oleochemicals such as free fatty acids, methyl esters, fatty alcohols, and fatty amines, with glycerol as a by-product. Modern synthesis methods have been recently applied extensively to fatty compounds for the selective modification of the alkyl chain [14]. In this regard, epoxidation of fatty acids has achieved significant attention. Epoxidation is the process of formation of an oxirane (epoxy) group, *i.e.,* cyclic ether in which the oxygen atom is contained in a three membered ring, by the reaction of peroxyacids (peracids) and olefinic double bonds. It is one of the most important and useful modifications using the double bonds of unsaturated fatty compounds, since epoxides are reactive intermediates that readily generate new

functional groups [15]. Owing to the high reactivity of the oxirane ring, epoxides can find application as raw materials for preparation of a variety of chemicals, such as alcohols, glycols, alkanolamines, carbonyl compounds, and polymers like polyesters, polyurethanes, and epoxy resins [16].

Table 1. 2: Properties of the major vegetable oils [7, 27]

Vegetable oil	**Iodine value (g I_2/100g oil)**	**Oxirane number**	**Viscosity (cps at 20 °C)**
Soybean oil	130	-	94
Vernonia oil	89	4.26	210
Palm oil	51	-	130
Canola oil	114	-	78
Sunflower oil	133.3	-	64
Linseed oil	182	-	88
Olive oil	29	-	85

The unsaturated fatty acid content is quantified by iodine value as double bonds react with iodine to form saturated compounds. The amount of iodine reacted indicate the number of double bonds and hence the unsaturated fatty acid concentration in the oil. In general terms, the iodine value can be defined as the amount of iodine (grams) reacted with 100 grams of oil or fat [99].

Epoxidised oils, currently manufactured from animal fats or vegetable oils treated with peracetic acid or from petrochemicals are widely used in oleochemical industry as

plasticizers and stabilizers for thermoplastic products (*e.g.* PVC), in reformulation of oil based paints, in cosmetics, and for pharmaceutical applications [17, 18]. Seeds from crops, such as soybeans and linseed, are currently used as major source of unsaturated oils, which are prescursors to epoxidized oils. However, the chemical epoxidation process is expensive as well as environmentally damaging. Extensive search to identify plants not competing with existing crops as new sources of industrial raw materials were carried out by the United States Department of Agriculture in the 1950s [5]. Among the many species examined, *vernonia galamensis* native to East Africa received increasing attention due to the high concentration of naturally epoxidized oil in its seed which could be used as a potential substitute for currently used epoxy oils [19].

1.3 *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). The genus *vernonia* is one of the largest groups in the family *Asteraceae*. 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 [20]. *Vernonia galamensis* is known to naturally grow as weed in fields or in wood lands under a wide range of agro-ecological conditions of Africa [20, 21]. Perdue identified this plant in 1964 for the first time in eastern Ethiopia some 7 km south east of Harar town, 9°14' N and 42° 35' E [5]. 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 [3].

Vernonia galamensis is an underexploited potential oil-producing annual plant. Vernonia, being an endemic tropical plant is most suited for dry land farming requiring drained soil and can grow under low rainfall [6]. The *vernonia galamensis* species complex is now recognized to include six subspecies, namely *galamensis, mutomoensis, naironbensis, afromomntana, gibbosa, and lushotoensis* [6]. The subspecies *galamensis* is the most widely distributed, highly diverse and has four botanical varieties, namely variety *galamensis*, variety *petitiana*, variety *australis* and variety *ethiopica* [3, 4, 6]. *Vernonia galamensis* ssp. *galamensis* var. *ethiopica* grows naturally in marginal areas with as little as 200 mm seasonal rainfall and at an elevation ranging from 700–2400 m in the southern and southeastern parts of Ethiopia [6]. The Ethiopian *vernonia* species is known to be superior in terms of oil yield and vernolic acid content [22].

Figure 1.3*: Vernonia galamensis* plant

Reports have witnessed that different parts of Ethiopia are habitats of *vernonia galamensis* plant. Some of the geographical locations and coordinates where *V. galamensis* subspecies *galamensis* varieties were found include [23, 24]; Yirgalem (06° 42’ N, 38° 21’ E), Areka (06° 48’ N, 37° 43’ E), Leku (06° 52’ N, 38° 27’ E), Awassa

(06° 52’ N, 38° 27’ E), Arsi-Negele (07° 00’ N, 38° 35’E), Gelemso (08° 49’ N, 40° 31’ E), Melkabelo (09° 12’ N, 41° 25’ E), Harar Zuria (09° 19’ N, 42° 07’ E), and Metta (09° 25’ N, 41° 34’ E).

Vernonia galamensis, a crop native to Ethiopia, is receiving increasing attention due to the high concentration of naturally epoxidized oil [3, 4, 21]. Very recently, the plant was found to be a potential crop for inclusion into the agricultural system in Ethiopia. Among other characters, a seed yield up to 4000 kg/ha and oil content of 40 % using unimproved local materials was obtained which is much higher than found elsewhere [3, 21]. *Vernonia galamensis* being most suitable for dry land areas, essentially noncompetitive with the existing crops and could be a primary source of income for farmers.

The seeds of *vernonia galamensis* are the source of an oil which is rich in the unusual fatty acid vernolic acid, *cis-12, 13-epoxy-cis-9-octadecenoic acid* [5]. It is the epoxy functionality in vernonia oil that makes it unique in comparison to other vegetable oils such as coconut oil, palm kernel oil, soybean oil, sunflower oil, etc., none of which contain the epoxy functionality. Oils rich in epoxy fatty acids are useful in the paint industry to reduce emissions of volatile organic compounds that produce smog as a result of using petroleum-based (alkyd-resin) paints [25]. Epoxy fatty acid oils are also useful in the manufacture of plasticizers, as additives to polyvinyl chloride, in polymer blends and coatings, and in cosmetic and pharmaceutical applications [17]. Currently, the requirement for epoxidized oils is met with petrochemicals or by chemical epoxidation of fats and vegetable oils such as soybean and linseed oil [26].

Vernonia oil is chemically similar to epoxidized soybean and linseed oils. Linoleic acid is the principal fatty acid present in soybean oil and linseed oil making up to 50 % and 57 % of the oils respectively, whereas vernonia oil is dominated by a single fatty acid known as vernolic acid (72-80 %). Furthermore, vernonia oil has several unique properties: it is a transparent homogenous liquid at room temperature with a low viscosity of about 300 cps at 10 °C and only 100 cps at 30 °C [27]. In contrast, epoxidized soybean oil and epoxidized linseed oils are highly viscous, with a viscosity of about 300-1500 cps; they are semi-solid at 10 °C and are non pourable below 0 °C [4].

Table 1.3: Iodine value, oxirane number and viscosity of vernonia oil, epoxidized soybean oil and epoxidized linseed oils [27, 28]

Vegetable oil	**Iodine number**	**Oxirane number**	**Viscosity (cps, 20 °C)**	**Density (g/cm^3, 25 °C)**
Soybean oil	130	0.00	94	0.9214
Fully epoxidized SO	0.81	6.41	543	0.9972
Linseed oil	190	0.00	88	0.9302
Fully epoxidised LO	4.69	8.76	990	1.0325
Vernonia oil	89.17	4.26	210	0.9630

SO = Soybean oil, LO = linseed oil

1.4 Composition of vernonia oil

Vernonia oil is a complex mixture of triacylglycerols, primarily made up of trivernolin, with the predominant acid moiety being (+)-(12S,13R)-epoxy-cis-9-octadecenoic (vernolic) acid and it has a homogeneous molecular structure [3, 4, 21, 27, 29, 30, 31].

A recent study reported by Ncube *et al.* [93] indicated that vernonia oil is composed of different triacylglycerols. The triacylglycerols of vernonia oil is composed of a low amount of non-epoxy triacylglycerols (9 %), monovernoloyl triacylglycerols (15 %), divernoloyl triacylglycerols (38 %) and trivernolin (38 %). Carlson *et al.* [26] also analyzed vernonia oil using gas chromatography, but they found a different ratio with trivernolin being the highest (59 %) followed by divernoloyl triacylglycerols (28 %), monovernoloyl triacylglycerols (9.5 %) and non-epoxy triacylglycerols (9 %).

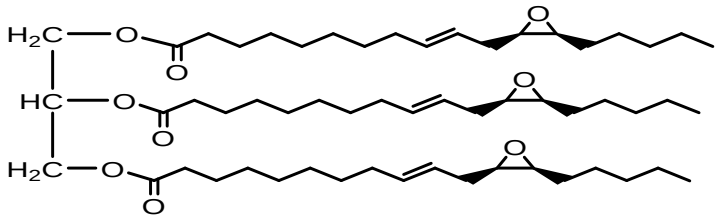

Figure 1.4*: Structure of trivernolin*

Vernonia oil, an inedible oil, is peculiar such that it is the only naturally epoxidized oil. Vernolic acid, which is a natural product is produced enantiomerically pure, as such unusual fatty acid may be considered as a key component of the chiral pool, providing an ideal starting material for the preparation of high value non-racemic products for the fine chemicals sector [92].

The seeds of *vernonia galamensis* plant contain approx. 40 % by weight of epoxy oil, which up on hydrolysis, yields different fatty acids with variable composition. Literature reports indicate that in addition to vernolic acid (72-80 %), *vernonia galamensis* oil is composed of fatty acids such as linoleic acid (C18:2) (11-12 %), oleic acid (C18:1) (4-6

%), stearic acid (C18:0) (2-3 %), palmitic acid (C16:0) (2-4%) and trace amount of arachidic acid (C20:0) [3, 27, 30, 32, 33].

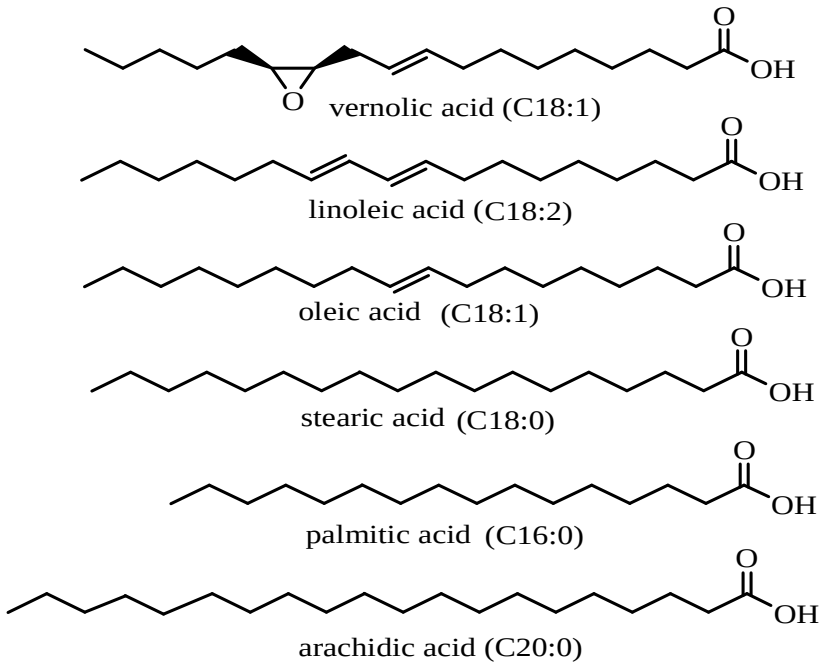

Figure 1.5*: Structures of fatty acids of vernonia oil*

1.5 Reactivity of vernonia galamensis oil and its economic importance

Several recent publications have focused on *vernonia galamensis,* a promising source of epoxy triglycerides and epoxy acids. Its importance lies in the unique properties of the seed oil, which make it interesting both economically and ecologically. The commercial importance of this oil includes its use as a reactive diluent in coatings, in plastic formulations, chemical coatings, epoxy resin, adhesives, plasticizers and stabilizers and as a chemical intermediate [20, 28], thus giving the species and other epoxy producing plants great economic importance [4,21].

Currently, huge industrial market is available for synthetically epoxidized vegetable oils such as linseed or soybean oils, but the chemical epoxidation process is expensive as well as environmentally damaging. Vernonia oil, on the other hand, is already epoxidized in nature by enzymatic action, and may be able to fill some of those market niches or could be used as a substitute for currently used epoxy oils [32]. As a major source of natural vernolic acid, there is no alternative to vernonia, hence, a wide market potential is available [19]. The potential of *vernonia galamensis* as a seed source of oil with inherent usefulness because of its oxirane content has been considered recently from both botanical and chemical viewpoints [17, 28]. The vernolic (76 %), oleic (4 %) and linoleic (13 %) acid levels in *vernonia galamensis oil* represent additional epoxidizable unsaturation, so that fully epoxidized VO could have an oxirane value near 10 % [26].

A wide variety of modification reaction characteristics of the ester group, the double bond, and the epoxy group could be conducted using the advantage of the unique and special structure of epoxy acid within the triglyceride structure of vernonia oil (Figure 1.6) [4, 17]. The presence of reactive epoxy groups and double bonds in the vernonia oil molecule makes it a unique starting material for a variety of potentially valuable synthetic transformations [34].

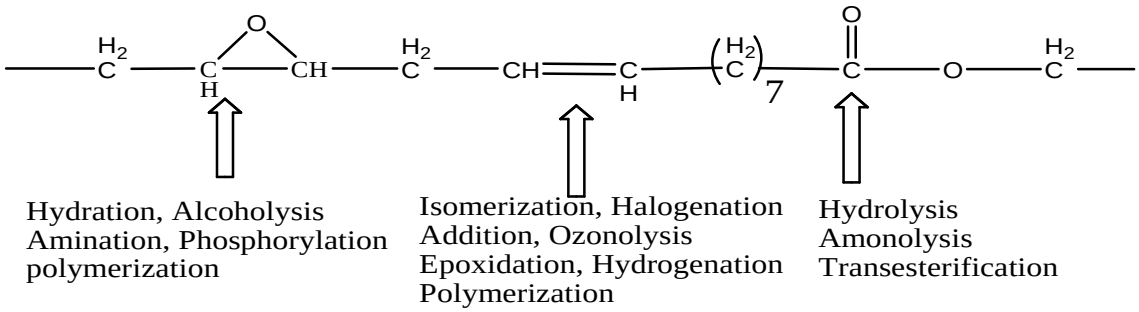

Figure 1.6*: Reaction characteristics of vernolic acid within the triglyceride*

The inherent epoxy group, the relatively low viscosity and polymerizing characteristics of vernonia oil makes it especially important as a solvent in coating and paint applications [35]. Oleochemical industries could benefit by using the long-chain unsaturated epoxy fatty acid present in vernonia oil in production of various value added products. Some of the products that are being developed from vernonia oil include; degradable lubricants and lubricant additives, epoxy resins, plastic formulations of polyvinyl chloride, adhesives, insecticides and insect repellants and reactive monomers in polymer synthesis [35, 36]. There are reports on the transformation of vernonia oil into key industrial raw materials such as dibasic acids [37]. These dibasic acids and their derivatives, which are used in the manufacture of polyurethanes, polyamides, alkyd resins, plasticizers, and elastomers, are obtained primarily from petroleum [38]. The tri-functionality of vernonia oil has been exploited to form cross-linked polymers with difunctional reagents, such as dibasic acids [39]. Dodecanedioic acid, currently available from complicated synthesis utilizing petrochemical feedstock, was synthesized from *vernonia galamensis* oil [40]. Vernonia oil has also been used for the synthesis of hydroxyl alkoxy fatty esters, epoxy secondary amide and interpenetrating polymer networks [41, 42, 43]. Reports also indicated that vernonia oil was used as a solvent/reactive diluent in styrene–acrylate copolymerization and UV curable acrylic polymer systems [44]. Mann, *et al.* [45] showed that vernonia oil has the potential to be used as a valuable, bio-based intermediate for synthesizing low viscosity resins.

1.6 Starch

Starch consists of two major components: amylose, a mostly linear α-D(1-4)- glucan and amylopectin, an α-D-(1-4)-glucan which has α-D(1-6) linkages at the branch point. The linear amylose molecules of starch have a molecular weight of 0.2–2 million Daltons, whereas the branched amylopectin molecules have molecular weights as high as 100–400 million Daltons [46].

Starch is highly abundant, relatively cheap, non-toxic and renewable natural resource that can be further modified [47, 48]. Native starches are readily available from different origins like maize, potato, cassava, etc. Cassava (*Manihot esculenta crantz*) has a stable yield of storage roots with high starch content (fresh weight 26–32 %) even under suboptimal growth conditions, making it the most important sources of carbohydrates in tropical regions. Cassava starch granules are generally round, with a flat surface on one side (truncated) and they range about 5–40 μm in size [49]. The amylose content of cassava ranges from 15-25 %, depending on cultivars and growth conditions [50]. Cassava starch is one of the most important starches in industry for synthesis of bio-based polymers.

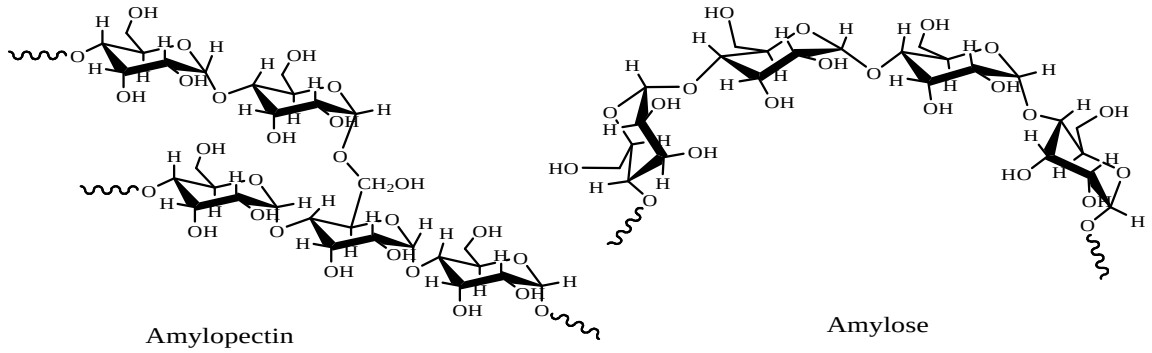

Figure 1.7*: Chemical structure of starch amylose and amylopectin*

The common areas where starches find wide applications include food industry, pharmaceutical, textile, paper, and packaging [48]. However, the highly hydrophilic nature, and poor mechanical properties have hindered its wider application [51]. Hence, modification of native starch is necessary to attain the required property and quality of product. Modified starch derivatives could be obtained by either glucosidic bond cleavage (acid modification) or forming new functional groups (carbonyl group formation during oxidation), or substitution of free available hydroxyl groups (by etherification or esterification), or bridging of molecular chains by cross-linking reactions [32]. These modifications overcome the limitations of native starch properties [28]. Starch based material may be used as potential substituent for petroleum-based plastic material especially in the packaging industries.

Esterification is one of the common methods of modification of starch to attain desired properties [52, 53].

1.7 Carbohydrate fatty acid esters

Carbohydrate fatty acid esters are composed of mono-, oligo- or polysaccharide esterified with fatty acids of various chain lengths. Starch modified with long chain fatty acids has biomedical applications such as materials for bone fixation and replacements, carriers for controlled release of drugs and other bioactive agents. These include starch-based biomaterials as scaffolds for the tissue engineering of bone and cartilage materials for bone fixation and replacement as well as for filling bone defects. They are used in many branches of industry as glues, adhesives and auxiliaries of a wide range of rheological and functional properties [54, 55, 56].

1.8 Chemical synthesis of carbohydrate fatty acid esters

Starch fatty acid esters can be synthesized either by chemical or enzymatic method. The chemical method of synthesis of starch esters could be attained by reacting the free hydroxyl groups of the anhydroglucose unit (AGU) monomers of starch with different fatty acid precursors such as fatty acid vinyl esters, fatty acid chlorides, carboxylic acids, or fatty acid methyl esters using toxic, expensive and environmentally unfriendly solvents such as pyridine, dimethylformamide, dimethylsulfoxide, or toluene in the presence of alkaline catalysts like sodium carbonate, potassium carbonate etc. has been reported [52, 53, 57, 58].

A major drawback of the existing synthetic methodology for fatty acid starch esters is the use of organic solvents such as pyridine and DMSO that have a negative environmental impact and may limit further commercialization of the process, particularly for food applications. Therefore, there is a strong incentive to develop green and environmentally friendly solvents such as ionic liquids as solvents instead of conventional organic solvents in the modification of carbohydrates [59]. Ionic liquids are simply salts and therefore entirely composed of ions that are liquid below 100 °C. Typical ionic liquids are normally based on organic cations, (*e.g*. 1,3-dialkylimidazolium, tetraalkylammonium etc.) paired with a variety of polyatomic inorganic anions that have a distributed negative charge (*e.g.* $[BF_4]^-$, $[PF_6]^-$, etc.), which results in low viscosity and easily handled materials with very interesting properties as solvents [60, 61].

In recent years, the application of ionic liquids (ILs) as alternative solvents and reaction media for a wide variety of synthetic processes has received significant attention owing to

their fascinating and intriguing properties, such as low melting points, wide liquid ranges, lack of vapor pressure and high ability to dissolve wide range of organic and inorganic compounds [60, 61]. They offer convenient media for carbohydrate reactions. In particular, the ILs bearing the imidazolium structure has been found to be a non-derivatizing medium capable to dissolve polysaccharides, such as cellulose and starch [62, 63]. Xie, W. *et al*. [48] recently reported that high fatty acid esters of corn starch were synthesized by reacting the starch with fatty acid methyl ester using 1-butyl-3-methylimidazolium chloride, ([BMIM]Cl) ionic liquid (IL) as reaction media. Chemical modification of polysaccharides, *e.g.* esterification reactions of mostly 1–8 wt % polysaccharide dissolved in IL, were achieved using five or more molar equivalents of carboxylic acid anhydride or carboxylic acid chlorides per anhydroglucose unit (AGU) resulting in highly-substituted polysaccharide esters [64].

1.9 Enzymatic synthesis of carbohydrate fatty acid esters

Starch fatty acid esters can also be synthesized by enzymatic methods. The enzymatic synthesis provides many advantages which includes 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, and easier separation. Other advantages such as lower energy requirement, improved quality of product and high catalytic efficiency are also very attractive in commercial applications [65, 66, 67].

Lipases are the most widely investigated of all enzymes. Owing to their greater stability at high temperatures, over a wide pH range, easy handling and repeated use, immobilized

lipases have been employed in a number of industrial reactions. The lipases are specific towards the ester bond and hence the formation of undesirable by-products is eliminated [68]. Novozym 435 is an immobilized lipase derived from *Candida antarctica* and used in a number of reactions.

Candida antarcitca lipase B (CAL-B), a biocatalyst known for its efficiency and high selectivity is used in a wide range of applications in industrial synthetic processes such as aminolysis, esterification, transesterification, hydrolysis in water, esterification in organic solvents, enantio- and regioselective transformations of many low molar mass and polymer substrates that possess a broad range of catalytic activities suitable for biotransformation [69, 70]. Beside other factors, the stability of the enzyme in ionic liquids affects its catalytic activity and the yield of the reaction. Many researchers have reported that a variety of enzymes in ionic liquids are capable of performing catalytic activities, which are generally comparable with or higher than those observed in conventional organic solvents.

Over the past two decades, studies have revealed 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 for use in organic solvents [65]. The use of enzymes in nonaqueous organic media for 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 [71].

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 non-polar character (*i.e.* fatty acid or fatty acid ester). As the result, finding the appropriate solvents to dissolve both the hydrophilic carbohydrate and the hydrophobic fatty acid, while at the same time maintaining enzyme activity is not an easy task [72]. In fact, there is no solvent compatible with enzyme activity that is both nontoxic and allows the solubilization of both substrates [73].

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 [71, 74]. 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 their partial or complete inactivation [75], and they are not compatible with applications in the food and pharmaceutical industry. 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 [75].

The search for a suitable solvent has driven some groups to investigate in the use of alternative non-conventional media for enzymatic reaction such as supercritical fluids, (SCFs), which have been used in a large number of studies. The main advantage of SCFs

is that they can be easily removed after the reaction by decreasing the pressure [76], 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 [77]. 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 supercritical fluids in biocatalytic processes [76, 78]. Very recently, the use of ILs as reaction medium for enzyme catalyzed reactions is attracting the focus of many research groups.

1.10 Enzyme catalyzed reactions in ionic liquids

Ionic liquids have emerged as exceptionally interesting non-aqueous reaction media for enzymatic transformations, and research interest in this area has increased widely in recent years. Room-temperature ionic liquids (RTILs) have become more and more important as desirable green solvents and reaction media for a wide variety of processes [79]. ILs could be an alternative to conventional organic solvents for enzyme-catalyzed synthesis because of their negligible vapor pressure, excellent solvent properties, high chemical and thermal stability, recoverability and recyclability [80]. 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, ionic liquids are non-volatile and therefore, the water generated during the reaction can be easily removed under vacuum. Kaar *et al.* [81] observed that the free *Candida rugosa* lipase was only active in hydrophobic

[BMIM][PF_6], but inactive in all hydrophilic ILs based on [NO_3]$^-$,[CH_3COO]$^-$ and [CF_3COO]$^-$ during the transesterification of methylmethacrylate with 2-ethyl-1-hexanol. They indicated that the latter three anions are more nucleophilic than [PF_6]$^-$, and thus could interact with the enzyme causing the protein conformational changes.

Studies on enzymes after incubation in ionic liquids have also revealed that enzymes are not only strongly tolerant against ionic liquids, but they can even be activated. The thermostability of *Candida antarctica* lipase B, either free (Novozym SP525) or absorbed on a macroporous acrylic support (Novozym 435), was investigated by incubating the enzyme preparation in anhydrous [BMIM][PF_6] at 80 ^{0}C for a specific period and then after dilution with water, measuring the residual activity in triacetin hydrolysis. Both enzyme forms showed a significant increase in their activities: 120 % increase for the free enzyme after incubation for 20–100 h, and 350 % increase for Novozym 435 after 40 h incubation [82].

It was observed that the use of these ILs as reaction media enhanced the selectivity and stability of enzymes, while the activity of enzymes in pure ILs was usually lower than those in conventional organic solvents used for the synthesis of sugar ester [83, 84]. 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]$^-$) have been reported to be good solvents for carbohydrate dissolution but least chosen in enzyme-catalyzed reaction due to the inactivation of most enzymes [78]. 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 [78]. 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 [85] but resulted in losing the stability and activity of enzymes. Several studies have indicated that the use of ILs and organic solvents mixtures or supersaturated sugar solution in ILs can significantly increase the enzyme activity in the synthesis of sugar ester [86, 87].

1.11 Polymers from renewable resources

Polymers are quantitatively the most important products of the chemical industry that are used in various applications in everyday life. Almost all current polymers are produced from fossil sources. However, due to their widespread use and non-biodegradable nature, polymers make a significant contribution to the increasing solid waste accumulation with a potential to damage the environment.

Renewable resource-based products can yield a promising platform to substitute petroleum-based products through innovative ideas in designing new value added materials such as bio-based polymers which can compete or even surpass the existing petroleum based materials on a cost-performance basis with added advantage of eco-friendliness [88]. Many oils are currently converted through more or less extensive oleochemical processing to value added products, such as lubricants, polymer additives, or surfactants. In most cases these products are based on fatty acids, their methyl esters or

fatty alcohol as intermediates, because the reactive functional group of these compounds is a ready candidate for chemical derivatization [88].

The active sites such as double bond present in the triglyceride molecules can be modified through chemical reactions. It is possible to polymerize triglycerides directly through the double bonds in their structure either by free radical or cationic mechanism [89], or to modify the triglyceride structure with polymerizable groups using the same synthesis techniques that have been applied in the synthesis of petroleum-based polymers. However, some vegetable oils contain naturally occurring functional groups, such as hydroxyl (*e.g.*, in castor oil) and epoxide (*e.g.*, in vernonia oil), which make them candidates for direct cross-linking with various hardeners to form interpenetrating polymer networks [4,39].

1.12 Ring opening polymerization of epoxidized oils

Ring opening polymerization has been focused in petroleum based epoxides, as ethylene oxide and propylene oxide for the synthesis of polyetherpolyols, which are particularly used for the preparation of polyurethanes. However, to alleviate environmental problems, vegetable oil-based epoxides are becoming renewable monomers of choice [90]. The epoxidized fatty acid derivatives from vegetable oils can be used directly in a broad range of polymer applications, such as, stabilizers of polyvinyl chloride or the direct photochemically initiated cationic polymerization and cross-linking with polyols, diamines or anhydrides [91]. Common uses of these derivatives are the synthesis of polyurethanes by reaction of the polyols with an isocyanate, or the radical polymerization in the case of the ring opening with acrylic acid.

Recent years have witnessed that there is a significant attention given to the use of renewable resources in the synthesis of multitude of value added products. However, vegetable oils need further modification to achieve the required quality of product. The use of naturally epoxidized vegetable oils, such as vernonia oil, could minimize the cost as well as environmental damages that may occur during derivatization. Vernonia oil, a renewable resource which is under exploited, has the potential to substitute petroleum based products. In this research project, derivatization of vernonia oil was conducted to prepare various value added products.

1.13. Objectives

In recent years, interest in finding environmentally friendly or green biodegradable polymers derived from natural resources as alternatives to non-biodegradable petroleum-based polymers is increasing dramatically [52] . Among others, sugar fatty acid esters, synthesised from renewable resources such as carbohydrates, have found broad applications in the food industry, cosmetics, detergents and medical supplies [94]. Ethiopia celebrates a huge diversity in natural resources, particularly endemic plants and this richness offers tremendous opportunities to pursue novel routes towards sustainability. *Vernonia galamensis* is a native plant original from the low land areas of Ethiopia whose seeds contain a significant concentration (approx 40%) of naturally epoxidised triglyceride oils [26, 95] . This type of moiety is particularly useful in cross-linking agents and synthetic building blocks and therefore, vernonia oil offers great potential for numerous applications such as for the preparation of base compounds for pharmaceuticals and powder coatings and the synthesis of biodegradable plastics [4 ,30, 36]. The industrial production of Vernonia oil has already started. However, its

application in North America has been significantly hindered due to high production costs and low seed yields [96, 97]. In this regard, tropical and sub-tropical regions such as Ethiopia have a distinct advantage as the plant thrives well in its "home territory", with high seed content and oil yield, to which competitive labour costs can be added. However, to maximise the commercialisation of this product, a better understanding of the extraction methods and a detailed exploration of the synthetic routes towards the manufacturing of valuable materials is needed. Investigation of synthetic routes for the polymerisation of the vernonia oil with sugars, such as the endemic cassava starch, could lead to the discovery of new and valuable biodegradable composite materials that will offer an environmentally benign alternative to petroleum based materials and will widen the scope of application of vernonia oil.

Currently, there are a number of reports on the synthesis of carbohydrate fatty acid esters. However, little attention has been focused on enzymatic synthesis of starch fatty acid esters using ionic liquids as solvents. Despite the high potential of the *vernonia galamensis* seed oil for synthesis of bio-based derivatives, the use of vernolic acid or methyl vernolate, derivatives of vernonia oil, in the esterification with renewable resources such as carbohydrates has not been reported. Therefore, this study focuses on the use of renewable resources such as glucose, endemic cassava starch, vernonia oil, and derivatives of vernonia oil (vernolic acid, methyl vernolate or vernanol) to synthesise value added products. Chemical and enzymatic synthesis of starch and glucose epoxy fatty acid esters in either ionic liquid or organic solvent as reaction medium were conducted. The use of enzyme as catalysts and ionic liquids as solvents in the processes

demonstrate the importance of green method of synthesis. The enzymatic transesterification of cassava starch or glucose with epoxy fatty acid methyl esters or vernolic acid could, in principle, be a valuable approach for the development of biodegradable materials that will offer an environmentally benign alternative to petroleum based materials.

The presence of multiple functional groups in vernonia oil increases the potential of the oil in the synthesis of additional derivatives such as epoxidized vernonia oil, vernanol (epoxy alcohol), polymerized vernonia oil methyl ester (VOME), acrylated vernanol, hydroxylated methyl vernolate and polyhydroxy vernonia oil (vernonia oil polyol). These possibilities widen the scope of application of vernonia oil and its derivatives in the synthesis of value added products.

The principal aim of this research project is the synthesis and characterization of new value added products based on vernonia oil derivatives and carbohydrates, such as endemic cassava starch and glucose. The use of these types of raw materials for the manufacture of valuable products will produce bio-materials with enhanced biodegradability and will also help reducing the dependence on fossil fuels. More specifically, this work deals with:

- Extraction of vernonia oil from *vernonia galamensis* seed
- Characterization of vernonia oil
- Derivatization of the oil into value added products such as carbohydrate fatty acid esters and other derivatives
- Physico-chemical characterizations of the new derivatives and value added products

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CHAPTER TWO: EXPERIMENTAL

2. MATERIALS AND METHODS

Cassava starch commercially obtained was dried for 24 h at 105 °C under vacuum to reduce the moisture content below 2 % before use. Vernolic acid and vernolic acid methyl ester (VOME) were prepared as per standard method [11] using vernonia oil extracted from *vernonia galamensis* seed kindly donated by Adet Agricultural Research Centre, Ethiopia. Novozym 435 (*Candida Antarctica* type B lipase immobilized on acrylic resin) was provided by *Instituto de Catalysis y Petroleoquimica*, CSIC, Madrid, Spain. The ionic liquids 1-Butyl-3-methylimidazolium hexafluorophosphate, [C_4mim][PF_6], 1-Butyl-3-methylimidazolium chloride, [C_4mim]Cl were obtained from the School of Chemistry, University of Nottingham, Analytical grade DMSO, 2-methyl-2-butanol (2M2B) and t-butyl alcohol were purchased from Sigma Aldrich Chemical Company.

2.1 Synthetic Procedures

Vernonia oil was extracted from vernonia galamensis seed obtained from Adet Agricultural Research Centre, Bahr Dar area. Once the oil is extracted using organic solvents such as n-hexane, purification to remove free fatty acids was carried out using standard procedures [11] and characterized with different techniques.

2.1.1 Extraction and purification of vernonia oil

2.1.1.1 Extraction of vernonia oil

Dried *V. galamensis* seeds were heated in an oven for 1 hour at 90 °C for lipase deactivation and powdered seeds of *V. galamensis* were extracted with n-hexane as a

solvent for three hours using Soxhlet extractor. Solvent was removed at reduced pressure and the crude oil was subjected to refining process.

2.1.1.2 Refining of vernonia oil

In addition to triglycerides, the crude oil contains variable amounts of impurties which must be removed to produce pure vernonia oil. Some of these impurities are of the non-glyceride type such as the FFA, which may be built up due to enzymatic processes (lipase) resulting from damage to the seed. The other impurities include moisture and solvent, pigment or coloring materials primary and secondary oxidation products, waxes and saponifiable materials.

2.1.1.3 Bleaching

Crude vernonia oil was refined with 5–8 % by weight of activated charcoal at a temperature of 60 °C by continuous stirring for one hour. The decolorized oil was isolated by hot filtration.

2.1.1.4 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 vernonia oil was degummed by stirring with 2.5-5 g by weight of distilled water, heated 60-70 °C for one hour followed by centrifugation at 5,000 rpm. Gum and oil were separated and the oil was dried at 60 °C on a rotary evaporator.

2.1.1.5 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 vernonia oil was mixed with sodium hydroxide solution and then heated to 40 °C 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 °C.

2.1.2 Transesterification of Vernonia Oil to methyl vernolate (VOME)

Transesterification of vernonia oil was carried out using reported procedure [8] with modification. 12.4 g (0.04 mol) of vernonia oil was transferred into a 500 mL round-bottom flask. Hexane (65 mL) was added, followed by 6.25 mL sodium methoxide in methanol (25 wt %). The mixture was allowed to rotate (approximately 240 revolutions/min) for about 80 min without heat or vacuum on a rotary evaporator. 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 then stripped to give 10.8 g of VOME (vernonia oil methyl ester).

2.1.3. Preparation of Vernolic Acid from Vernonia Oil

The preparation of vernolic acid from vernonia oil was conducted using reported procedure [2] with slight modification. To a 250 mL distilling flask, equipped with magnetic stirrer bar, 50 mL methanol and 5 g (0.125 mol) sodium hydroxide were added.

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 5.12g (5.5 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 substance 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 vacuum filtered immediately 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 vernolic acid.

Purification of the acid was accomplished by low-temperature recrystallization. Hexane (50 mL) was added to a 150 mL beaker containing the crude vernolic acid, and the beaker was placed in a freezer for 24 h. The resulting solid was vacuum-filtered and rinsed with an additional 50 mL ice-cold hexane to give pure vernolic acid and characterized by NMR and FTIR.

2.1.4 Preparation of epoxy alcohol from VOME

The preparation of epoxy alcohol, vernanol was carried out reported procedure [8] with modification. In a 500 mL, three necked round bottom flask equipped with air condenser and separatory funnel, 60 mL of hexane was added to dissolve 0.9 g of $LiAlH_4$. Then 2.5

g VOME was added with stirring. After the reaction mixture was stirred for 2 h, 5 mL of deionised water was added slowly and stirred for an additional 15 min. The product was vacuum filtered, the filtrate stripped off with rotary evaporator to yield a white solid product.

2.1.5 Synthesis of starch vernolate

The esterification of starch has also been carried out applying both chemical and enzymatic methods in organic solvents as well as mixture of organic solvents and ionic liquids. Chemical and enzyme catalyzed reactions of cassava starch and vernonia oil methyl ester or vernolic acid were conducted under different conditions.

2.1.5.1 Chemical synthesis of starch vernolate in organic solvents

The synthesis of starch vernolate was carried out using the reported procedure by Junistia *et al.* [6] with modifications. Cassava starch (2.0 g, 0.012 mol, anhydroglucose unit, AGU) was first gelatinized in DMSO (20 mL) at 70 °C for 3 h. To the transparent solution obtained, methyl vernolate (3mol/mol anhydroglucose units in starch, i.e., 0.036 mol, 11.25 g) and 0.23 g (2 % w/w, with respect to starch) of potassium carbonate (catalyst) were added and the mixture was stirred at 110 °C for 12 h. After cooling, the product was precipitated under vigorous stirring using methanol (100 mL) and separated from the liquid phase by decantation. The product was further washed three times with methanol (30 mL and 15 mL, 10 mL respectively). Finally, the product was dried in an oven (75 °C) and a brown solid product obtained was kept for characterization.

2.1.5.2 Enzymatic synthesis of starch vernolate in ionic liquids

The procedure of synthesis of starch vernolate was developed by modifying the method of *Wenlei Xie and Yingbin Wang* [7]. Dried cassava starch was dissolved in 20 % DMSO and 80 % 1-Butyl-3-methylimidazolium hexafluorophosphate, [C_4mim][PF_6] in a three-neck round flask fitted with a magnetic stirrer, and then heated and stirred thoroughly at the 70 °C reaction temperature. After the reaction mixture became a homogeneous solution, vernonia oil methyl vernolate (VOME), (3 mol/mol with respect to the AGU), and the catalyst Novozyme SP-435 were added according to the conditions required by an experimental design, and then stirred for 72 h at 40 °C. Upon completion of the reaction, the reaction mixture was cooled to room temperature, filtered off to remove the lipase enzyme, and then the starch esters were subsequently precipitated by addition of absolute ethanol under vigorous stirring followed by centrifugation. The precipitate was rewashed further three times with absolute ethanol to remove the residual IL and unreacted reagents, and then oven-dried at 60 °C under vacuum for 24 h. The products obtained as yellowish powders were used for testing.

2.1.5.3 Chemical method of synthesis of starch vernolate in ionic liquids

Dried cassava starch 1.0 g (6.17 mmol) was dissolved in 10.5 g (60 mmol) 1-Butyl-3-methylimidazolium chloride, [C_4mim]Cl, in a three-neck round flask fitted with a magnetic stirrer, and then heated and stirred thoroughly at the 70 °C reaction temperature. After the reaction mixture became a homogeneous solution, 5.74 g, (18.5 mmol) of methyl vernolate (3 mol/mol with respect to the AGU), and 2.9 g (37 mmol, 6 equiv/AGU) pyridine as a catalyst were added according to the conditions required by an

experimental design, and then stirred for 24 h at 110 °C. Upon completion of the reaction, the reaction mixture was cooled to room temperature and the starch esters were subsequently precipitated by addition of absolute ethanol under vigorous stirring followed by centrifugation. The precipitate was rewashed further three times with absolute ethanol to remove the residual IL and unreacted reagents, and then oven-dried at 60 °C under vacuum for 24 h. The products obtained as yellowish powders were used for testing.

2.1.5.4 Enzymatic synthesis of starch vernolate in organic solvent

Dried cassava starch 1.0 g (6.17 mmol) was dissolved in 20 mL of Dimethylformamide in a three-neck round-bottom flask fitted with a magnetic stirrer, and then heated and stirred thoroughly at the 50 °C reaction temperature. After the reaction mixture became a homogeneous solution, 3.65 g, (12.33 mmol) of vernolic acid (2 mol/mol with respect to the AGU), and Novozyme SP-435 lipase as a catalyst were added according to the conditions required by an experimental design, and then stirred for 72 h at 55 °C. Upon completion of the reaction, the reaction mixture was cooled to room temperature and filtered of to remove the lipase and then the starch esters were subsequently precipitated by addition of absolute ethanol under vigorous stirring followed by centrifugation. The precipitate was rewashed further three times with absolute ethanol to remove the residual IL and unreacted reagents, and then oven-dried at 60 °C under vacuum for 24 h. The products obtained as yellowish powders were used for testing.

2.1.6 Enzymatic Esterification of Glucose

2.1.6.1 Enzyme catalyzed synthesis of glucose vernolate in [C_4mim]Br

The first enzymatic synthesis to mention is one that was carried out using glucose dissolved in ionic liquid as solvent. However, the solubility of glucose in IL used was low. Hence we used 20 % DMSO as co-solvent. The following is short summary of procedure applied.

The esterification of glucose was carried out using the procedure of Ganske *et al.* [3] with slight modifications. In 100 mL round bottom flask with magnetic stirrer by dissolving 0.5 g (2.78 mmol) of D-glucose in 20 % DMSO and 80 % 1-Butyl-3-methylimidazolium bromide, [C_4mim]Br and 2.46 g (8.33 mmol) of vernolic acid. The reaction started by adding 0.2 g of lipase from *C. antarctica* (CAL-B), Novozym SP-435 as catalyst and 0.4Å molecular sieve to remove the byproduct methanol. The reaction temperature was kept at 50 °C for 72 h.

After completion of the reaction, the product was isolated by washing first with water to remove the ionic liquid, unreacted glucose and filtered off to remove the lipase and molecular sieve. Then the product was further extracted with acetone and chloroform. The white solid product obtained was dried in oven at 60 °C for 24 h.

2.1.6.2 Enzymatic synthesis of glucose vernolate in DMSO and 2M2B

The esterification reaction was carried out according to procedure of K.Sakaki *et al*. [4] with minor modifications. In 100 mL round bottom flask with magnetic stirrer 0.5 g (2.78 mmol) of glucose was dissolved in 10 % DMSO and 90 % 2-methyl-2-butanol (2M2B)

and equimolar of i.e., 0.86 g (2.78 mmol) of vernonia oil methyl ester (VOME). The reaction started by adding 0.2 g of Lipase from *C. antarctica* (CAL-B), Novozym SP-435 as catalyst and 0.4Å molecular sieve to remove the by product methanol. The reaction temperature was kept at 50 °C for 72 h.

After completion of the reaction, the product was isolated by washing first with water to remove unreacted glucose and filtered off to remove the lipase and molecular sieve. Then the product was further extracted with acetone and chloroform. The brown product obtained was dried in oven at 60 °C for 24 h.

2.1.6.3 Enzyme catalyzed synthesis of glucose vernolate in DMSO and t-butyl alcohol

The esterification reaction was carried out according to procedure of K.Sakaki *et al*. [4] with minor modifications. In 100 mL round bottom flask with magnetic stirrer by dissolving 1.0 g (5.55 mmol) of glucose in 20 % DMSO and 80 % t-butyl alcohol and 4.93 g (16.7 mmol) of vernolic acid. The reaction started by adding 1.0 g of Lipase from *C. antarctica* (CAL-B), Novozym SP-435 as catalyst and 0.4 Å molecular sieve to remove the by product water. The reaction temperature was kept at 50 °C for 72 h.

After completion of the reaction, the product was isolated by washing first with water to remove unreacted glucose and filtered off to remove the lipase and molecular sieve. Then the product was further extracted with acetone and chloroform. The brown product obtained was dried in oven at 60 °C for 24 h.

2.1.6.4 Synthesis of alkenyl glycoside

The synthesis of alkenyl glycoside was carried by modifying the reported procedure [141]. Glucose pentacetate (1.0 g, 2.5 mmol) was dissolved in anhydrous dichloromethane (20 mL) and stirred for 1-2 h with 1.0 g molecular sieves (0.4 nm) under an argon atmosphere. The solution was treated with tin (IV) chloride (1.0 mL) and immediately treated with the vernanol, epoxy alcohol, (0.8 g, 2.5 mmol) dissolved in anhydrous dichloromethane (10 mL). After 6 h of reaction time, the mixture was poured into the saturated sodium hydrogen carbonate solution (20 mL), the organic layer separated, and the aqueous phase extracted with dichloromethane (3 x 20 mL). The combined organic phases were washed twice with water (2 x 20 mL), filtered over Celite, and evaporated in vacuum. The resulting product was deacetylated with sodium methoxide in methanol to remove the acetate moiety.

2.1.7. Further modifications of vernonia oil

Besides the above synthesis and characterizations, additional modifications of vernonia oil and characterization of resulting products were carried out. These modifications include ring opening polymerization of vernonia oil, ring opening polymerization of vernonia oil methyl ester (VOME), epoxidation of vernonia oil, reduction of vernonia oil methyl ester, preparation of vernonia oil polyol and dihydroxylation reactions.

2.2 Instrumentation

The products obtained in this study were fully characterized using the following techniques.

^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 MHz Bruker 400 Ultra-Shield spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) with reference to residual traces in the commercial deuterated solvent of protonated dimethyl sulfoxide (δ_H 2.54) and $(CD_3)_2SO$ (δ_C 40.45) at ambient temperature.

The Mass Spectrometry (MS) spectrum were acquired on a Bruker Apex IV ICR-MS, equipped with an electron ionisation (EI) source, utilising external ionisation of the sample at 70 eV. The ion source was operated at a temperature of 130 °C, although several samples required heating of the Direct Insertion Probe (DIP) in order to volatilise them. A typical maximum temperature of the DIP was 300 °C. The mass range acquired was 50-1000, with free induction decay (FID) data set size of 512K. Four spectra were additively combined prior to performing the Fourier Transform, in order to enhance signal-to-noise. Spectra were acquired using Bruker XMass instrument control software, and the resultant data files processed off-line with Bruker Data Analysis v4.0.

Fourier Transform Infrared Spectroscopy analysis was performed on a Nicolet model Protégé 460 Magna IR spectrometer. The analysis of the cassava starch and starch vernolate was carried out with analytical grade KBr pellets using the transmission mode. All the spectra were recorded with 4 cm^{-1} resolution over 100 scans.

Thermal stability was evaluated using a Perkin Elmer TGA 7 in a temperature range between 50 and 500 °C, with a heating rate of 10 °C/min and nitrogen gas flow of 50 mL/min.

The morphology of the cassava starch particles and the starch vernolate were observed using a scanning electron microscope Philips XL30 SEM. The samples were mounted on SEM stubs with double sided adhesive tapes and then coated with platinum under vacuum before analysis in order to make the samples conductive. The accelerating voltage used was 15kV.

Powder X-ray Diffraction studies were performed using a PANalytical X'Pert Pro MPD in Bragg-Brentanogeometry, with monochromated $CuK\alpha_1$ (λ = 1.5406Å, 40 kV, 40 mA) radiation, automated divergence and receiving slits (10 mm illuminated length), 10 mm beam mask, 0.04 rad soller slits and a step size of 0.08° in the angular range of 2-70° (2θ).

MALDI MS were performed in a Bruker Ultraflex III, a TOF instrument equipped with a Matrix-Assisted Laser Desorption Ionization (MALDI) source operated in reflectron mode. The matrix used was trans-2[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene] malononitrile, commonly referred to as DCTB. The sample solution and the DCTB in acetonitrile were mixed together to give about 10:1 excess of matrix. 0.5 μl of this mixture was spotted onto a stainless steel target plate and allowed to evaporate to dryness before introduction into the mass spectrometer. Polarity and mass range were dependent on the sample, but the mass range always went up to at least 50 % above the expected molecular weight. The mass spectrometer was operated in reflectron mode to maximize

the mass resolution and the power of the irradiating laser was adjusted experimentally to achieve a satisfactory spectrum at the lowest possible laser power. The laser operates at a wavelength of 337nm. External calibration of the data was performed in the FlexControl software.

Solid state ^{13}C CP-MAS (cross polarization magic angle spinning) spectra were collected in a Bruker advance DSX 200 MH at room temperature by using a standard Bruker wide-band MAS probe. Dry samples were packed in 4 mm zirconia rotors, with sealed Kel-Fe caps and spun at a speed of 5 kHz. The spectra were acquired with a contact time of 3 ms and recycle delay of 5 s. The free induction decay was subjected to standard Fourier transformation and phasing. The chemical shifts were externally referenced to the solid adamantane peak at 38.56 ppm. A total of 5000 scans were averaged for each spectrum.

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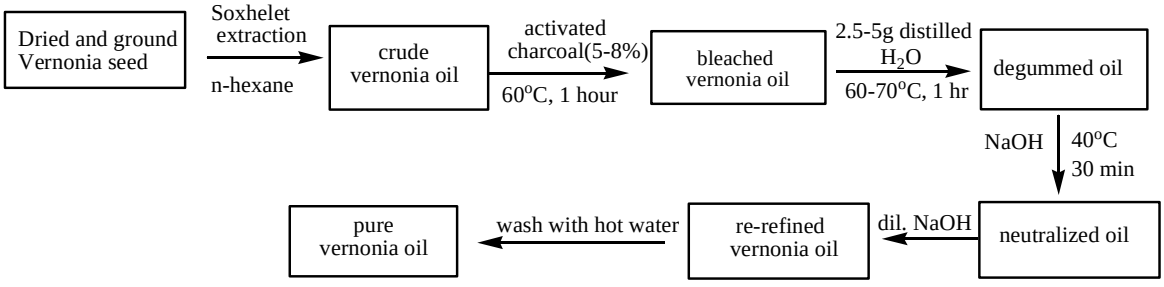
CHAPTER 3: RESULTS AND DISCUSSION

3.1 EXTRACTION, PURIFICATION AND CHARACTERIZATION OF VERNONIA OIL AND ITS DERIVATIVES

3.1.1 Vernonia oil

Vernonia galamensis is a biorenewable resource that grows as wild plant in tropical Africa [1]. The growing interest in *vernonia galamensis* seed oil (vernonia oil) derives from its naturally epoxidized triglyceride fatty acids, giving it the potential of being a unique oil for industrial raw materials used in plastic formulations, adhesives, chemical coatings, plasticizers, stabilizers, and syntheses of industrial chemical intermediates [2, 3].

In this study, the seed of *vernonia galamensis variety ethiopica* collected from Adet Agricultural Research Centre was used (fig. 3.1.1). The first stage of our research was extraction and purification of vernonia oil from *vernonia galamensis* seed. The following scheme shows a summary of steps used in the extraction and purification of vernonia oil.



Scheme 3.1*: Scheme for extraction and purification of vernonia oil*

The extraction of the oil from the *vernonia galamensis* seed was carried out using Soxhlet method, a reported procedure [4] with modification. The set up of the Soxhlet extraction is presented in fig.3.1 below. The active lipase commonly present in vernonia seed exhibits acidolysis and hydrolysis selectivity properties toward vernolic acid moiety in vernonia oil triglycerides. The lipolytic activity that could be induced when the seeds of *vernonia galamensis* are milled, results in high free fatty acid levels in the extracted oil and resultant meal [111]. Therefore, the extraction of the oil was performed under lipase deactivation conditions by heating the seed at temperature of 90 °C for one hour [110]. After milling of the seeds, the oil was extracted applying standard Soxhlet extraction procedure in which pre-distilled n-hexane was used as a solvent. The n-hexane solution containing the extracted oil was concentrated in a rotary evaporator *in vacuo* and the crude concentrated oil was refined by heating over activated charcoal followed by filtering [4].

Figure 3.1*: Set up of Soxhlet extraction*

Figure 3.1.1 Seeds of *vernonia galamensis*

Golden yellow colored oil was obtained after degumming by stirring with warm distilled water followed by centrifugation. Room temperature extraction by soaking powdered seeds and stirring in n-hexane was also carried out. However, it was found that Soxhlet extraction yielded more oil (32 %) than room temperature extraction (25 %). The oil obtained by room temperature extraction contained less free fatty acids.

The seed of vernonia galamensis has been identified as potential source of epoxy oil [9]. The oil content was calculated as a ratio of weight of the oil to its respective sample mass expressed in percentage (%). The type of the seed (ecotype), the solvent used, the particle size of the extracted materials (the fineness and coarseness of the powder), and the time of extraction are among the major factors that could influence the oil yield of vernonia seed. The oil content may be different based on the difference in the degree of grinding. Fine powder is more exposed towards the solvent and the solvent is more effective in leaching the oils. Therefore, the fine powder results in higher oil content than the coarse one [5].

The seed oil content obtained in this study was on average 32 %. This value is higher than the value reported by Angelini *et al*. [6]. They reported that the *Vernonia galamensis* accessions they used in their study had an oil content varying between 22.10 and 31.20 % with the mean of 26.70 %. In another investigation carried out by Baye et al. [7] on the performance of seed characters of eight *vernonia galamensis* var. *ethiopica* accessions sown at Haramaya, Harar and Babile, higher seed oil contents averaging at 38.70, 39.50 and 34.70 % for each respective location were obtained. Mebrahtu *et al*. [9] also indicated that oil content of *vernonia galamensis* seed they collected from Eritrea

and Ethiopia ranged from 14.9- 29.5% with a mean of 24 %. Therefore, the oil content of vernonia galamensis seed used in our current investigation was in good agreement with reported values.

Figure 3. 2*: Purified vernonia oil*

Characterization of Vernonia oil

The purified oil was characterized using NMR and FTIR spectroscopy as well as ESI-MS.

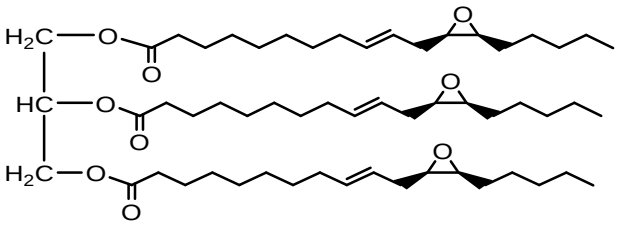


Figure 3.3: *Trivernolin structure of vernonia oil*

^{1}H NMR data (ppm) (400 MHz, $CDCl_3$): The ^{1}H NMR data of the vernonia oil shows the presence of olefinic protons (CH=CH) and glyceral proton (CH) at 5.34-5.43, glyceral proton (CH_2) at 4.16 and 4.31, epoxy protons (O-C-H) at 2.77-2.92 characteristic of vernolic acid moiety in the trivernolin structure, protons of methylene attached to olefinic group (-CH_2-CH=CH-CH_2-) at 2.04-2.34, protons of methylene groups (CH_2)n at 1.26-1.63, protons of methyl (CH_3) group at 0.89. The results obtained indicate that the major functional groups such as double bond, epoxy group and triglyceride ester characteristic components of vernonia oil remained intact though out the extraction and purification processes.

The ^{13}C NMR data (ppm) (400 MHz, $CDCl_3$): The ^{13}C NMR of the vernonia oil indicates the presence of the vernolyl carbonyl carbon (C=O) at 173.04, olefinic carbons (CH=CH) at 123.99-132.56, glyceral (CH) at 68.95, and glyceral (CH_2) at 62.04-64.99, epoxy carbons (O-C-H) at 56.62-57.28, methylene carbons (CH_2)n at 22.64-34.03, and methyl carbon (CH_3) at 14.06. This data is in agreement with the literature report [8].

The peak areas of the methyl protons of crude VO shown in the table below, is too high because of the presence of other saturated triglyceride oils corresponding to fatty acids such as stearic acid, palimitic acid and arachdic acids. This result was confirmed by the presence of a peak at 173.23 ppm due to the ester carbonyl carbon of VO on ^{13}C NMR spectrum. 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 ^{13}C NMR spectrum of crude VO corresponding to the olefinic carbons (CH=CH).

Integral peak area of different functional groups of vernonia oil			
Compound	**Double bond**	**Epoxy**	**Methyl**
Crude oil	1	0.68	1.62
Purified oil	1	0.64	1.43
Theoretical value	1	1	1.5

The integral peak area of the functional groups of the crude and purified VO 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 the hydration that may occur during neutralization and washing of the oil which affect the epoxy groups on the triglyceride structure of VO.

The Electron Spray Ionization (ESI) Mass Spectrometry study revealed that vernonia oil is mainly composed of trivernolin structure due to $[M+Na^+]$, m/z = 949.69 ($C_{57}H_{98}O_9Na$) and the sodiated vernolic acid peak corresponding to $C_{18}H_{32}O_3Na$ appearing at m/z = 319.23.

Intensity (%)	m/z	Theoretical mass	Proposed formula
100	319.23	319.13	$C_{18}H_{32}O_3Na$
28	928.21	926.72	$C_{57}H_{98}O_9$
36	949.69	949.53	$C_{57}H_{98}O_9Na$

Table 3.1*: ESI-MS of vernonia oil*

FTIR spectrum analysis

The infrared region of the electromagnetic spectrum extends from 14 000 to 50 cm^{-1} and is divided into three areas: the far-infrared from 400 to 50 cm^{-1}; the mid-infrared region from 4000 to 400 cm^{-1}, which is a very interesting region of the spectrum for the study of organic compounds, because the absorption bands are due to the vibration of a particular functional grouping; and the near-infrared (NIR) from 14 000 to 4000 cm^{-1}.

The table below summarizes the frequencies of the characteristic bands or shoulders, their assignment to functional groups, their vibration mode and their intensity in the spectrum of samples of vernonia oil.

No	Frequency (cm^{-1})	Assigned group	Mode of vibration	Intensity
1	3008	=C-H (*cis*-)	Stretching	m
2	2925	-C-H (CH_3)	Stretching (a)	m
3	2890	-C-H (CH_2)	Stretching (s)	vs
4	1740	-C=O (ester)	Stretching	vs
5	1654	-C=C- (cis-)	Stretching	vw
6	1465	-C-H (CH_2, CH_3)	Bending (Scissoring)	m
7	1377	-C-H (CH_3)	Bending (s)	m
8	1259	-C-O, -CH_2-	Stretching, bending	m
9	1170	-C-O, -CH_2-	Stretching, bending	st

10	1100, 1097, 1033	-C-O	Stretching	m
11	840	-C-O (epoxy group)	stretching	w
12	823	-C-O (epoxy group)	stretching	w
13	723	$-(CH_2)_n$,	Bending (rocking)	m

w = weak, vw= very weak, m= medium, vs= very strong, st= strong

Table 3.2*: Summary of FTIR data of vernonia oil*

In the spectrum, the functional groups characteristics of the triglyceride structure of vernonia oil were clearly identified. Among these, the peak at 823, 840 cm^{-1} correlated to the epoxy (or oxirane) group, the C=O stretching band obtained at 1740 cm^{-1} and the peak at 1654 cm^{-1} corresponding to C=C stretching mode can be mentioned. The presence of very weak peak at 3400 cm^{-1} in the spectrum of vernonia oil indicates partial hydrolysis of the epoxide. The FTIR spectrum of vernonia oil is in a good agreement with the literature reports [4, 10, 11, 12].

The spectral data analysis result indicates that the vernonia oil is pure. The neutralization process was successful and no free fatty acid peaks were observed in both the NMR and FTIR spectra. The oil content of the vernonia galamensis seed was on average 32 %, which is in good agreement with published reports [6, 9].

Viscosity and thermal stability of VO

The viscosity of vernonia oil ranges from 100-110 cps 30 °C and 300 cps at 10 °C [8]. The viscosity of VO decreases on increasing temperature. In our study the purified VO had viscosity of 150 cps at 25 °C and 20 cps at 90 °C. However, Wamalw *et al.* [13] reported that at higher temperature (188 °C), the oil became increasingly viscous on heating which might be due to thermally driven ring opening polymerization.

The thermal stability of vernonia oil was also analyzed using TGA. The TGA profile obtained in our study, shows only 6 % loss in weight at 200 °C which might be due to loss of water. It is also found that the onset temperature for decomposition of vernonia oil was 219 °C (see fig. 3.4). Therefore, VO is thermally stable at 200 °C.

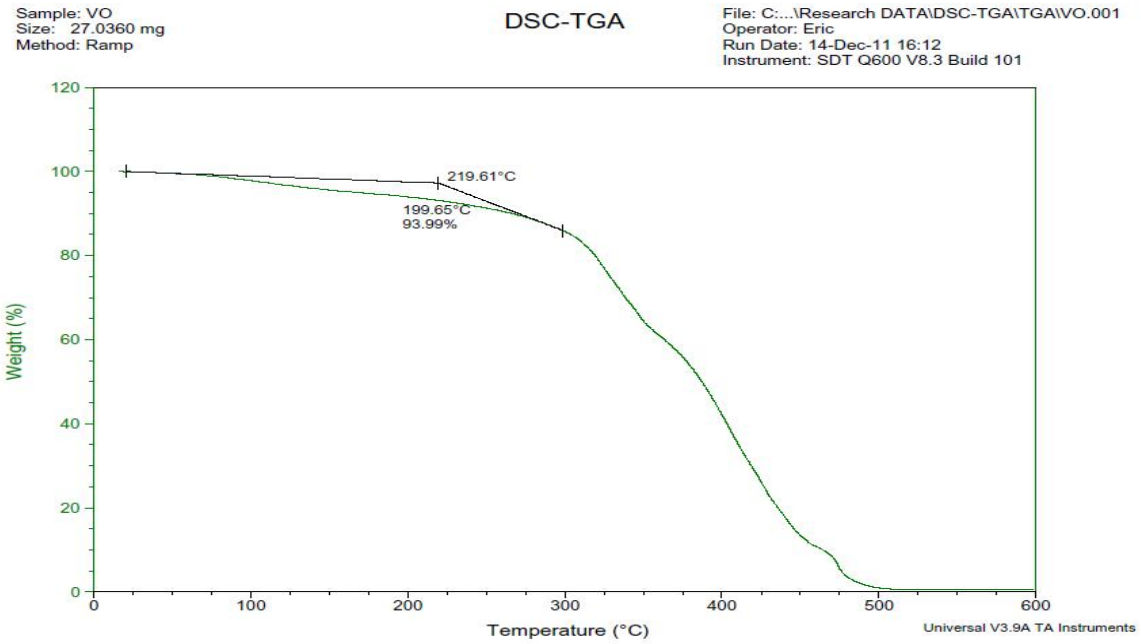
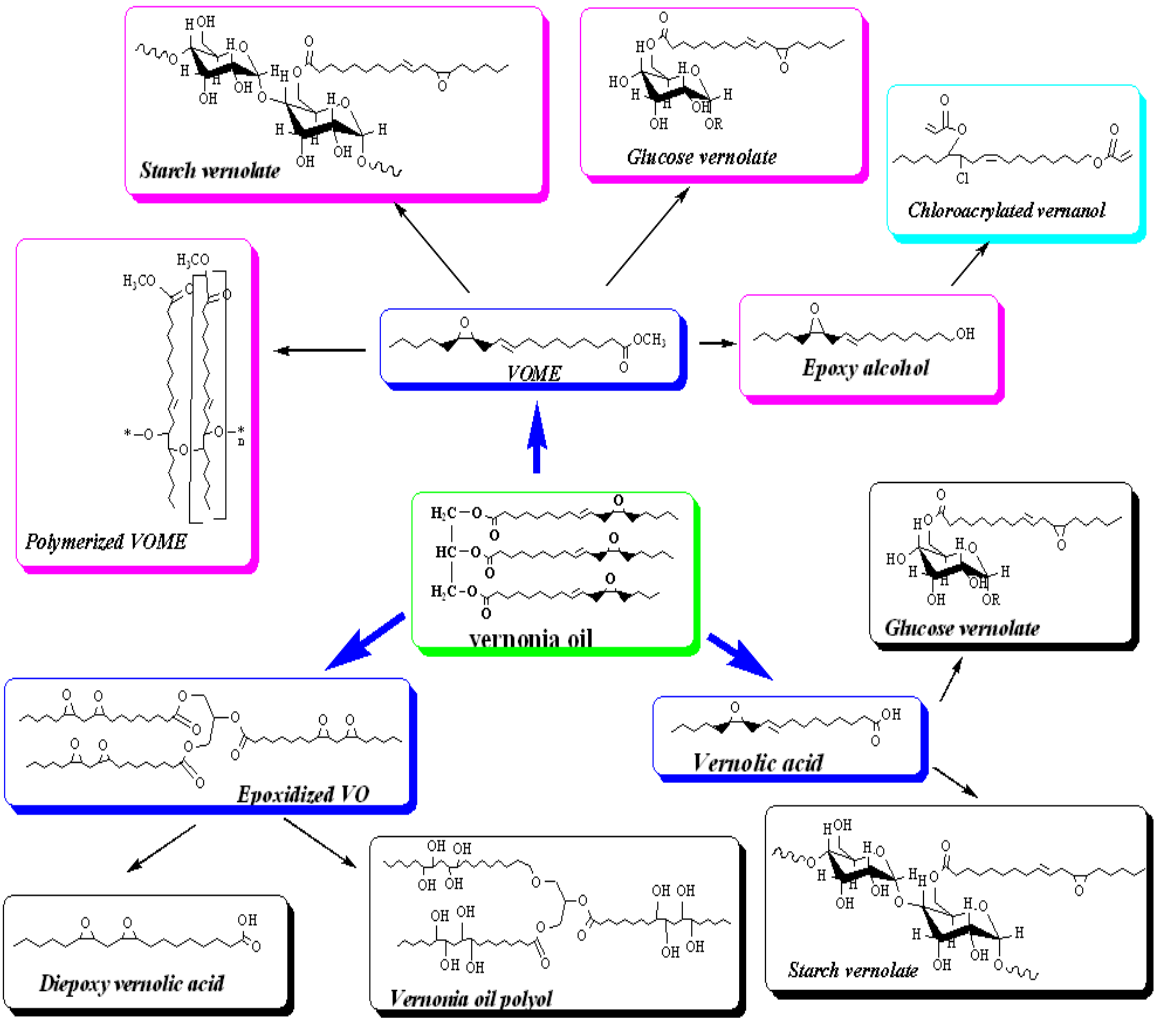


Figure 3.4*: TGA thermogram of vernonia oil*

After purification and characterization of vernonia oil, various modifications were carried out. The following scheme presents the major derivatization of vernonia oil.

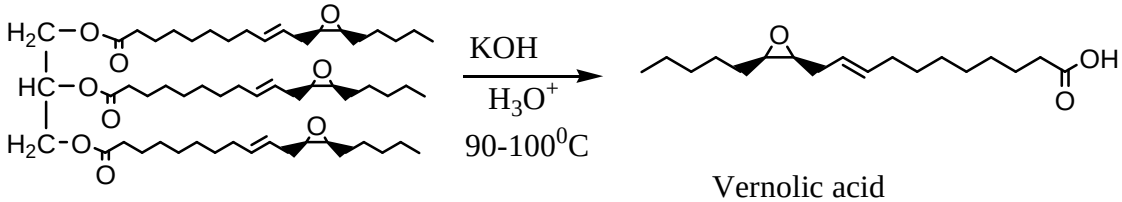


Scheme 3.2: *Schematic representations of derivatives of vernonia oil*

3.1.2 Vernolic acid (cis-12, 13-epoxy-cis-9-octadecenoic acid)

Nowadays, there is growing interest in preparing new derivatives and intermediates from functionalized oil. At present, standard methodologies are available for derivatizing vernonia oil to vernolic acid [14]. Vernolic acid (12-Epoxyoctadeca-cis-9-enoic acid) is a C_{18} fatty acid has a unique structure and potential which makes it convenient for further modification attributed to the presence of an epoxy group. Seed oils of a number of *Asteraceae and Euphorbiaceae* species are enriched in vernolic acid, which is of great industrial value [15].

The preparation of epoxy fatty acid (vernolic acid), which is a precursor for the intended synthesis of carbohydrate fatty acid esters, was carried by saponification of vernonia galamensis seed oil using potassium or sodium hydroxide [16]. The white solid product (yield of 73 %) obtained after purification was characterized by different spectroscopic techniques.



Vernonia oil

Vernolic acid

Scheme 3.3*: Hydrolysis of vernonia oil*

The ^{1}H and ^{13}C NMR spectrum obtained confirmed that the hydrolysis of vernonia oil was successfully carried out resulting in vernolic acid. The presence of the major functional groups, characteristic of vernolic acid, in their respective positions was indicated in the spectrum. Accordingly, the ^{1}H NMR ($CDCl_3$) data of the vernolic acid

indicates the presence of hydroxylic protons (-OH) at 12.11 ppm, olefinic protons (CH=CH) at 5.32-5.48 ppm, epoxy protons (O-C-H) at 2.75-2.93 ppm, protons of methylene attached to olefinic group (-CH_2-CH=CH-CH_2-) at 2.02 - 2.30 ppm, protons of methylene groups $(CH_2)_n$ at 1.24-1.60 ppm, protons of methyl (CH_3) group at 0.89 ppm. The new peak at 12.11 ppm corresponding to (O-H) peak of the vernolic acid and the disappearance of the glyceryl proton peak of vernonia oil in the region 4.00-4.34 ppm after the hydrolysis, were additional confirmation for the success of hydrolysis of vernonia oil.

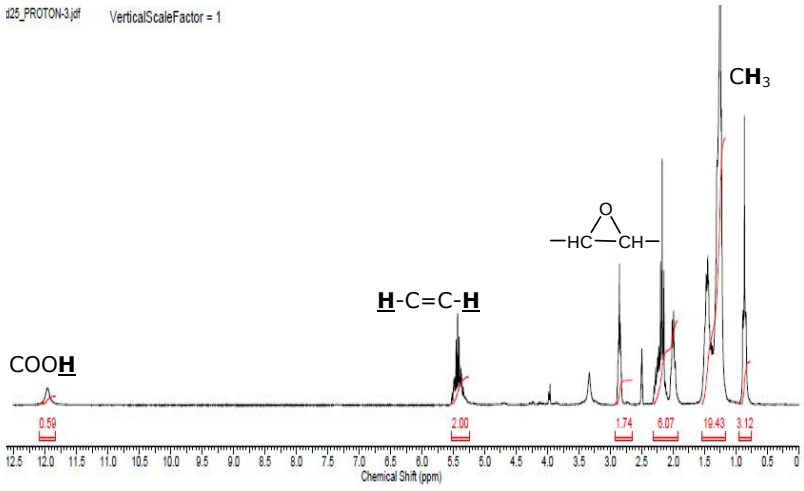


Figure 3.5*: ^1H NMR spectrum of vernolic acid*

The ^{13}C NMR ($CDCl_3$) spectrum of the vernolic acid shows the presence of the carbonyl carbon (O=C) at 175.5 ppm, olefinic carbons (CH=CH) at 125.05-132.43 ppm, epoxy carbons (O-C-H) at 56.02-56.59 ppm, methylene carbons $(CH_2)_n$ at 22.60-34.21 ppm, and methyl carbon (CH_3) at 14.41 ppm which are characteristic peaks of vernolic acid.

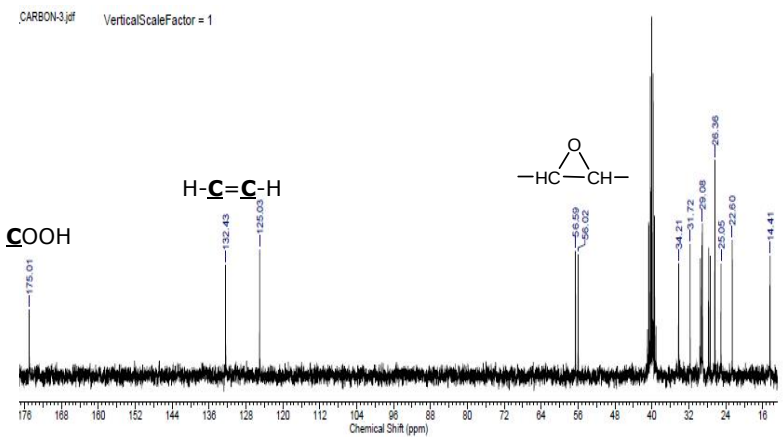


Figure 3.6: *^{13}C NMR spectrum of vernolic acid*

The ESI-MS data obtained revealed that M^+ ion is at m/z= 295.22 with a loss of H^+ ion from the vernolic acid ($C_{18}H_{32}O_3$) structure.

The FTIR spectrum recorded showed the major functional groups in the vernolic acid.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3489	O-H	stretching	w
2	3008	=C-H (*cis*-)	Stretching	m
3	2960	-C-H (CH_3)	Stretching (a)	m
4	2890	-C-H (CH_2)	Stretching (s)	vs
5	1711	-C=O (acid)	Stretching	vw
6	1654	-C=C-	Stretching	vw

7	1465	-C-H (CH_2, CH_3)	Bending	m
8	1377	-C-H (CH_3)	Bending (s)	m
9	1243	-C-O, -CH_2-	Stretching, bending	m
10	1033-1118	-C-O	Stretching	m
11	823, 840	-C-O (epoxy group)	Stretching	w
12	723	-$(CH_2)_n$,	Bending (rocking)	m

Table 3.3*: Summary of FTIR data of vernolic acid*

From the FTIR spectrum, the observation of the hydroxyl (O-H) stretching band at 3489 cm^{-1}, carbonyl (C=O) stretching band characteristic of an acid functionality at 1711 cm^{-1} and the presence of epoxy group peak at 823 and 840 cm^{-1} strongly support that the saponification of vernonia oil results in vernolic acid.

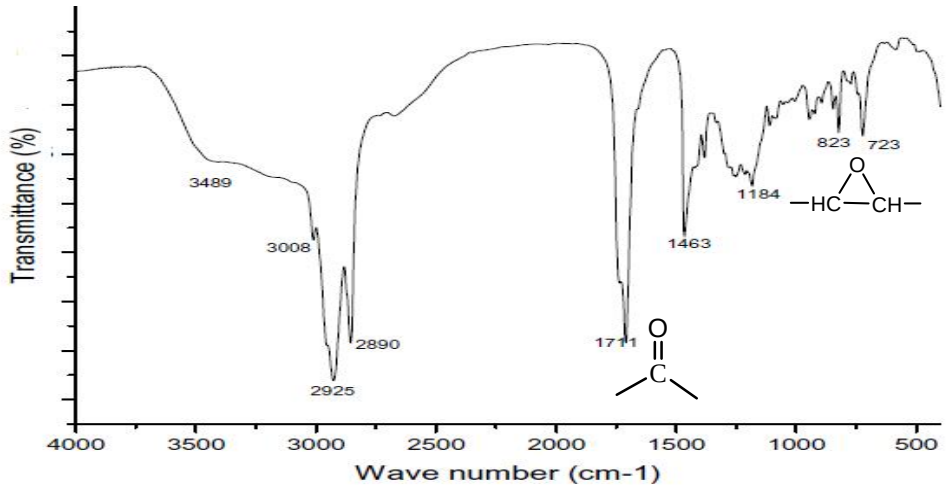
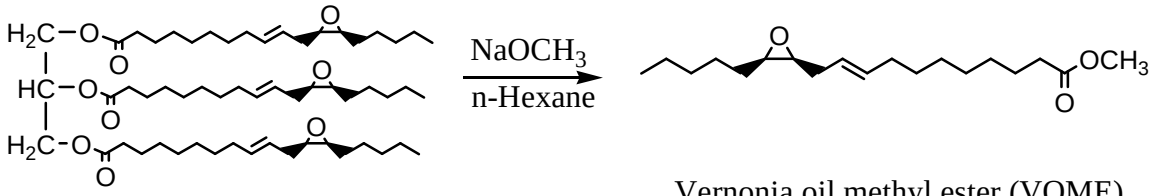


Figure*: FTIR spectrum of vernolic acid*

The presence of the major functional groups characteristic of vernolic acid in ^{1}H NMR ^{13}C NMR, FTIR spectral data as well as stable vernolic acid molecular ion obtained in ESI-MS analysis conducted confirm that the hydrolysis of vernonia oil was successfully carried out and pure vernolic acid was obtained. The vernolic acid was used for esterification reactions with glucose and cassava starch.

3.1.3 Vernonia oil methyl ester (VOME)

Transesterification of the triglyceride structure of vegetable oils with an alcohol in the presence of a strong acid or base, results in a mixture of fatty acids alkyl esters and glycerol [20]. In this study, base catalyzed synthesis of vernonia oil methyl ester by transesterification of vernonia oil to vernonia oil methyl ester was carried out. The prepared ester, vernonia oil methyl ester (methyl vernolate), was purified by column chromatographic technique with a glass column using silica gel (60–120 mesh size) as stationary phase and the eluent consisting of hexane and ethyl acetate. The polarity of the solvent was increased gradually starting with pure hexane to 10 % ethyl acetate in hexane. The methyl vernolate was much easier to purify since the epoxy group introduces a larger polarity difference relative to the other oil components. This method was used for the separation of methyl vernolate from the product containing unreacted acylglycerols and other methyl esters. The transesterification of vernonia oil resulted in a pure yellowish colored vernonia oil methyl ester (methyl vernolate) with a yield of 93 %.



Vernonia oil

Vernonia oil methyl ester (VOME)

Scheme 3.4*: Reaction scheme of synthesis of VOME*

The product, methyl cis-12, 13-epoxy-cis-9-octadecenaote, (methyl vernolate) was characterized using ^{1}H and ^{13}C NMR, FTIR, ESI-MS spectroscopic techniques.

The ^{1}H NMR (ppm) (400 MHz, $CDCl_3$) data of the indicated the presence of olefinic protons (CH=CH) at 5.22-5.37, methoxy protons (OCH_3) at 3.62, epoxy protons (O-C-H) at 2.65-2.77, protons of methylene attached to olefinic group ($-CH_2-CH=CH-CH_2-$) at 2.16 -2.17, protons of methylene groups $(CH_2)_n$ at 1.15-1.50, protons of methyl (CH_3) group at 0.77. In the ^{1}H NMR spectrum, there is no trace of glycerol moieties indicating that the transesterification has resulted in cleavage of the triglyceride structure of vernonia oil.

The ^{13}C NMR data of the VOME shows the presence of the carbonyl carbon (O=C) at 173.64, olefinic carbons (CH=CH) at 123.89-132.17, epoxy carbons (O-C-H) at 56.14-56.76, ($O-CH_3$) at 50.96, methylene carbons $(CH_2)_n$ at 22.53-33.77, and methyl carbon (CH_3) at 13.86. Based on the analysis of characteristic peaks obtained in the ^{1}H and ^{13}C NMR spectral data, it was confirmed that VOME successfully prepared.

The FTIR spectrum also shows the presence of the major functional groups typical of vernonia oil methyl ester. The peak at 1742 cm^{-1} corresponding to the C=O stretching band of the ester functionality, and epoxy peak band at 823 cm^{-1} are among the characteristic peaks confirming the successful preparation of vernonia oil methyl ester.

No	Frequency (cm^{-1})	Assigned group	Mode of vibration	Intensity
1	3008	=C-H (*cis*-)	Stretching	m
2	2927	-C-H (CH_3)	Stretching (asym)	m
3	2890	-C-H (CH_2)	Stretching (sym)	vs
4	1742	-C=O (ester)	Stretching	vs
5	1654	-C=C-	stretching	vw
6	1461	-C-H (CH_2, CH_3)	Bending (scissoring)	m
7	1377	-C-H (CH_3)	Bending (sym)	m
8	950-1250	-C-O	Stretching	m
9	823	-C-O (epoxy group)	Stretching	w

Table 3.4: FTIR spectrum of VOME

The ESI-MS data obtained also confirmed the product in which the corresponding signals of m/z = 311 is due to $C_{18}H_{32}O_3Na$ where as m/z= 333.23 is corresponding to sodiated methyl vernolate, $C_{18}H_{32}O_3Na$.

The base catalyzed transesterification is a mild reaction condition in which the oxirane ring cleavage as well as reaction of the epoxy group could be avoided as compared to acid catalyzed transesterification procedure. Spectrometric characterizations revealed that the base catalyzed transesterification of vernonia oil to prepare VOME was successful. The epoxy methyl ester could serve as chemical intermediate for new bio-based products.

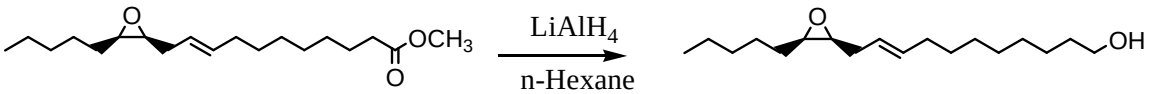
Chemical and enzymatic transesterification reactions of glucose and cassava starch were carried out using the pure VOME obtained as a precursor.

3.1.4 Synthesis of cis-12, 13-epoxy-cis-9-octadecenol (Vernanol)

Fatty alcohols are among the most widely used oleochemicals, after fatty acids, and efforts focusing on preparing new derivatives and applications for the alcohols are significantly growing. The synthesis of fatty alcohols involves direct reduction of fatty esters with lithium aluminum hydride (LAH) [17]. The reduction of carbonyl functionality in aldehydes, ketones, and esters to the corresponding alcohols; without affecting the carbon-carbon double bond or carbon-carbon triple linkages could be achieved by using LAH, which is among the most powerful complex metal hydride reducing agents [18]. Vernanol is one of the derivatives of vernonia oil. The procedure used in this synthesis was adapted from Elhilo, E.B. *et al*., [19] with modification. In a 500 mL, three necked round bottom flask equipped with air condenser and separatory funnel, 60 mL of hexane was added to dissolve 0.9 g of $LiAlH_4$. Then 2.5 g VOME was added with stirring. After the reaction mixture was stirred for 2 h, 5 mL of deionised water was added slowly and stirred for additional 15 min. The product was vacuum filtered, the filtrate stripped off with rotary evaporator to result in white solid product. In this study, the reduction of vernonia oil methyl ester (VOME) in the presence of $LiAlH_4$ catalyst resulted in epoxy alcohol (vernanol), cis-12, 13-epoxy-cis-9-octadecenol, with a yield of 85 %. During the reaction of $LiAH_4$ with vernonia oil methyl ester, the reduction of the ester functionality proceeds by nucleophilic attack of the hydride ion on the

carbonyl group, leading to the formation of intermediate alkoxide salts from which the alcohol is generated upon acidification.

The schematic representation of the reaction is given below.



Vernonia oil methyl ester

Vernanol (epoxy alcohol)

Scheme 3.5*: Reduction of VOME*

The ^{1}H NMR, IR and ESI-MS analysis carried out showed that the synthesis of vernanol was successful.

The ^{1}H NMR (ppm) (400 MHz, $CDCl_3$) data of the epoxy alcohol indicates the presence of the olefinic protons (CH=CH) at 5.35 and 5.60, methylene protons (O–CH_2) at 3.61, epoxy protons (O–C–H) at 2.9, proton of the hydroxyl group at (-C-OH) at 2. 21, protons of methylene attached to olefinic group (–CH_2–CH=CH–CH_2–) at 1.94–2.45, protons of other methylene groups (CH_2)*n* at 1.21–1.72 and protons of methyl (CH_3) groups at 0.89. This clearly signals that the reduction of vernonia oil methyl ester has resulted in an epoxy alcohol (vernanol). This is also supported by the ^{13}C NMR spectrum data.

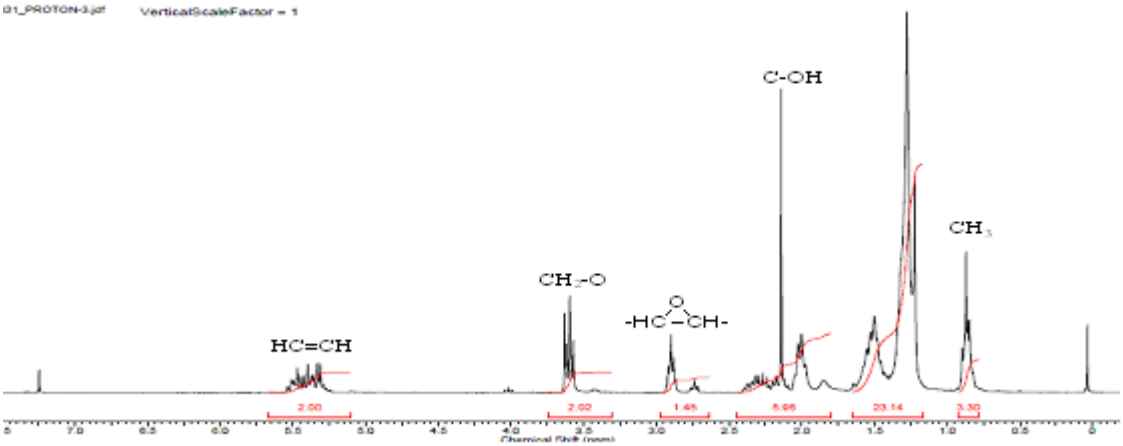


Figure: *[1]H NMR spectrum of vernanol*

The ^{13}C NMR (ppm) (400 MHz, $CDCl_3$) data also shows indicates the presence of the major peaks characteristic of epoxy alcohol. Among these, the olefinic carbons of the epoxy alcohol (CH=CH) at 132.73 and 123.90, epoxide carbons (O–C–H) at 56.68 and 57.35, carbon attached to the hydroxyl group (C–OH) at 63.04, methylene carbons $(CH_2)n$ at 22.66–32.83, and methyl carbon (CH_3) at 14.06 can be mentioned.

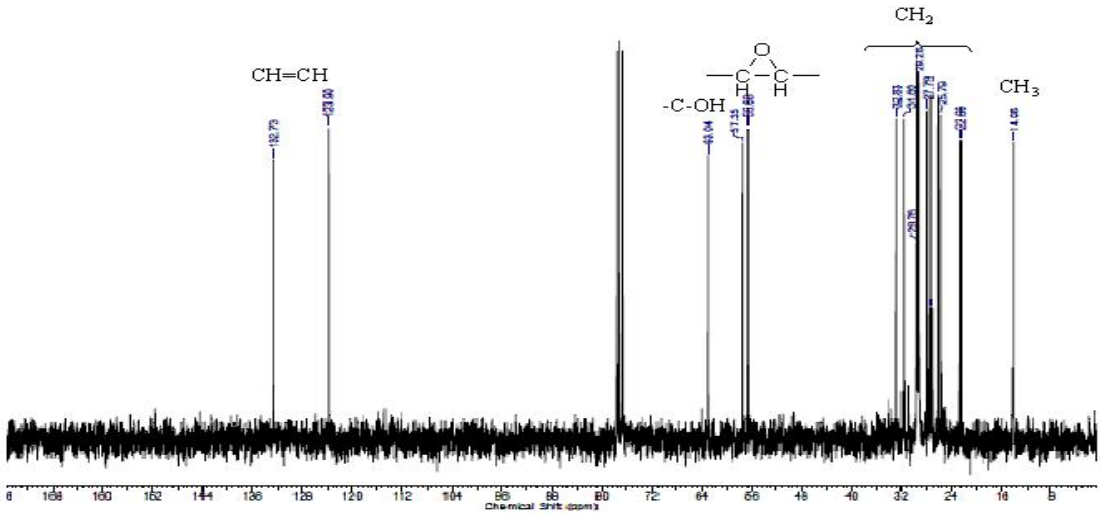


Figure: *[13]C NMR spectrum of vernanol*

FTIR spectrum analysis

The FTIR spectrum recorded shows the presence of the major functional groups in the vernanol.

No	Frequency (cm^{-1})	Assigned group	Mode of vibration	Intensity
1	3386	O-H	stretching	w
2	3008	=C-H (*cis*-)	Stretching	m
3	2927	-C-H (CH_3)	Stretching (a)	m
4	2856	-C-H (CH_2)	Stretching (s)	vs
5	1465	-C-H (CH_2, CH_3)	Bending (scissoring)	m
6	1377	-C-H (CH_3)	Bending (s)	m
7	950-1250	-C-O	Stretching	m
8	823	-C-O (epoxy group)	Stretching	w

Table 3. 5*: Summary of FTIR data of vernanol*

The FTIR spectrum recorded shows the major functional groups characteristic of vernanol (epoxy alcohol). The hydroxyl (O-H) peak at 3386 cm^{-1}, C-H stretching band at 3008, 2856, 2927 cm^{-1}, C-O-C stretching band in the range 950-1250 cm^{-1} and the epoxy peak at 823cm^{-1} were clearly observed. The band at 1654 cm^{-1} corresponding to (-C=C-, stretching), peak at 1465 cm^{-1} due to -C-H (CH_2, CH_3, bending (scissoring) and band at 1377 cm^{-1} due to -C-H (CH_3), bending (sym) were also indicated. This confirms that synthesis of vernanol from vernonia oil methyl ester was successfully carried out.

The ESI-MS data obtained confirmed that M^+ ion exists as a stable moiety at m/z= 305.24, corresponding to sodiated epoxy alcohol, $C_{18}H_{32}O_2Na$.

3.2 SYNTHESIS AND CHARACTERIZATION OF STARCH VERNOLATE

3.2.1 Synthesis of Starch Vernolate in Organic Solvents

Starch is one of the most widely used biopolymers because it is cheap, abundant and renewable. However, the hydrophilic nature of starch has limited the scope of its application. Many industrial applications require the modification of native starches. Therefore, the modified starch derivatives could be obtained by either glucosidic bond cleavage (acid modification) or forming new functional groups (carbonyl group formation during oxidation), or substitution of free available hydroxyl groups (by etherification or esterification), or bridging of molecular chains by cross-linking reactions [21]. These modifications overcome the limitations of native starch properties [22]. Starch based materials may be used as potential substituent for petroleum-based plastic materials, particularly in the packaging industries [23].

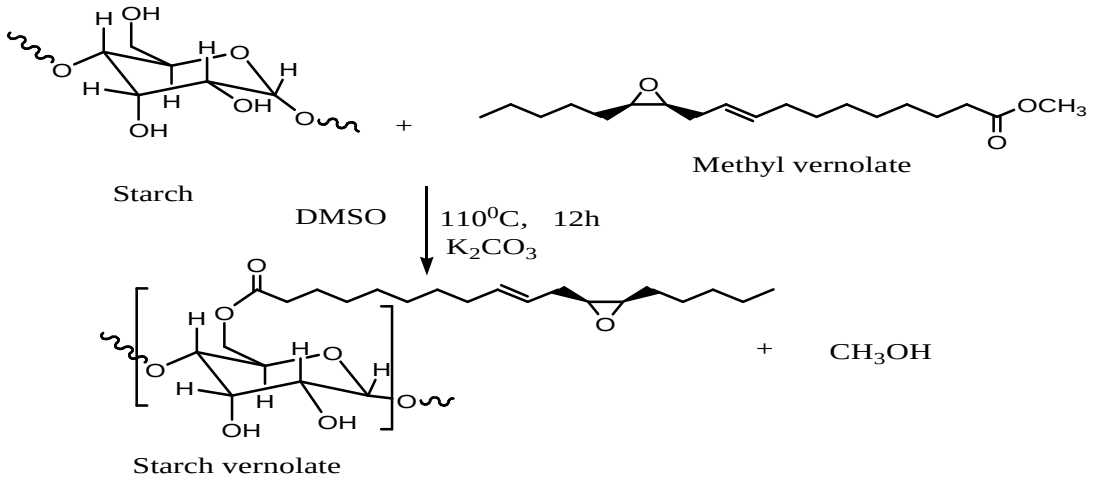
Starch is commonly modified by chemical and enzymatic treatment. Starch derivatives can be prepared through chemical modification of starch which is based on reaction of the free hydroxyl groups of the anhydroglucose unit (AGU) monomers with different functional groups. Esterification is one of the most common methods of starch modification involving the hydroxyl groups. Starch fatty acid esters can be prepared either by chemical or enzymatic method. Literature reports on esterification of starch indicate that most studies gave emphasis to synthesis of short chain fatty acid esters specially starch acetates from carboxylic acid (C_1-C_4) [24]. Furthermore, chemical methods of production of starch fatty acid esters by reacting starch with carboxylic acids

(C_4-C_{16}) are reported [25]. Junistia *et al.* [26] reported their success in the synthesis of long-chain fatty esters of corn starch with a broad range in degree of substitution (DS = 0.24-2.96) by reacting the starch with fatty acid vinyl esters (e.g. vinyl laurate, vinyl stearate) in the presence of basic catalysts in DMSO. Aburto *et al*. [27] also reported that they prepared longer-chain fatty esters (C_8–C_{18}) of potato starch and corn amylose using fatty acid chlorides and pyridine. In another study, synthesis of starch fatty acid esters from 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 at high temperatures and in polar aprotic solvents has been reported [28]. Polar solvents such as pyridine or DMSO are commonly used to dissolve starch to make the hydroxyl groups accessible towards esterification [41].

Literature reports indicate that there is large number of research being conducted on modification of starch using short or long chain alkyl substituents. However, investigations on the use of fatty acids containing multiple functionalities such as double bond or epoxy ring in their structure for the modification of starches are at their infancy. The use of vernonia oil methyl ester (VOME) or vernolic acid, derivatives of naturally epoxidized vernonia oil, as row material is of great advantage in minimizing cost as well as hazards that could arise from using synthetic methods of epoxidation. As there are no reports on the introduction of long chain fatty acid containing epoxy ring structure on starch, in this study grafting of starch with epoxy ring containing methyl vernolate derived from naturally epoxidized vernonia oil has been carried out. Inter and/or intra-molecular ring opening polymerizations that could arise from the epoxy group of the

grafted vernolate moiety can be used as opportunity to improve the application of starch esters as value added material.

In this study, novel starch vernolate (epoxy starch fatty acid ester) was successfully prepared by esterification of endemic cassava starch with long chain epoxy fatty acid methyl ester i.e., vernonia oil methyl ester (VOME) in the presence of basic catalyst, potassium carbonate in DMSO as a solvent.



Scheme 3. 6*: Schematic representation esterification of cassava starch with VOME*

The cassava starch was gelatinized in dimethyl sulfoxide at 70 °C for 3 h before the addition of the vernonia oil methyl ester to make the starch OH groups more accessible for reaction. The dissolution of starch in DMSO might have caused in the cleavage of the hydrogen bonds of starch and simultaneous destruction of the crystalline nature of starch to form a homogeneous solution making the hydroxyl functional group accessible for effective esterification of the starch with vernonia oil methyl ester. The reaction was carried out 12 h at temperature of 110 °C using basic salt, K_2CO_3 as the catalyst. Rubbery brownish solid product was obtained after product precipitation and washing with

methanol. The K_2CO_3 used was found to be effective catalyst in the transesterification of cassava starch with vernonia oil methyl ester. This agrees with reported observations. Junistia *et al*. [26] indicated that sodium acetate and K_2CO_3 were considerably active catalysts than Na_2HPO_4 and resulted in product with higher DS value during the synthesis of long-chain fatty acid esters of corn starch with vinyl laurate and vinyl stearate using alkaline catalysts.

Products Characterization

Starch fatty acid esters are commonly soluble in organic solvents. However, starch vernolate obtained in this method was rubbery brown solid insoluble in most organic solvents. This may be due to ring opening polymerization that could arise from epoxy (oxirane) ring of the vernonia oil methyl ester moiety. Hence, it was impossible to characterize the product using liquid state NMR analysis technique. Therefore, ^{13}C CP/MAS NMR (solid state NMR) analysis was used instead of the liquid state NMR technique. Furthermore, the product was characterized by FTIR, Electron Scanning Microscopy, X-ray powder diffraction and DSC/ TGA techniques.

The determination of the degree of substitution (DS)

The degree of substitution (DS) of a starch derivative is defined as the number of hydroxyl (OH) groups substituted per D-glucopyranose structural unit of the starch polymer. Since each glucose unit possesses three reactive hydroxyl groups, the maximum possible DS value is 3. The DS varies with the source of starch, amylose and amylopectin fractions, stoichiometric amounts and reaction time [29].

The degree of substitution was determined using titrimetric method. The principle of the method is that if modified starch is saponified with a known amount of hot aqueous NaOH, the ester bonds will be hydrolysed and sodium acylates will form. When this solution is back-titrated with a standard strong acid (*e.g*. HCl), the amount of NaOH used for saponification, can be calculated and consequently the acyl group substitution can be quantified. In this particular case, the DS of starch vernolate was determined using reported method [30] with minor modification. Approximately 0.5 g of dry starch vernolate was weighed and added into a 50 mL conical flask. Then 3ml water and 5ml of 1.0 M NaOH was added, and the conical flask was agitated with a magnetic stirrer at room temperature for 48 h. After the indicator (phenolphthalein) was added, the excess alkali was titrated with 0.5 M hydrochloric acid. The starch reference sample and duplicates were treated in a similar way.

The vernolyl content (*A* %) was calculated according to the following equation:

$$A\% = \frac{[(V_o - V_n) \times Molarity\ of\ HCl \times M_{vernolyl} \times 10^{-3} \times 100]}{M}$$

Where V_0 in mL is the volume of 0.5 N HCl used to titrate the blank; *Vn* in mL is the volume of 0.5 N HCl used to titrate the samples; *N* is the concentration of the used HCl (mol/L); *M* in g is the amount of dry starch vernolate sample; 279 is the formula weight of vernolyl groups. The vernolyl content (*A* %) was used to calculate the degree of substitution, DS, according to the following equation:

$$DS = \frac{(162 \times A\%)}{(M_{vernolyl} \times 100) - ((M_{Vernolyl} - 1) \times A\%)}$$

Where 162 is the molecular weight of glucose units and 279 is the formula weight of vernolyl group. The DS of starch vernolate in this case was found to be 1.24

FTIR spectrum Analysis

The major functional groups of starch vernolate in the FTIR spectrum presented in the table below show that transesterification of cassava starch with vernonia oil methyl ester (VOME) under the reaction conditioned employed was successful.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3437	O-H	stretching	m
2	2927	-C-H (CH_3)	Stretching (a)	m
3	2855	-C-H (CH_2)	Stretching (s)	vs
4	1740	-C=O (ester)	Stretching	vs
5	1461	-C-H (CH_2, CH_3)	Bending (scissoring)	m
6	1377	-C-H (CH_3)	Bending (s)	m
7	954-1200	-C-O (anhydroglucose unit)	Stretching	m
8	841	epoxy carbon	Stretching	w

Table 3. 6*: Summary of FTIR data of starch vernolate*

In a native starch FTIR spectrum, strong broad bands in the region 900–1250 cm^{-1} particularly, the peaks at 1197, 1024, and 954 cm^{-1} are noticeable characteristic of anhydroglucose ring O-C stretching vibrations bands for a polysaccharide [31]. These bands were also observed in the spectrum of starch vernolates with better resolution confirming replacement of some of the hydrogen bonds in the native starch which is a good signal for success of the transesterification reaction. Another distinctive band observed between 3000 and 3500 cm^{-1} was assigned to the hydroxyl group stretching vibrations [32, 33, 34]. The appearance of the two peaks in the spectrum of starch vernolate with relatively strong intensities at 2927 and 2855 cm^{-1} correspond to the methyl and methylene C-H stretching associated with epoxy fatty acid chain substituents [23]. The grafting of the vernolyl moiety can also be observed by the appearance of a new peak at 841 cm^{-1} referring to epoxy ring functional group from the vernonia oil methyl ester [35].

The successful esterification of the starch with VOME was confirmed by the appearance of new peak at 1740 cm^{-1} which was absent in the spectrum of both cassava starch and methyl vernolate. The intensity of the bands in the range 3000-3500 cm^{-1} which corresponds to hydroxyl peaks in the native starch has decreased as expected due to esterification [36]. A shift was also observed in the hydroxyl peak's maximum from 3381 cm^{-1} for native cassava starch to 3437 cm^{-1} for esterified starch.

Thermal Analysis

Differential Scanning Calorimetry (DSC) Analysis

Differential Scanning Calorimetry (DSC) is a technique used to determine thermophysical properties of materials (*e.g.* polymers). It involves the simultaneous application of a heat flow to the sample and a reference while measuring the differential heat flow between the sample and a reference. DSC is typically used to detect and measure the melting temperatures of polymers and biomaterials through measurement of enthalpic changes undergone by these materials during phase transitions produced by changes in temperature.

The melting temperatures of the native and starch vernolates were determined by differential scanning calorimetry studies. Glass transition, Tg is the glass-rubbery transition that occurs in amorphous polymers and Tm (melting point) occurs when the ordered regions of a polymer fall apart upon heating. Gelatinization is a complex process which includes the disruption of the crystalline regions (*Tm*) within the granules, while the phenomenon of the *Tg* occurs just to amorphous materials. The differences in temperatures for the endothermic peaks among different starch samples are associated therefore to the starch composition (amylase to amylopectin ratio), granular architecture (crystalline to amorphous ratio) and Mw (molecular weight) as well as polydisperisty of the chains [37]. In general, DSC results are entirely dependent on the moisture content (mc), or plasticizer as well as thermal conductivity. Septo [38] reported that in sealed DSC pans with potato starch only one peak corresponding to the endothermic transition was detected at 75 °C with 45 % moisture content and 150 °C at 12 % moisture content.

The midpoint in the DSC curve was taken as *Tm*, as it gives the average temperature at which most of macromolecules undergo the melting. In the DSC curves of the native starch as well as that of the graft copolymers, only one endotherm was observed in the temperature range used for the study. *Tm* of native cassava starch in this study having about 10-13 % moisture was 139 °C as shown in Figure 3.5 below. This value agrees with reported results. According to Jyothi, N., *et al*. [39] the *Tm* of cassava starch with 8-10 % moisture content was found to be 162 °C [40]. In another study, Rajan, A. *et al*., [41] indicated that the glass transition temperature of native cassava starch which was 139 °C has decreased during solution state enzymatic esterification with recovered coconut oil.

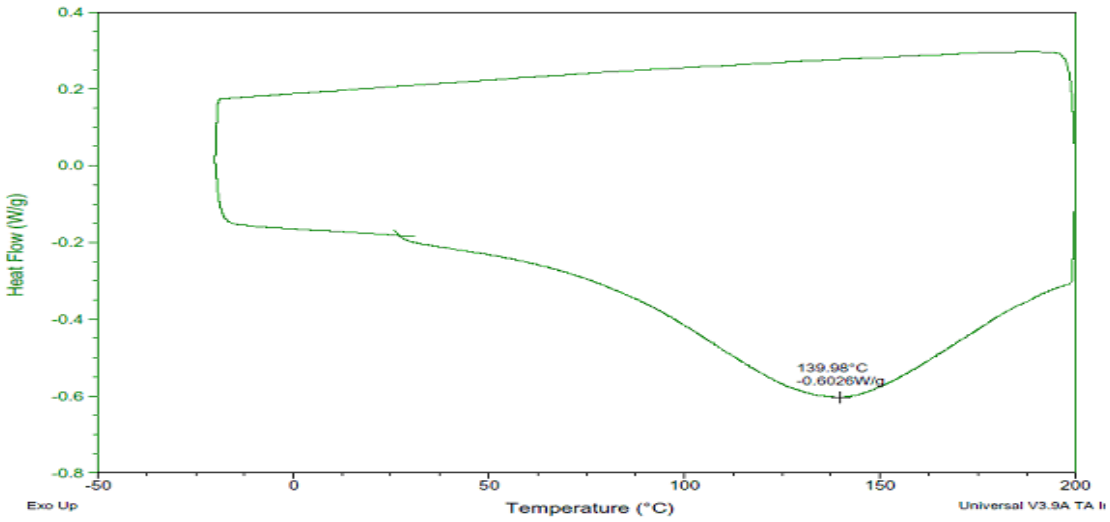


Figure 3. 5*: DSC thermogram of Cassava starch*

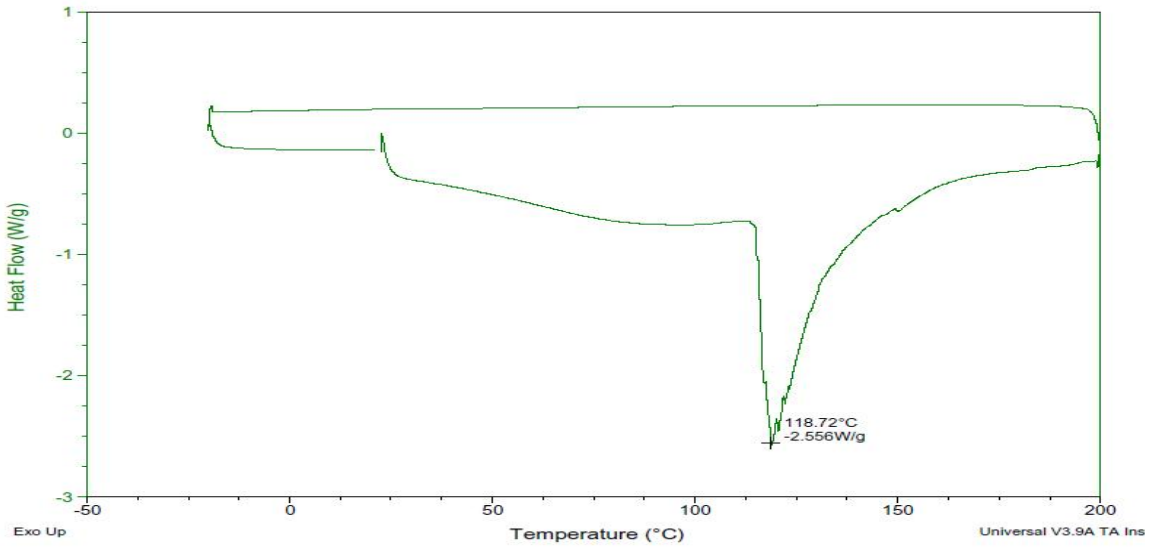


Figure 3.6*: DSC thermogram of Starch vernolate*

DSC has been extensively used to study the *Tm* (melting point) on starch granules and *Tg* (glass transition temperature) on amorphous materials. *T*g and *T*m of the native starch were changed by the esterification reaction [42]. In Figures 3.5 and 3.6 above, it is shown that *T*m of the native starch was 139 °C and that of the starch vernolate was 118 °C, respectively. In other words, *Tm* of the modified starch is lower by 21 °C than its unmodified counterpart. *Tm* of starch decreased with the increasing degree of esterification. This agrees with the results of Shogren [43]. These changes can be explained by the fact that the intermolecular hydrogen bonds, which stiffen the macromolecular chain, decrease with the partial replacement of hydroxyl groups by vernolyl groups. Moreover, the increase in the free volume within the molecules due to the introduction of bulk groups that facilitates more molecular mobility also contributes to the decrease in *T*m of starch after esterification [41, 44].

Thermogravimetric Analysis (TGA)

The thermal stability of native cassava starch and esterified cassava starch has been investigated by using TGA. The TG profiles were used to determine the weight loss of native cassava starch and starch vernolate as they were heated, cooled or held isothermally [35]. The initial weight loss in the starch started at lower temperature around 70-100 °C corresponding to loss of water absorbed as natural starch is hydrophilic and can absorb moisture under normal room conditions. The other major degradation (i.e., more than 85 %) of native starch began at 306.5 °C.

Although the initial of degradation of starch vernolate started at lower temperature, it leaves more residues, i.e. 27 % at 420 °C, which is more than cassava starch with a residue of only 13 % at 420 °C. This is an indication of an overall higher thermal stability for starch vernolate [45, 46]. The thermogram also shows difference in degradation products between the starch and starch vernolate, which could be another signal for the modification of starch i.e., esterification.

The improved thermal stability of starch esters as compared to native starch is probably due to the low content of hydroxyl groups in the former. Accordingly, the relatively higher degree of substitution achieved in this method of synthesis could have improved the thermal stability of the esterified starch. From TGA analysis, it can be concluded that, esterified starch is thermally more stable than native starch.

Scanning Electron microscopy (SEM)

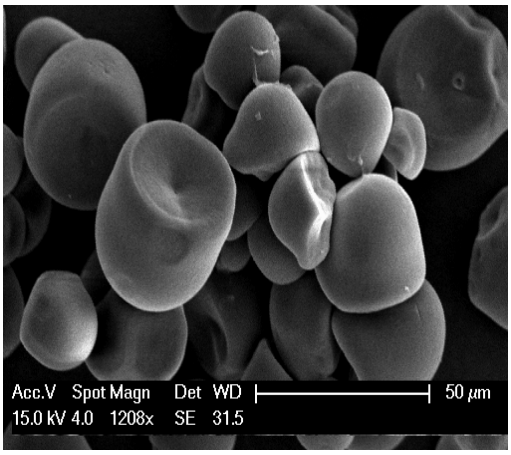
Scanning electron microscope (SEM) uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens [113]. It enables the investigation of specimens with a resolution down to the nanometer scale. Here an electron beam is generated by an electron cathode and the electromagnetic lenses of the column and finally swept across the surface of a sample [113].

Various signals are generated as result of the impact of the primary electrons (PE) of the electron beam and the specimen's bulk which are collected to form an image or analyse the sample surface. These include secondary electrons (SE) and backscattered electrons (BSE) and X rays [113]. They originate from an interaction volume in the specimen which varies in diameter according to different energies of the primary electrons (typically between 200 eV and 30 keV). The SE come from a small layer on the surface and yields the best resolution, which can be realized with a scanning electron microscope. The BSE come from deeper regions of the material under study thus giving a lower resolution. In the conventional scanning electron microscope, which operates in high vacuum, the specimen has to be electrically conductive or has to be coated with a conductive layer such as Platinum, Carbon or Gold [113,114].

Information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample could be obtained from the signals that derive from electron-sample interactions [113,114].

In this study, the SEM images obtained show that there is a remarkable difference in appearance between native cassava starch and starch vernolates prepared by the method mentioned. Native cassava starch granules had spherical and truncated hemi-spherical shape [47, 48]. However, the cassava starch granules which were spherical and truncated hemi-spherical ones have lost their individuality and smooth surface texture after esterification due of replacement of hydroxyl groups. The entire loss of granular nature of the starch confirms relatively high degree of substitution.

According to the SEM profiles, the starch granules were mostly converted from their crystalline structure into amorphous state during the dissolution processes, which led to the uniform esterification reaction conducted under conditions involved [49, 80]. The solvent DMSO used as reaction medium during the modification reaction was responsible for disruption the hydrogen bond of starch, sequentially led to the destruction of the crystalline structure of starch granules. This can be the cause for enhanced chemical reagent access to the hydroxyl groups of starch and increase starch reaction with the reagent. The introduction of vernolyl groups disrupt the ordered structure of native starch and hamper the re-association of amylose and amylopectin structures in starch, leading to the change in morphological properties. The morphology of starch vernolates were changed as compared to native cassava starch as a result of esterification. The successful grafting of long chain epoxy fatty acid on to starch using the method mentioned has also been confirmed from the SEM analysis.



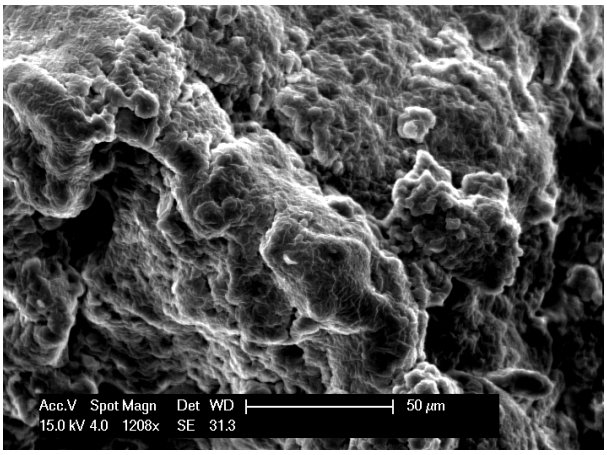


Figure 3.7*: SEM image of starch*

Figure 3. 8: *SEM image of starch vernolate*

Powder X-ray Diffraction

X-ray diffraction has been widely used to detect and characterize the crystalline patterns of starch granules [50]. Starch has a definite crystalline nature and the crystallinity has been assigned to the well-ordered structure of the amylopectin molecules inside the granules [51]. Powder XRD studies provide much of the information about starch granule crystalline properties. Different starches can be classified into A, B or C patterns. *A* form starch is mainly present in cereal starches, such as maize starch and wheat starch. The XRD patterns of these starches give the stronger diffraction peaks at around 15, 17, 18 and 23°. The B form starch is usually available in tuber starch such as potato and this type of starch gives the strongest diffraction peak at 2θ of 17°. There were also few small peaks at around 2θ values of 20, 22 and 24°. The C pattern starch is a mixture of both A and B types, characteristic of smooth seeded pea starch and various bean starches [52].

In starch structure, linear amylose composed of α-1,4-glucopyranose was responsible for the amorphous region, while large amylopectin composed of both α-1,4 and α-1,6-glucopyranose contributed to the crystalline region. The X-ray powder diffraction pattern obtained for native cassava starch and representative starch vernolate are presented in Figure 3.9 and Figure 3.10 below. The native cassava starch powder had crystalline structure, with a strong diffraction pattern. In this study, the diffractogram of native cassava starch exhibited a crystalline pattern, giving three peaks at 2θ of 15.34°, 17.24°, 18.31° and 23.36°. The occurrence of these peaks confirms that the cassava starch used in our study had an A pattern [53, 54]. This data also agrees with the report by Paulos *et al*. [48] which shows that cassava starch obtained from cassava tubers collected from three different regions in Ethiopia, namely, Gamo Gofa, Illubabor and Wollega were found to have A-type crystallinity.

After esterification, the diffraction pattern showed broad peak, an indication for the amorphous character of the starch vernolate. The highly ordered crystalline structure of cassava starch is related to intra- and intermolecular hydrogen bonds. However, upon esterification, some of the hydroxyl groups on starch backbone were replaced by vernolyl moiety. This has minimized the formation of intermolecular hydrogen bonding and, thereby, disturbing and reducing the orderly crystalline structure of native cassava starch [55, 56]. The relatively higher degree of substitution (DS = 1.24) obtained in our study indicates that much of the hydroxyl groups were substituted by vernolyl group. The samples, as expected, gave an amorphous pattern with a new broad peak appearing around 20°. The presence of this new peak, which was not observed in the diffractogram

characteristic of native cassava starch, was another confirmation for the modification of starch by esterification with long chain epoxy fatty acid methyl ester. These broad peaks may have originated from smaller size starch crystals as the esterification reaction continued. Similar results were reported by Zhang *et al.* [57] and Luo, Z. *et al.* [80].

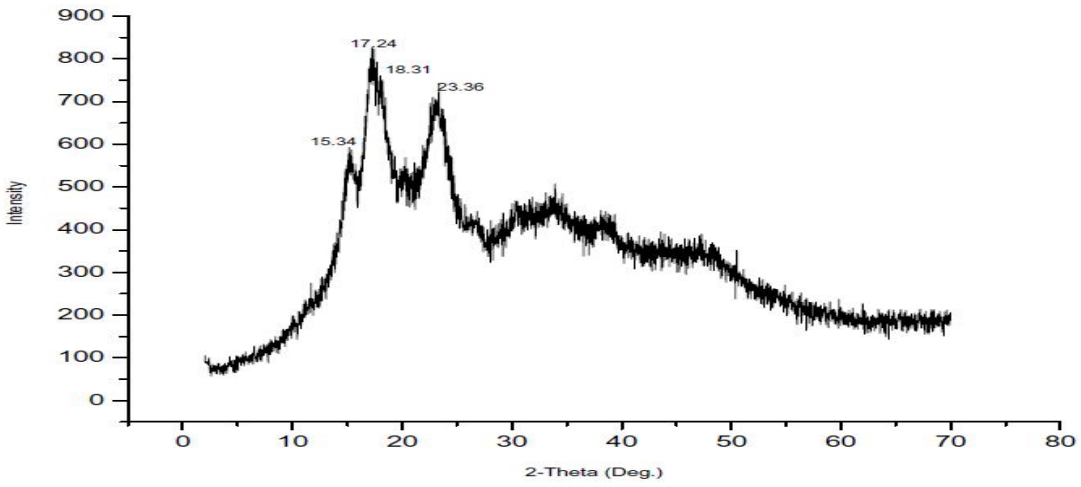
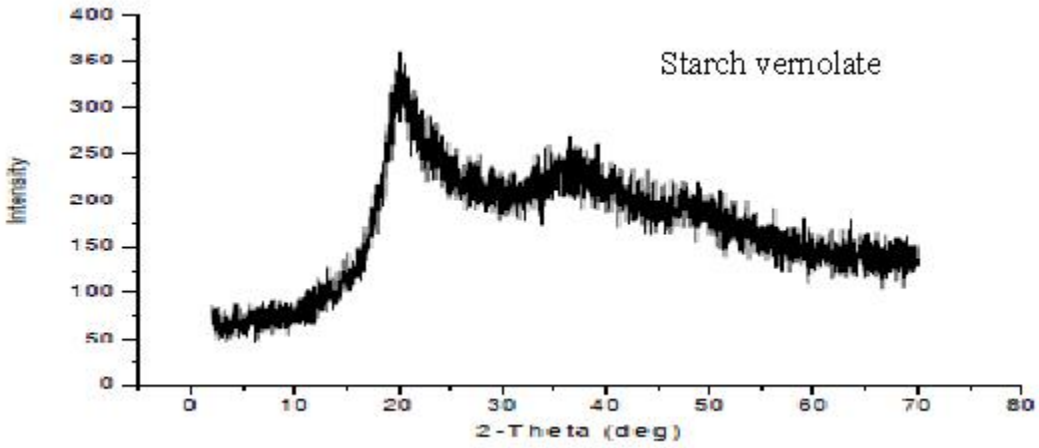


Figure 3.9*: XRD diffractogram of cassava starch*



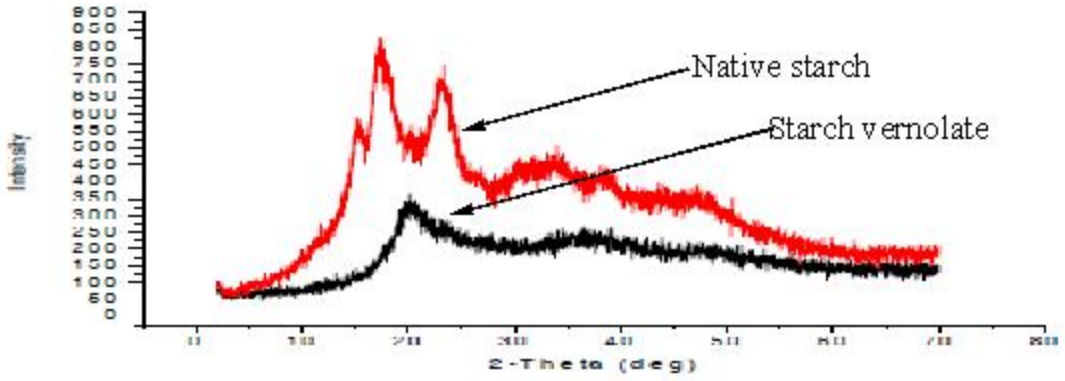


Figure 3. 10: *XRD diffractogram of starch vernolate*

Solid state ^{13}C CP-MAS (Cross Polarization Magic Angle Spinning) spectrum

^{13}C CP-MAS NMR is sensitive to substances at low humidity content (rigid state with low molecular mobility). This technique has been used to obtain structural information of granular starches and starch vernolate. The spectrum shows the position of each one of the resonances at the respective carbons located in the glucopyranose ring and fatty acid moieties.

The epoxy starch fatty acid ester obtained in this case was an insoluble solid. This made liquid state NMR analysis was impossible. Therefore, the esterification reaction of cassava starch was also characterized by ^{13}C CP-MAS NMR spectroscopy. In the spectrum, the noticeable signals in the region between 50 to 110 ppm are mostly attributed to the different carbons of starch [58, 59, 60]. The resonance line at 104.08 ppm is assigned to the C-1. The peaks at 89.8 and 83.36 ppm are related to the C-4 of crystalline starch and disordered starch, respectively. A similar trend can be seen in the

signal to C-6, namely, the signal at 62.00 ppm is attributed to the crystalline starch and the peak at 62.65 ppm is assigned to disordered or crystal surfaces starch. In this region there were also two peaks at 73.01 and 78.6 ppm which belong to the C-2−C-5. In addition, the two representative peaks at 179.8 and 21.5 ppm in spectrum, which belong to C=O and CH_3 in vernolyl group, indicated the occurrence of esterification reaction [59, 60]. Increased intensity of peaks at 179 ppm (C=O) and 14.5 ppm (CH_3) and the decrease of strength for peaks at 70.5, 78.6 ppm (C-2−C-5) and 62.8 (C-6) in spectrum indicated that the extent of esterification.

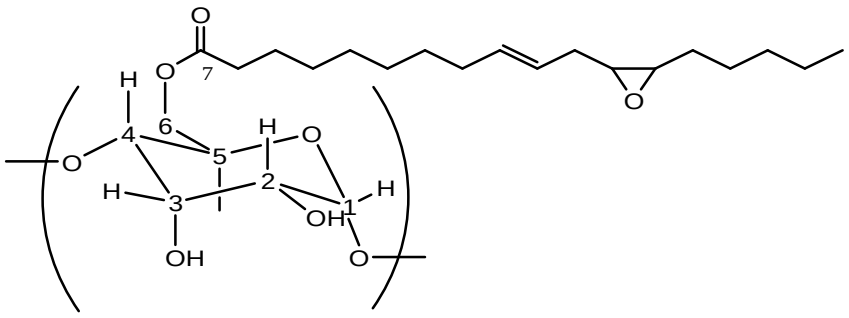


Figure 3.11*: Structure of starch vernolate*

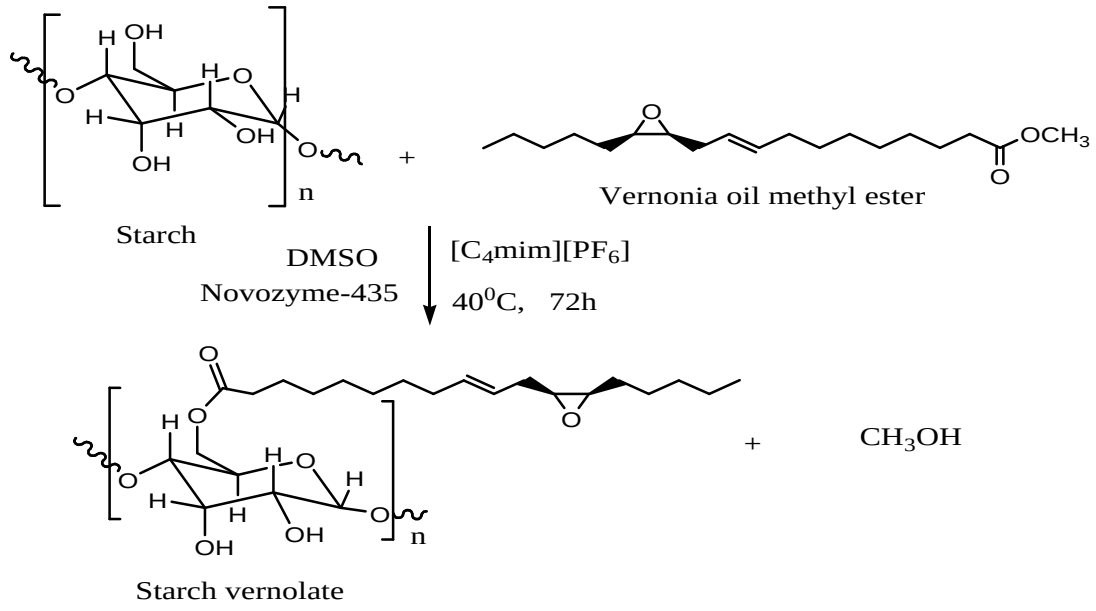
3.2.2 Enzymatic Synthesis of Starch Vernolate in Ionic Liquid

Fatty acid esters of starches could be prepared by reacting starch with fatty acid chlorides, fatty acid vinyl esters, carboxylic acids, or fatty acid methyl esters as reactants in organic solvents such as pyridine, toluene, or DMSO [26, 44]. However, volatility, toxicity, expensiveness, economical as well as environmental impacts associated with the use of organic solvents has greatly limited the industrial application of starch esters, especially in food industries. Accordingly, there is a strong incentive to search for an alternative and environmentally benign process for the synthesis of fatty acid starch ester [61]. Recently, synthesis of starch esters in ionic liquids as reaction media is becoming area of interest [62, 63, 64].

Enzymatic esterification process is an environmentally friendly method which occurs under milder conditions. *Candida antarctica* lipase B (CALB) immobilized in acrylic macroporous resin is commercialized as Novozym-435 and is one of the most used enzymes in bio-catalysis being employed in industrial processes as catalyst for the synthesis of simple esters, amides, alcohols, and amines [66]. Lipase enzymes showed superior activity in esterification reactions carried out using ILs such as [EMIM][BF_4], [EMIM][$(CF_3SO_2)_2N$], [BMIM][PF_6] and [BMIM][$(CF_3SO_2)_2N$] [68]. The activation and deactivation of enzymes can also be considered as a solubility-based phenomenon of substrates that are incompatible or compatible with the ionic liquids used. Enzymes can be dehydrated, in particular in polar solvents, and consequently lose their function. However, water-repellent ionic liquids are advantageous in terms of enzyme activity as they not dehydrate enzymes and therefore structure and function are maintained [68, 69].

However, little or no attention has been given to enzymatic synthesis of epoxy starch fatty acid esters in the presence of ionic liquid solvents.

In this study, immobilized lipase (Novozyme-435) catalyzed preparation of epoxy starch fatty acid ester (starch vernolate) was carried out from cassava starch and methyl vernolate using ionic liquid, 1-butyl-3-methylimidazolium hexafluorophosphate, [BMIM][PF_6] in the presence of DMSO as co-solvent.



Scheme 3.7 *: Schematic representation of esterification of starch with methyl vernolate*

The product yield was determined from the weight of recovered starch vernolate and calculated on the basis of 100 % conversion of vernonia oil methyl ester to starch ester. Accordingly, an optimum yield of 78 % was obtained at temperature of 40 °C and reaction time of 72 h. The use of DMSO, which is preferable solvent to dissolve starch, in this case is to improve the solubility of cassava starch which is low in pure ionic liquid. The ionic liquid used in this case was viscous. The viscosity of an IL limits enzyme

activity by affecting mass transfer of substrates. Viscosity of IL can be lowered by adding organic solvent [65]. Accordingly, we found that adding DMSO (20 % v/v) as co-solvent improved the esterification reaction by decreasing the viscosity of ionic liquids and improving activity of the lipase enzyme. The dissolution of starch by the ionic liquids and co-solvent might have resulted in the cleavage of the hydrogen bonds of starch and simultaneous destruction of the crystalline nature of starch to form a homogeneous solution necessary for effective esterification of starch with vernonia oil methyl ester. The result obtained and success of the enzymatic esterification in ionic liquids in our study is in good agreement with published reports. Chen, Z.G. *et al*. [65], reported that in Novozym 435 catalyzed acylation of konjac glucomannan, using ionic liquids. They found that enzymatic acylation in water miscible ionic liquids ($[C_2MIM][BF_4]$ or $[C_4MIM][BF_4]$) gave products with higher degree of substitution than those in water immiscible ILs ($[C_6MIM][BF_4]$ or $[C_4MIM][PF_6]$). They also reported that the use of t-butyl alcohol as co-solvent in enzymatic acylation of starch using ILs showed enhanced DS. However, with too much organic solvent the enzyme activity dropped significantly. In another study, starch was solubilized by 1-butyl-3-methylimidazolium chloride, [BMIM]Cl [62] and 1-butyl-3-methylimidazolium dicyanimide, [BMIM][DCA] and reacts with acetic anhydride in pyridine to give starch acetates [63]. It is also reported that high fatty acid esters of starch were synthesized by reacting the corn starch with fatty acid methyl ester using 1-butyl-3-methylimidazolium chloride [BMIM]Cl ionic liquid as reaction media [64]. Generally, ILs with 1, 3-dialkylimidazolium cations is generally recognized as the most valuable for biocatalytic applications.

The Novozym-435 lipase catalysis applied in our study showed better compatibility, stability and catalytic activity in the ionic liquid, [BMIM][PF_6]. It is also observed that Novozym-435 lipase used was active at the reaction temperature of 40 °C in this study. Similar observations were presented in various studies that enzyme-catalyzed reaction in ILs provides better results as compared to those obtained in conventional organic solvents [65, 67].

Products characterization

Starch fatty acid esters usually tend to soluble in organic solvents. However, starch vernolate obtained in this method was white solid powder insoluble in most of organic solvents. This may be due to ring opening polymerization that could arise from epoxy (oxirane) ring of the vernonia oil methyl ester moiety. Hence, the use of ^{13}C CP-MAS NMR (solid state NMR) technique was found to be crucial for solid product analysis as it was impossible to characterize the product using liquid state NMR analysis. Furthermore, the product was characterized by FTIR spectroscopy, Electron Scanning Microscopy, X-ray Powder Diffractometry and DSC/ TGA techniques.

The determination of the degree of substitution (DS)

The degree of substitution (DS) of starch vernolate prepared was determined using the method [30] with minor modification. Approximately 0.5 g of dry starch vernolate was weighed and added into a 50 mL conical flask. Then 3 mL water and 5 mL 1.0 M NaOH was added, and the conical flask was agitated with a magnetic stirrer at room temperature for 48 h. After the indicator (phenolphthalein) was added, the excess of alkali was titrated

with 0.5 M hydrochloric acid. The starch reference sample and duplicates were treated in a similar way.

The vernolyl content (*A* %) was calculated according to the following equation:

$$A\% = \frac{[(V_o - V_n) \times Molarity\ of\ HCl \times M_{vernolyl} \times 10^{-3} \times 100]}{M}$$

Where V_0 in mL is the volume of 0.5 N HCl used to titrate the blank; *Vn* in mL is the volume of 0.5 N HCl used to titrate the samples; *N* is the concentration of the used HCl (mol/L); *M* in g is the amount of dry starch vernolate sample; 279 is the formula weight of vernolyl groups. The vernolyl content (*A* %) was used to calculate the degree of substitution, DS, according to the following equation:

$$DS = \frac{(162 \times A\%)}{(M_{vernolyl} \times 100) - ((M_{Vernolyl} - 1) \times A\%)}$$

Where 162 is the molecular weight of glucose units and $M_{vernolyl}$ = 279 is the formula weight of vernolyl group. The DS of starch vernolate in this case was found to be 0.95.

FTIR Spectrum Analysis

The important peaks in the FTIR spectrum of esterified starch obtained were presented in the table below.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3434	O-H	stretching	m
2	2927	-C-H (CH_3)	Stretching (a)	m
3	2855	-C-H (CH_2)	Stretching (s)	vs
4	1728	-C=O (ester)	Stretching	m
5	1647	-C=C-	stretching	vw
6	1377	-C-H (CH_3)	Bending (s)	m
7	954-1200	-C-O (anhydroglucose unit)	Stretching	m
8	848	-C-O (epoxy group)	Stretching	w

Table 3.7*: summary of FTIR data of starch vernolate*

The main difference between the spectrum of the cassava starch and epoxy starch ester is attributed to the appearance of new bands at 1728, 1373, 1240, and 848 cm^{-1}, characteristic of the vernolate group introduced during the esterification process [57, 70].

The presence of unreacted hydroxyl group is indicated by the vibrations in the range 3000-3500 cm^{-1} implies that the DS is less than 3. The peak at 1647 cm^{-1}corresponds to C=C double bond of the ester group [71]. After introduction of the vernolyl moiety, the intensity of the hydroxyl vibration bands observed in starch vernolate has decreased as compared to that of the native cassava starch due to esterification. It was also clearly

indicated in the spectrum that the hydroxyl peak's maximum of native cassava starch which was observed at 3381 cm^{-1} has shifted to 3434 cm^{-1} in the esterified starch. A new absorption band recorded at 1728 cm^{-1} is attributed to the characteristic band of carbonyl (C=O) groups in the starch ester structure and therefore could be considered as a signal for the esterification of starch with vernonia oil methyl ester. With increasing degree of substitution, the intensity of the carbonyl peak increases. In this study, as there are still hydroxyl groups remaining unsubstituted on the cassava starch structure, carbonyl peak with medium intensity was obtained corresponding to DS of 0.95. This was also confirmed by the presence of broad hydroxyl (O-H) stretching band in the starch vernolate structure which appeared at 3434 cm^{-1}. The observation of a small peak at 848 cm^{-1} characteristic of the epoxidized vernolate group was an additional confirmation for the success of esterification reaction.

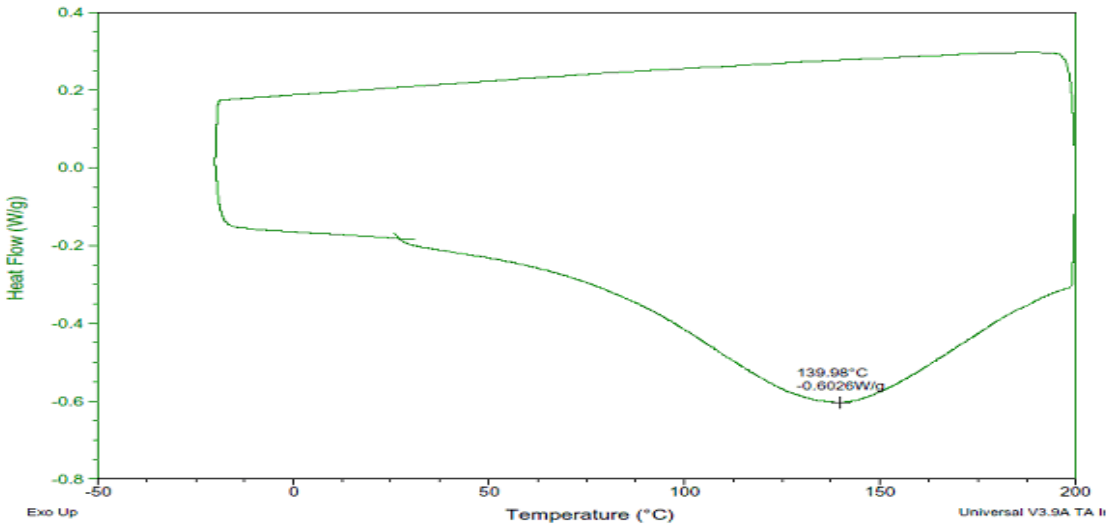
Thermal Studies

Differential Scanning Calorimetry (DSC)

Thermal studies of both cassava starch and starch vernolate were carried out using DSC. In the figure below, it is shown that *Tm* of native cassava starch in this study having about 10-13 % moisture was 139 °C and that of the starch vernolate was 87 °C, which is 52 °C lower than its unmodified cassava starch. The *Tg* of native cassava starch was found to be 107 °C and the *Tg* of modified starch vernolate became 69 °C. Jyothi *et al*. [39] reported *Tm* cassava starch with 8-10 % moisture content to be 162 °C [40]. Rajan, A. *et al*. [41] also reported that the glass transition temperature of native cassava starch which was 139 °C has decreased during solution state enzymatic esterification with

recovered coconut oil. Xu, W. *et al.* [56] reported in their study that they found that *Tg* of the native starch was 273 °C and that of the starch acetate with a DS of 2.95 was 226 °C. This is lower by 47 °C than its unmodified counterpart. The replacement of hydroxyl groups with long chain epoxy fatty acid methyl ester has led to loss of crystallinity of native starch**.** *T*m of starch decreased considerably with the increasing degree of esterification. This agrees with the results of literature report [43, 72].

Introduction of ester groups into starch backbone enhances plasticization and hence the transition temperatures of starch esters were considerably lowered through chemical modification. On the other hand, esterification of long-chain fatty acid decreases the number of hydroxyl groups and interferes with the hydrogen bonding. Moreover, the grafted vernolyl side chains damage the crystalline phase presented in the native cassava starch. As a result of the increasing size of bulky flexible side-chain groups, free volume of the polymer increases. This could lead to enhanced mobility of amylose and amylopectin molecules [44]. Hence, as expected the esterification of cassava starch with vernonia oil methyl ester resulted in a product i.e., epoxy starch fatty acid ester with decreased melting point as compared to native cassava starch.



. Figure 3. 12*: TGA thermogram of cassava starch*

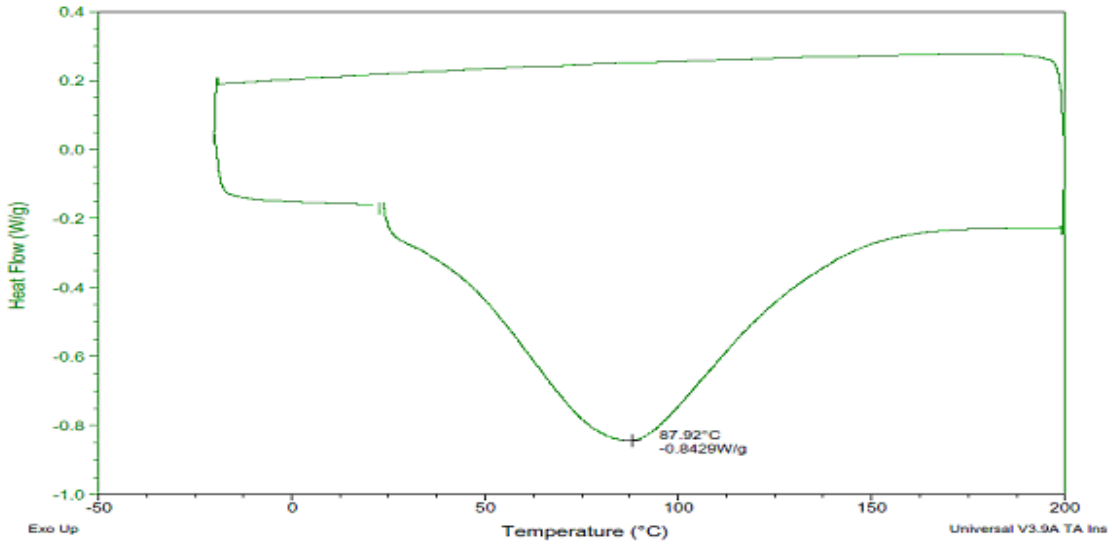


Figure 3. 13*: DSC thermogram of starch vernolate*

Thermogravimetric analysis (TGA)

The thermogravimetric curves of the native starch and starch vernolates obtained by using TGA technique were used to examine the changes in thermal stability caused by esterification and to determine the weight loss of the material on heating. The TGA curves of the native cassava starch and starch vernolate material are shown in Figures

3.14 and 3.15. The native starch showed a two-stage weight loss below 500 °C, with the first minor one corresponding to the loss of water around 60-100 °C and the other one corresponding to its decomposition with an onset temperature of 306 °C. Examining the decomposition thermogram of starch vernolate in this particular case, it can be seen that the decomposition took place in three degradation stages; the first one was due to starch decomposition while the second and the third parts, observed relatively at higher temperature, corresponds to the decomposition of the newly introduced ester part [57]. Although the initial degradation temperature of the starch vernolate is reduced, there is an overall increase in thermal stability compared to native cassava starch as indicated by a in the second and third degradation by broadening of shoulder in the thermogram. This observation was in good agreement with reported results by Luo, Z. *et al.* [80] and Zhang *et al*. [57].

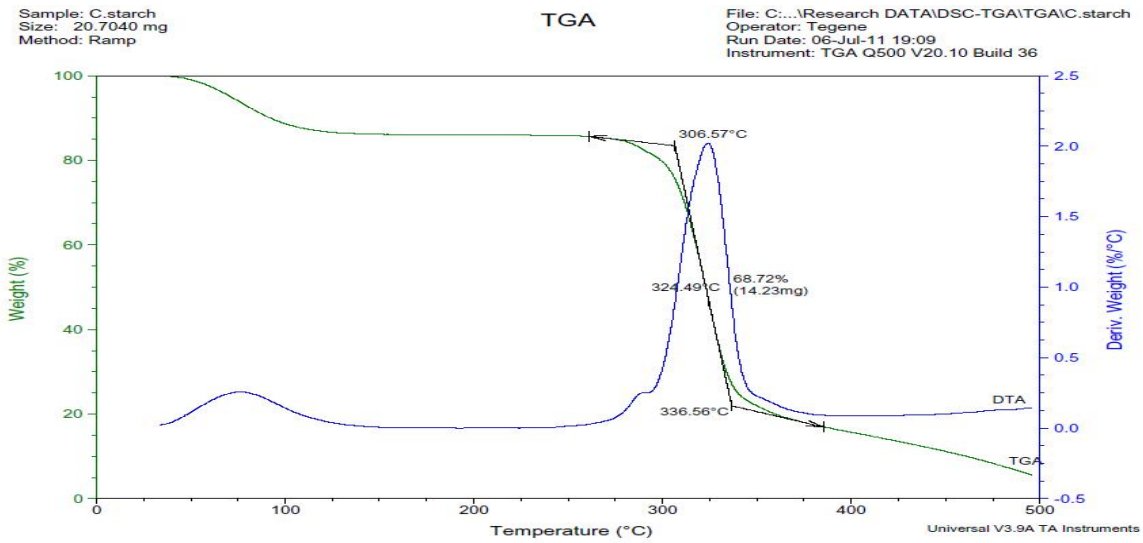


Figure 3. 14*: TGA thermogram of cassava starch*

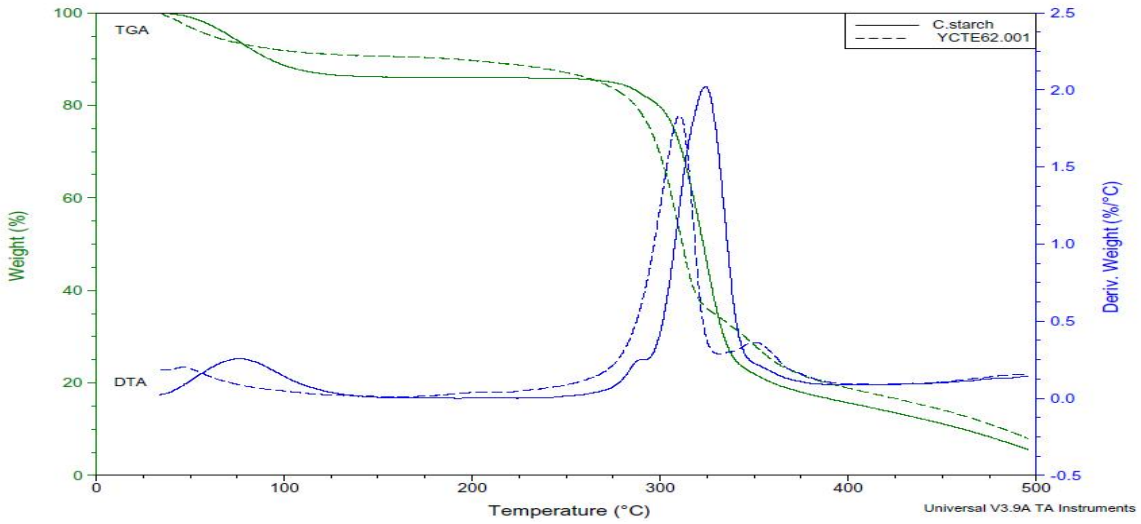


Figure 3. 15*: TGA thermogram of starch (-) and starch vernolate (---)*

Scanning Electron Microscopy (SEM)

In this study, the SEM micrographs show that there is a significant difference in appearance between native cassava starch and starch vernolate. The morphology of starch esters were considerably changed as compared to native cassava starch which was another confirmation for the success of transesterification between cassava starch and vernonia oil methyl vernolate. This can be noticed from the fact that the spherical and truncated hemi-spherical granules of cassava starch [47, 48] have lost their individuality and smooth surface texture after esterification due of replacement of hydroxyl groups present in the native cassava starch with vernolyl group. The entire loss of granular nature of the starch in this study confirms achievement of relatively high degree of substitution.

The disruption in the hydrogen bond of starch, and the sequent destruction of the crystalline structure of starch granules, were attributed to the polar nature and starch dissolving capacity of the imidazolium based ionic liquid used as reaction medium during the modification reaction. This can be the cause for enhanced chemical reagent access to the hydroxyl groups of starch and increase starch reaction with the reagent. During the dissolution processes, the starch granules have lost their crystalline structure and changed into amorphous state, which led to the uniform esterification reaction, conducted under conditions involved [49, 80]. This observation is in agreement with other literature reports [63, 64].

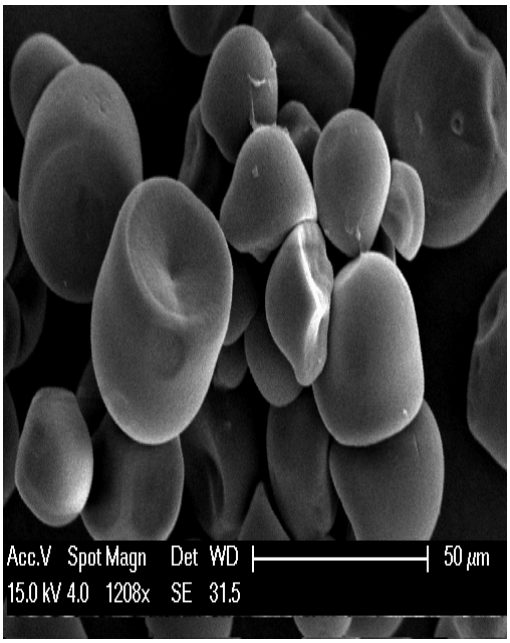


Figure 3. 16*: SEM image of starch*

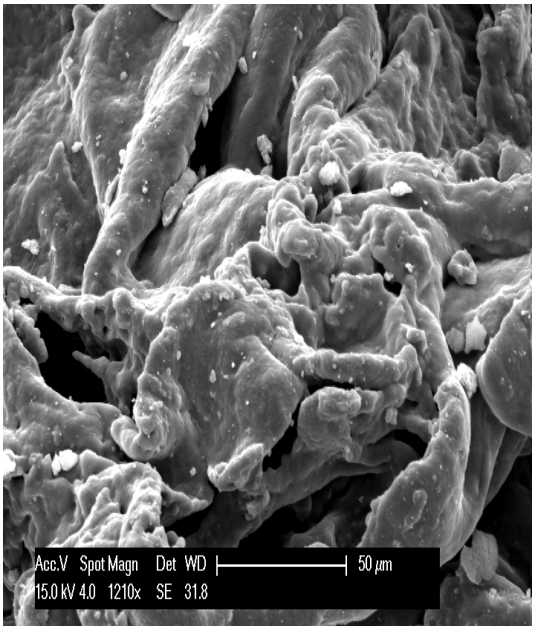


Figure 3. 17*: SEM image of starch vernolate*

The SEM micrograph clearly presents the change in morphology of native starch due to the esterification reaction. The profile is also in close agreement with starch vernolate

prepared by chemical method. This indicates that enzymatic synthesis of starch fatty acid esters is a promising method and the ionic liquid involved has relatively high starch dissolving ability that enhanced the access of the hydroxyl group of the starch facilitating the high degree of substitution achieved comparable to the one attained using chemical catalysis (section 3.2.1).

Powder X-ray Diffraction Study

X-ray diffraction measurements were performed to check if chemical modification altered the crystallinity of starch. The X-ray diffractogram of native starch and esterified starches are presented in figure below. The native cassava starch powder in this study had crystalline structure, with an intense and sharp diffraction pattern giving three peaks at 2θ of 15.34°, 17.24°, 18.31° and 23.36°. This corresponds to typical A-type pattern [53].

After esterification, the diffraction peak pattern characteristic native cassava starch was completely changed and became much broader, indicative of the increase in the amorphous character of the starch vernolate as a result of the grafted vernonia oil methyl ester moiety. The intra- and intermolecular hydrogen bonds were responsible for the highly ordered crystalline structure of starch. However, during esterification some of the hydroxyl groups on starch backbone were replaced by vernolyl group. This had reduced the formation of intermolecular hydrogen bonding and, thereby, damaged and decreased the orderly crystalline structure of native cassava starch [57, 80]. The relatively higher degree of substitution (DS = 0.95) obtained in our study indicates that much of the hydroxyl groups are substituted by vernolyl group. These broad peaks may have derived from smaller size starch crystals as the esterification reaction continued. As can be seen

from the diffractogram, the loss of crystallinity could be attributed to the effect of IL dissolution process which made the hydroxyl group of starch more accessible for reagent to undergo the esterification reaction. This observation was in good agreement with the SEM results.

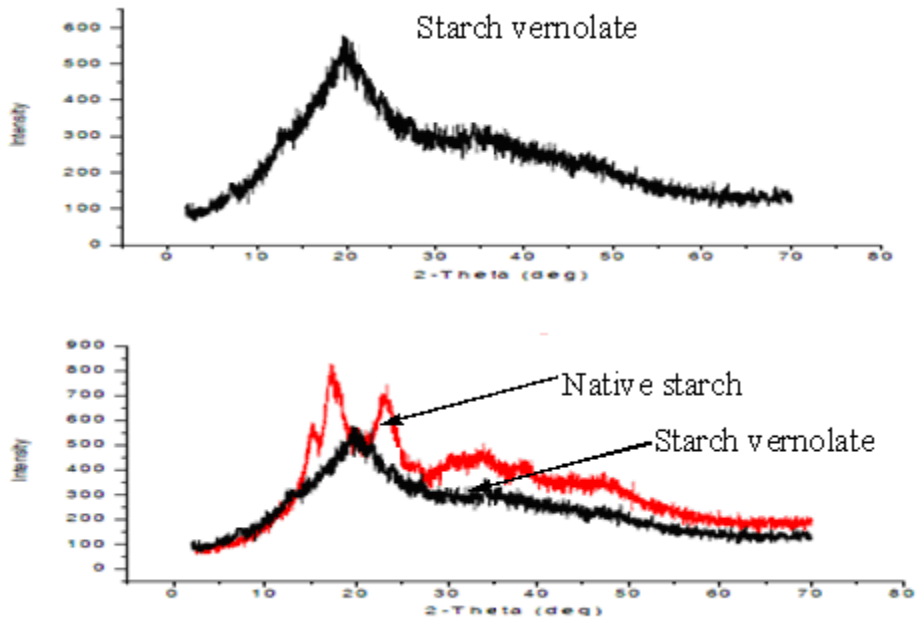


Figure 3. 18*: XRD diffractogram of starch ester*

The powder X-ray diffraction study revealed that starch was modified using enzymatic catalysts and the imidazolium based ionic liquid. This has been confirmed from the loss in the crystallinity of starch and introduction of amorphous character in the diffractogram of epoxy starch fatty acid ester. This observation agrees with the data obtained from the chemical synthesis of starch ester (section 3.2.1). Therefore, enzymatic synthesis in ionic liquid as a solvent could be used for starch modification and could be a good replacement

for environmentally damaging chemical synthesis methods in which organic solvents and chemical catalysis are mostly used.

Solid state ^{13}C CP/MAS NMR Analysis

The esterification of starch with methyl vernolate in ionic liquid as a solvent has been successfully carried out which was also confirmed by solid state, ^{13}C CP-MAS NMR spectroscopy, analysis. In the CP-MAS ^{13}C-NMR spectrum, the noticeable signals in the region between 50 to 110 ppm are mostly attributed to the different carbons of starch [58, 59]. In addition, the two representative peaks at 179.8 and 21.5 ppm in spectrum, which belong to C=O and CH_3 in vernolyl group, indicates the occurrence of esterification reaction. The varying intensity of peaks at 179.8 ppm (C=O) and 13.87 ppm (CH_3) and the strength the peaks at 70.5, 78.6 ppm (C-2−C-5) and 62.8 (C-6) in spectrum is related to the degree of esterification. The higher the intensity at 179 ppm and 13.87 ppm indicates relatively higher degree of substitution. The solid state NMR analysis result obtained in our study is in a good agreement with report by Carvalho *et al.* [58]. In their study on transesterification of the dextrin with vinyl acrylate (VA) in anhydrous dimethylsulfoxide (DMSO), the ^{13}C CP/MAS NMR spectra revealed that the glucopyranosyl carbons appear in the 58–110 ppm region and the acrylate carbons in the 120–175 ppm region. Signals at 90–105 ppm and 58–65 ppm are attributed to C1 and C6 carbons in hexapyranoses, respectively.

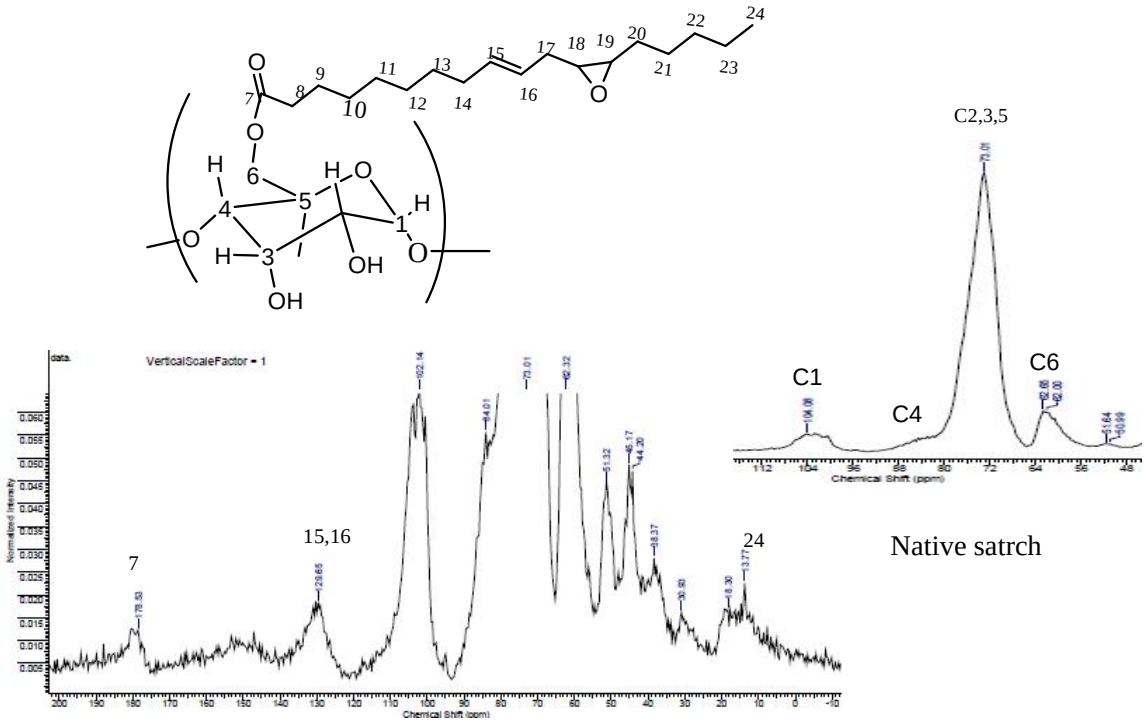


Figure 3. 19: *^{13}C CP/MAS NMR spectrum of native starch and starch vernolate*

This study clearly demonstrated that vernonia oil derivatives could be prepared using ionic liquids in enzymatic synthesis. The capacity of ionic liquids to dissolve biopolymers such as starch and its compatibility with enzymes has been used as synthetic advantage. It is also clearly seen that enzyme catalyzed esterification of long chain epoxy fatty acids could be conducted in ionic liquids. The relatively high dissolution of starch in the mixtures of imidazolium based ionic liquid and small amount of organic solvent was responsible for the access of the hydroxyl groups of starch to undergo esterification

reaction. Accordingly, novel starch epoxy fatty acid esters with relatively higher degree of substitution were synthesized.

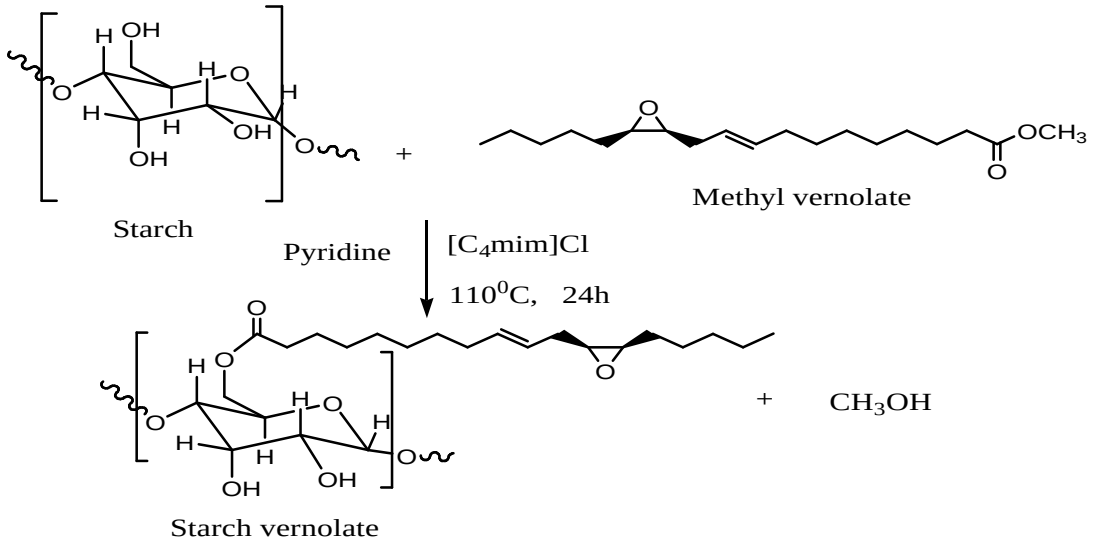
The SEM micrographs and X-ray powder diffractograms obtained were strong confirmation on the change of the morphology of starch granule and loss in its crystallinity, respectively as compared to the starch vernolate.

The results proved that the use of ionic liquid as a green solvent, enzymatic catalysis and the renewable resources such as naturally epoxidized vernonia oil derivatives, and native cassava starch, is a promising environmentally benign method of sythesis providing products with potential substitute for petroleum based products.

3.2. Synthesis of Starch Vernolate in Ionic Liquids

In this study, epoxy fatty acid esters of cassava starch were synthesized by reacting cassava starch with vernonia oil methyl ester using 1-butyl-3-methylimidazolium chloride, [BMIM]Cl as solvent and reaction medium and pyridine as basic catalyst at reaction temperature of 110 °C with in 24 h of reaction time.

The schematic representation of the reaction is presented below.



Scheme 3.8*: Chemical synthesis of starch vernolate*

The study revealed that 1-butyl-3-methylimidazolium chloride, [BMIM]Cl, was a good solvent for dissolution of starch promoting the pyridine catalyzed esterification of cassava starch with epoxy fatty acid methyl ester. Reports also show that there has been a growing interest in use of ILs as solvents and reaction media in the modification of starches [73]. Owing to their ionic character, ionic liquids appear to be highly polar resulting in their enhanced biopolymer dissolving capacity. Room-temperature ionic liquids (RTILs) have been regarded as desirable, alternative, and green solvents for a

broad variety of synthesis and catalysis due to their attractive and fascinating properties, such as low melting points, wide liquid ranges, and low or negligible vapor pressure [74]. ILs containing the imidazolium structure has been found to be a non-derivatizing medium with a potential to solubilize polysaccharides, such as cellulose and starch [75]. The ionic liquid, 1-butyl-3-methylimidazolium chloride, [BMIM]Cl, is extensively used in the homogeneous derivatization of starch owing to its capacity to dissolve up to 15 % w/w concentration at 80 °C [64, 73]. Xie, W. *et al*. [49] have recently reported that their study on the synthesis of starch acetate, starch succinate, and starch phosphates in the [BMIM]Cl medium, has revealed this IL to be a feasible solvent for the chemical derivatization of starches. However, the melting point of [BMIM]Cl is high (>75 °C) which makes it inconvenient for enzymatic reactions. The high nucleophilicity of the $[Cl]^-$ anion in [BMIM]Cl ionic liquid, with the potential to coordinatively bind to positive site of the enzyme and changing its conformation, is responsible for low enzyme activity of *Candida antarctica* lipase in this ionic liquid. Hence, the esterification reaction in this particular procedure was carried out using pyridine as catalyst instead of lipase, Novozym 435.

The degree of substitution (DS) was determined using reported method [30] with minor modification. Approximately 0.5 g of dry starch vernolate was weighed and added into a 50 mL conical flask. Then 3 mL water and 5 mL of 1.0 M NaOH was added, and the conical flask was agitated with a magnetic stirrer at room temperature for 48 h. After the indicator (phenolphthalein) was added, the excess of alkali was titrated with 0.5 M hydrochloric acid. The starch reference sample and duplicates were treated in a similar way.

The vernolyl content (*A* %) was calculated according to the following equation:

$$A\% = \frac{[(V_o - V_n) \times Molarity\ of\ HCl \times M_{vernolyl} \times 10^{-3} \times 100]}{M}$$

Where V_0 in mL is the volume of 0.5 N HCl used to titrate the blank; *Vn* in mL is the volume of 0.5 N HCl used to titrate the samples; *N* is the concentration of the used HCl (mol/L); *M* in g is the amount of dry starch vernolate sample; 279 is the formula weight of vernolyl groups. The vernolyl content (*A* %) was used to calculate the degree of substitution, DS, according to the following equation:

$$DS = \frac{(162 \times A\%)}{(M_{vernolyl} \times 100) - ((M_{Vernolyl} - 1) \times A\%)}$$

Where 162 is the molecular weight of glucose units and $M_{vernolyl}$ = 279 is the formula weight of vernolyl group. Accordingly, the DS of starch vernolate in this case was found to be 1.03.

Esterification of cassava starch with vernonia oil methyl ester (VOME) in this study resulted in product with degree of substitution of 1.03 which is higher than value reported in another study. Wenlei Xie and Yingbin Wang [64] reported the production of high fatty acid corn starch esters using a fatty acid methyl esters using ionic liquid as the solvent. They indicated that products with a broad range of DS values (0.06–0.32) were obtained with pyridine being more active catalyst and gave product with higher DS value than other basic catalysts.

Product characterization

The starch vernolate obtained in this method was white solid powder insoluble in most organic solvents. This may be due to ring opening polymerization that could arise from epoxy (oxirane) ring of the vernonia oil methyl ester moiety. It was impossible to characterize the product using liquid state NMR analysis. Hence, the use of ^{13}C CP-MAS NMR (solid state NMR) technique was found to be decisive for the solid product analysis. Furthermore, the product was characterized by FTIR spectroscopy, Electron Scanning Microscopy, X-ray Powder Diffractometry and DSC/ TGA techniques.

FTIR Spectrum Analysis

The FTIR spectrum recorded was analyzed and the presence of the major characteristic peaks of cassava starch epoxy ester presented in the following table shows that the esterification of cassava starch with methyl vernolate was successful.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3385	O-H	stretching	m
2	2923	-C-H (CH_3)	Stretching (a)	m
3	2853	-C-H (CH_2)	Stretching (s)	vs
4	1744	-C=O (ester)	Stretching	s
5	1377	-C-H (CH_3)	Bending (s)	m
6	954-1200	-C-O (AGU)	Stretching	m
7	855	-C-O (epoxy group)	Stretching	w

Table 3. 8*: Summary of FTIR of starch vernolate*

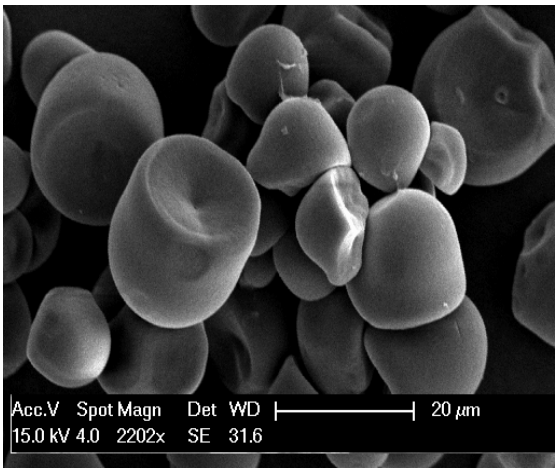
The comparison of FTIR spectra of starch and starch vernolate, indicate that the structure of the original polysaccharide remained intact. The strong hydroxyl peak at 3381 cm^{-1} in the spectrum of unmodified starch, decreased in intensity and also shifted in position in the spectrum of starch vernolate following esterification which is an indication that most of hydroxyl groups in the starch backbone took part in the reaction. The appearance of two peaks at 2923 and 2853 cm^{-1}in the spectrum of starch vernolate with relatively strong intensities, corresponds to methyl and methylene C-H stretching associated with incorporated epoxy fatty acid (vernolyl) group. In comparison with the spectrum of the unmodified cassava starch, the major change was the presence of a new peak at 1744 cm^{-1} corresponding to carbonyl group. This new absorption peak can be assigned to the characteristic ester group from methyl vernolate in the starch vernolate structure. This could be considered as an argument for the transesterification of methyl vernolate with cassava starch because the carbonyl groups from the ester are located in this region [35]. The ester band becomes more intense as the hydroxyl stretching gets weaker. A small peak at 855 cm^{-1} characteristic of the methyl vernolate (epoxy ester) also appears in the spectrum due to the presence of epoxy group in the starch ester. Therefore, the FTIR spectrum data recorded reveal the appearance of new bands in the spectrum of starch vernolate which could be considered as a signal for the esterification of cassava starch with vernonia oil methyl ester. In this method of synthesis, starch epoxy fatty acid ester with relatively higher DS value, *i.e*., 1.03 was obtained. This implies that ionic liquid, [BMIM]Cl, was effective solvent in dissolving cassava starch, making the hydroxyl group accessible for esterification reaction and the catalyst pyridine was relatively an

effective catalyst in the esterification of cassava starch with vernonia oil methyl ester (VOME).

Scanning Electron Microscopy (SEM)

In this study, the results of Scanning Electron Microscopy (SEM) analysis presented below indicate the difference in appearance between cassava starch and starch vernolate prepared by the method mentioned. The SEM micrograph of native starch shows typical cassava starch granules with spherical and truncated hemi-spherical shapes [47, 48]. However, after the esterification reaction, they lost their individuality and smooth surface texture due of substitution of hydroxyl groups by the vernolate moiety. The complete loss of granular nature of the starch confirms relatively high degree of substitution.

According to the SEM profiles, the starch granules were significantly changed into amorphous state during the dissolution processes, which led to the uniform esterification reaction conducted under reaction conditions involved [49]. The morphology of starch esters were changed as compared to native cassava starch as a result of esterification. The SEM micrographs showed that there is a remarkable difference in appearance between native cassava starch and starch vernolates. The solvent IL used as reaction medium during the esterification reaction was mainly responsible for the destruction of the crystalline structure of starch granules by disrupting the hydrogen bond of starch backbone. This can be the cause for enhanced chemical reagent access to the hydroxyl groups of starch and increase starch reaction with vernonia oil methyl ester. This observation is in agreement with other literature reports [63, 64].



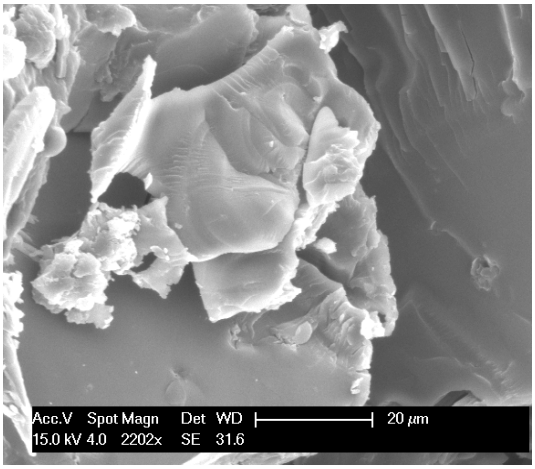


Figure 3. 20*: SEM image of starch*

Figure 3. 21*: SEM image of starch vernolate*

Powder X-ray Diffraction

The native cassava starch powder had crystalline structure, with an intense and sharp diffraction pattern, with major peaks appearing at 2θ of 15.34°, 17.24°, 18.31° and 23.36°, typical of an A-type starch [53]. The intra- and intermolecular hydrogen bonds were responsible for the highly ordered crystalline nature of starch. The esterified cassava starch (starch vernolate) only had a dispersive broad peak and showed no crystal peak of starch which implies that the crystallinity of native starch was damaged completely during the starch modification. As a result of the esterification, some of the hydroxyl groups on the starch backbone were replaced by vernolyl group which minimized the formation of intermolecular hydrogen bonding and, thereby, destructing and reducing the well-organized crystalline structure of native cassava starch. As can be seen from the diffractogram, the loss of crystallinity could be attributed to the effect of IL dissolution

process which led to more access for reagent to react with the hydroxyl group of starch [57, 76, 80]. This observation was in good agreement with the SEM results

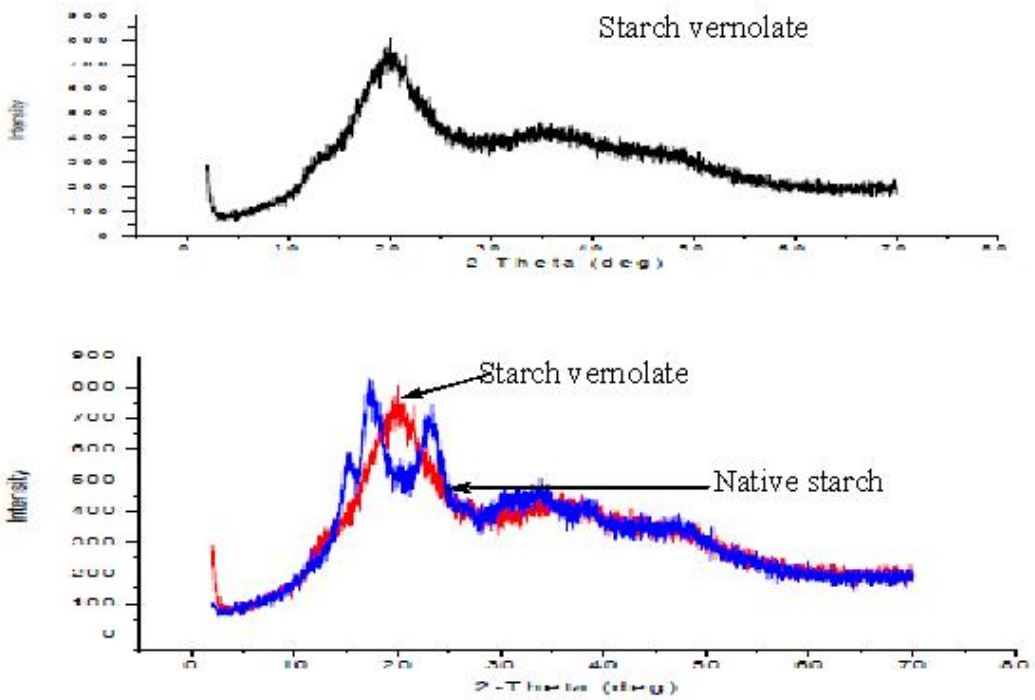


Figure 3. 22*: XRD diffractogram of starch vernolate*

DSC thermogram of starch vernolate

Native cassava starch had Tm of 139 °C whereas that of starch vernolate was 111 °C. The *Tg* of native cassava starch was found to be 107 °C and the *Tg* of modified starch vernolate became 87 °C. The replacement of hydroxyl groups with long chain epoxy fatty acid methyl ester has led to loss of crystallinity of native starch**.** *T*g of starch decreased dramatically with the increasing degree of esterification. This agrees with the results of literature report [43, 72, 80]. Wang, Xu *et.al*. [56] reported in their study they found that

Tg of the native starch was 273 °C and that of the starch acetate with a DS of 2.95 was 226 °C, which is 47 °C lower than its unmodified counterpart.

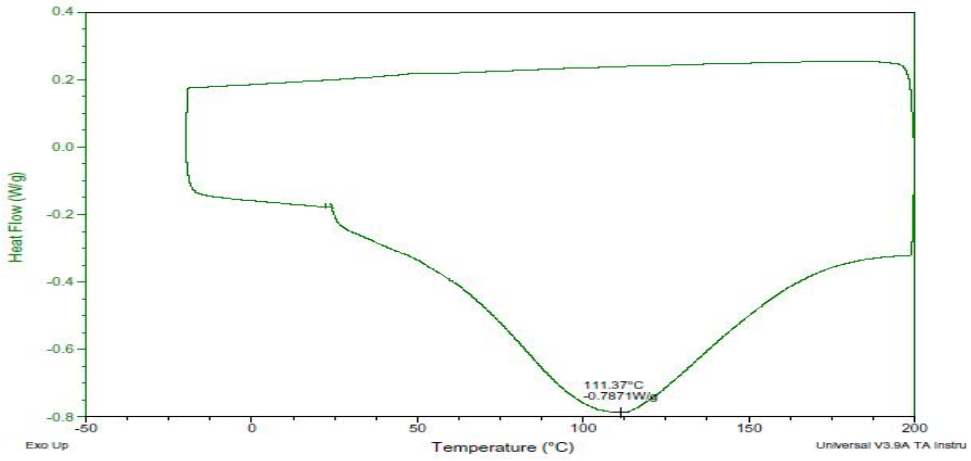


Figure 3. 23*: DSC thermogram of starch vernolate*

Solid state (CP/MAS ^{13}C-NMR) Analysis

The esterification of starch with methyl vernolate in ionic liquid as a solvent has been successfully carried out which was also confirmed by solid state, CP-MAS ^{13}C-NMR spectroscopy, analysis. In the CP/MAS ^{13}C-NMR spectrum, the noticeable signals in the region between 50 to 110 ppm are mostly attributed to the different carbons of starch [58, 59]. In addition, the two representative peaks at 179.8 and 21.5 ppm in spectrum, which belong to CO and CH_3 in vernolyl group, indicated the occurrence of esterification reaction. The intensity of peaks at 179 ppm (CO) and 13.87 ppm (CH_3) and the strength the peaks at 70.5, 78.6 ppm (C-2−C-5) and 62.8 (C-6) in spectrum is related to the degree of esterification. The higher the intensity in at 179 ppm and 13.87 ppm indicates relatively higher degree of substitution (1.03) in this case. This spectral data agrees with report in another study by Carvalho et al. [58].

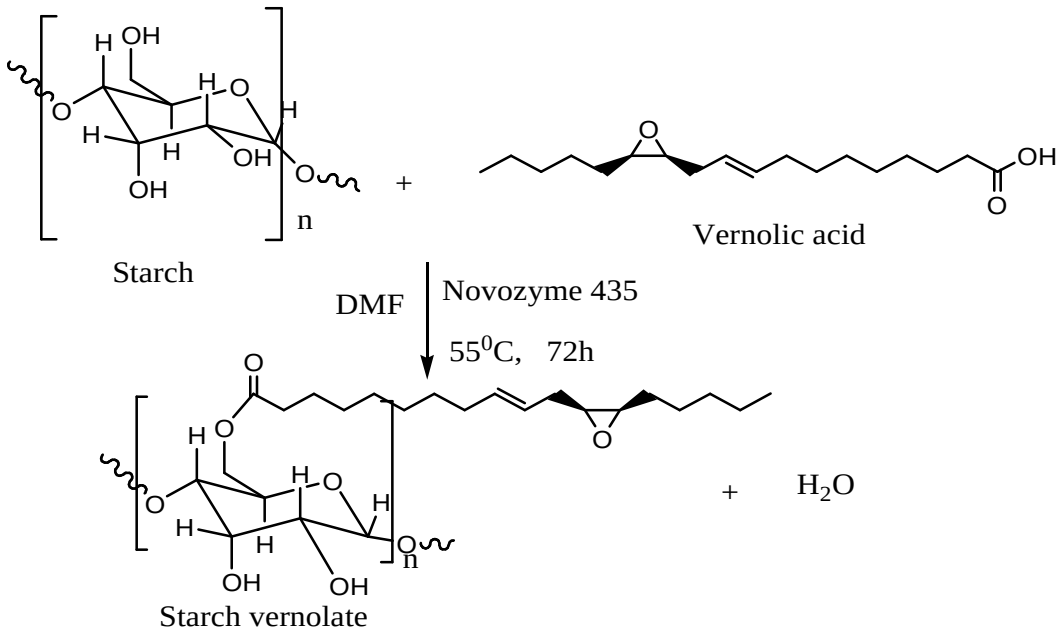
3.2.4 Enzymatic Synthesis of Starch Vernolate in Organic Solvent

In the previous report we have discussed the successfulness of enzyme catalyzed esterification of cassava starch with methyl vernolate (epoxy ester) in the presence of ionic liquids as solvents. We have also carried out esterification of starch with methyl vernolate in organic solvents using chemical method where basic catalysis was employed.

There are only few reports regarding enzyme catalyzed esterification of starch. Rajan A. *et al.* [77] indicated that starch esters could be obtained with hydrolyzing enzymes like lipase by liquid state esterification and through microwave radiation heating. Lipase obtained from *Candida rugosa* (lipase AYS) catalyzed esterification of cassava starch with recovered coconut oil (lauric acid) using microwave heating gave degree of substitution (DS) 1.1 and liquid state esterification with palmitic acid gave a degree of DS 1.04 using organic solvents DMSO or DMF.

In enzymatic modification of carbohydrates, the major challenge is to find out a solvent, which can dissolve sugar and retain the enzyme activity, simultaneously. Organic solvents such as pyridine, tert-butanol, 2M2B and DMF or their mixtures are the most suitable solvents providing a more comfortable environment for several lipases and proteases [71, 78]

In this particular esterification procedure, Novozyme-435 lipase catalyzed esterification of cassava starch with epoxy acid, vernolic acid, was carried out in organic solvent, dimethylformamide (DMF).



Scheme 3.9*: Enzymatic synthesis of starch vernolate in organic solvent*

Degree of substitution

The degree of substitution (DS) of starch vernolate prepared by this method was determined using the method [30] with minor modification. Approximately 0.5 g of dry starch vernolate was weighed and added into a 50 mL conical flask. Then 3ml water and 5 mL of 1.0 M NaOH was added, and the conical flask was agitated with a magnetic stirrer at room temperature for 48 h. After the indicator (phenolphthalein) was added, the excess of alkali was titrated with 0.5 M hydrochloric acid. The starch reference sample and duplicates were treated in a similar way.

The vernolyl content (*A* %) was calculated according to the following equation:

$$A\% = \frac{[(V_o - V_n) \times Molarity\ of\ HCl \times M_{vernolyl} \times 10^{-3} \times 100]}{M}$$

Where V_0 in mL is the volume of 0.5 N HCl used to titrate the blank; *Vn* in mL is the volume of 0.5 N HCl used to titrate the samples; *N* is the concentration of the used HCl (mol/L); *M* in g is the amount of dry starch vernolate sample; 279 is the formula weight of vernolyl groups. The vernolyl content (*A* %) was used to calculate the degree of substitution, DS, according to the following equation:

$$DS = \frac{(162 \times A\%)}{(M_{vernolyl} \times 100) - ((M_{Vernolyl} - 1) \times A\%)}$$

Where 162 is the molecular weight of glucose units and $M_{vernolyl}$ = 279 is the formula weight of vernolyl group. The DS of starch vernolate in this case was found to be 0.34

Products Characterization

The product obtained was characterized using different techniques. The limited solubility of the product in conventional organic solvents has made the use of liquid state NMR analysis impossible. Therefore, ^{13}C CP-MAS NMR (solid state NMR) analysis was used instead of the liquid state NMR technique. Furthermore, the product was characterized by FTIR, Electron Scanning Microscopy, X-ray powder diffraction and DSC/TGA techniques.

FTIR Spectrum Analysis

FTIR spectrum recorded for the starch before and after esterification was analyzed.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3438	O-H	stretching	m
2	2929	-C-H (CH_3)	Stretching (a)	m
3	2853	-C-H (CH_2)	Stretching (s)	vs
4	1737	-C=O (ester)	Stretching	w
6	1377	-C-H (CH_3)	Bending (s)	m
7	954-1200	-C-O (anhydroglucose unit)	Stretching	m
8	860	Epoxy Carbon	Stretching	w

Table 3. 9*: summary of FTIR spectrum of starch vernolate*

The FTIR signals of the starch and the corresponding epoxy starch fatty acid ester indicate that the structure of the original polysaccharide remained intact. The very strong O-H stretching band at 3381 cm^{-1} i.e., typical of unmodified cassava starches, has decreased in intensity following esterification which is an indication that the O–H groups of starch are reacted. The peak has shifted to 3438 cm^{-1} in the spectrum of starch vernolate. The two peaks at 2929 cm^{-1} and 2853 cm^{-1} in the spectrum of starch vernolate

with relatively strong intensities, are attributed to methyl and methylene C-H stretching associated with vernolyl group. In comparison with the spectrum of the unmodified cassava starch, the major change is the presence of a new peak at 1737 cm^{-1} corresponding to carbonyl group. This new absorption peak can be attributed to the characteristic ester group from methyl vernolate in the starch vernolate structure. This could be considered as an argument for the transesterification of methyl vernolate with cassava starch because the carbonyl groups from the ester are located in this wavelength region. A small peak at 860 cm^{-1} characteristic of the vernolic acid (epoxy ester) has also appeared in the spectrum due to the presence of epoxy group in the starch ester. The carboxyl (C-O) peak of the vernolic acid which appears at 1712 cm^{-1} has shifted to 1737 cm^{-1} in the (C-O) ester group of the starch vernolate. Therefore, the FTIR spectrum data recorded reveal the appearance of new bands in the spectrum of starch vernolate which could be considered as a signal for the esterification of cassava starch with vernolic acid. However, the strong similarity between the spectrum starch and starch vernolate observed in this method of synthesis indicates the relatively lower degree of substitution (DS=0.34). This low DS could be attributed to low solubility of starch in DMF and deactivation of enzyme in the organic solvent, DMF used in this case. In another study, using ionic liquids [BMIM][PF_6] and [BMIM]Cl as solvents, we obtained starch vernolates with DS of 0.95 and 1.03 respectively.

Differential Scanning Calorimetry (DSC)

Thermal studies of both cassava starch and starch vernolate were carried out using DSC. In figure below, it is shown that *Tm* of the native starch was 139 ℃ and that of the starch vernolate was 107 ℃, which is 32 ℃ lower than its unmodified cassava starch. In another study, starch esters of higher DS value we synthesized using ILs showed lower *Tm* value. Wang Xu *et al*. [56] reported in their study they found that *T*g of the native starch was 273 ℃ and that of the starch acetate with a DS of 2.95 was 226 ℃, which is 47 ℃ lower than its unmodified counterpart. The replacement of hydroxyl groups with long chain epoxy fatty acid methyl ester has led to loss of crystallinity of native starch**.** *T*m of starch decreased dramatically with the increasing degree of esterification. This agrees with the results of literature report [33, 68].

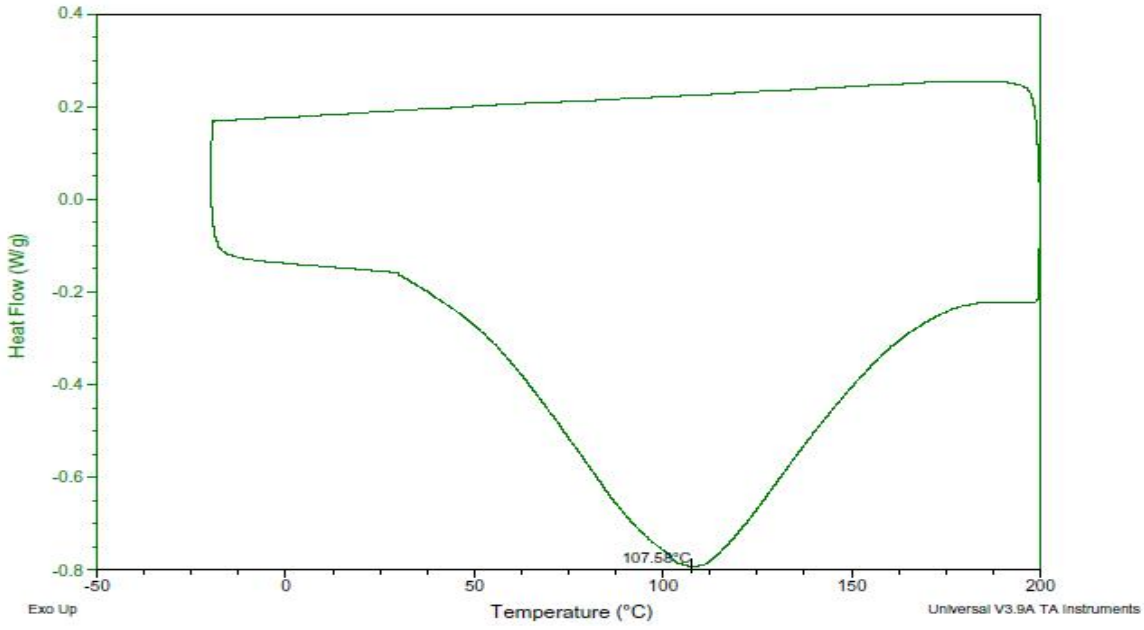


Figure 3. 24 *: DSC thermogram of starch vernolate*

Thermogravimetric analysis (TGA)

The thermal stability of esterified cassava starch has been investigated by using TGA. The TG spectral profiles were used to determine the weight loss of native cassava starch and starch vernolate as they were heated, cooled or held isothermally [35]. The initial weight loss in the starch started just at lower temperature around 70-100 °C corresponding to water absorption as natural starch is hydrophilic and can absorb moisture under normal room conditions. The other major degradation of native starch began at 306.5 °C.

The thermogram also shows slight difference in degradation products between the starch and starch vernolate, which could be another signal for the modification of starch i.e., esterification. The TGA thermogram analysis revealed that starch esters prepared by this method didn't show better thermal stability of compared to native starch is probably due to the lower degree of substitution. The lower DS obtained is also confirmed by the observation similarity in TGA analysis between starch and starch ester. This data agrees with PXRD and SEM results.

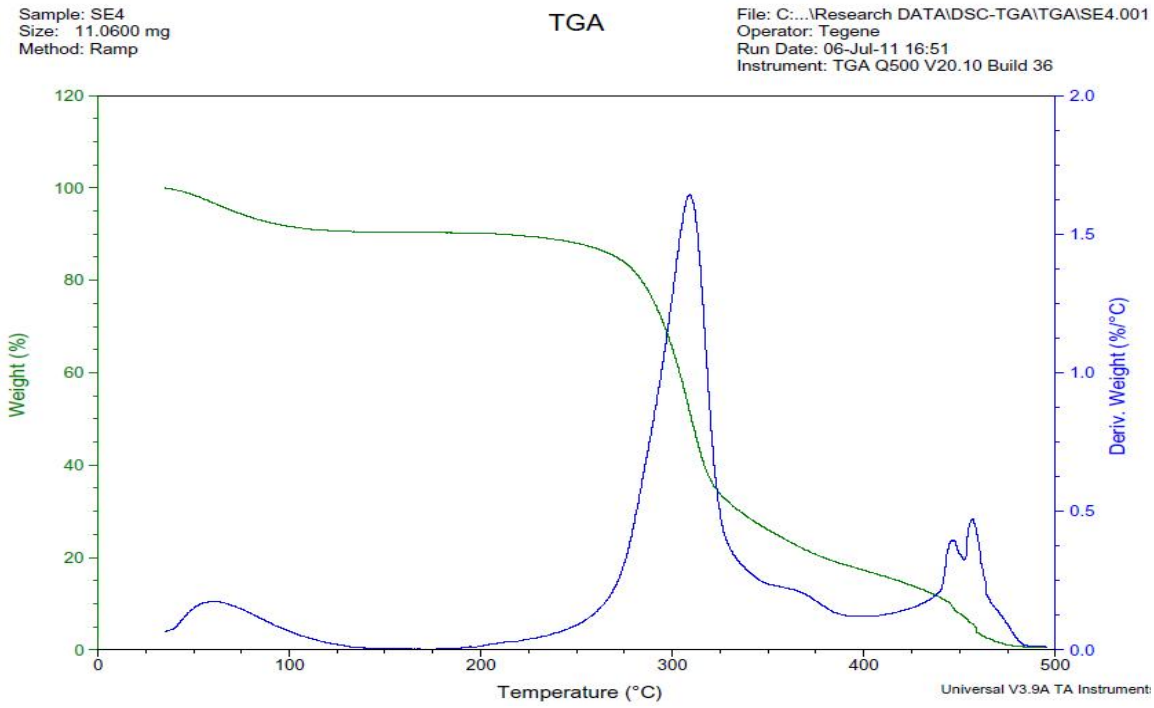


Figure 3. 25*: TGA thermogram of starch vernolate*

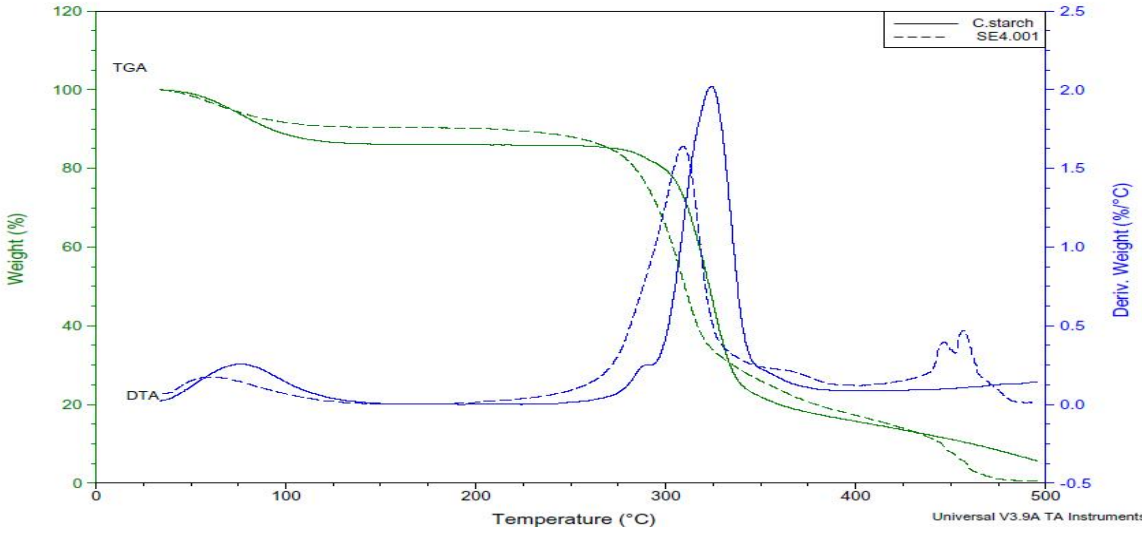
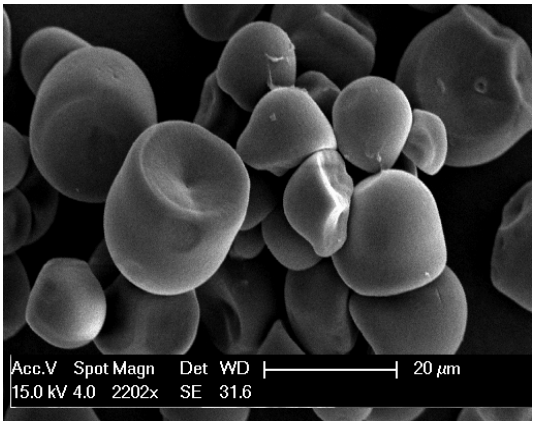


Figure 3. 26*: TGA thermogram of starch (-) and starch vernolate (--)*

Scanning Electron Microscopy (SEM)

The investigation of the molecular characteristics was supplemented with SEM of starch vernolates obtained. The SEM micrograph native starch showed typical granules of cassava starch, with spherical and truncated hemi-spherical shape. According to the SEM profiles, during the dissolution processes the starch granules showed minor change from their crystalline structure. It is also shown that there was very small difference in appearance between native cassava starch and starch vernolates. In this reaction process it is observed that minimum loss in the granular structure of the native starch confirming relatively low degree of substitution. As compared to esterification reactions carried out in ILs, the enzyme catalyzed esterification in DMF as solvent resulted in lower degree of substitution. It can be deduced from this observation that the limited solubility of starch in DMF (which was sort of suspension in contrast to clear solution obtained in the case of dissolution of starch in DMSO) could be the cause for the low substitution of hydroxyl groups of starch due to their limited accessibility. This observation was in close agreement with published reports. Rajan A. *et al*. [77] reported preparation of cassava starch ester with DS ranging from 0.33-1.1 using Candida rugosa (lipase AYS) in the presence of organic solvent, DMF. Zhang *et al*. [57] reported preparation of starch acetated with various range of degree of substitution. They observed that as the starch acetates reached the higher degree of substitution, the destruction degree on the surface of the starch granules was high i.e., the starch almost fell into pieces, which reveals that the esterification not only happened on the surface but also happened in the inner structure of the starch. In another study, M. Lukasiewicz and S. Kowalski, [79] reported that low

power microwave assisted enzymatic esterification of starch using DMSO and DMF as solvents gave products with varying degree of substitution.



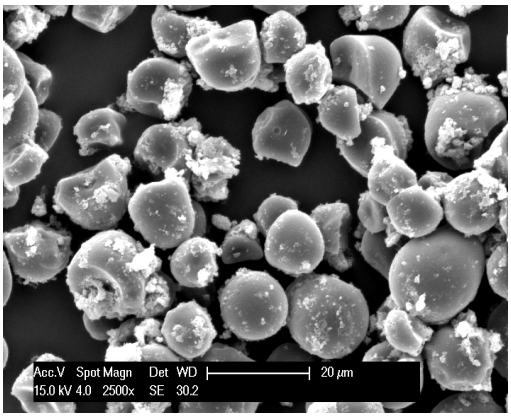


Figure 3. 27*: SEM image of starch* Figure 3. 28*: SEM image of starch vernolate*

Powder X-ray Diffraction

The native cassava starch powder had crystalline structure, with an intense and relatively sharp diffraction peaks. The diffractogram of native cassava starch exhibited an A-type crystalline pattern, with the major peaks appearing at 2θ of 15.34°, 17.24°, 18.31° and 23.36°. It is obvious that during the esterification process, the vernolyl groups replace some of the hydroxyl group on starch backbone. This phenomenon reduced the formation of intermolecular hydrogen bonding and, thereby, disrupted the orderly crystalline structure of native cassava starch. However, the pattern of diffraction in diffractogram of starch vernolate synthesized by this particular method showed minor difference from that of native cassava starch. This indicates that the crystalline nature of starch was mostly retained and the amorphous character of the starch vernolate was relatively small as compared to starch vernolates prepared using other methods where ionic liquids were

employed as solvents. This observation confirms the fact the hydroxyl groups were partially replaced. Therefore, the crystalline peaks observed in the starch vernolate were attributed to the remaining hydroxyl groups which had the opportunity to form hydrogen bonds. The Powder XRD analysis carried out confirms that the starch vernolate prepared using enzyme catalyzed esterification in the presence of organic solvent, DMF ended up with product of low degree of substitution. Besides the limited solubility of starch in DMF, lipase enzymes are known to be deactivated in such solvents, which could be the major reason for the low degree of substitution observed in this case. Therefore, we can conclude that enzymatic synthesis of starch vernolate using ionic liquids as solvents are more effective and gave products with higher degree of substitution. This observation was in good agreement with the SEM results.

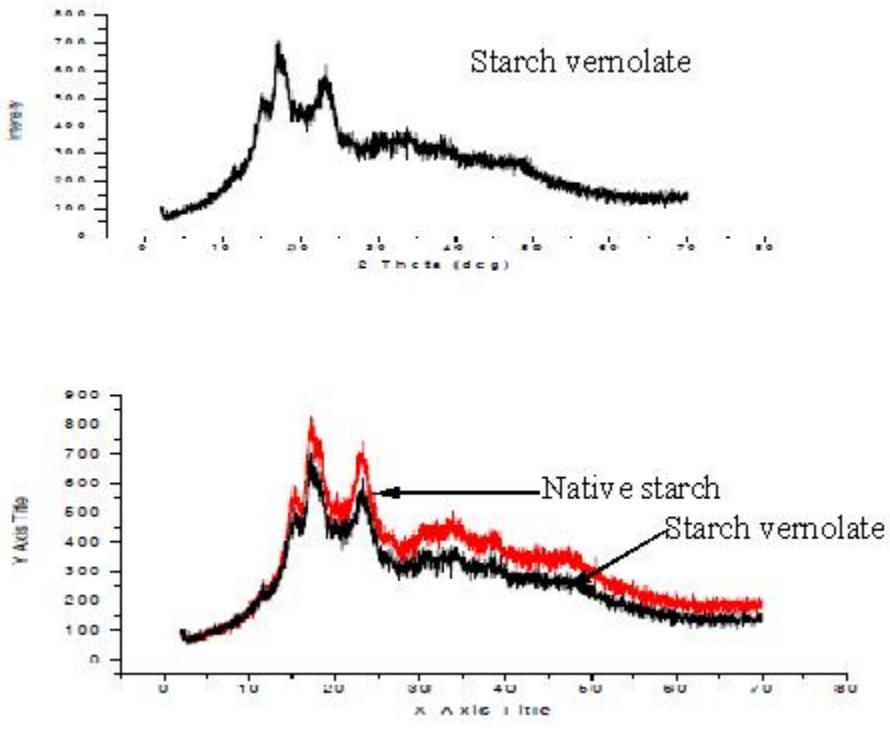


Figure 3. 29: XRD diffractogram of starch vernolate

Comparison of starch vernolates prepared by different methods

In this study, synthesis of epoxy starch vernolates (starch esters) were carried out using different methods and procedures developed. The characterization of the products indicated that starch vernolates of different degree of substitution the method were obtained.

Compound	Reagent	Solvent (s)	Catalyst	DS
Starch vernolate	Starch and VOME	DMSO	K_2CO_3	1.24
Starch vernolate	Starch and VOME	DMSO & [BMIM][Cl]	Pyridine	1.03
Starch vernolate	Starch and VOME	DMSO & [BMIM][PF_6]	Novozym-435	0.95
Starch vernolate	Starch and VOAc	DMF	Novozym-435	0.34

Table 3. 10 : Comparison of Starch vernolates

The results indicate that the high solubility of starch was responsible for the higher degree of esterification of starch with vernonia oil methyl ester (VOME). In a similar manner, synthesis of starch vernolate in ionic liquid, 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) as solvent in 20 % DMSO, and Novozym-435 lipase catalyst was found to be interesting in that it is relatively a green method and gave a product of high DS comparable to that of the chemical method in which pyridine was used as a catalyst.

This observation was confirmed by the data obtained from other characterization techniques employed. The X-ray powder diffraction study showed clearly that the decrease in crystalline nature of starch was observed in starch vernolates with increasing degree of substitution. Accordingly, starch vernolate with degree of substitution of 1.24 had more amorphous pattern than starch vernolate with low degree of substitution i.e., 0.34. In the latter, starch granules have almost retained their crystalline structure. It can be seen also that enzymatic synthesis of starch vernolate in ionic liquids was found to be promising with a potential to substitute the environmentally damaging chemical method of synthesis.

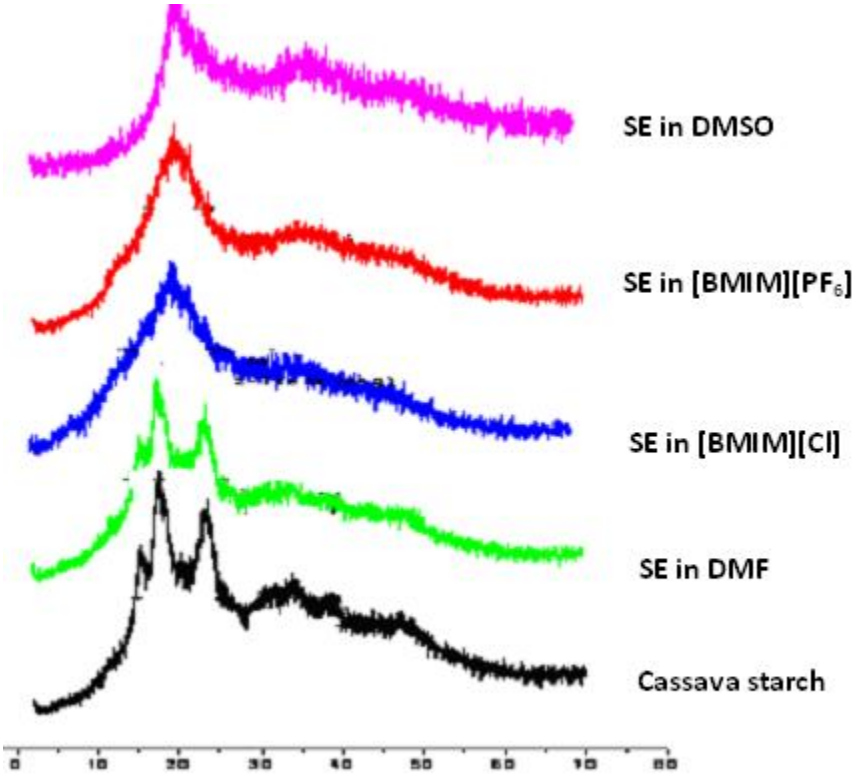
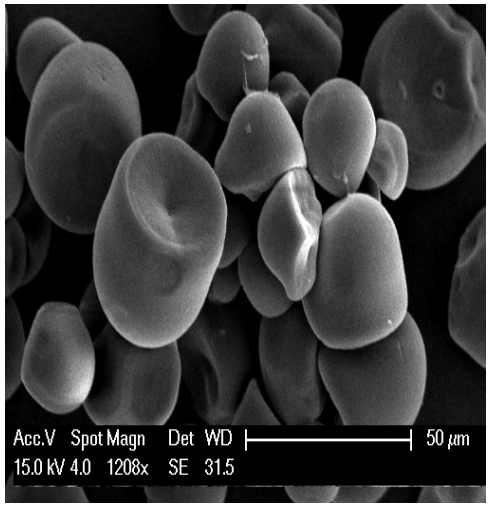


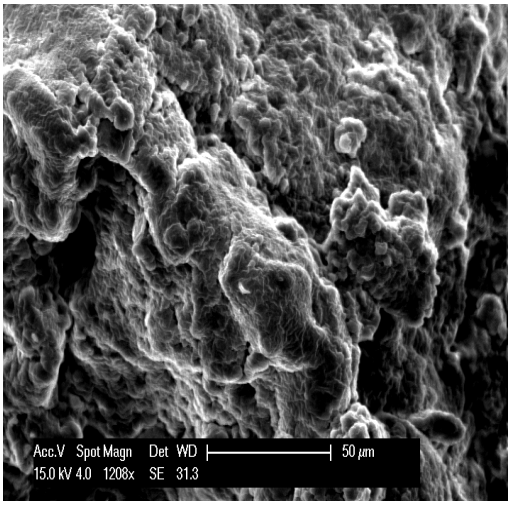
Figure 3. 30: *X-ray powder diffractogram of starch vernolates prepared by different methods*

The SEM micrograph profile of starch vernolates has clearly presented the difference in the morphology of native starch and esterified starch. It is evident from the micrographs that due to the dissolution of starch with the solvents used and the subsequent esterification with VOME or vernolic acid, the spherical and truncated hemi-spherical starch granules have lost their individuality and smooth surface. This could be attributed to the disruption of the hydrogen bond of starch followed by substitution with the vernolyl moieties**.**

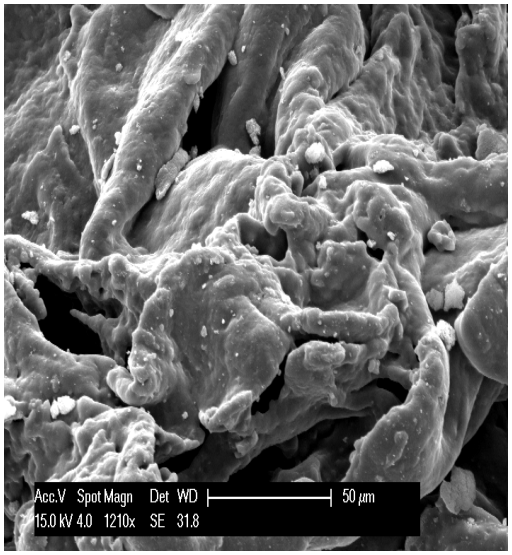
As in the case of the X-ray powder diffraction study, the SEM images obtained also confirmed that there is a difference in morphologies between starch vernolates synthesized using different methods. Starch vernolates with high DS value had showed a remarkable difference in appearance as compared to native starch whereas those with lower DS presented slight change of morphology.



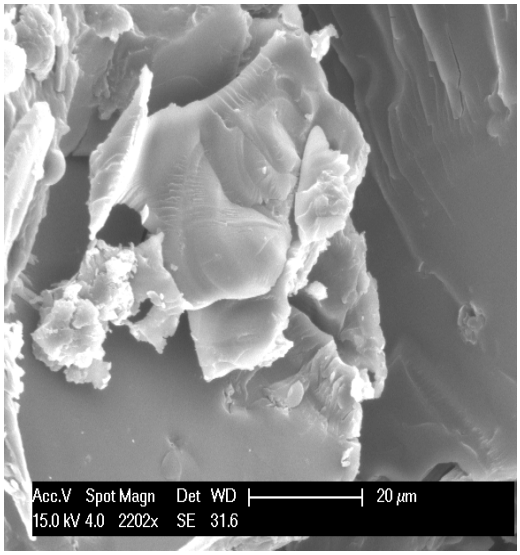
SEM image of Cassava starch



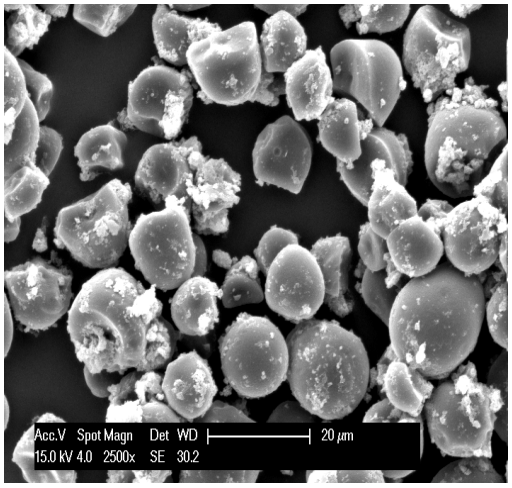
SEM image of SE in DMSO (DS =1.24)



SEM image of SE in [BMIM][PF_6] (DS=0.95)



SEM image of SE in [BMIM][Cl] (DS =1.03)



SEM image of SE in DMF (DS = 0.34)

Figure 3. 31: SEM images starch vernolates with varying DS values

3.3 SYNTHESIS AND CHARACTERIZATION OF GLUCOSE VERNOLATE

Sugar fatty acid esters (SFAE) represent a large group of compounds and consist of two abundant agricultural raw materials, namely; sugars and fatty acids derived from fats or oils. Synthesis of sugar fatty acid esters can be carried out either chemically or enzymatically. The chemical process occurs with a low selectivity and leads to a mixture of sugar esters with different degrees of esterification. Furthermore, the reactions involve the use of toxic organic solvents and high temperatures, which could cause coloration of the final products. Nowadays, the use of a biological catalyst such as lipase in the synthesis of sugar esters has become a promising method to overcome these problems. The main advantage of enzymatic synthesis is that its high regioselectivity leads mainly to monoester production. In addition, the enzymatic method can be performed under mild reaction conditions; thus, denaturation of substrate and/or products can be avoided [81].

Enzymatic sugar ester synthesis is based on esterification reactions catalyzed by lipases. Because esterification is a reversible reaction, the esterification reaction products such as water in the media should be removed to shift the equilibrium of the reaction away from hydrolysis to obtain a maximum yield of sugar ester [82]. Furthermore, the enzyme activity and/or stability are negatively affected by a high concentration of water [83]. In addition, the particles of an immobilized enzyme can be covered by a water layer preventing a lipophilic substrate (*i.e.*, a fatty acid) access to the enzyme [84]. To remove

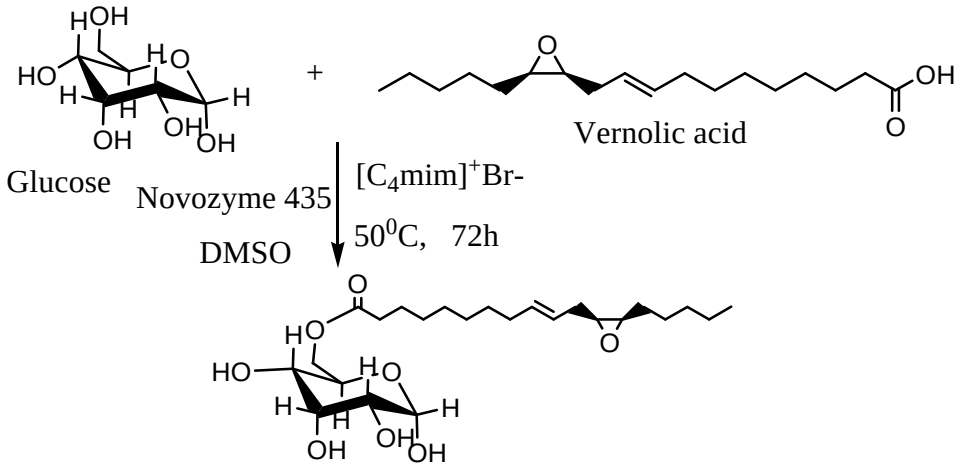
the water liberated by the reaction, evaporation under reduced pressure [85] and azeotropic distillation [86], and use of molecular sieve [87] were reported.

The most challenging issue in enzymatic synthesis of sugar esters is selection of suitable solvents. Solvents that can dissolve the sugar might have deactivating effect on the enzyme or solvents compatible with enzyme may have less sugar dissolving capacity [88]. Accordingly, less harmful organic solvents such as acetonitrile, acetone, t-butanol, and 2-methyl-2-butanol or mixtures of organic solvents are commonly used in the enzyme-catalyzed synthesis of fatty acid sugar esters with a partially dissolved or a solid-phase system [71, 89]. Recent studies show the attention given to the use of ionic liquids as solvents in the enzymatic synthesis of sugar fatty acid esters [88, 90]. It was reported that anhydrous ILs containing $[BF_4]^-$, $[PF_6]^-$, and dicyanamide, $[DCA]^-$ can be used alternative reaction media for biotransformation of sugars [88]. It was also observed that the use of these ILs as reaction media enhanced the selectivity and stability of enzymes, while the activity of enzymes in pure ILs was usually lower than those in conventional organic solvents used for the synthesis of sugar ester [91].

There are a number of reports on the synthesis and characterization of sugar esters. However, less attention has been given to synthesis of epoxy fatty acid sugar esters. In our present study, after preparing the precursors such as vernolic acid, vernanol (epoxy alcohol) and vernonia oil methyl ester, synthesis and characterization of glucose fatty acid esters were carried out using various reaction conditions.

3.3.1 Enzymatic Synthesis of Glucose Vernolate in Ionic Liquids

In this study, *Candida antarctica* lipase B (CALB) catalyzed synthesis of glucose vernolate by reacting glucose with vernolic acid, (cis-12, 13-epoxy-cis-9-octadecenoic acid) in the presence of ionic liquid, 1-Butyl-3-methylimidazolium bromide, [BMIM]Br and DMSO as solvents was successfully carried out.



Scheme 3.10 *: Schematic representation of synthesis of glucose vernolate*

Lipase B from *Candida antarctica* (CAL-B) is the most active and frequently used enzyme for sugar ester synthesis in organic solvents [91]. It was also found to be active in solvent systems containing ionic liquids [92]. Ganske, F. and Bornscheuer, U.T. [88] also reported that commercially available CAL-B showed no activity in the synthesis of sugar esters in pure ionic liquids. To improve the solubility of glucose in pure ionic liquids, DMSO was used as co-solvent which has led to increased solubility of glucose. We also found that adding DMSO as co-solvent improved the reaction by decreasing the viscosity of ionic liquids and improving activity of the lipase enzyme. The positive effects of minimum DMSO concentrations on the enzyme activity observed may be explained by

an increased solubility of glucose rather than by a direct effect of DMSO on the enzyme [91]. To remove the water produced as byproduct molecular sieves were used. Higher conversion was obtained using the immobilized enzyme as catalyst. The white solid product obtained was insoluble in most solvents. Hence, analysis of the product with liquid state NMR spectroscopy was impossible.

The impact of enzyme load, reaction time and temperature on product were studied. The optimum enzyme load in this study was 0.2 g. A yield of 76 % was obtained after 72 h of reaction time. The reaction temperature was maintained at 50 °C. Increasing temperature above 50 °C showed no increase in the product which might be due to inactivation of the enzyme at higher temperatures. This observation is in close agreement with another study carried out by Sung *et al*. [93] in which they reported that the increasing enzyme load up to 100 mg/mL increased the conversion at 2 h, but further increase of enzyme load had little effect on conversion during lipase-catalyzed glucose ester synthesis in ionic liquids and increasing temperature resulted in decreasing conversion as the highest conversion they obtained was at 50 °C indicating enzyme instability above 60 °C.

The characterization of the product was carried out using FTIR spectroscopy, Solid state NMR spectroscopy, TGA and DSC.

FTIR Spectrum of glucose vernolate

The FTIR analysis result indicates that the enzyme catalyzed esterification of glucose dissolved in ionic liquid and DMSO as co-solvent mentioned was successful. Some of the major IR (cm^{-1}) peaks are listed in the table below.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3458	O-H	stretching	m
2	2961	-C-H (CH_3)	Stretching (a)	m
3	2853	-C-H (CH_2)	Stretching (s)	vs
4	1731	-C=O (ester)	Stretching	w
6	1377	-C-H (CH_3)	Bending (s)	m
7	954-1200	-C-O, C-C (in sugar ring)	Stretching	m
8	844	-C-O (epoxy group)	Stretching	w

Table 3. 11: Summary of FTIR spectrum of glucose vernolate

From the FTIR spectrum, the carbonyl (C=O) peak obtained at 1731 cm^{-1} is clear indication of the introduction of an ester functionality in to the glucose structure. The presence of epoxy C-O-C stretching band observed at 844 cm^{-1}, characteristic of vernolic acid, could be another confirmation for the grafting of epoxy fatty acid on to the glucose structure. The OH peak intensity of the glucose vernolate has decreased as compared to unmodified glucose which could be a supporting evidence for the high degree of

substitution of glucose hydroxyl groups by vernolyl moiety achieved. As compared to the starting vernolic acid, the C=O peak in the spectrum is characteristic of an ester functionality with a shift from 1710 cm^{-1} (for vernolic acid) to 1731 cm^{-1} in the glucose vernolate.

Thermogravimetric (TGA) Analysis

TGA curves were used to examine the changes in thermal stability caused by esterification. The TGA thermogram of glucose and glucose vernolate is shown below. The initial weight loss in the glucose started just at lower temperature corresponding to water absorption as glucose is hydrophilic and can absorb moisture under normal room conditions. The other major degradation of glucose starts at 203 °C.

The thermogram reveals that glucose vernolate with the degradation onset temperature of 341 °C, has more thermal stability than glucose. This implies that esterification of glucose has increased thermal stability of glucose.

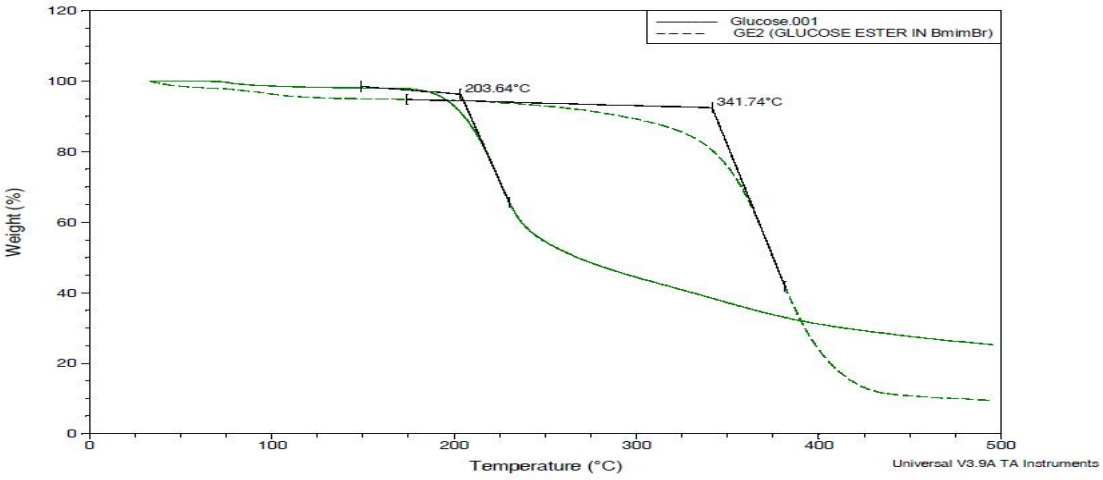
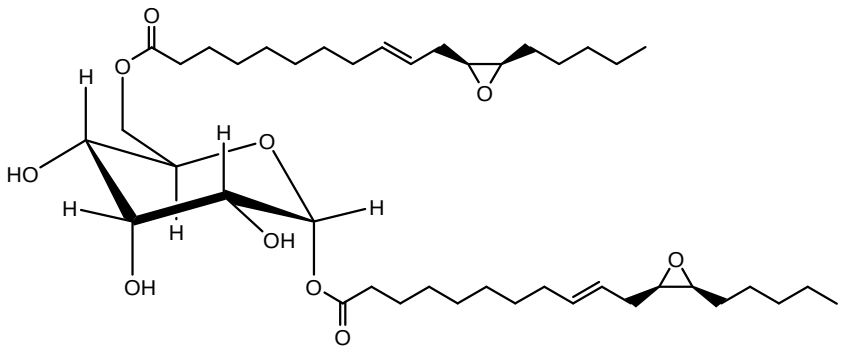


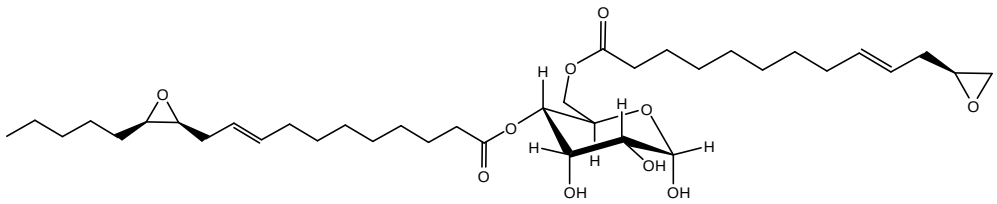
Figure 3. 32 *: comparison of TGA of glucose with glucose vernolate*

The mass spectrometry analysis carried out using MALDI-TOF MS technique showed fragmentations corresponding to m/z at 739.4 which is molecular ion $[M+2H]^+$, $C_{42}H_{72}O_{10}$ indicating the glucose is di-substituted with vernolyl group.



$C_{42}H_{72}O_{10}$
Mol. Wt.: 737.02

The m/z = 720 is due loss of H_2O from the molecular ion. The other masses at m/z = 666.88 corresponds to $[M+2H]^+$-C_5H_{12} which has the formula $C_{37}H_{62}O_{10}$



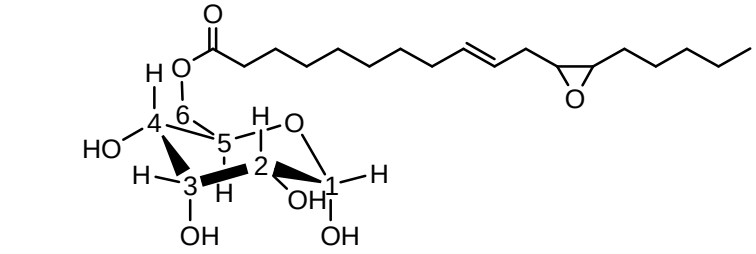
$C_{37}H_{62}O_{10}$

Mol. Wt.: 666.88

The m/z = 313 is due to ammoniated vernolic acid ($C_{18}H_{32}O_3$)

Solid state (CP/MAS ^{13}C-NMR) spectrum

The solid state NMR spectrum of the product showed the success of the synthesis of glucose vernolate in which carbon peaks from both glucose and the introduced vernolyl moiety were found in their respective positions. Peaks in the range of 64-103 ppm correspond to carbons of glucose while those in the range 14-58, 128-132, and 178 ppm are due to carbon of vernolyl moieties esterified on glucose.



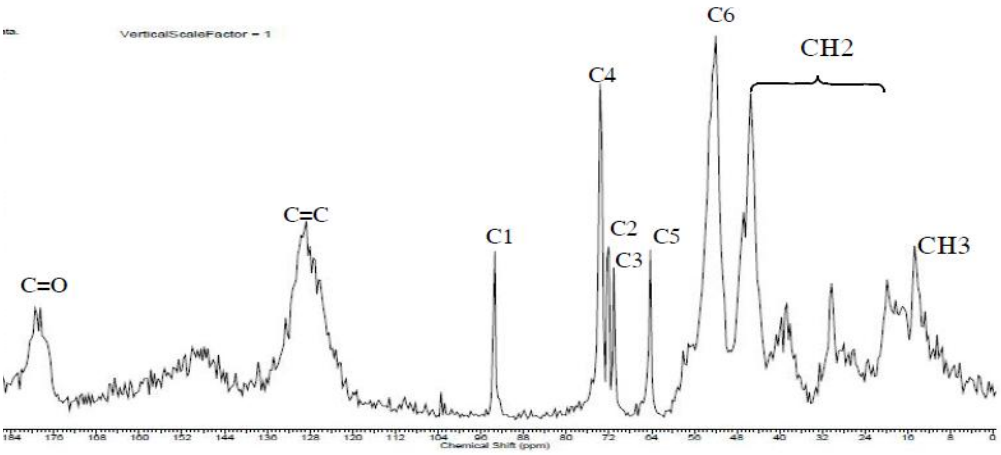
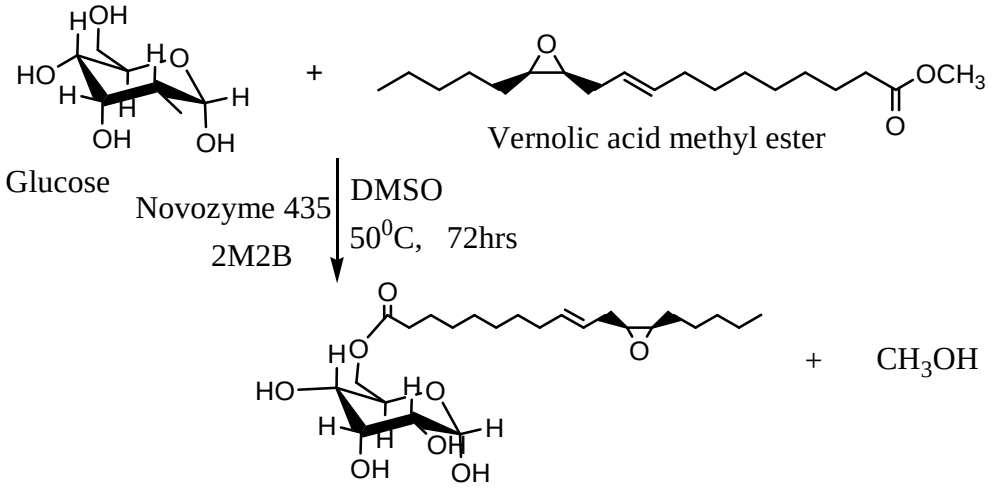


Figure: 13C CP/MAS ^{13}C-NMR spectrum of glucose vernolate

3.3.2 Enzymatic Synthesis of Glucose Vernolate in 2M2B and DMSO

In this study, Novozym SP 435, immobilized lipase B from *Candida antarctica* catalyzed transesterification of glucose with long chain epoxy fatty acid methyl ester (methyl vernolate) was carried out using a mixture of two miscible organic solvents, 2-methyl-2-butanol (2M2B) and DMSO. The glucose was dissolved in a low amount of a hydrophilic solvent (dimethylsulfoxide), and then was added to a tertiary alcohol, namely 2-methyl-2-butanol. This increased the solubility of the carbohydrate, thus allowing the acylation to proceed. As the reaction medium is mostly composed of 2-methyl-2-butanol (where most lipases are significantly stable), the inactivation of the biocatalyst is greatly reduced [94, 95, 96, 97].



Scheme 3. 11*: reaction scheme of synthesis of glucose vernolate*

Several studies have been undertaken on the role of suitable organic solvents on enzymatic synthesis of fatty acid sugar esters. Published results regarding lipase Novozym 435 from *Candida antarctica* B catalyzing synthesis of glucose palmitate showed similar behavior. The best results after 48 h of reaction performance at 60 °C

were achieved in acetone, but considerably lower conversion was found for t-butanol [89].

In this chemoenzymatic method, glucose vernolate was synthesised by esterification of glucose and vernonia oil methyl ester using immobilized lipase form *Candida antarctica* to give a white solid product with 68 % yield. The reaction medium contained 10 % DMSO in 2-methyl-2-butanol which was an optimum condition for synthesis of glucose epoxy fatty acid ester. The solubility of glucose was 0.6 g/l at 30 °C in 2-methyl-2-butanol [98]. However, its solubility increases with temperature, 1.5 g/l at 60 °C [112]. In the presence of DMSO as a co-solvent, and the solubility of glucose was found to be higher (i.e., 1.7 g/l at 50 °C) in 2-methyl-2-butanol at the reaction temperature of 50 °C maintained in our study. The by-product, methanol was removed from the reaction system by using molecular sieve.

After 72 h of reaction at 50 °C using 0.2 g of lipase catalyst, the highest yield of 68 % was obtained. This was in agreement with literature report indicating that direct esterification of equimolar solution of fructose and palmitic acid in 2-methyl 2-butanol performed at 60 °C, resulted in a conversion of 75 % in 72 h of reaction [96]. In another study by Ferrer, M., *et al*. [99], sucrose conversion of 70 % to 6-O-laurylsucrose in 24 h using 50 mg/ml of biocatalyst in the presence of mixture of organic solvents (2-methyl-2-butanol/DMSO 4:1 v/v) was also reported.

The product was characterized by FTIR, TGA, MALDI-TOF MS and Solid state NMR techniques.

FTIR Spectrum Analysis

FTIR analyses results indicate that the enzyme catalyzed esterification of glucose in the organic solvent mentioned were successful

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3601	O-H	stretching	m
2	2946	-C-H (CH_3)	Stretching (asym)	m
3	2856	-C-H (CH_2)	Stretching (sym)	vs
4	1731	-C=O (ester)	Stretching	w
5	1663	-C=C-	stretching	w
6	1377	-C-H (CH_3)	Bending (sym)	m
7	954-1200	-C-O, C-C (in sugar ring)	Stretching	m

Table 3. 12: Summary of FTIR of glucose vernolate

In the FTIR spectrum, the introduction of vernolyl moiety into glucose unit through esterification was clearly shown. Among the major peaks, the band at 1731 cm^{-1} corresponding to (C=O carbonyl, stretching), at 1663 cm^{-1} due to (C=C, stretching), and at 1006 cm^{-1} due to (C–O, stretching of sugar ring) can be mentioned. The decrease in the OH peak intensity implies high degree of substitution of glucose hydroxyl groups by vernolyl moiety [100]. The decrease in the intensity of epoxy ring peak may be attributed to ring opening polymerization of the product.

Thermogravimetric Analysis (TGA)

Thermal analysis of glucose and glucose vernolate (epoxy fatty acid ester of glucose) was performed to understand the trend of change in thermal property. From the thermogram presented in the figures below it can be seen that the initial weight loss in the glucose started just at lower temperature (80-100 °C) corresponding to water loss as glucose is hydrophilic and can absorb moisture under normal room conditions. The other major degradation of glucose began at 203 °C leaving around 25 % residue at 500 °C. However, the degradation of glucose vernolate took place in two steps, the first one at lower temperature was due to removal of water whereas the second degradation has an onset temperature of 350 °C corresponding to the degradation of the grafted ester group i.e., vernolyl moiety. The thermogram clearly shows that glucose vernolate had higher thermal stability than glucose. This was confirmed from the fact that more than 56 % of glucose vernolate left undegraded at the maximum temperature of 500 °C set for the analysis. Therefore, this phenomenon along with the difference in the degradation pattern between glucose and glucose vernolate could be a strong evidence for the successful modification of glucose with the vernonia oil methyl ester under the reaction condition involved. We can also conclude that esterification of glucose has improved its thermal stability.

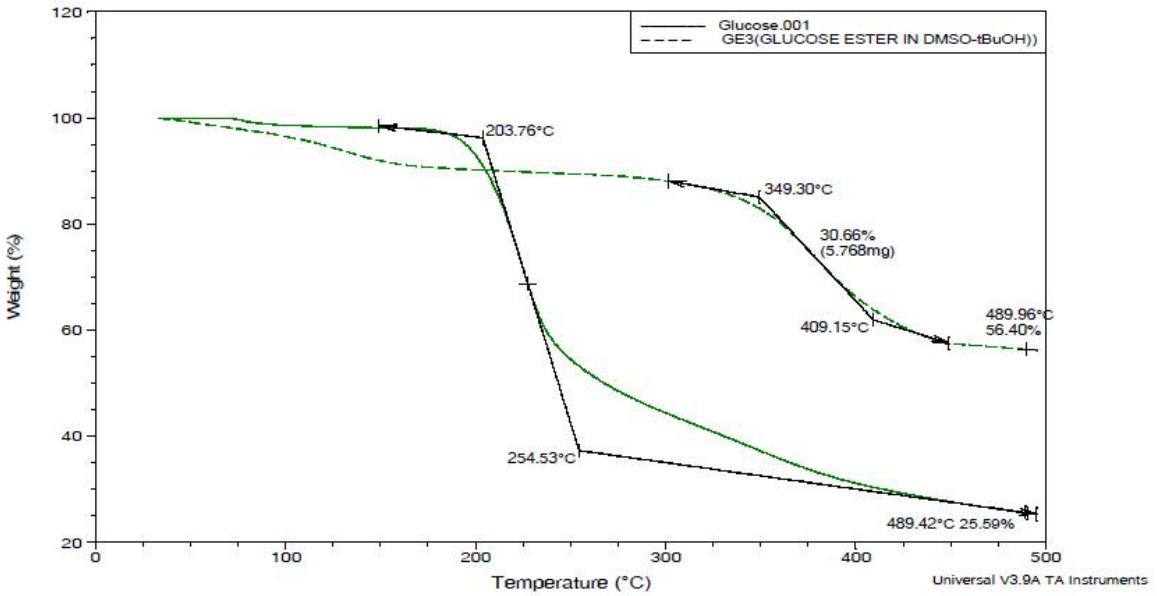
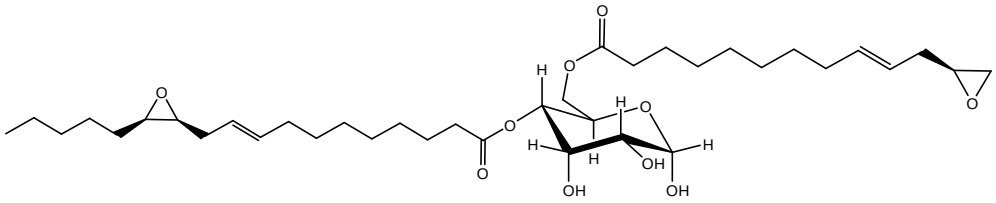


Figure 3. 33*: Comparison of TGA thermogram of glucose and glucose vernolate*

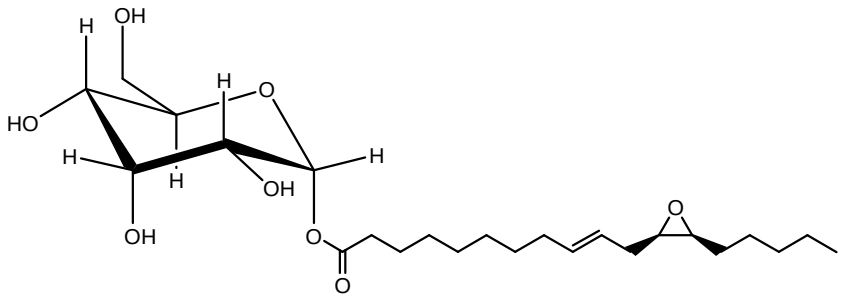
The MALDI-TOF MS analysis showed fragmentations corresponding to m/z at 739.4 which is the molecular ion $[M+2H]^+$, $C_{42}H_{72}O_{10}$ indicating the glucose is di-substituted with vernolyl group. The m/z = 720 is due loss of H_2O from the molecular ion. The other masses at m/z = 666.88 refers to $[M+2H]^+$-C_5H_{12} corresponding to the formula $C_{37}H_{62}O_{10}$



$C_{37}H_{62}O_{10}$

Mol. Wt.: 666.88

The m/z = 459 is due to loss of vernolic acid



$C_{24}H_{42}O_8$
Mol. Wt.: 458.59

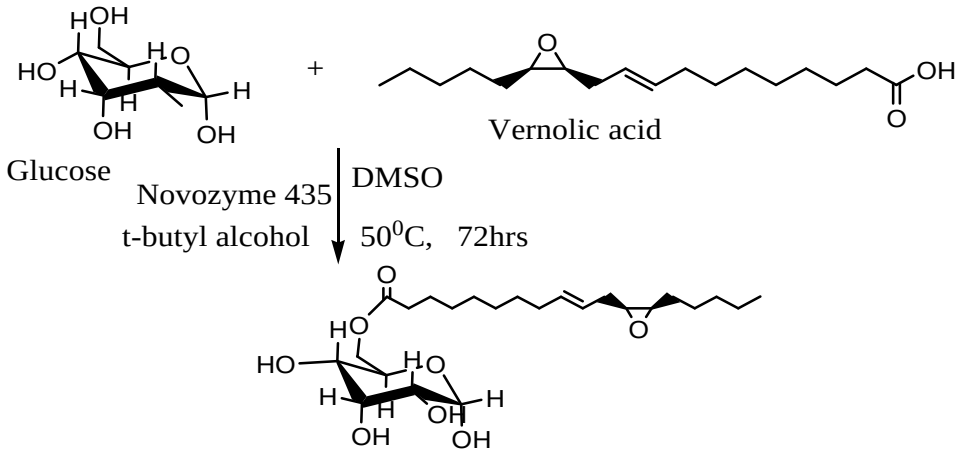
The other m/z at 376 is due to loss of $C_6H_{14}O$ from the mono-substituted glucose, which corresponds to the formula $C_{18}H_{28}O_7$

Solid state (^{13}C CP/MAS NMR) spectrum

The solid state NMR spectrum of the product shows the synthesis of glucose vernolate in which carbon peaks from both glucose and the introduced vernolyl moiety are found in their respective positions. Peaks in the range of 64-103 ppm correspond to carbon of glucose while those in the range 14-58, 128-132, and 178 ppm are due to carbon of vernolyl moieties esterified on glucose. The intensities of glucose carbon peaks are small indicating higher degree of substitution of glucose vernolate in this particular method of esterification.

3.3.3 Enzymatic Synthesis of Glucose Vernolate in DMSO and t-butyl alcohol

In this study Novozym SP 435, immobilized lipase B from *Candida antarctica* catalyzed esterification of glucose with epoxy fatty acid (vernolic acid) was carried out using a mixture of two miscible organic solvents, 20 % dimethyl sulfoxide in t-butyl alcohol.



Scheme 3. 12*: Schematic representation of synthesis of glucose vernolate*

The ability of a solvent system to solubilize glucose is more important for enzyme activity than the type of solvent used. It is also well documented that *Candida antarctica* lipase catalyses carbohydrate fatty acid ester synthesis from carbohydrates and free fatty acids in organic media. Degn *et al*. [78] previously reported that using an immobilized *Candida antarctica* lipase, they have efficiently synthesized 6-Omyristate- D-glucopyranose in *tert*-butanol. However, more polar solvents such as DMSO have the potential to inactivate enzymes and hence limit the feasibility of the reaction. Reports indicate that using mixture of solvents containing smaller proportion of the more polar solvents has alleviated such problems. Degn *et al*. [91] showed that the solvent systems

with pure DMSO and mixtures of acetone and DMSO containing more than 20 % DMSO, the enzyme showed no residual activity indicating a complete deactivation of the enzyme.

In this particular study, the glucose was dissolved in a low amount of a hydrophilic solvent, dimethylsulfoxide, and then was added to tert-butyl alcohol. This increased the solubility of glucose, thus favored the esterification to proceed. The reaction medium, mostly composed of t-butyl alcohol in which lipase enzymes are relatively stable, has created conducive environment for the esterification reaction; hence the inactivation of the biocatalyst is greatly reduced [101].

In this chemoenzymatic method, glucose vernolate was synthesised by esterification of glucose and vernolic acid using immobilized lipase form *Candida antarctica* to give a white solid product with 78 % yield. The relatively higher yield (78 %) achieved in this method of synthesis is attributed to the improved solubility of glucose in mixture of solvents used and the stability of enzyme in tertiary alcohol. Similar observation has been reported by Degn *et al*. [91] and Ferrer *et al*. [99].

FTIR Spectrum

The FTIR analysis result indicates that the enzyme catalyzed esterification of glucose in organic solvent mentioned was successful. Some of the major IR (cm^{-1}) peaks are listed in the table below.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3368	O-H	stretching	m
2	2943	-C-H (CH_3)	Stretching (asym)	m
3	2873	-C-H (CH_2)	Stretching (sym)	vs
4	1731	-C=O (ester)	Stretching	w
5	1665	-C=C-	stretching	w
6	1377	-C-H (CH_3)	Bending (sym)	m
7	954-1200	-C-O, C-C (in sugar ring)	Stretching	m

Table 3. 13: Summary of FTIR spectrum of glucose vernolate

In the FTIR spectrum, the introduction of vernolyl moiety into glucose unit through esterification was clearly shown. Among the major peaks, the band characteristic of the ester functionality at 1731 cm^{-1} corresponding to (C=O carbonyl, stretching), at 1665 cm^{-1} due to (C=C, stretching), and at 1002 cm^{-1} due to (C–O, stretching of sugar ring) can be mentioned. This data is in agreement with reported values for glucose fatty acid ester [91]. The decrease in the intensity of epoxy ring peak is due to ring opening polymerization of the product which is also confirmed by presence of broad band hydroxyl peaks with a shifted position.

Thermogravimetric (TGA) Analysis

In similar pattern as in the previous case the glucose vernolate prepared using this method also exhibits more thermal stability than the starting glucose. In the thermal study carried out, the initial weight loss in the glucose started just at lower temperature corresponding to water loss as glucose is hydrophilic and can absorb moisture under normal room conditions. The other major degradation of glucose began at 203 °C. In this case the degradation of glucose vernolate took place in two steps, the first one at lower temperature where as the second one began at 338.9 °C. However, glucose vernolate shows strong thermal stability than glucose as still more than 65 % of it remains not degraded at the set of temperature used during the analysis. Therefore, esterification of glucose improves its thermal stability.

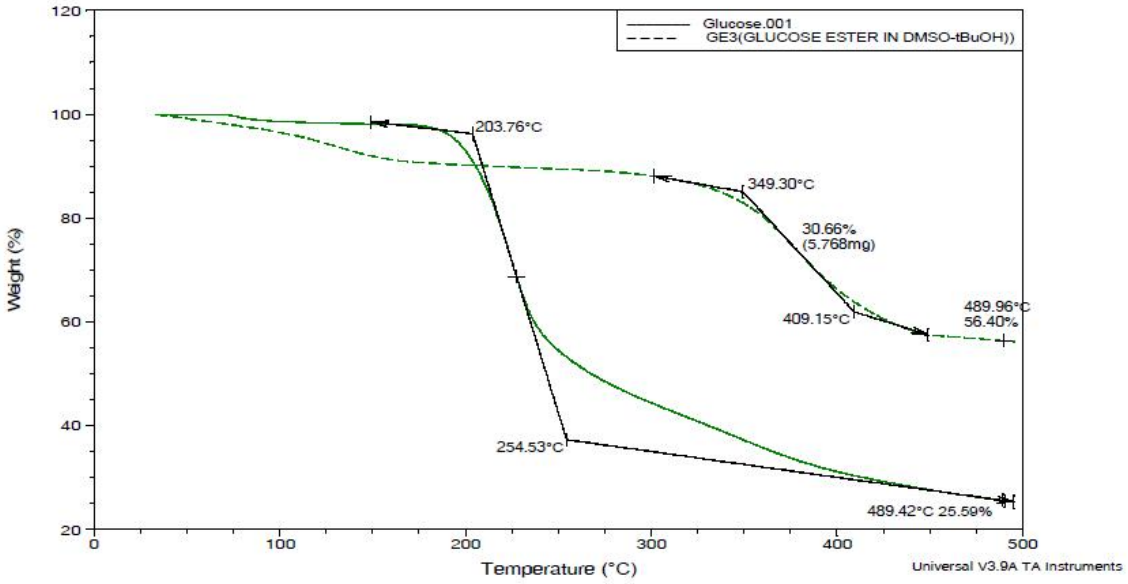


Figure 3. 34*: Comparison of TGA of glucose and glucose vernolate*

MALDI-TOF MS of Glucose vernolate in DMSO/ t-BuOH

The MALDI-TOF MS analysis showed fragmentations corresponding to m/z at 737.4 which is is molecular ion $[M+2H]^+$, $C_{42}H_{72}O_{10}$ indicating the glucose is di-substituted with vernolyl group. The m/z = 720 is due loss of H_2O from the molecular ion. The other masses at m/z = 666.88 refers to $[M+2H]^+$-C_5H_{12} corresponding to the formula $C_{37}H_{62}O_{10.}$ The m/z = 459 corresponds to $C_{24}H_{42}O_8$. This is due to loss of vernolic acid from the disubstituted moiety.

3.3.4 Epoxy alkenyl polyglycosides from vernanol

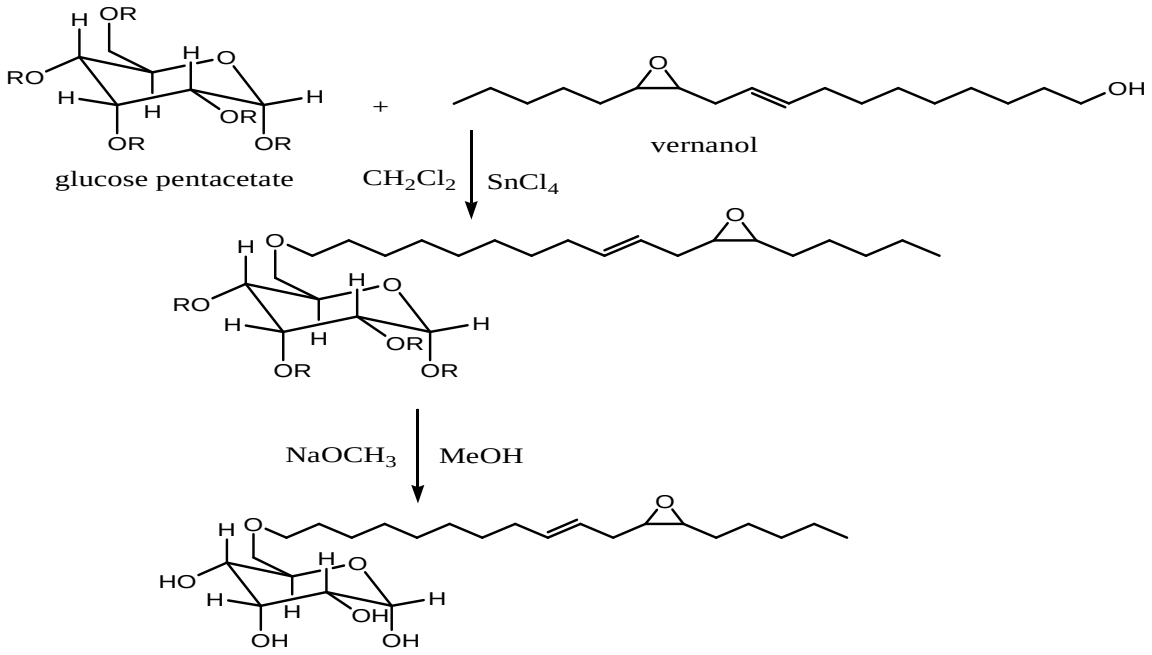
Alkyl glycosides (AG) and Alkyl polyglycosides (APGs) are a new generation of highly effective carbohydrate-based surfactants that are low in toxicity, ecologically safe, made from renewable resources at low cost [102]. The first alkyl glucoside was synthesized and identified in the laboratory by Emil Fischer more than 100 years ago [103]. So far as the industrial production of alkyl polyglycosides is concerned, processes based on the Fischer synthesis have been successfully adopted. Their development began about twenty years ago and has significantly accelerated in the past ten years.

The raw materials for APGs are carbohydrates and fatty alcohols, which are naturally occurring and renewable. During the synthesis of glycosides, a polyfunctional sugar component is combined with a nucleophile, such as alcohol, carbohydrate, or protein.

The processes for the reaction of carbohydrates to alkyl polyglycosides by the Fischer synthesis can be attributed to two process variants, namely direct synthesis and the transacetalization process. Direct synthesis is simpler from the equipment point of view. In this case, the carbohydrate reacts directly with the fatty alcohol to form the required long-chain alkyl polyglycosides. The two-stage transacetalization process involves more equipment than the direct synthesis. In the first stage, the carbohydrate reacts with a short-chain alcohol (for example n-butanol or propylene glycol) and optionally depolymerizes. In the second stage, the short-chain alkyl glycoside is transacetalized with a relatively long-chain alcohol (C-OH) to form the required alkyl polyglycosides. If the molar ratios of carbohydrate to alcohol are identical, the oligomer distribution obtained in the transacetalization process is basically the same as in the direct synthesis [104, 105].

Transacetalization reactions using acid catalysis has been reported. Noller and Rockwel [106] prepared alkyl polyglycosides by a method in which sugars were first peracetylated and then converted to the acetobromo sugars. The alkyl group was introduced by performing a reaction of the desired alcohol with the brominated peracetate in the presence of silver oxide, and deacetylation was accomplished by a treatment with sodium methoxide. Boether [107] reported later an alternative synthesis of alkyl glycosides involving a double alcohol interchange in the presence of an acid catalyst. Glucose was first converted to methyl glycoside, then to butyl glycoside, and finally to the desired alkyl glycoside. Recently, Zoran Markovic *et al*. [108] reported the synthesis of C_7-C_{16}-alkyl maltosides in the presence of tin (IV) chloride as a Lewis acid catalyst

In this study, synthesis of alkyl glycoside using glucose pentacetate and epoxy vernanol, obtained from derivatization of naturally epoxidized vernonia oil, was carried out in the presence of tin (IV) chloride a Lewis acid catalyst. The procedure applied was developed by modifying reported method [108]. Glucose pentacetate (1.0 g, 2.5 mmol) was dissolved in anhydrous dichloromethane (20 mL) and stirred for 1-2 h with molecular sieves (1.0 g) under an argon atmosphere. The solution was treated with tin (IV) chloride (1.0 mL) and immediately treated with the vernanol, epoxy alcohol, (0.8 g, 2.5 mmol) dissolved in anhydrous dichloromethane (10 mL). After 6 h of reaction time, the mixture was poured into the saturated sodium hydrogen carbonate solution (20 mL), the organic layer separated, and the aqueous phase extracted with dichloromethane (3 x 20 mL). The combined organic phases were washed twice with water (2 x 20 mL), filtered over Celite, and evaporated in vacuum. The resulting product was deacetylated with sodium methoxide in methanol remove the acetate moiety.



Scheme 3.13*: Schematic presentation of synthesis of epoxy alkyl glycoside*

The DS was obtained from the ratio of the area of the proton peak at 0.865 ppm to that of the proton peak between 3.10 and 5.12 ppm. Thus, the DS could be determined from the ratio of the integrated intensities of the signals of three protons of the terminal methyl group of the vernolyl chain to seven protons of the anhydroglucose units according to equation [32].

$$DS = \frac{I\,vernoyl/n}{\sum I\,Agu/7}$$

Where n is the number of protons from the signal of the methyl protons (n = 3) and AGU is the anhydroglucose unit.

Hence, $DS = \frac{I\,vernoyl/n}{\sum I\,Agu/7} = \frac{3/3}{8.69/7} = 0.81$ this implies that nearly three hydroxyl group of every four glucose was etherified with vernanol moiety.

The product obtained was purified and characterized using spectroscopic techniques. The ^{1}H NMR showed that proton peaks in the structure of vernolyl glycoside are obtained in their respective positions. 0.86 ppm (CH_3), 1.25 ppm $CH_3(CH_2)$, 3.37 ppm ,$O–CH_2–(CH_2)$, 2.7-2.9 ppm epoxy protons, 3.04–4.29 ppm , (glycoside protons), 4.8 ppm (glycoside OH). The ^{13}C NMR spectrum also indicated that the introduction of vernanol in to the glucose structure has been carried out successfully. The peaks at 168 ppm (C-O), 123-132 ppm (C=C), 56-57 ppm (epoxy carbon), 68-97 ppm (glycoside carbon) are characteristic of the product obtained. This also agrees with literature report [109].

FTIR analysis

The FTIR spectrum recorded showed the presence of the major functional groups in the alkyl glycoside. The band at 3375cm^{-1}corresponds to unsubstituted hydroxyl (O-H) groups in glucose, band at 986 cm^{-1} (C–O in sugar ring), C-H stretching band at 2864, 2925 cm^{-1}, C-O-C stretching band in the range 900-1250 cm^{-1} among the major peaks obtained. The peak at 1461cm^{-1} due to -C-H (CH_2, CH_3, bending (scissoring) and band at 1377 cm^{-1} due to -C-H (CH_3), bending (sym) were observed. The appearance of the epoxy band at 823 cm^{-1} indicates that the introduction of the vernanol moiety in the glucose structure was successfully carried out. The disappearance of the ester (C=O) band at 1731 cm^{-1} revealed that the deacetylation of glucose acetate after etherification with vernanol was successful.

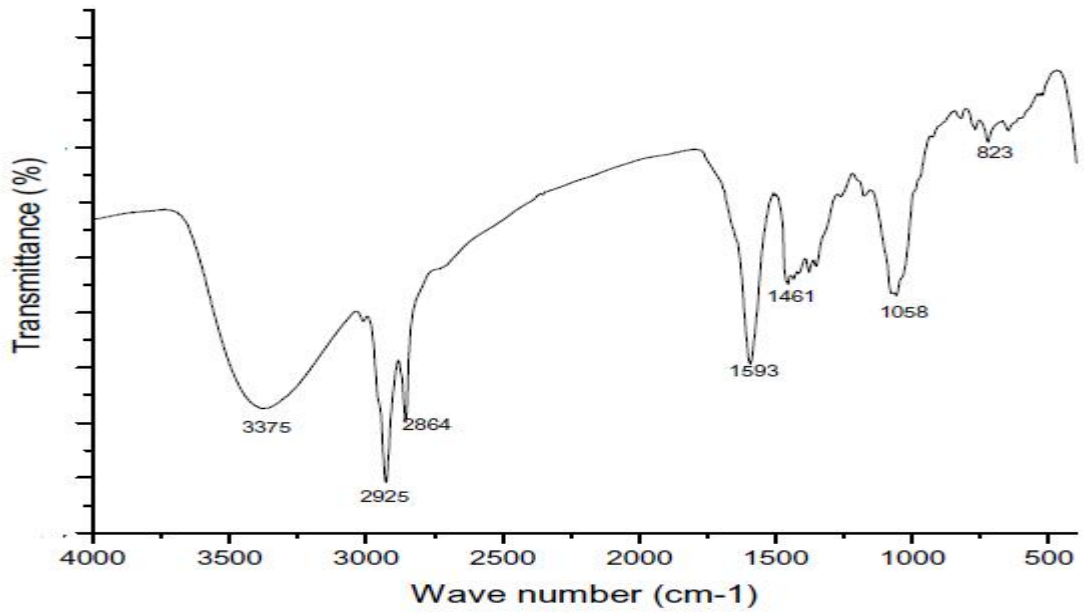


Figure 3. 35*: FTIR spectrum of epoxy alkyl glycoside*

The alkenyl glycoside synthesised in this study is a new product containing functionalized groups that can further be modified to prepare polymeric or cross linked surfactants.

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CHAPTER FOUR

4. POLYMERIZATION OF VERNONIA OIL AND VOME

Ring opening polymerization has been traditionally focused in petroleum based epoxides, as ethylene oxide and propylene oxide for the synthesis of polyetherpolyols, which are especially used for the production of polyurethane foams. However, due to environmental concerns, ease of synthesis and availability of the starting unsaturated triglycerides, the polymerization of vegetable oil-based epoxides monomers has become a topic of recent studies [1].

Vegetable oils containing unsaturated fatty acids are used in polymerizations to make bio-based polymers [2]. Three main routes can be followed for the preparation of polymers from plant oils. The first is the direct polymerization through the double bonds or other reactive functional groups present in the fatty acid chain. This method results in materials of different hardness [3]. The second route consists of the chemical modification of the double bonds or introducing functional groups which are easier to polymerize. The double bonds can easily converted into an epoxide group in several ways such as using m-chloroperbenzoic acid, Prilezhaev epoxidation or, more environmentally friendly, chemo-enzymatic epoxidation. Owing to the high reactivity of the oxirane ring, epoxides can find application as raw materials for preparation of polymers like polyesters, polyurethanes, and epoxy resins [1, 4, 5, 6], and the third is the chemical transformation of plant oils to produce platform chemicals which can be used to produce monomers for the polymer synthesis [1]. Epoxidised oils such as epoxidized soyabean oil (ESO) are an important intermediate that can be converted into polyols that are used in

polyurethane materials [7] and polymer synthesis [8]. These materials may have potential uses in paints, coatings, medicine, and many other areas.

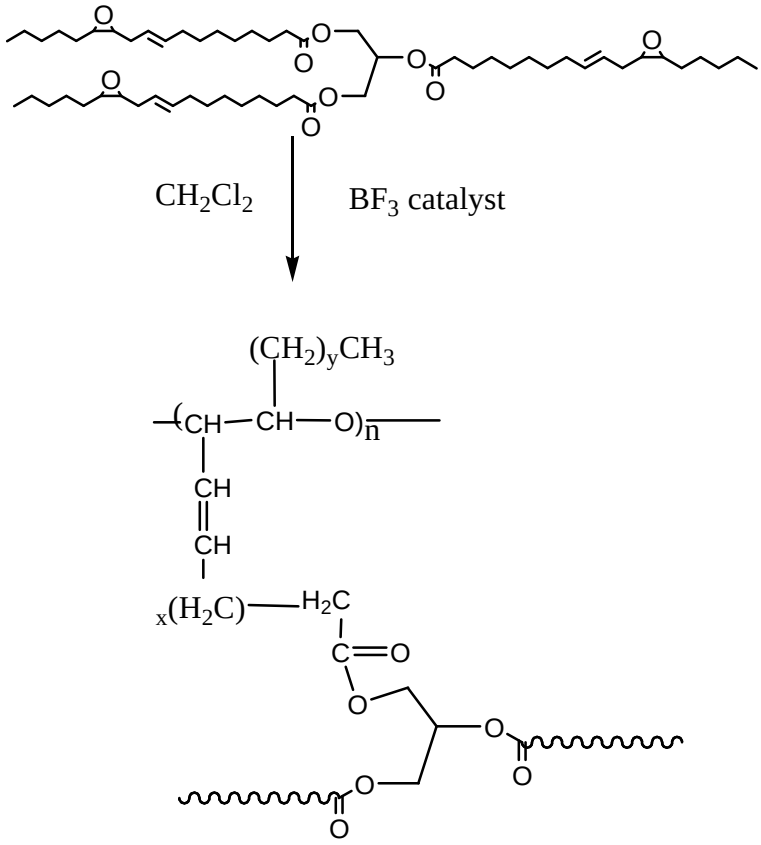
Polymerization of epoxides in the presence of acid catalysts occurs very fast, forming ethers. Lewis acid catalysts have been used for curing of epoxides. One group of such catalysts is boron trifluoride (BF_3) based catalysts. BF_3 is a gas and it is complexed with various alcohols, ethers or amines to give a liquid catalyst [9]. The cationic polymerization of biological oils by using boron trifluoride (BF_3) as the catalyst is described in two patents. Uloth [10] reported that soybean oil was polymerized at 130 °C in the presence of 2.8 % BF_3 as catalyst. A viscous product was obtained, with viscosity about five times higher than the initial viscosity of soybean oil. Under similar conditions, cottonseed oil was polymerized over six hours at 130 °C in the presence of 4 % BF_3 as catalyst. The second patent [11] describes the polymerization of soybean oil at 70 °C in the presence of 2 % BF_3 as a catalyst during 50-80 h. Liu, Z., *et al* [12] reported the ring-opening polymerization of ESO catalyzed by the Lewis acid, BF_3-OEt_2, in methylene chloride gave polymerized ESO (PESO). However, polyethers from long chain or functionalized epoxides are rare because of the lack of reactivity regarding their high steric hindrance that prevents the interaction with the catalyst sites.

Currently, the need for epoxidized oils has been met by the chemical epoxidation of vegetable oils such as linseed or soybean oils. However, the chemical epoxidation process is expensive as well as environmentally damaging. Vernonia oil, a naturally epoxidized seed oil, has very interesting functional groups like double bond, ester and inherent epoxide in its structure that can be further modified to achieve wider

applications. The epoxide ring of vernonia oil can be opened and polymerization reactions of different types such as self assembly or cross linking could be carried out.

4.1 Ring Opening Polymerization of Vernonia oil

In this particular study, we report the ring-opening polymerization of *vernonia galamensis* oil catalyzed by the Lewis acid, BF_3-OEt_2, in methylene chloride gave polymerized vernonia oil. We used procedure developed by Liu *et al*. [12] with modification. 3 g of VO and 50 mL of CH_2Cl_2 were added to a 250 mL round bottom flask with stirrer and fitted with condenser and dropping funnel. The solution was cooled to 0 ^{0}C with an ice bath and 0.14 g of the catalyst, BF_3-OEt_2, was added drop wise over 2 min. Then the solution was stirred at 0 °C for 3 h. After 3 h, ethanol (5 mL) was added to the mixture to deactivate the catalyst. The dichloromethane was removed using rotary evaporator and the remaining product was dried in vacuum oven.



Scheme 4.1*: Schematic representation of synthesis of polymerized vernonia oil*

The characterization of the ring opening polymerization product of vernonia oil was carried out using ^{1}H NMR, ^{13}C NMR and MALDI-TOF MS spectroscopic techniques. The ^{1}H-NMR spectrum of the extracted soluble substances showed that the epoxy protons in the 2.8-3.0 ppm region had nearly disappeared. The methine proton of –CH_2(O)–CH(O)–CH_2(O)– glycerol structure at 5.0–5.3 ppm, and methylene proton of –CH_2(O)–CH(O)–CH_2(O)– glycerol structure at 4.0–4.3 ppm were observed showing that the glycerin structure was present in the material. A very small peak at 5.4 ppm is attributed to the olefin hydrogen and the multiple peaks at 2.7 ppm assigned to the methylene protons between two carbon–carbon double bonds and these were observed due to the double bond of vernonia oil. A strong signal at 2.3 ppm assigned to the methylene

protons adjacent to the carbonyl groups was observed and supports the supposition that an oil ester structure remains. From the ^{13}C-NMR spectrum it can be seen that the peaks at 55–57 ppm assigned to epoxy carbons have disappeared. Peaks at 70 and 64 ppm assigned to the methine and methylene carbons of the glycerin structure were observed

FTIR Spectrum Analysis

The FTIR spectrum recorded shows the major functional groups in the polymerized vernonia oil. The hydroxyl (O-H) peak at 3373 $cm^{-1}$of the ring opened vernonia oil moiety, C-H stretching band at 3008, 2890, 2960 cm^{-1}, (C=O) stretching at 1737cm^{-1}, C-O-C stretching band in the range 900-1250 cm^{-1} among the major peaks obtained. The band at 1654 cm^{-1} corresponds to (-C=C-, stretching), peak at 1465 cm^{-1} due to -C-H (CH_2, CH_3, bending (scissoring) and band at 1377 cm^{-1} due to -C-H (CH_3), bending (sym) were observed. The carbonyl carbon peak at 1737 cm^{-1} characteristic of the ester functionality remains intact in the triglyceride structure of polymerised vernonia oil. However, the epoxy peak bands at 820, 840 cm^{-1} initially present in vernonia oil disappear indicating that the ring opening polymerization has taken place.

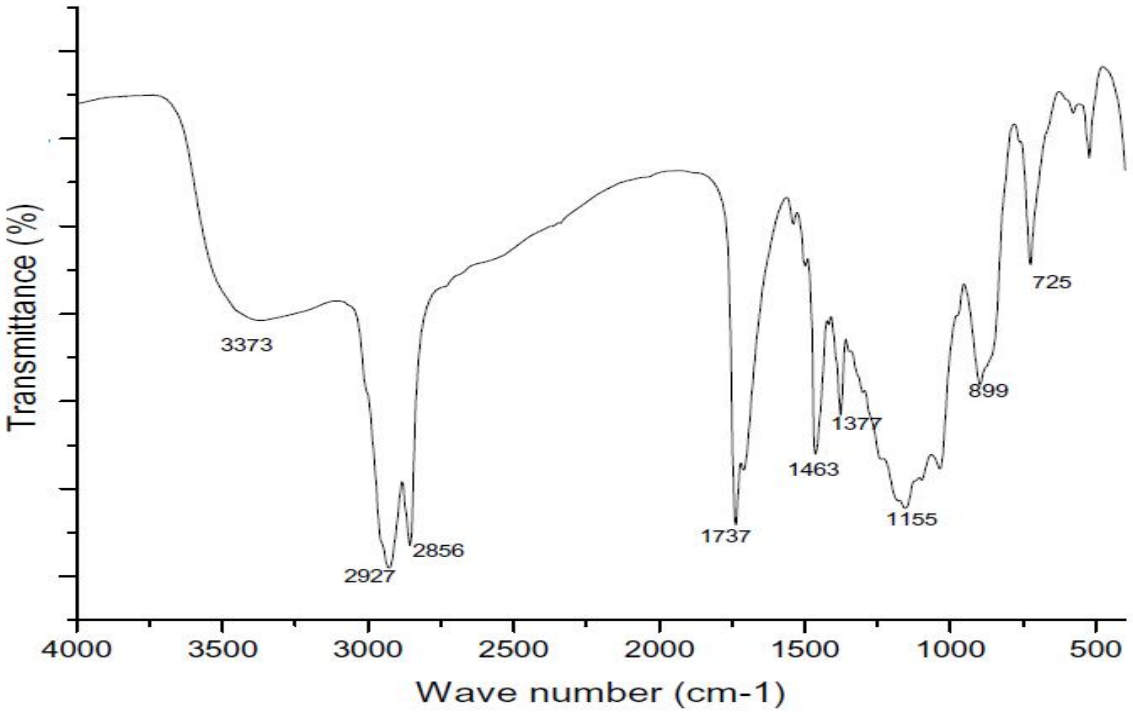
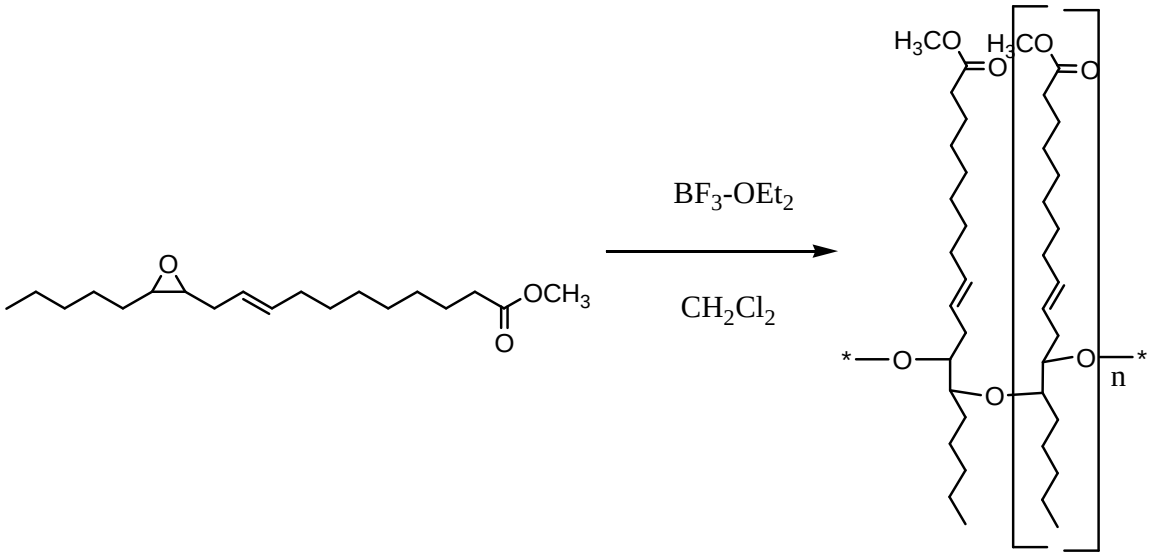


Figure 4. 1*: FTIR spectrum of polymerized vernonia oil*

4. 2 Ring Opening Polymerization of VOME

In addition to ring opening polymerization of epoxidized vegetable oils, polymerization of fatty acids and of methyl esters of fatty acids can be conducted using Lewis acids as catalysts, mainly boron trifluoride (BF_3) or boron trifluoride diethyl etherate (BF_3. Et_2O). In another study, Lligadas *et al*. [13] have reported that they obtained oligomeric polyether polyols through the acid-catalyzed ring-opening polymerization of epoxidized methyl oleate and the subsequent partial reduction of ester groups to give primary alcohols. Del Rio *et al*. [14] reported that the polymerization of internal epoxidized fatty acid derivatives such as methyl epoxy oleate (MEO) proceed in a more difficult way because of the higher steric demand of the epoxy groups.

Vernonia oil methyl ester is an epoxidized ester which contains an inherent epoxy ring in its structure. In this study, the polymerization of vernonia oil methyl ester (VOME) was carried out in the presence of boron trifluoride diethetate (BF_3-OEt_2) as a catalyst. The procedure for the ring-opening polymerization of VOME was prepared by modifying the method reported [12] as follows: 3 g (9.6 mmol) VOME and 50 mL methylene chloride were added to a 250 mL round-bottom flask fitted with a mechanical stirrer, condenser, thermometer, argon atmosphere and dropping funnel. The solution was cooled to 0 °C with an ice bath and BF_3-OEt_2, 0.141 g (1 mmol) was added drop-wise over 2 min. The solution was stirred at 0 °C for 3 h and ethanol (2 mL) was added to the mixture to deactivate the catalyst. The methylene chloride was removed using a rotary evaporator. The reaction was repeated using excess of the catalyst. The result was characterized using ^{1}H and ^{13}C NMR as well as ESI-MS.



Scheme 4. 2*: Scheme of polymerization of VOME*

The ^{1}H NMR and ^{13}C NMR analyses of the product showed that the opening of the epoxy ring has taken place as the epoxy proton peak around 2.7-2.9 ppm in ^{1}H NMR and the corresponding peak at 56-57 ppm in ^{13}C NMR nearly disappear. However, the methoxy proton peak at 3.6ppm in ^{1}H NMR and at methoxy carbon peak at 51 ppm in ^{13}C NMR are retained indicating the ester functionality is intact. The low intensity of hydroxyl peaks also shows that the ring opened epoxy group are cross linked. However, the ESI-MS data recorded shows that the product is oligomer instead of polymer. The m/z at 643 is the mass due to sodiated dimmer of VOME. While the fragment at m/z = 333 could be due to sodiated VOME. Therefore, the result obtained in this study is in good agreement with literature report that low molecular weight polyether attained is attributed to the backbiting as well as stearic hindrance long chain internal epoxy containing fatty acid methyl esters or fatty acids [13, 14].

FTIR spectrum Analysis

The FTIR spectrum recorded shows the presence of the major functional groups in the polymerized VOME. Weak band of the hydroxyl (O-H) stretching peak observed at 3384 cm^{-1}corresponds to the ring opening in the oxirane of vernonia oil methyl ester. However, the weak intensity of the hydroxyl peak indicates that the ring opened oxirane has crosslinked. C-H stretching band at 3008, 2890, 2927 cm^{-1}, (C=O) stretching at 1741 cm^{-1}, and the C-O-C stretching band in the range 900-1250 cm^{-1} among the major peaks obtained. The band at 1654 cm^{-1} corresponds to (-C=C-, stretching), peak at 1461 cm^{-1} due to -C-H (CH_2, CH_3, bending (scissoring) and band at 1377 cm^{-1} due to -C-H (CH_3), bending (sym) were observed. The carbonyl carbon peak at 1741 cm^{-1} characteristic of

the ester functionality remains intact in the structure of polymerised vernonia oil methyl ester. However, the epoxy peak band at 823 cm^{-1} initially present in vernonia oil methyl ester has disappeared indicating that the ring opening polymerization has taken place. The appearance of the –C=C- double bond peak at 1654 $cm^{-1}$is another confirmation that the polymerization has taken place through oxirane ring opening instead of polymerization through the double bond.

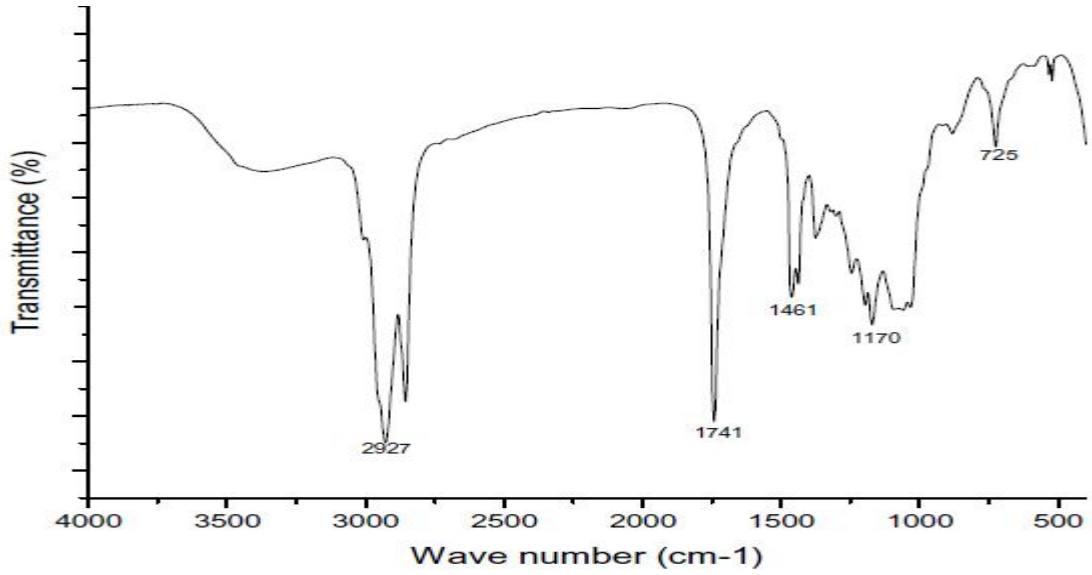


Figure 4. 2*: FTIR spectrum of polymerized VOME*

The ESI-MS spectrum obtained indicated, the sodiated M^+ ion, (M^+ + Na) with m/z = 333.3 refer to $C_{19}H_{33}O_3Na$ and m/z at 463.49 refers to dimmerized VOME, $C_{38}H_{67}O_5Na$

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CHAPTER FIVE

5. SYNTHESIS AND CHARACTERIZATION OF OTHER DERIVATIVES OF VERNONIA OIL

In addition to the epoxy glucose fatty acid esters and epoxy starch fatty acid esters, further derivatization of vernonia oil and characterization of the following products obtained were carried out.

5.1 Epoxidation of vernonia oil in acidic ion exchange resin

There are several methods for producing epoxides from vegetable oils, fatty acids and methyl esters. These includes epoxidation with percarboxylic acid generated *in situ* in the presence of acids or enzymes as catalysts [1], epoxidation with organic and inorganic oxidants such as potassium peroxomonosulphate, meta-chloroperoxybenzoic acid and ethyl methydioxirane [2, 3], epoxidation with halohydrins for the epoxidation of olefins with electron-deficient double bonds and epoxidation with molecular oxygen [4].

From process, environmental safety and efficiency point of view epoxidation of vegetable oils in one step, i.e., with peroxy acid generated *in situ* from carboxylic acid (formic/acetic acid) with hydrogen peroxide in the presence of acid catalyst is widely used on an industrial scale. However, the use of a mineral acid as catalyst in epoxidation is inefficient because of problems associated with separation of the catalyst from the reaction product. The process can be made competitive with the use of ion-exchange catalyst instead of traditional homogeneous one in epoxidation of unsaturated compounds [5].

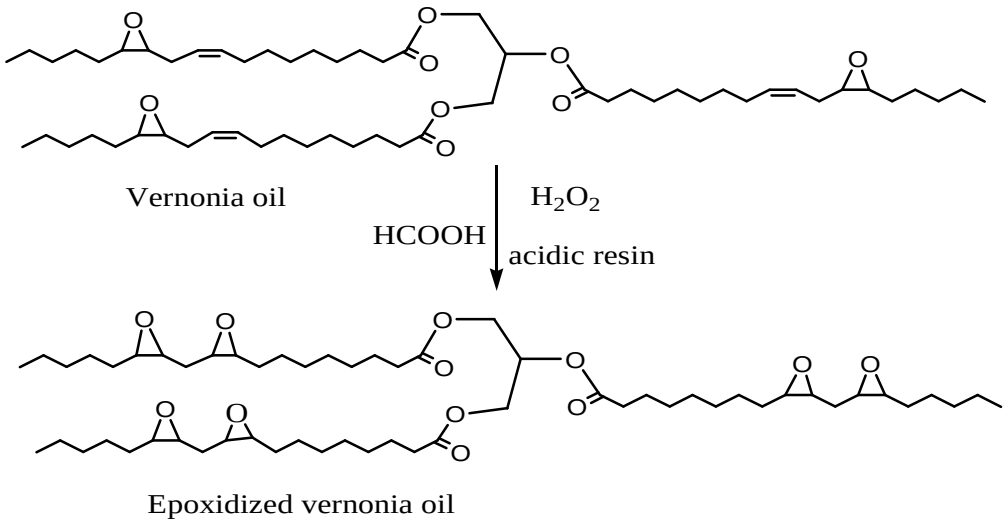
Epoxidized vernonia oil (EVO) was produced by the epoxidation of vernonia oil using peroxyformic acid, formed *in situ* by the reaction of hydrogen peroxide and formic acid in the presence of an acidic ion exchange resin (Amberlite IR-120 hydrogen form) as the catalyst. The Amberlite IR-120H catalyst is a copolymer based on styrene (98 wt %) crosslinked by divinylbenzene (2 wt %). Its acidity is generated by sulfonic acid groups attached to the polymer skeleton.

A solution of vernonia oil, formic acid 0.67 mol and Amberlite IR-120 (15 wt %) were added to a three-necked round bottom flask equipped with a mechanical stirrer and thermometer. The reactor contents were mixed for five minutes prior to drop wise addition of hydrogen peroxide (35 %) for one hour. The reaction was then performed over a period of seven hours at 110 rpm and 75 °C. On completion of the reaction the solution was washed with water three times (cool, hot, cool) to remove the residual peroxyacetic acid and then filtered to remove the catalyst. The resulting product was characterized.

The ^{1}H NMR data of the shows the presence of glyceral proton (C-H) at 5.34 -5.43, epoxy protons (O-C-H) at 2.78 - 2.92, protons of methylene groups $(CH_2)_n$ at 1.261-1.63, protons of methyl (CH_3) group at 0.89.

The ^{13}C NMR data also indicated the presence of the vernolyl carbonyl carbon (O=C) at 173.06, glyceral (CH) at 68.95, and glyceral (CH_2) at 62.04-64.99, methylene carbons $(CH_2)_n$ at 22.64 - 34.03, and methyl carbon (CH_3) at 14.06. However, the result also showed that the intensity of the epoxy group has increased where the double bond peaks

has nearly disappeared in both ^{1}H and ^{13}C NMR. The triglyceride structure remained intact after epoxidation.



Scheme 5. 1*: Epoxidation of vernonia oil using acidic ion exchange resin*

This method of epoxidation has resulted in product with about 78% conversion. Acid exchange resin catalyzed epoxidation reactions are found to be promising in minimizing oxirane ring opening [6]. The resulting epoxidized vernonia oil is a promising intermediate for synthesis of bio-lubricants. The epoxide, or oxirane, group can be easily functionalized for production of lubricant base stocks from acid-catalyzed oxirane ring-opening reactions followed by esterification of hydroxyl groups [7].

FTIR spectrum analysis

For VO the characteristic peaks at 3008, 1650 and 723 cm^{-1} are attributed to the stretching vibration of the double bonds: =C-H, C=C, *cis*-CH=CH, respectively. Hernandez-Lopez *et al.* [8] reported the diminution of peak, C=C-H stretching at 3008 cm^{-1} after epoxidation reaction, which supports our study where the almost complete disappearance of double bonds band at 3008 cm^{-1} at 75 °C after 7 h was observed. This

confirms that almost all the C=C-H had taken part in the epoxidation reaction. Also, there is decrease in the intensity of the other important unsaturated bond signals in comparison with the unreacted oil, giving reliable support of its chemical transformation to an oxirane ring. The presence of new peaks in the FTIR spectrum of EVO at 820-843 cm^{-1}, attributed to the epoxy group, confirmed the success of the epoxidation reaction of VO. Vleck and Petrovic [9] reported the presence of epoxy groups at 822–833 cm^{-1}, which agrees well with this study. The other new peak at the 3449 cm^{-1} was attributed to the hydroxyl functional group, derived from the epoxy functional group via partial epoxy ring opening reaction. The intensity of this band indicates the extent of hydrolysis of epoxidized vernonia oil (EVO). The epoxy ring opening reaction could occur either by acid catalysis in the presence of water associated with aqueous solution of H_2O_2 used [10]. The hydrolysis of the ester groups during epoxidation reaction in oils is the main side reaction. In the case of hydrolysis, a carboxylic acid functional group is formed and this carbonyl group will appear near but differentiable of the ester carbonyl stretching C=O in the glyceride moiety at 1737 cm^{-1}. However; in the course of the epoxidation reaction carried out in our study, no evidence of the carbonyl from carboxylic acid group signal was observed [11].

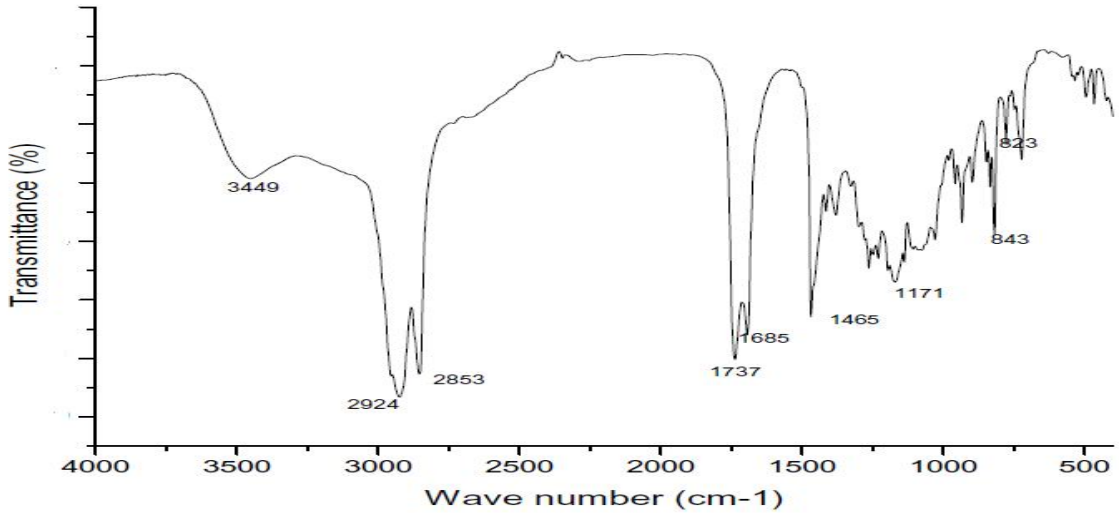


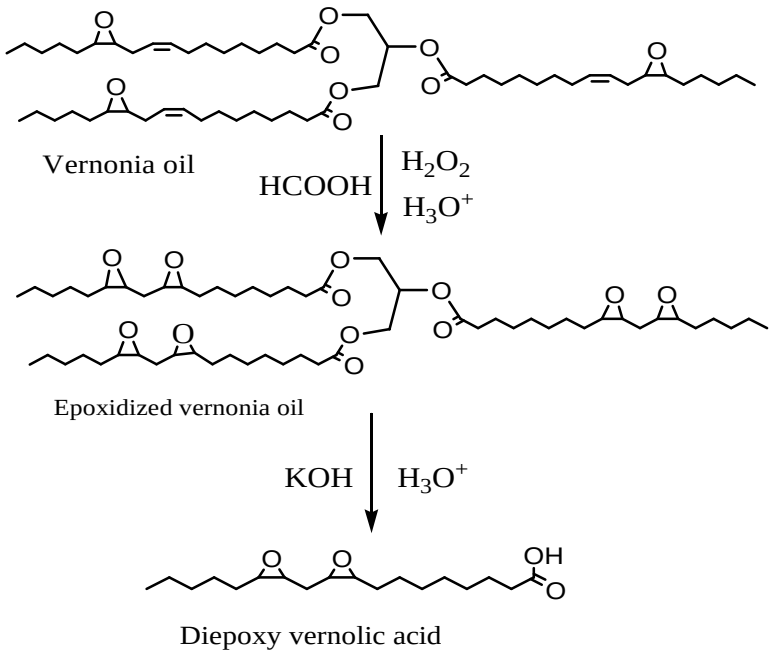
Figure 5.1*: FTIR spectrum of epoxidized vernonia oil*

5.2 Epoxidized vernolic acid

Epoxidation consists of the formation of an oxirane (epoxy) group by the reaction of peroxyacids (peracids) and olefinic double bonds. Epoxidation reactions can be carried out either chemically or enzymatically. In general, peracetic or performic acids are used in epoxidation process for oxygen transfer to the double bonds [12]. The use of these acids is prone to loss of yield and side reactions as acid catalyzed ring opening of the oxirane group.

In this study, we report the synthesis of epoxidized vernolic acid using chemical method in which the epoxidation of vernonia oil followed by hydrolysis has resulted in the epoxidized vernolic acid with over 88 % conversion. The characterization of the product using NMR, ESI-MS indicated a successful epoxidation and hydrolysis has been carried out. The increased intensity of the epoxy group in both the ^{1}H and ^{13}C NMR spectrum,

along with the ESI-MS recording with molecular ion $[M+Na]^+$ at m/z = 335.22 reveals the presence of stable sodiated di-epoxy vernolic acid.



Scheme 5. 2*: Schematic representation of synthesis of di-epoxy vernolic acid*

The ^{1}H NMR (ppm) (400 MHz, $CDCl_3$) data from the epoxidized vernolic acid indicated methylene protons (O–CH_2) at 3.64, epoxy protons (O–C–H) at 2.9, protons of other methylene groups $(CH_2)_n$ at 1.25–1.76, and protons of methyl (CH_3) groups at 0.90. The presence of small peak of the olefinic protons (CH=CH) at 5.35 and 5.60 in spectrum indicated that the conversion was incomplete (88 %).

The ^{13}C NMR data also showed epoxide carbons (O–C–H) at 56.68 and 57.35, carbon attached to the carboxylic group (-COOH) at 179.04, methylene carbons $(CH_2)_n$ at 22.66–32.83, and methyl carbon (CH_3) at 14.06. The increased intensity of the epoxy peak

confirms that the epoxidation of the vernonia oil accompanied by the hydrolysis was successful.

FTIR analysis

The FTIR spectrum recorded shows the presence of the major functional groups in the vernolic acid.

No	Frequency (cm^{-1})	Functional group	Mode of vibration	Intensity
1	3489	O-H	stretching	w
2	3008	=C-H	Stretching	m
3	2960	-C-H (CH_3)	Stretching (a)	m
4	2890	-C-H (CH_2)	Stretching (s)	vs
5	1711	-C=O (acid)	Stretching	vw
6	1465	-C-H (CH_2, CH_3)	Bending (sci.)	m
8	1377	-C-H (CH_3)	Bending (s)	m
9	900-1250	-C-O,	Stretching, bending	m
10	823, 840	-C-O (epoxy gr.)	Stretching	m
11	723	-$(CH_2)_n$,	Bending (rocking)	m

Table 5. 1: *Summary of FTIR spectrum of diepoxy vernolic acid*

The hydroxyl (O-H) peak at 3489 $cm^{-1}$of the vernolic acid moiety, C-O-C stretching band in the range 900-1250°Cm^{-1} and the epoxy peak at 820, 840 cm^{-1} are among the major peaks obtained. The band at 1654 cm^{-1}, corresponds to (-C=C-, stretching), disappeared due to epoxidation of the vernolic acid. The increased intensity of epoxy peak signals the successful epoxidation of vernolic acid to result in di-epoxy compound.

The ESI-MS spectrum data recorded revealed that m/z at 313.5 corresponds to epoxidized vernolic acid, $C_{18}H_{32}O_4$, where as the m/z at 335.21 refers to sodiated epoxy vernolic acid, $C_{18}H_{32}O_4Na$.

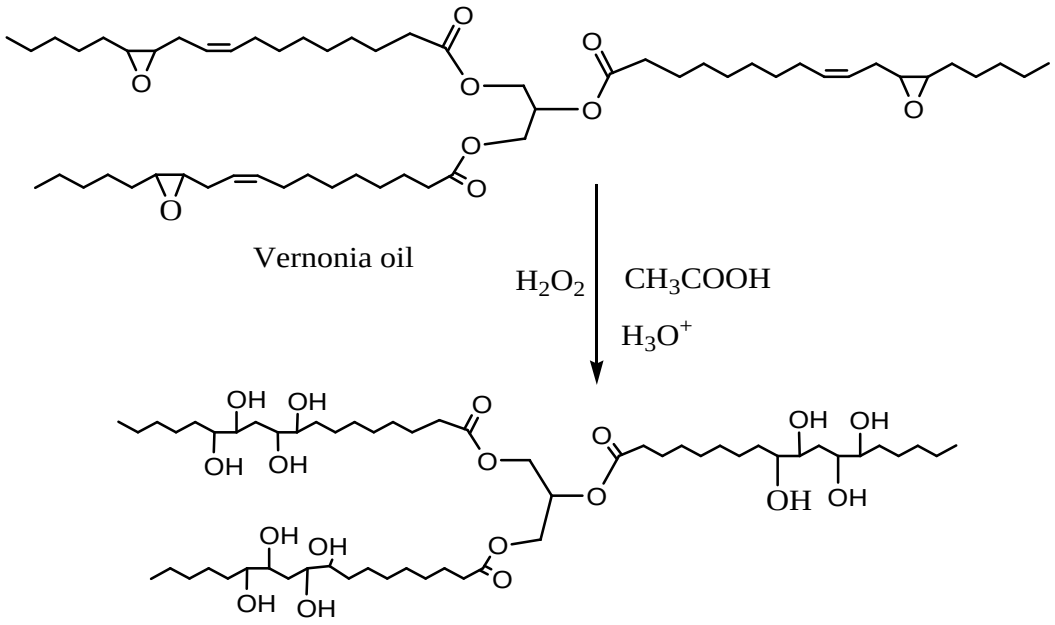
The di-epoxy vernolic acid prepared can be used in the synthesis of fatty acid based polymers, and epoxy resin.

5.3 Synthesis of Vernonia Oil Polyol

The double bonds present in fatty acids represent excellent starting points for modification of the hydrocarbon chain for the production of new types of feedstocks. For example, plant oils can be treated with a variety of chemicals to convert the double bonds of fatty acids into hydroxyl groups, and the resulting ‘polyols’ can be mixed with compounds such as isocyanates to form polyurethanes. These environmentally friendly, renewable alternatives to petroleum-derived polyurethane have excellent physical characteristics and perform well in a variety of applications, such as spray insulating foams, rigid foams, flexible foams such as interior car parts, coatings, sealants, adhesives and elastomers [13].

One of the early methods used for preparing polyols from various vegetable oils was based on transesterification of the fatty acid in triglycerides with a polyol such as glycerin, glycerol [14]. The main disadvantage of this process was the long reaction time and the occurrence of premature degradation due to high temperature. Hydroformylation is another synthetic strategy that has been explored for the preparation of polyols [15]. In this approach, the double bonds of the vegetable oil are first converted to aldehydes using suitably chosen catalysts. The aldehydes are subsequently hydrogenated to alcohols. The mechanical properties of the polyurethanes prepared from these polyols were however, found to vary significantly with the choice of the hydroformylation catalyst [15]. More recently, epoxidation of soy-oil and subsequent opening of the epoxide rings either by heating or by hydroxylation with polyfunctional alcohols, have been explored for devising more efficient methods for soy-polyol synthesis [16].

In this study, we report that chemical epoxidation of vernonia oil with excess of H_2SO_4 catalyst has led to ring opening of the vernonia oil and resulted in polyhydroxy vernonia oil (polyol). The epoxidation stage of this synthesis was carried out using the procedure of Okieimen F.E. *et al*. [17], with modification. 10 g VO was dissolved in 3 mL of glacial acetic acid. To this solution 5 mL of 35 % H_2O_2 was added drop wise with in 10 min. at 30 °C. Excess H_2SO_4 (4 %) was used as a catalyst. Then the temperature was raised to 60 °C and reaction continued for 24 h. Purification of product was done by extraction with 25 mL ethyl acetate. Then the ethyl acetate was removed and product dried in a rotary evaporator.



Scheme 5.3*: Synthesis of vernonia oil polyol*

The effect of temperature and catalysis concentration on rate of epoxidation was studied. It was found that as temperature increased, the epoxidation rate increased. At lower temperatures (30 °C), the relative percentage conversion to oxirane continuously increased within the experimental time limit. However, the relatively maximum percentage conversion to oxirane was attained at higher temperature (60 °C), after which it gradually decreased. The relative percentage conversion to oxirane showed a continuous decrease at 80 °C, after some increase during the initial phase of the reaction. This indicates that an increase in temperature not only increased the epoxidation rate but also increased the rate of hydrolysis (oxirane cleavage) of the product.

Increasing concentration of the catalyst (H_2SO_4) above 3 % resulted in epoxy ring opening and simultaneous conversion to hydroxyl functionality (polyol). This observation was in agreement with literature report that there is optimum concentration of catalyst for epoxidation, and with increased concentration the epoxide ring began to cleave [18]. In general, with an increase in acid concentration, oxirane conversion increased. They also found that increasing acid concentration from 1-2 % had reduced reaction time taken to reach the maximum conversion of oxirane value. The optimum conversion to oxirane was obtained at 2 % loading of H_2SO_4. However, when the catalyst loading was increased to 3 %, higher oxirane cleavage was observed with a correspondingly lower oxirane value.

The functional groups of the synthesized vernonia oil polyol were further confirmed from the ^{1}H NMR spectrum in the figure below. Vernonia oil has clear signature of the double bond hydrogen (olefin proton -H-C=C-H-) between 5.0-5.5 ppm. The other significant peaks are; methylene proton in the glyceryl group (4.0-4.5 ppm,), methylene protons adjacent carbonyl group (2.0-2.5 ppm) allyl methylene proton (~ 2 ppm), methylene protons from carbonyl group (~ 1.6 ppm), and methylene protons on saturated carbon atom (1-1.5 ppm) and the terminal methyl group. The signature of the double bond, the olefinic proton peak at 5.0–5.5 ppm, is observed to almost disappear in the spectrum of vernonia oil polyol. Further, the spectrum of polyol shows the appearance of new peaks between 3.2-4.1 ppm, which correspond to the signature of the methylinic proton (-CH=) and the proton associated with the OH groups. This confirms the incorporation of hydroxyl groups. Both the ^{1}H NMR and ^{13}C NMR indicate that the epoxy ring has been broken and additional hydroxyl peaks 3.2-4.1 ppm in ^{1}H NMR and 62-85 ppm in ^{13}C

NMR appeared. The characteristic ester carbonyl carbon peak at 173 ppm indicates that the triglyceride structure remained intact during the reaction.

FTIR spectrum

The FTIR spectrum study clearly indicated that the characteristic peaks of the double bonds, C=C-H stretch at ~ 3010 cm^{-1} and C=C stretch at ~1654 cm^{-1} (that were present in the spectrum of VO) completely disappear in the spectrum of vernonia oil polyol. In comparison to the spectrum of vernonia oil, the epoxy groups (CO twin bands at 823 and 840 cm^{-1}) nearly disappear in the spectrum of polyol confirming the oxirane opening. Most importantly, the spectrum of polyol show very prominent signature of the broad hydroxyl-stretching peak at around 3417 cm^{-1}, confirming the incorporation of the hydroxyl groups.

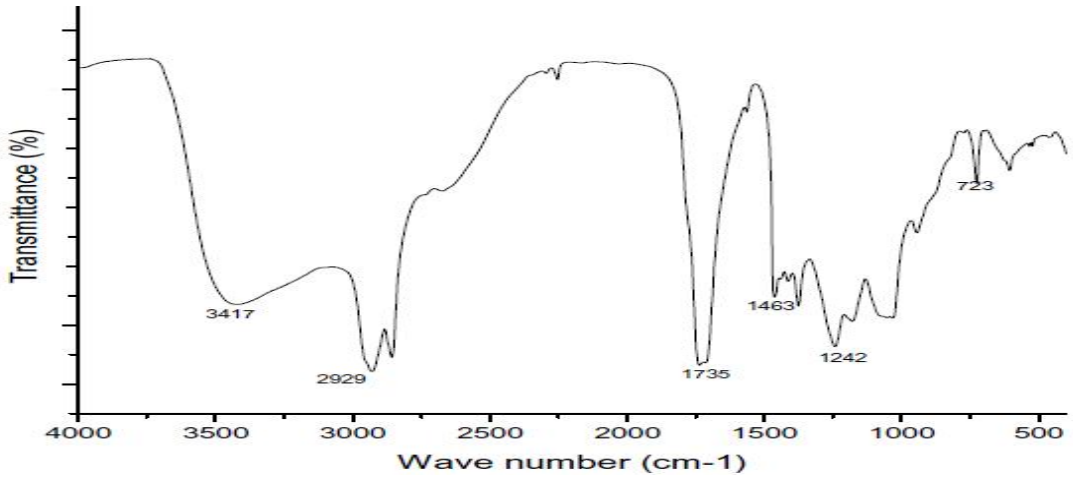


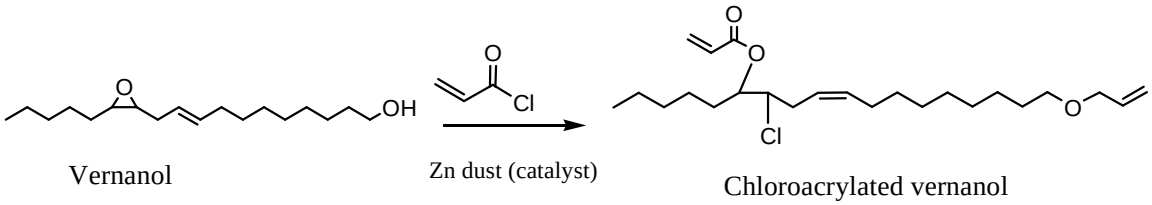
Figure 5.2*: FTIR spectrum of vernonia oil polyol*

5.4 Synthesis and Characterization of Acrylated vernanol

Recently epoxidized fatty oils and their derivatives have showed a promising effect when used as reactive resins, since the vernonia oil contains unsaturation and epoxy group that can be chemically modified through simple reactions. These reactions permit to introduce polymerizable groups as epoxy or acrylates which are available to give products with practical useful properties and characteristics. Acrylated vernanol can be used as thermosetting resin. It can be cured alone and also used in epoxy formulations, and as precursor in the preparation of bio-based composites and for coating purposes.

The other modification carried out was on the epoxy structure of vernanol. The introduction of acryloyl group in to the structure of vernanol was carried out using modified version of a reported procedure [24]. 2 g of vernanol and 0.75 g acryloyl chloride were taken in 50 mL round bottom flask. To this 0.14 g zinc dust was added and stirred at 30 °C for few seconds. After completion of the reaction, 25 mL diethyl ether was added and the organic phase was washed successively with saturated $NaHCO_3$ (10 mL), water (10 mL X 2) and dried over anhydrous Na_2SO_4 and product concentrated in rotary evaporator.

From the spectrum data evidence it is observed that the acryloyl moiety has substituted the terminal hydrogen of the hydroxyl functionality and with ring opening of the epoxy, anther acryloyl and a chloride group were attached. These were confirmed by the disappearance of the epoxy group in both the ^{1}H and ^{13}C NMR spectrum. The FTIR spectrum also indicated the disappearance of the broad band of hydroxyl group which was present in vernanol.



Vernanol Chloroacrylated vernanol

Scheme 5.4*: Schematic representation of synthesis of acrylated vernanol*

The ^{1}H NMR spectrum of acrylated vernanol showed the typical resonances. The signal at 0.8 ppm was attributed to the three methyl hydrogen atoms and that at 2.3 ppm to the methylene protons. Peaks at 4.0 ppm refers to (–CHCl) and signal at 4.05 - 4.11 ppm is due to [–O-CH_2-CH(O–)-CH_2-O–]. The acrylated vinyl protons appeared between 5.7 and 6.4 ppm and the fatty acid double bond protons at 5.2–5.4 ppm. The disappearance of the epoxy peak in the range 2.7-2.9 ppm signals the success of acrylation. The ^{13}C NMR data showed carbon attached to the carbonyl group (-C=O) at 168 ppm, methylene carbons $(CH_2)_n$ at 22.66–32.83, and methyl carbon (CH_3) at 14.06. The vinyl and fatty acid double bond carbons were obtained at 123-132 ppm. The absence of epoxide carbons (O–C–H) at 56.68 and 57.35 and new peaks around the 62 to 75 ppm area due to (–C=O)OCH_2–, (–C=O)-OCH–, and –CHO(C=O)-CH=CH_2 can be seen. These are presumed to be due to the ring opening reaction of the epoxide group followed by acrylation of the epoxide ring.

The ESI-MS spectrum obtained indicated, the sodiated M^+ ion, ($M + Na^+$) with m/z = 449.24 refer to the chloroacrylated compound.

The FTIR spectrum of acrylated vernanol shows that the hydroxyl group is successfully converted to the acrylated functionality through condensation esterification. The peak at 3386 cm^{-1} corresponding to hydroxyl stretching of vernanol has disappeared after the

introduction of the acryloyl group into the structure of vernanol. This is also indicated from the presence of a strong absorption band for the carbonyl group, C═O of the acrylated compound at 1727 cm^{-1}. Another significantly different absorption band is observed in the spectrum at 1636 cm^{-1} indicating the presence of vinyl functionality (–CH=CH_2–). The presence of vinyl functionality of the acrylated product is also supported by the absorption peak at 984 cm^{-1} corresponding to [CH_2=CH-(CO)–O]. The disappearance of the epoxy band at 820 and 840 cm^{-1} show that ring opening followed by the acrylation of epoxy alcohol (vernanol) has taken place.

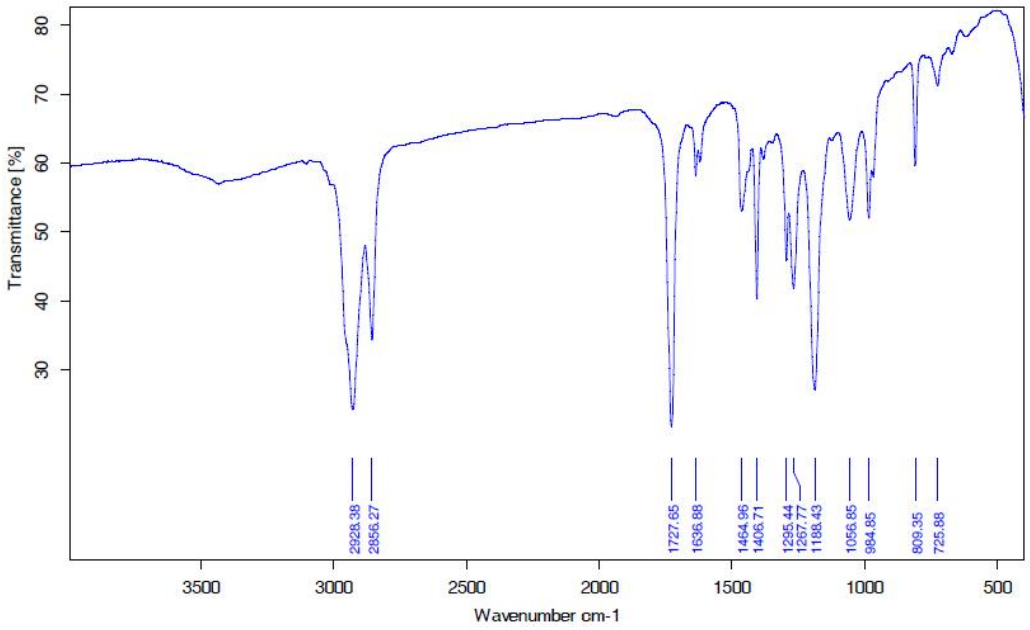
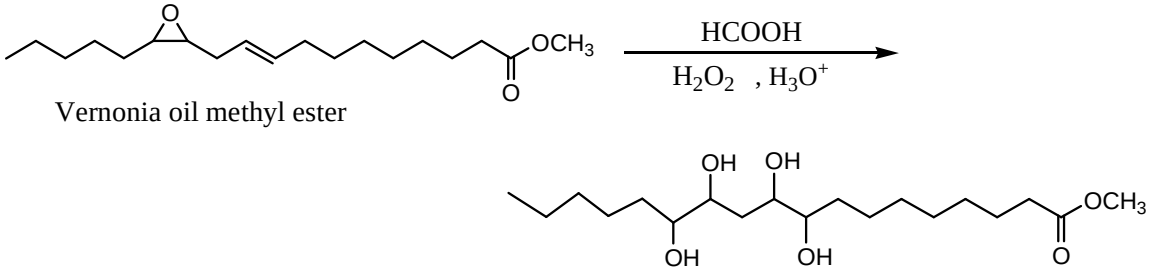


Figure 5.3*: FTIR spectrum of chloroacrylated vernanol*

The acrylated vernanol could be used to prepare UV curable resin which is a potential substitute for coatings that are widely formulated from oleo-resins such as clear varnishes, industrial enamels, printing inks and epoxy paints.

5.5 Synthesis of hydroxylated VOME

Hydroxy fatty acids with more than one hydroxyl groups can be obtained by chemical functionalization of vegetable oils. They can also be prepared by epoxidation of unsaturated fatty acid or fatty acid methyl esters followed by catalytic opening of the oxirane ring in the presence of water. In this study, we report the synthesis of di-hydroxylated VOME using the procedure of Okieimen *et al*. [17], with slight modification. VOME (2 g) was reacted with a mixture of formic acid (1.0 g), aqueous H_2O_2 (1.6 g 35 wt %), and 3 % H_2SO_4 in a three-necked round bottom flask equipped with a magnetic stirrer at RT for 24 h. Subsequently, the organic phase was extracted with diethyl ether (50 mL) and washed several times with brine solution until no peroxide was left in the mixture. The organic layer was centrifuged to remove the remaining water and dried over $MgSO_4$. Diethyl ether was removed by vacuum distillation using a rotary evaporator. The Prilezhaev method using *in situ* generated performic acid from hydrogen peroxide and formic acid in the presence of excess catalyst sulfuric acid gave as colorless product, methyl 9,10,12, 13-tetrahydroxyoctadecanoate.



Vernonia oil methyl ester

methyl 9,10,12, 13-tetrahydroxyoctadecanoate

Scheme 5.5*: Synthesis of hydroxylated VOME*

^{1}H NMR showed that the carbon–carbon double bond conversion was essentially quantitative. Resonances of the –CH–OH units appear in the region of d 3.33–3.97 ppm. Interestingly, also some peaks are present in the region d 8.0– 8.3 ppm. These are typical for formyl branches, known to be formed when the intermediate epoxide is reacting with formic acid instead of water. Based on peak areas, the selectivity to diols is about 90 and 10% for formyl branches. The ^{13}C NMR data obtained also supports the fact that the epoxy ring has disappeared and additional peaks corresponding to the carbon attached to the hydroxyl group has been observed. The loss of C=C double bond peak at 123-132 ppm shows that the epoxidation stage was successful. The retention of the methoxy carbon was visible from strong signal at 51 ppm. Therefore, we can conclude that epoxidation of vernonia oil methyl vernolate followed by catalytic ring opening results in hydroxylated VOME.

FTIR Spectrum analysis

The FTIR spectrum recorded showed the major functional groups in the hydroxylated vernonia oil methyl ester (VOME). The hydroxyl (O-H) peak at 3421 $cm^{-1}$of the ring opened vernonia oil methyl ester moiety, C-H stretching band at 2890, 2960 cm^{-1}, (C=O) stretching at 1728 cm^{-1}, C-O-C stretching band in the range 900-1250 cm^{-1} among the major peaks obtained. The peak at 1465 cm^{-1} due to -C-H (CH_2, CH_3, bending (scissoring) and band at 1377 cm^{-1} due to -C-H (CH_3), bending (sym) were observed. The band at 1654 cm^{-1} corresponding to (-C=C-, stretching) of the vernonia oil methyl ester has disappeared due to epoxidation of the double bond. The carbonyl carbon peak at

1728 cm^{-1} characteristic of the ester functionality of the methyl ester remains intact. However, the disappearance of the epoxy peak band at 823 cm^{-1} initially present in vernonia oil methyl ester indicate that hydroxylation took place on the double bond and epoxy ring positions of structure of vernonia oil methyl ester.

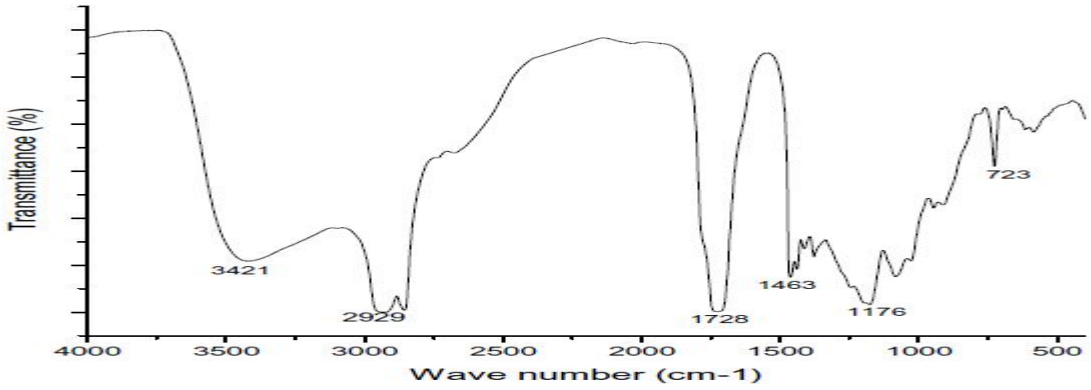


Figure 5.4*: FTIR spectrum of hydroxylated VOME*

Reference:

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CHAPTER SIX

CONCLUSIONS AND FUTURE WORKS

In this study, vernonia oil has been extracted and characterized using ^{1}H NMR, ^{13}C NMR, FTIR, MALDI-TOF MS, as well as thermal analyses methods (DSC/TGA). The results show that pure vernonia oil was obtained. The oil content of vernonia galamensis seed in this study was 32 % and it was also observed that a high percentage of the oil is naturally epoxidized with vernolic acid content of 71 %.

Vernolic acid, vernonia oil methyl ester and , vernanol, were used as precursors in the synthesis of various epoxy glucose fatty acid esters and epoxy starch fatty acid esters. These derivatives were also well characterized using available spectroscopic techniques such as using ^{1}H NMR, ^{13}C NMR, FTIR, and ESI-MS techniques.

One of major studies in this research project was synthesis and characterization of cassava starch fatty acid esters using naturally epoxidized vernolic acid or vernonia oil methyl ester. Different conditions were employed in the synthesis of starch ester. Enzymatic synthesis of starch ester in organic solvents, chemical synthesis of starch ester in organic solvents, enzymatic synthesis of starch ester in ionic liquids and chemical synthesis of starch ester in ionic liquids as solvents were successfully carried out.

The synthesis of starch vernolate in the ionic liquid, 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) as solvent in 20 % DMSO, and Novozym-435 lipase catalyst was found to be interesting in that it is relatively a green method and gave

a product of high DS comparable to that of the method in which chemical catalysts were used.

The products obtained were fully characterized using various techniques such as FTIR, Powder XRD, TGA, SEM, and solid state NMR techniques. Starch esters of different degree of substitution (DS 0.34-1.24) were obtained. From the FTIR spectrum obtained it was confirmed that the introduction of epoxy ester into glucose structure was successful. The SEM and XRD data also showed that the crystalline nature of starch was destroyed to different extent (based on the solvent used and degree of substitution) due to dissolution and simultaneous esterification processes. Hence, introduction of the epoxy ester group was recognized from the different morphologies recorded. In the solid state NMR study, it is clearly showed that esterification of starch was effective.

Synthesis and characterization of different epoxy glucose fatty acid esters (Glucose vernolates) were successfully carried out. In this study, we prepared glucose esters in organic solvents using enzyme catalysis. Similarly, enzymatic synthesis of epoxy glucose esters, using ionic liquid solvents were done and products obtained were characterized. Novel, epoxy glucose fatty acid esters were prepared by esterification of glucose and naturally epoxidized vernolic acid or vernonia oil methyl ester. Similarly, synthesis of epoxy alkyl glycoside was conducted using glucose pentaacetate and vernanol. FTIR of products confirmed that esterification of glucose was successful under reaction conditions employed. The thermal analyses showed that the glucose vernolate prepared had higher thermal stability than glucose indicating that esterification of glucose increases its thermal stability. Enzymatic synthesis in ionic liquids, were found to be successful and

effective. Optimum reaction conditions for enzymatic synthesis in both organic solvents and ionic liquid solvents were also obtained in the study carried out.

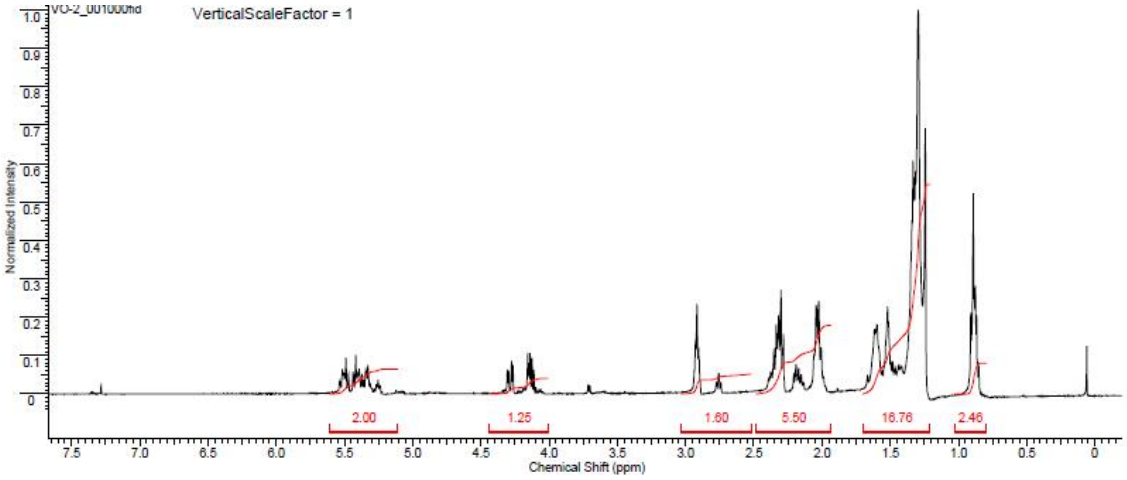
In addition to the glucose and starch fatty acid derivatives, modification of vernonia oil in to epoxidation of vernonia oil, polymerization of vernonia oil, polymerization of vernonia oil methyl ester, hydroxylation of vernonia oil, hydroxylation of vernonia oil methyl ester, preparation of vernonia oil polyol were among the major syntheses conducted in our study. In a similar manner, characterizations of the products were successfully done. In all cases, ^{1}H NMR, ^{13}C NMR, FTIR, ESI-MS results obtained confirmed the syntheses of the intended products.

Generally, this study clearly demonstrated that the naturally epoxidized vernonia oil has a great potential to be used in industry. The multifunctional vernonia oil can be used to prepare a number of value added products. Therefore, vernonia oil is a potential candidate as renewable resource to replace the current products obtained from depleting, high cost petroleum based products.

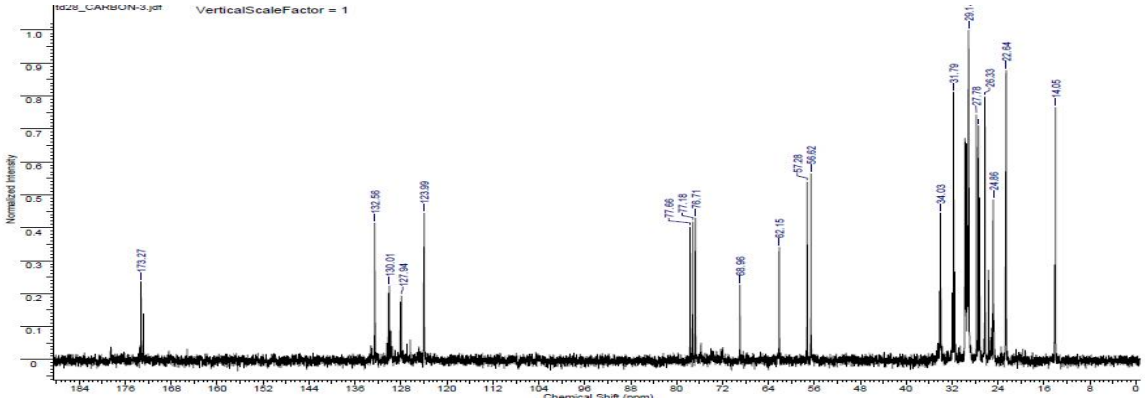
As a future work we recommend that reaction conditions for synthesis of starch and glucose epoxy fatty acid esters could be optimized to obtain products with higher DS substitution. The enzymatic syntheses in ionic liquids as solvents could be further improved using mixture of ionic liquids.

Appendices

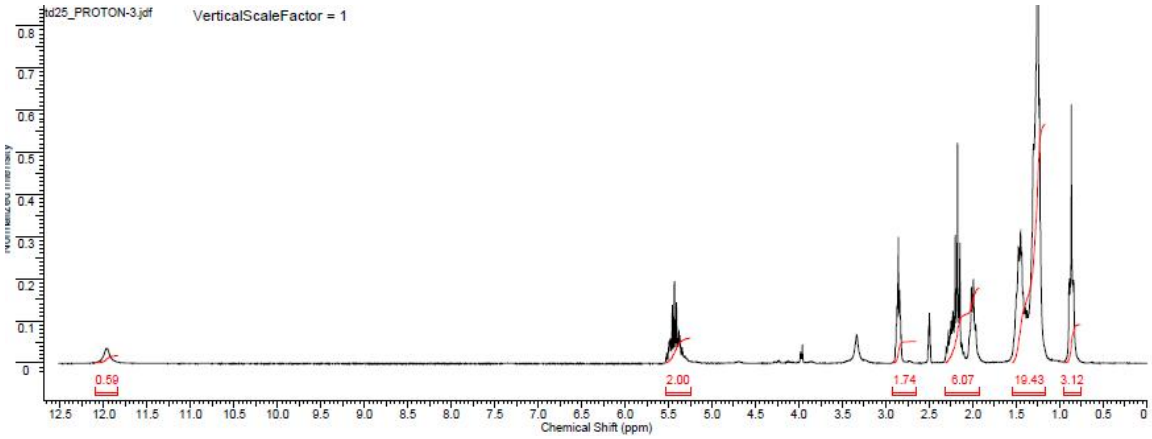
Appendix 1: 1H and 13C NMR spectra



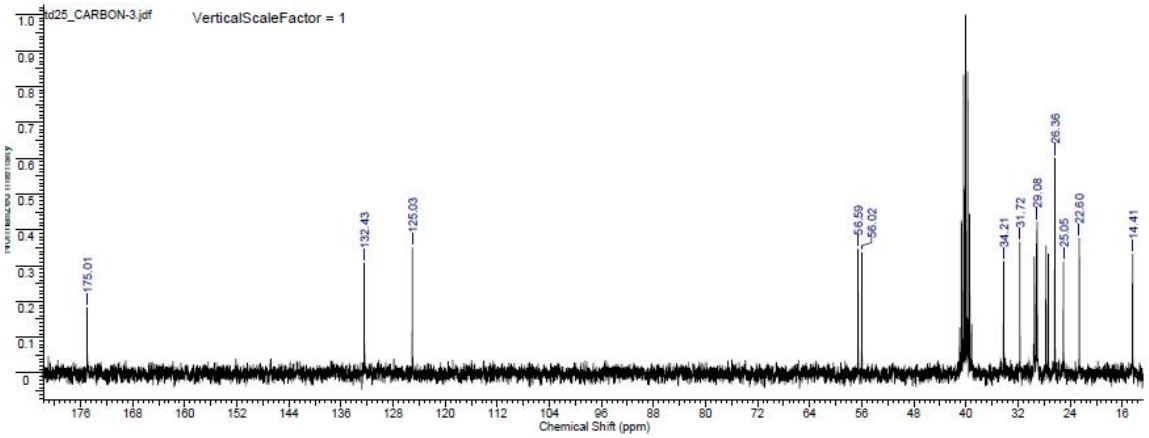
^{1}H NMR spectrum of vernonia oil



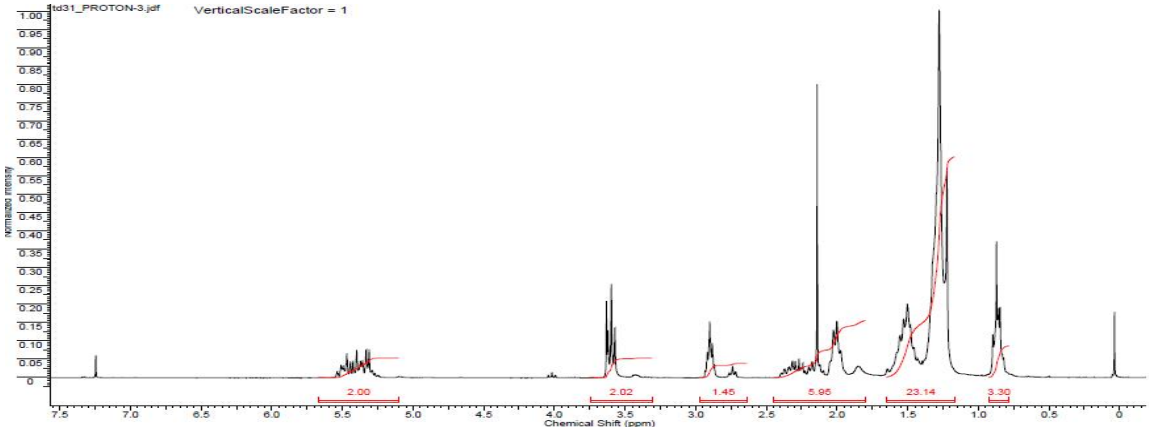
^{13}C NMR spectrum of vernonia oil



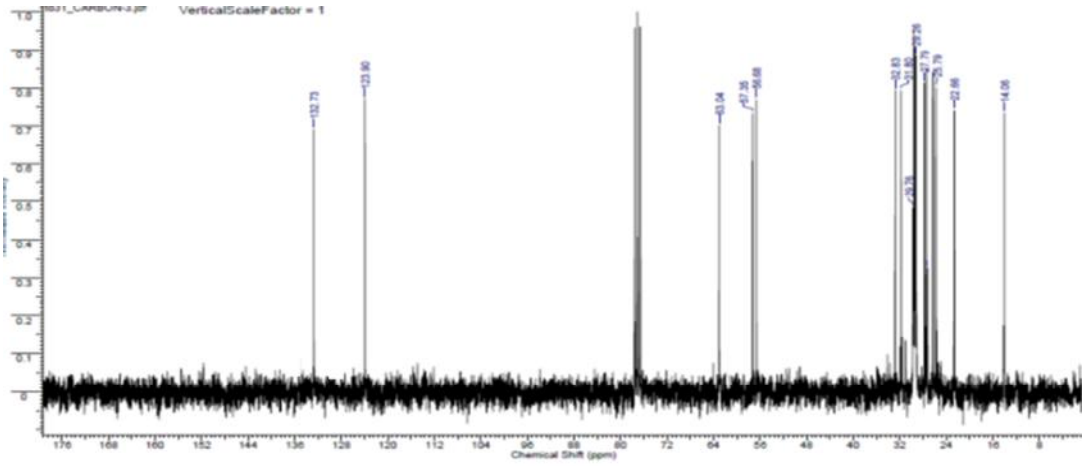
[1]H NMR spectrum of vernolic acid



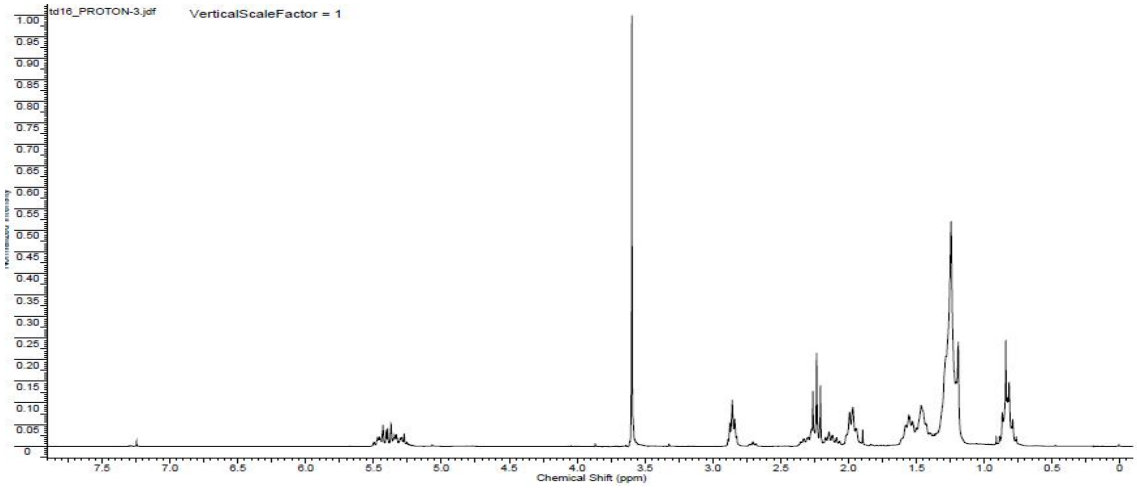
[13]C NMR spectrum of Vernolic acid



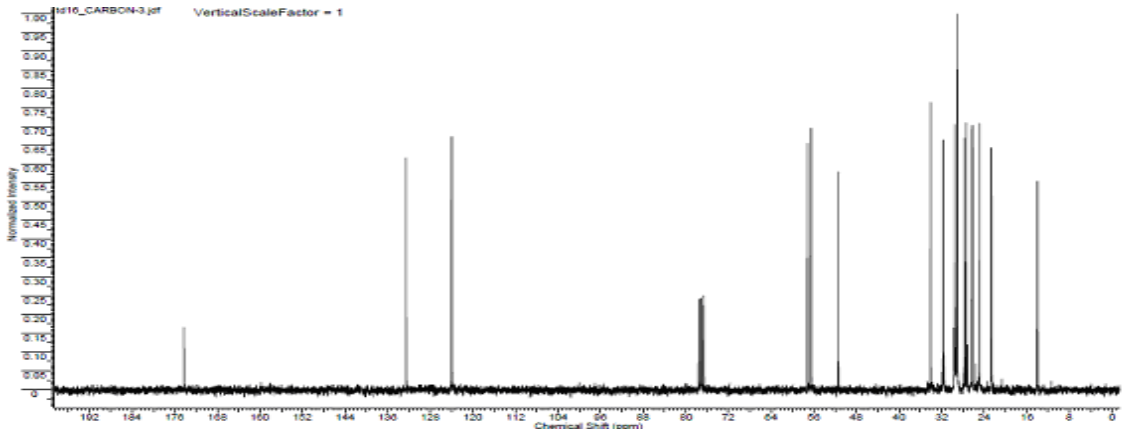
[1]H NMR spectrum of vernanol



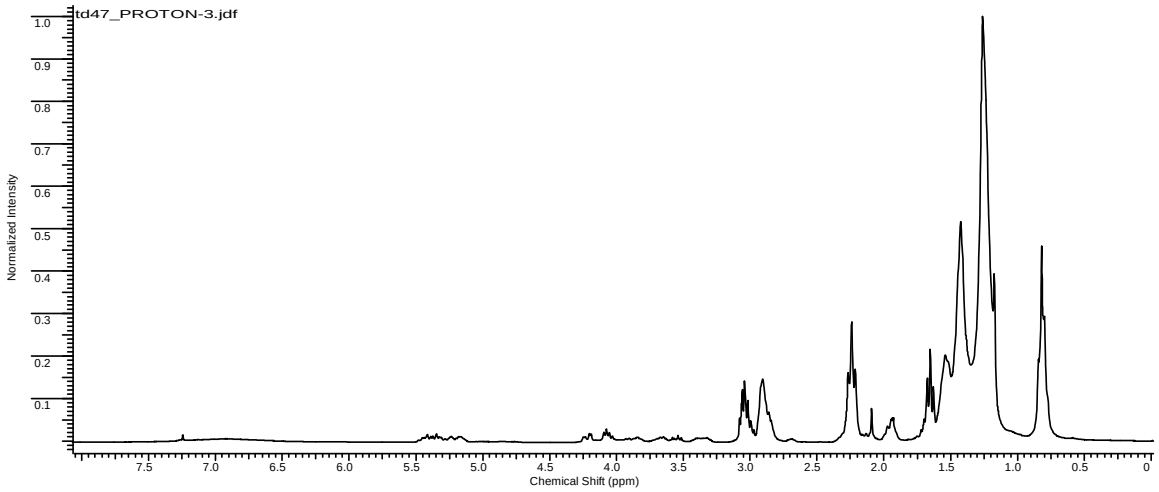
^{13}C NMR spectrum of vernanol



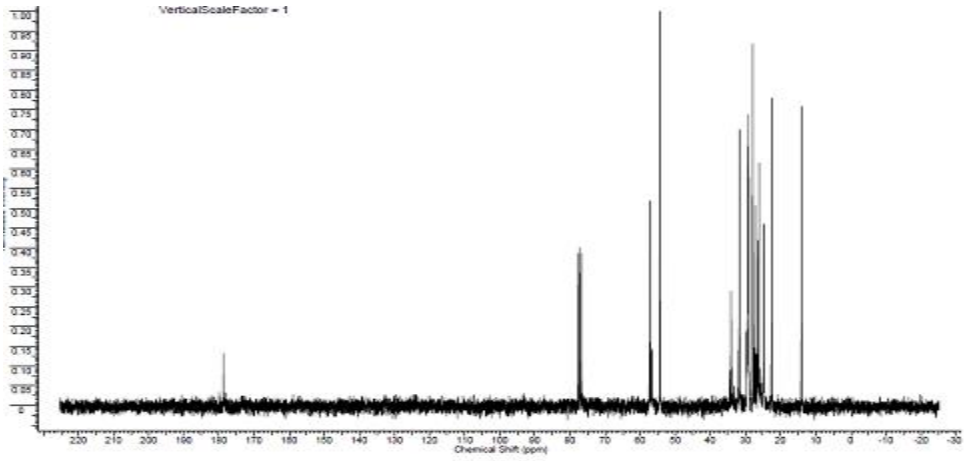
^{1}H NMR spectrum of VOME



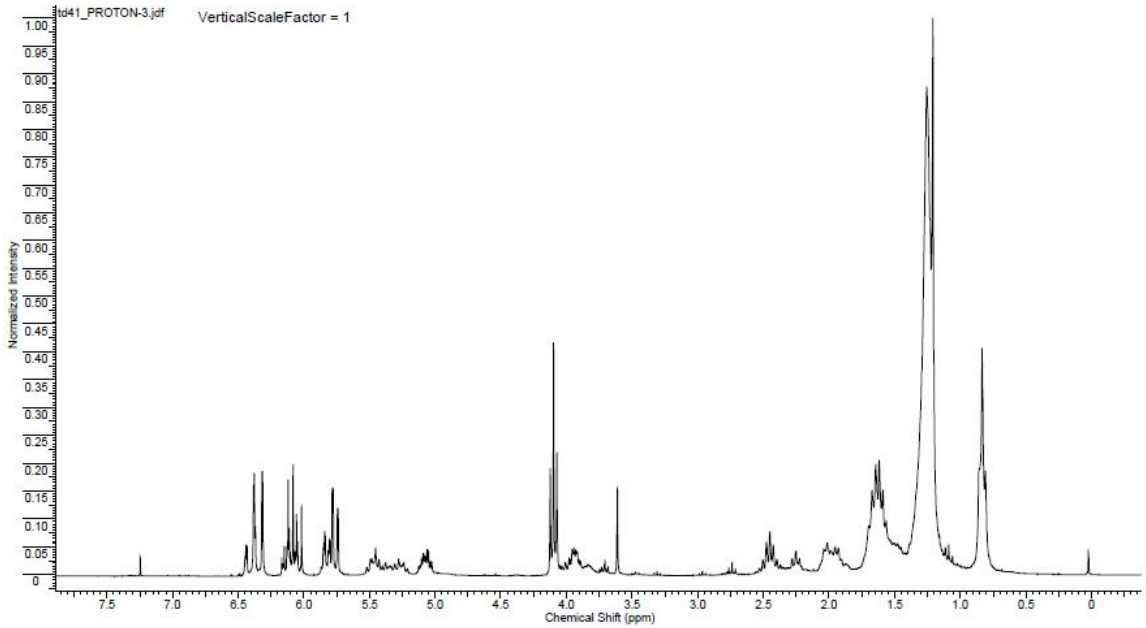
^{13}C NMR spectrum of VOME



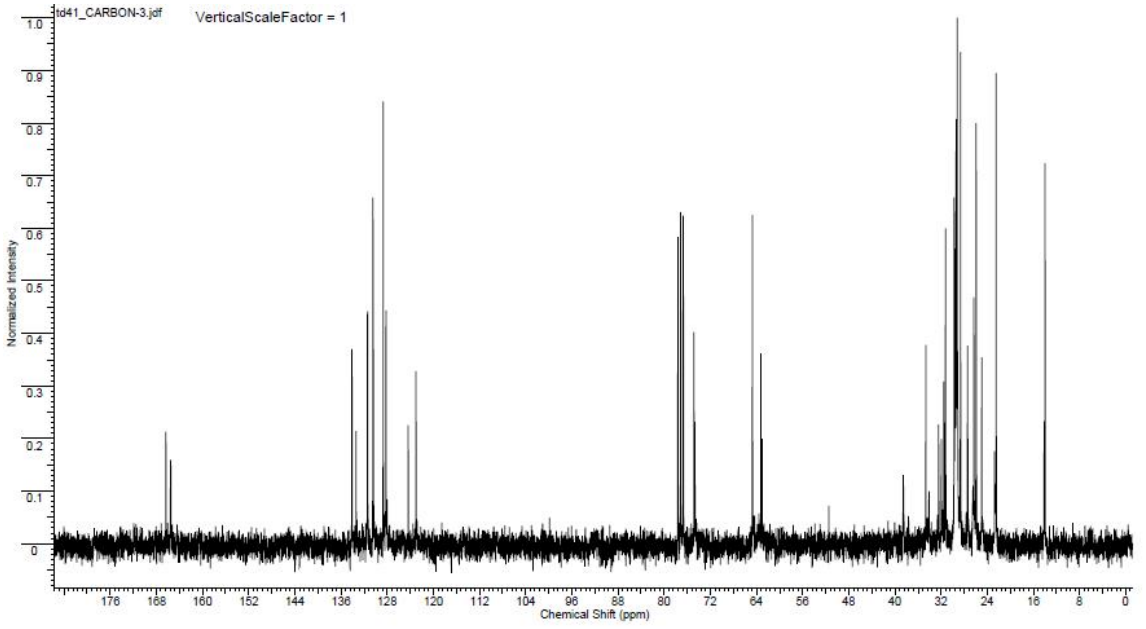
^{1}H NMR spectrum of epoxidized vernolic acid



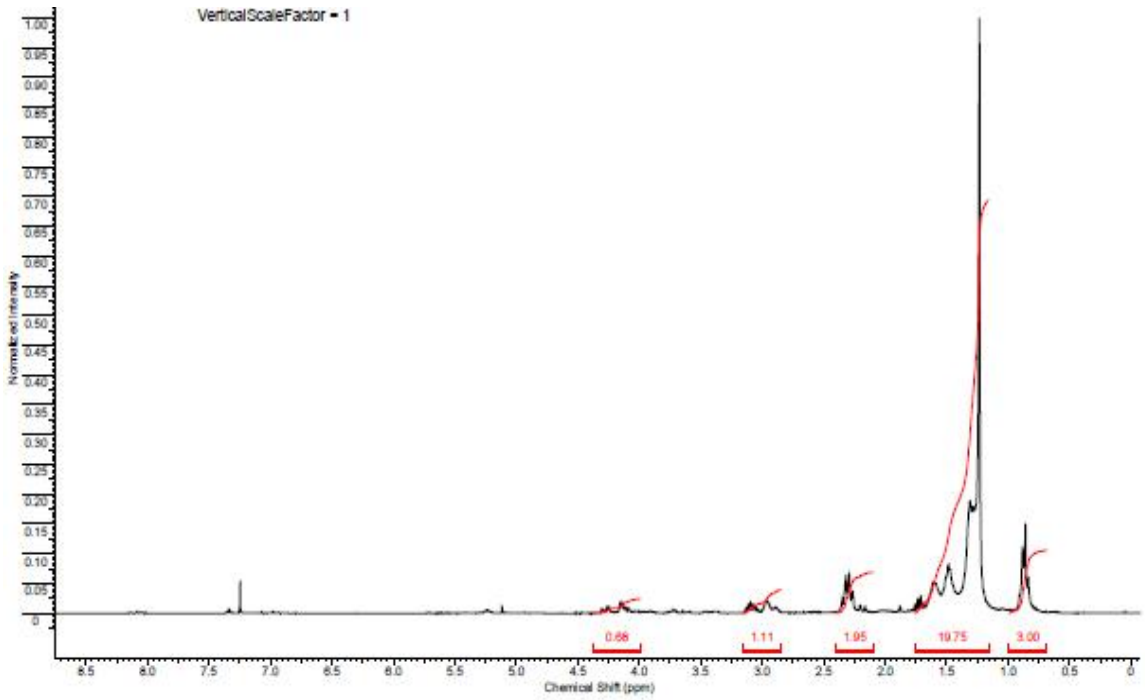
^{13}C NMR spectrum of epoxidized vernolic acid



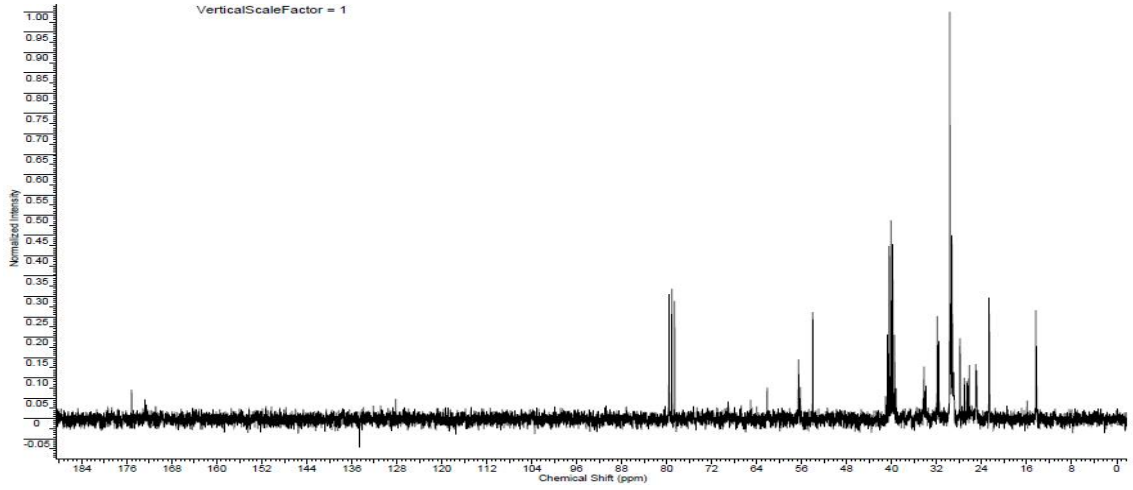
[1]H NMR spectrum of chloroacrylated vernanol



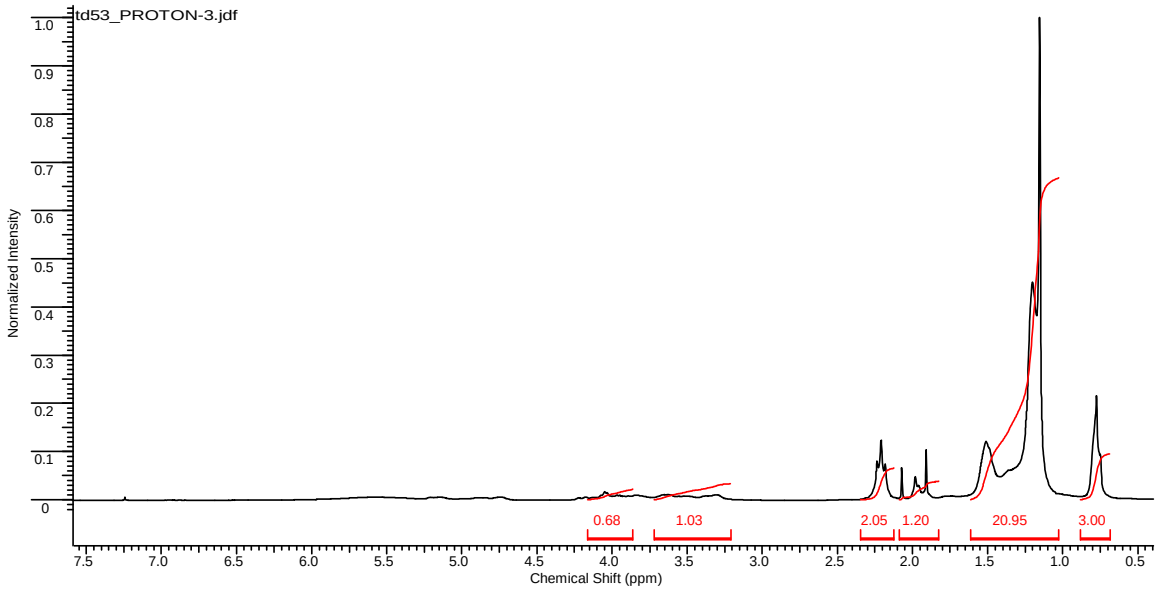
[13]C NMR spectrum of chloroacrylated vernanol



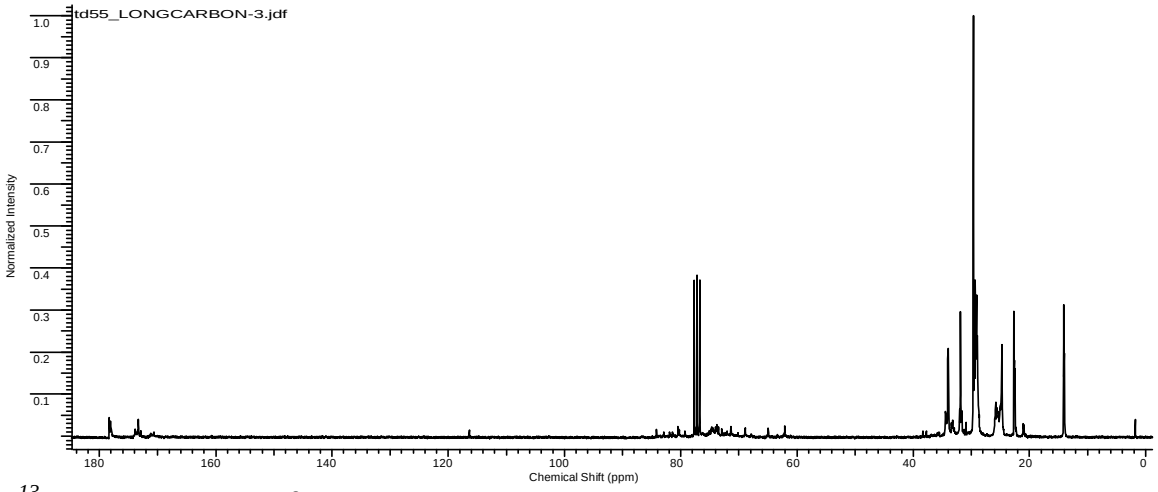
[1]H NMR spectrum of epoxidized vernonia oil



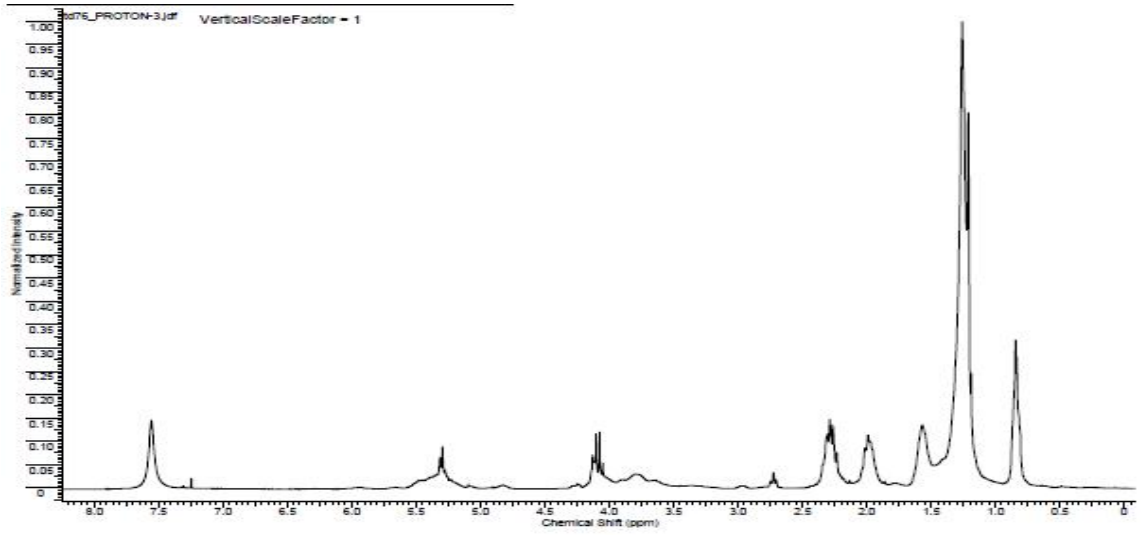
[13] NMR spectrum of epoxidized vernonia oil



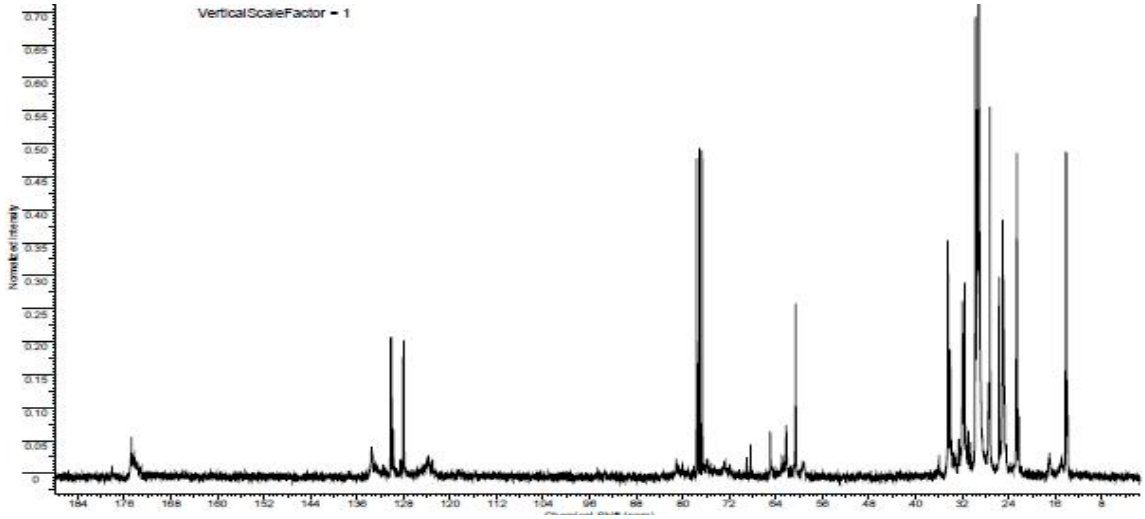
^{1}H NMR spectrum of Vernonia oil polyol



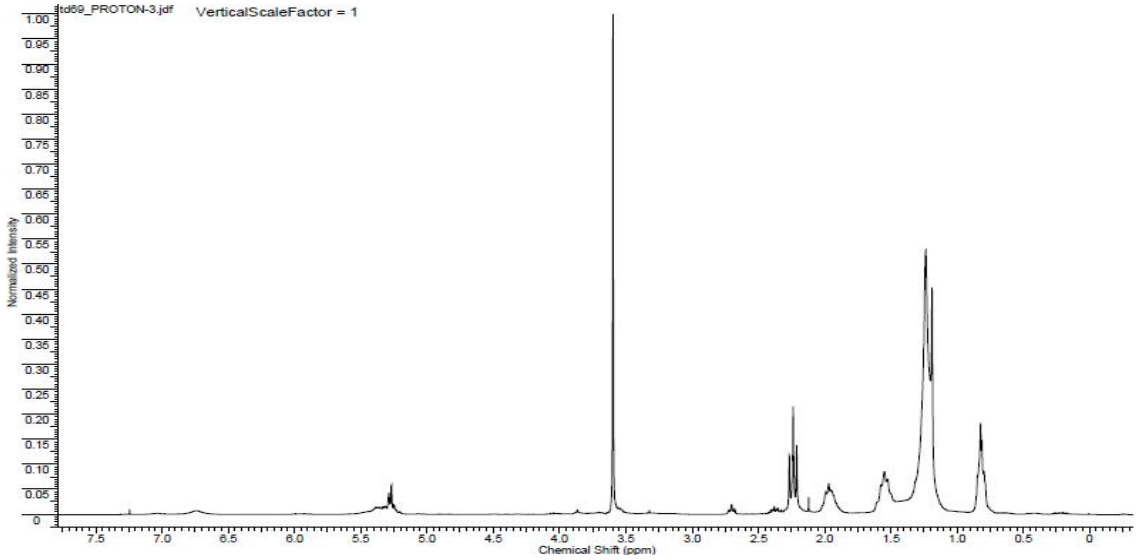
^{13}C NMR spectrum of Vernonia oil polyol



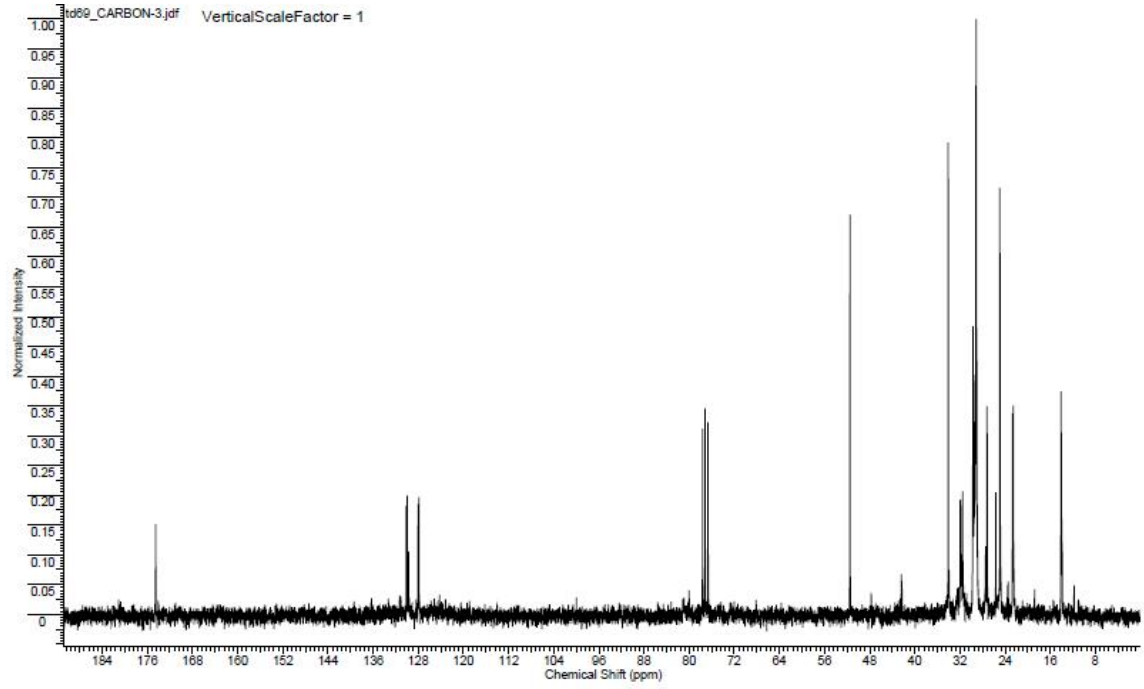
^{1}H NMR spectrum of polymerized VO



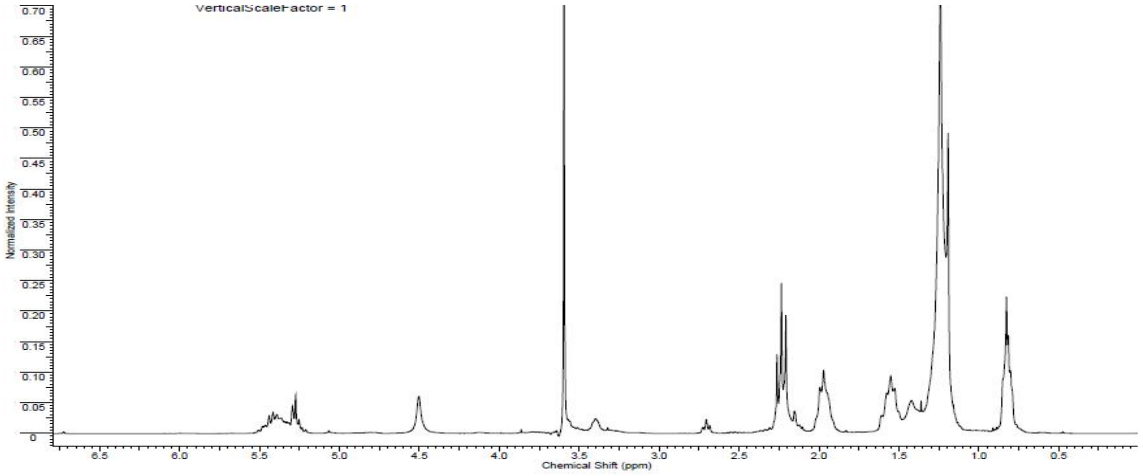
^{13}C NMR spectrum of polymerized VO



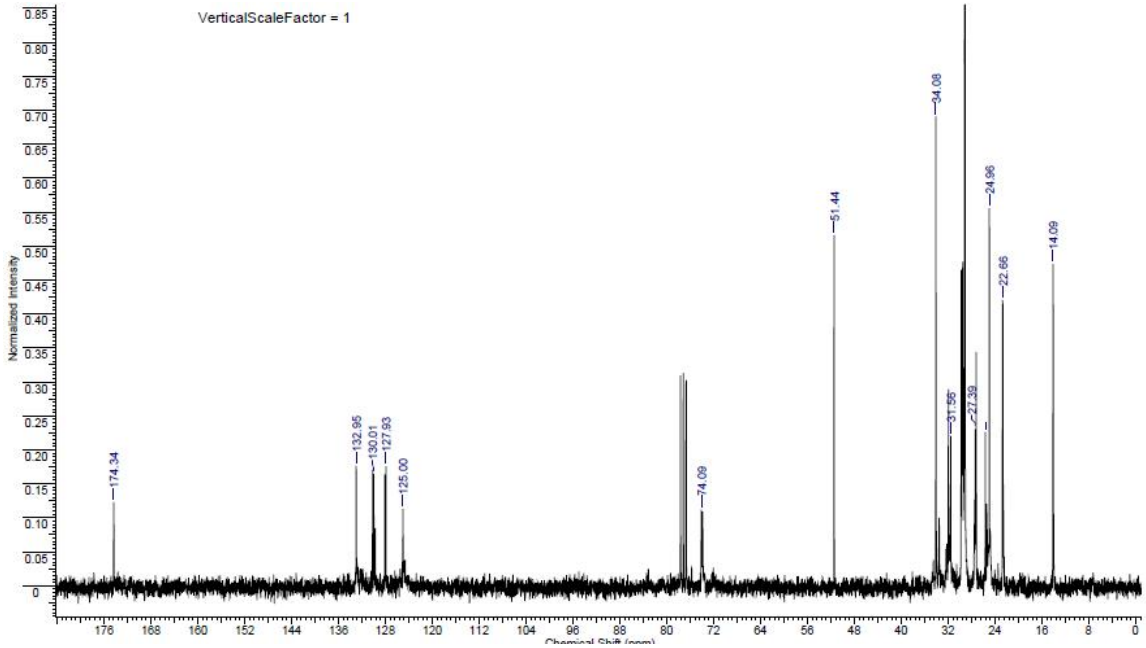
^{1}H NMR of polymerized VOME



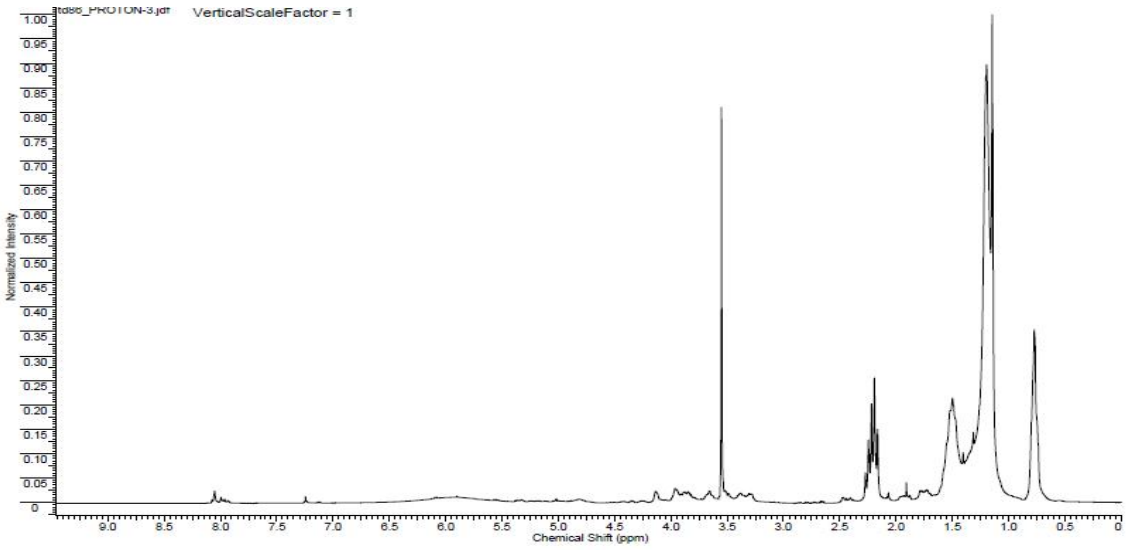
^{13}C NMR of polymerized VOME



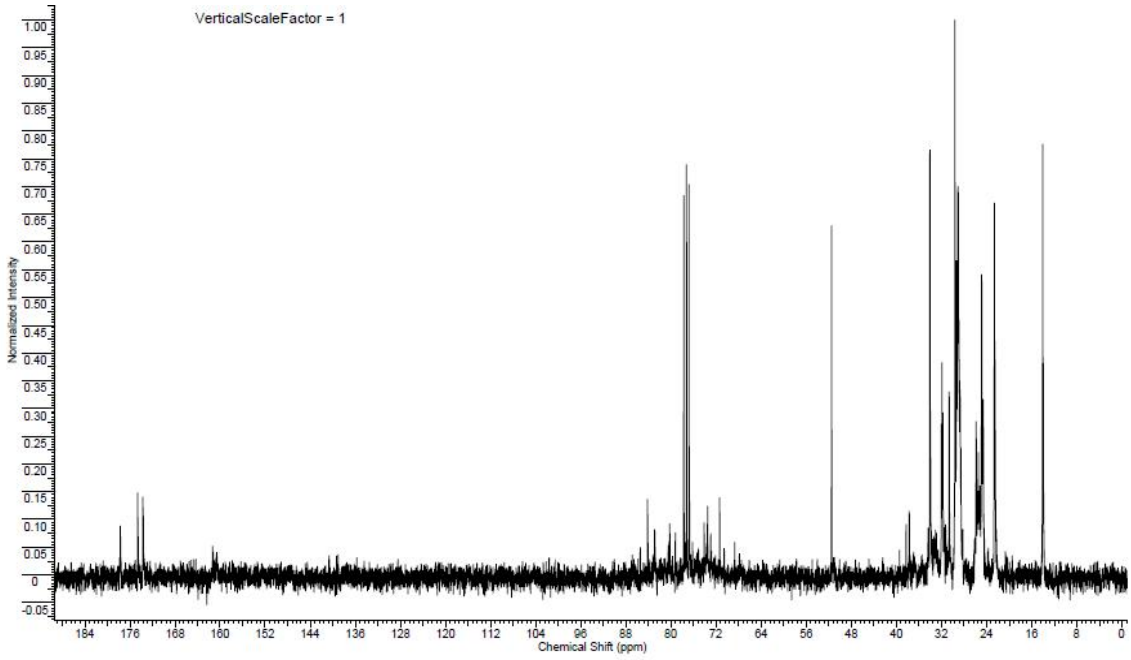
^{1}H NMR spectrum of polymerized VOME in super acid



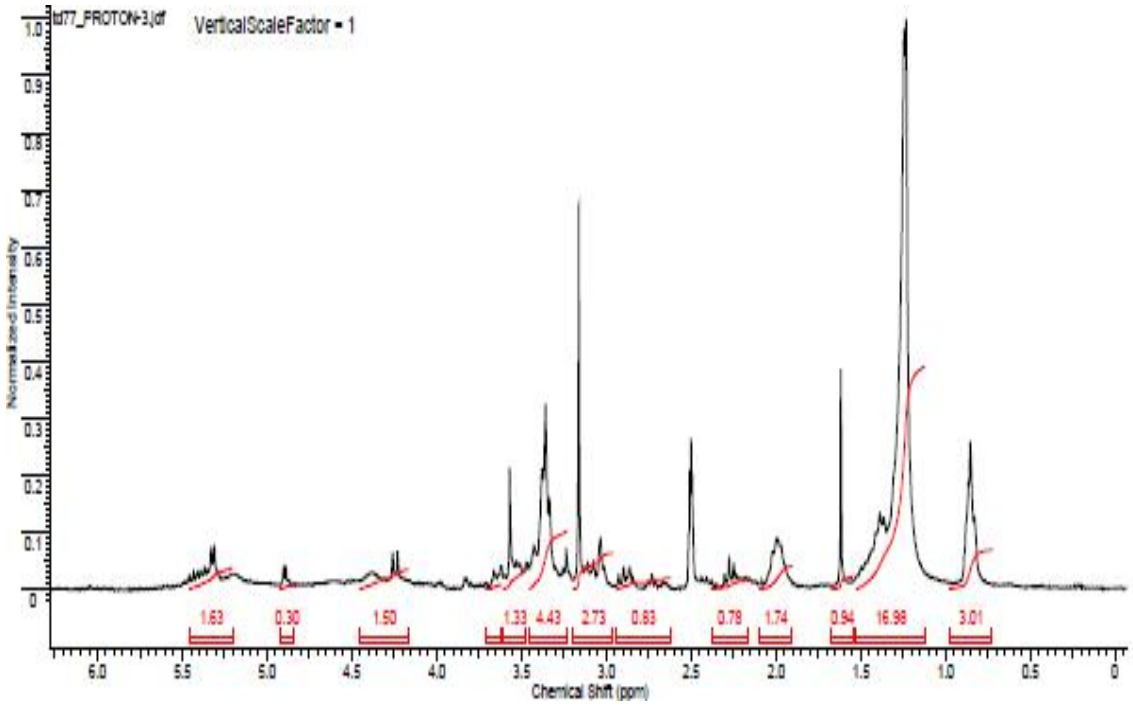
^{13}C NMR spectrum of polymerized VOME in super acid



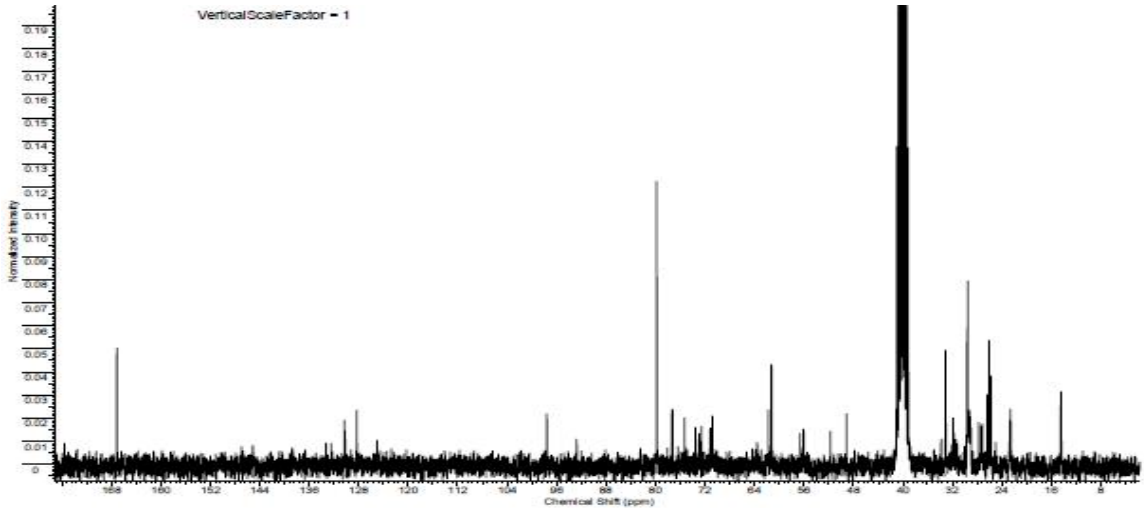
[1]H NMR spectrum hydroxylated VOME



[13]C NMR spectrum of hydroxylated VOME

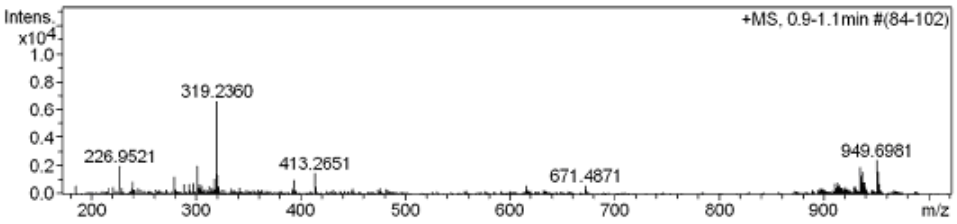


^{1}H NMR spectrum of epoxy alkyl glycoside



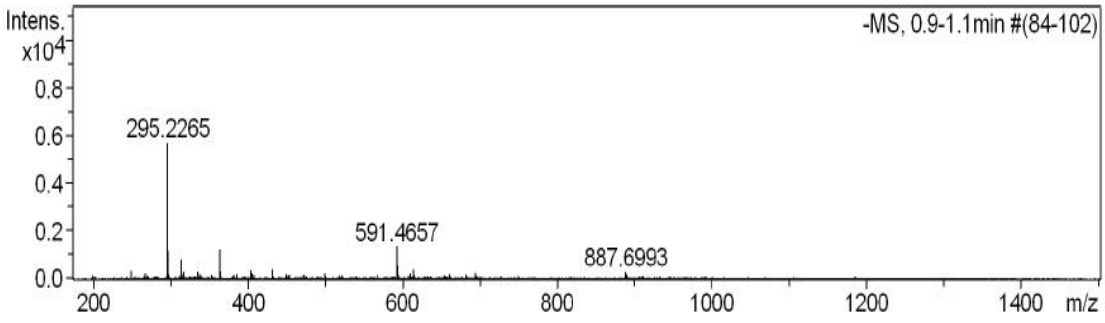
^{13}C NMR spectrum of epoxy alkyl glycoside

Appendix 2: ESI-MS spectra

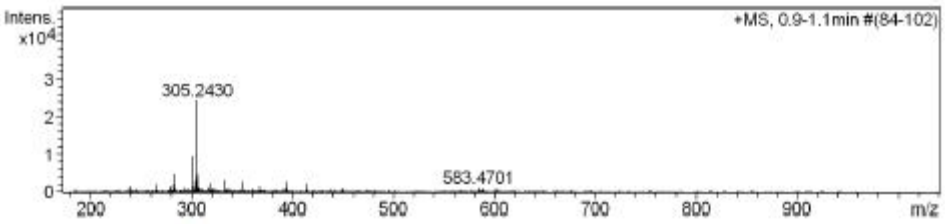


#	m/z	I %
1	226.9521	29.4
2	239.1584	13.0
3	279.2251	18.0
4	297.2586	11.0
5	301.1383	29.7
6	317.2427	15.4
7	319.2360	100.0
8	320.2391	20.3
9	393.2907	14.6
10	413.2651	22.1
11	909.7119	10.2
12	911.7176	11.6
13	933.7033	28.2
14	934.7063	18.5
15	935.7090	23.2
16	936.7204	12.0
17	937.7371	12.3
18	949.6981	36.0
19	950.7024	23.4
20	951.7032	10.0

ESI-MS spectrum of vernonia oil

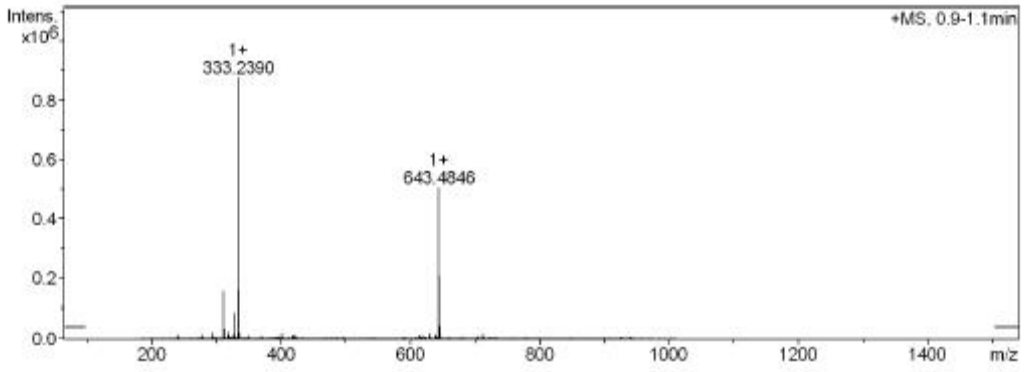


ESI-MS spectrum of vernolic acid

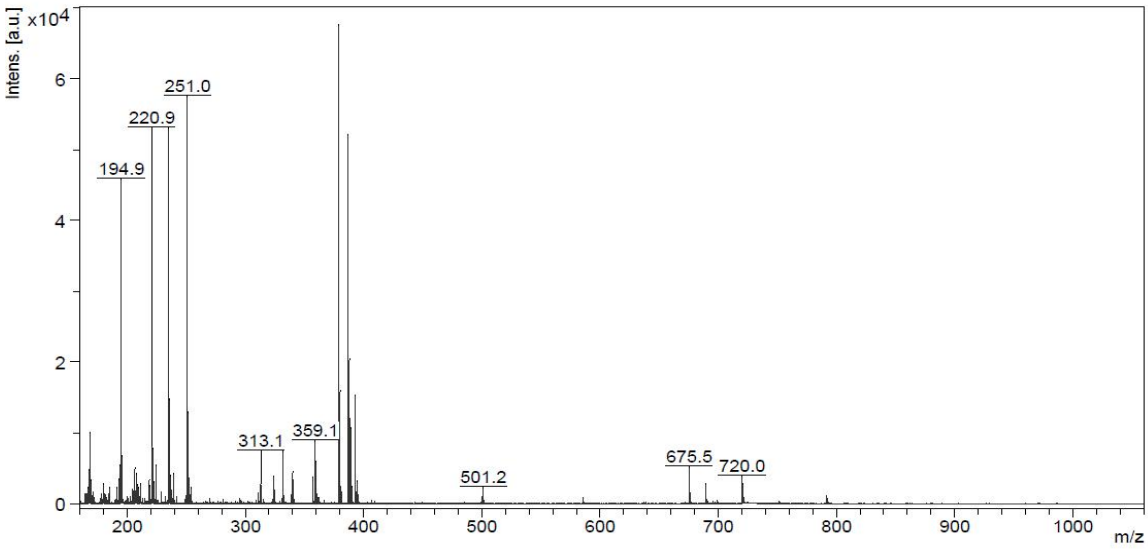


#	m/z	I %
1	239.1577	6.3
2	265.2482	8.2
3	279.2126	5.9
4	282.2765	7.8
5	283.2623	18.9
6	293.2358	4.7
7	301.1398	38.3
8	302.1489	6.6
9	304.2613	12.0
10	305.2430	100.0
11	306.2475	18.2
12	307.2485	4.6
13	317.2379	4.5
14	319.2535	9.4
15	333.2391	12.7
16	351.2445	11.0
17	367.2145	6.5
18	393.2879	11.1
19	413.2642	8.5
20	583.4701	4.4

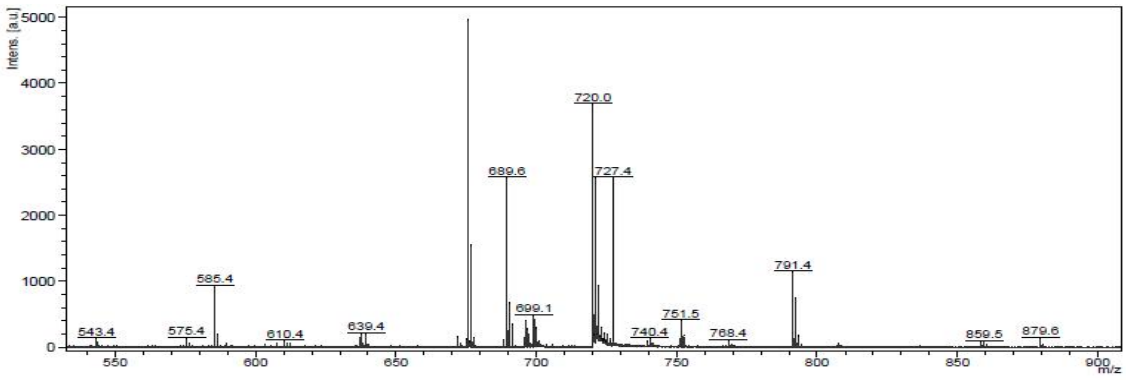
ESI-MS spectrum of vernanol



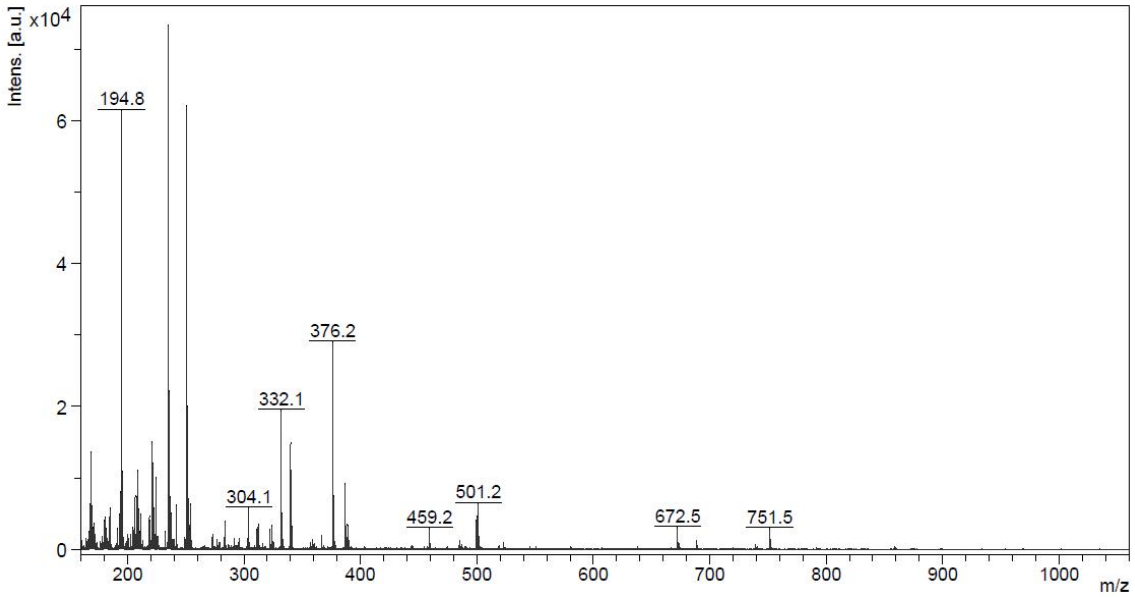
ESI-MS spectrum of VOME



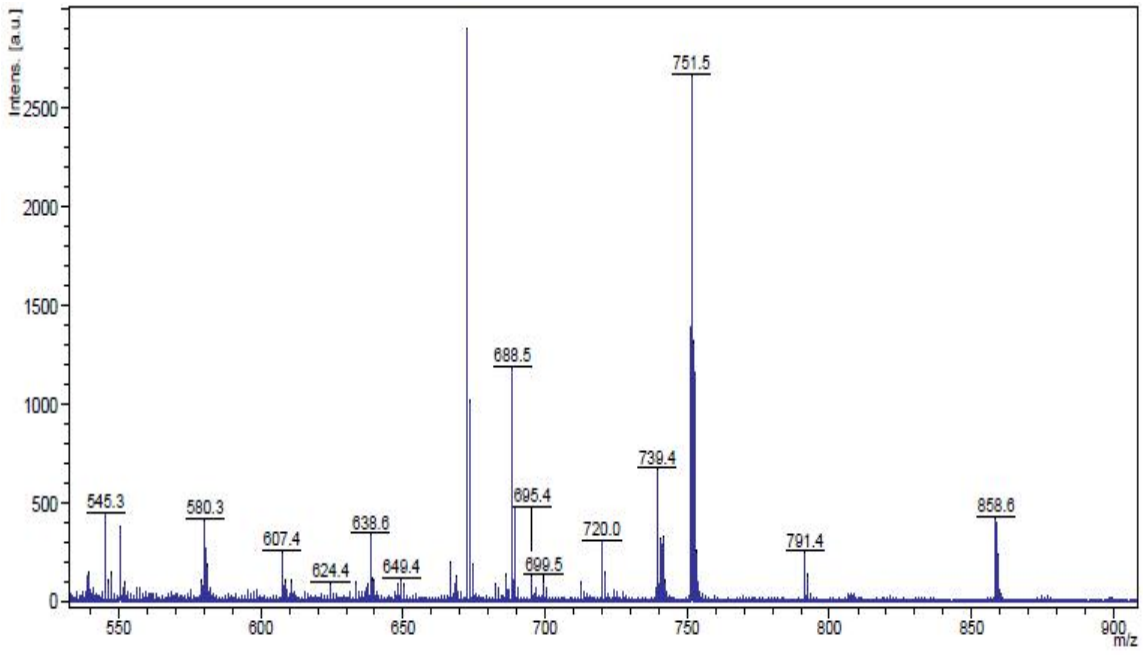
MALDI-TOF MS of glucose vernolate (GE in ILs)



MALDI-TOF MS of glucose vernolate (GE in ILs)

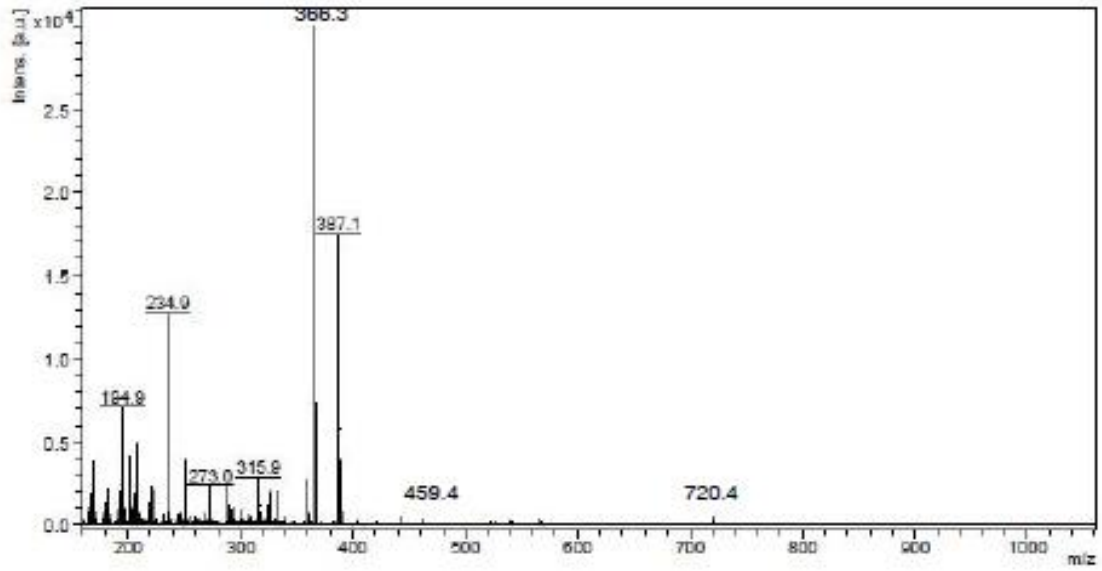


MALDI-TOF MS spectrum of glucose vernolate in DMSO/2M2B

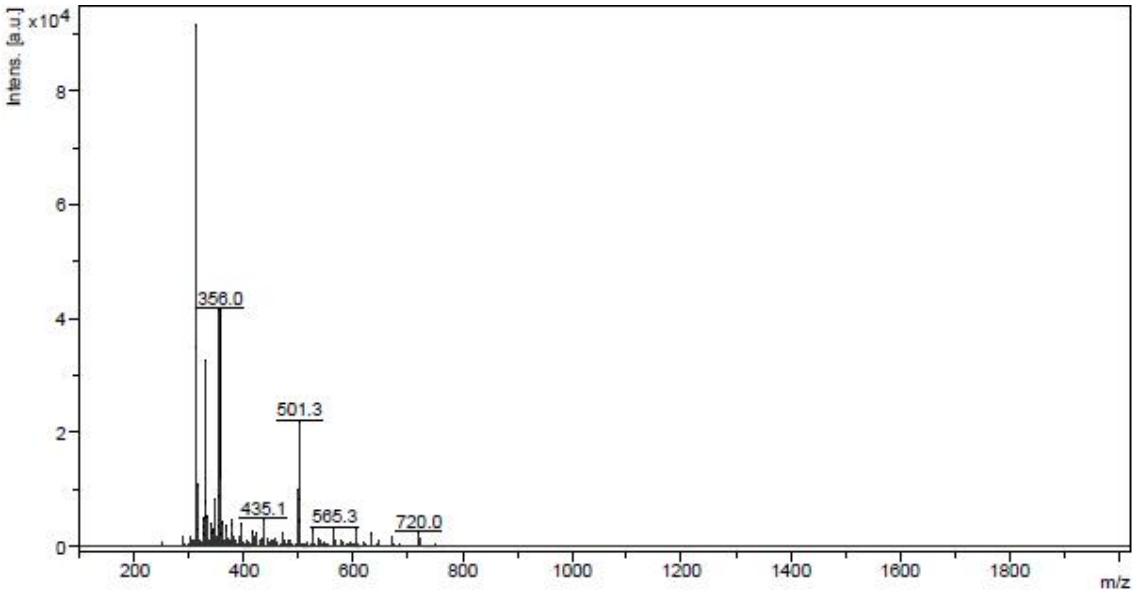


Expanded spectrum for the region m/z 520-900 of glucose vernolate in DMSO/2M2B

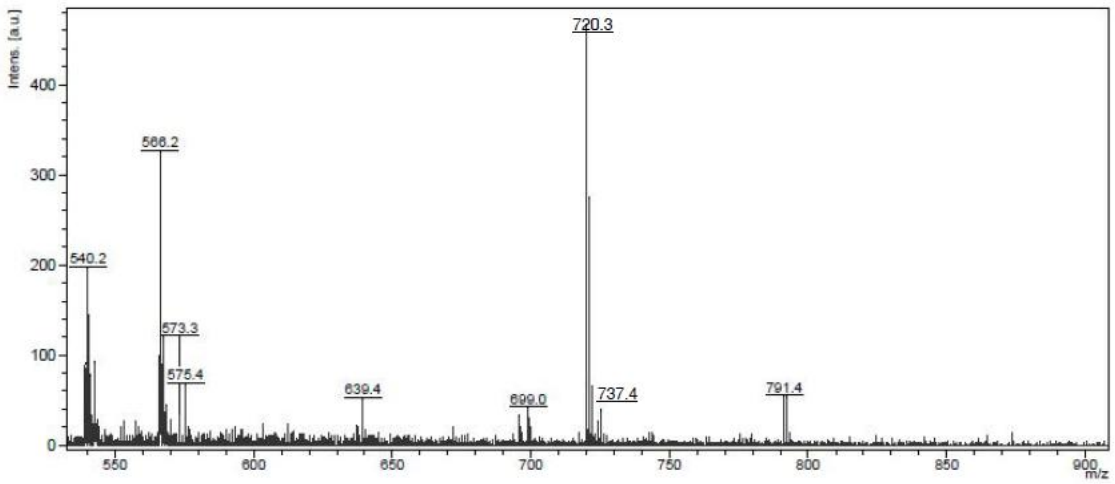
Positive ion MS



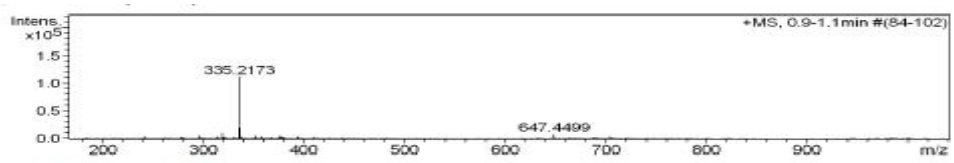
Negative ion MS



MALDI-TOF MS spectrum of glucose vernolate in DMSO/t-butanol



Expanded MS in the range of m/z = 420- 900 in DMSO/t-butanol



#	m/z	I %
1	242.2846	3.5
2	277.2140	2.5
3	295.2245	4.9
4	313.2356	3.4
5	319.2280	9.1
6	321.2384	2.3
7	330.2622	2.4
8	335.2173	100.0
9	336.2210	17.5
10	337.2208	2.7
11	351.1894	4.8
12	357.1983	3.5
13	367.1563	2.0
14	375.1494	4.5
15	377.1493	2.7
16	393.2862	3.4
17	409.2560	2.1
18	647.4499	6.9
19	648.4539	2.7
20	703.4741	2.7

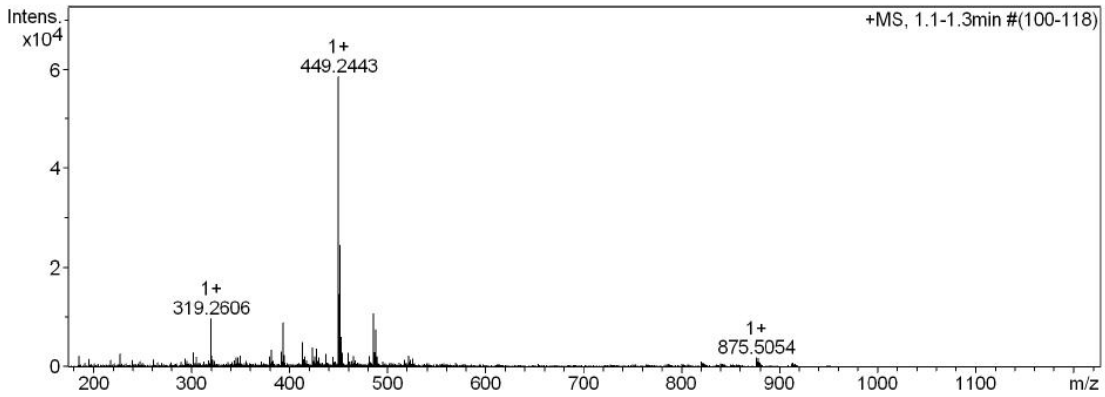
Generate Molecular Formula Parameters

Charge	Tolerance	sigma limit	H/C Ratio	Electron Conf.	Nitrogen Rule	Chrom.BackGround	Calibration
+1	6 ppm	0.08	3 - 0	both	false	false	TRUE

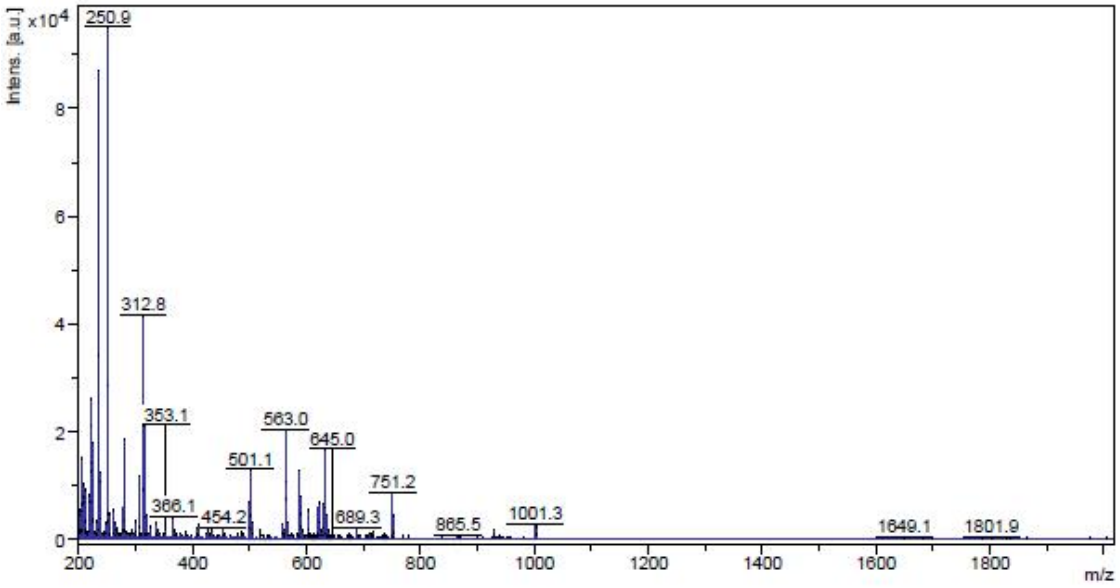
Expected Formula C18 H32 O4 **Adduct(s):** H, Na, NH4, radical

#	meas. m/z	theo. m/z	|Err|[ppm]	Sigma	Formula	Adduct	Adduct Mass
1	313.2356	313.2373	5.50	0.0377	C18H33O4	M+H	1.0078
1	335.2173	335.2193	5.80	0.0134	C18H32NaO4	M+Na	22.9898
1	330.2622	330.2639	5.00	0.0699	C18H36NO4	M+H4N	18.0344

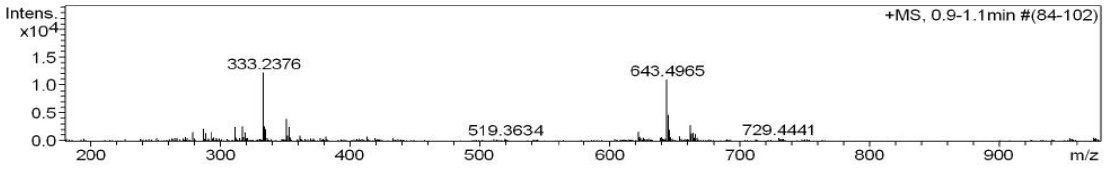
ESI-MS spectrum of epoxidized vernolic acid



ESI-MS spectrum of chloroacrylated vernanol

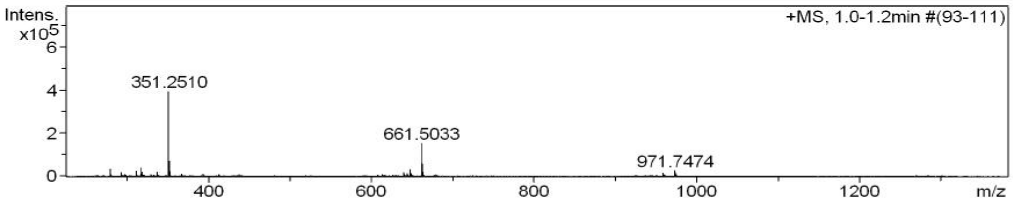


MALDI-TOF MS Spectrum of polymerized VO



#	m/z	I %
1	279.2276	12.5
2	286.9629	17.5
3	288.9577	11.1
4	293.2442	13.1
5	311.2554	20.0
6	317.2428	20.7
7	319.2550	12.1
8	333.2376	100.0
9	334.2412	21.1
10	335.2436	16.8
11	351.2473	31.6
12	353.2443	20.0
13	621.5146	13.8
14	643.4965	89.6
15	644.4992	37.5
16	645.4992	16.4
17	661.5042	23.1
18	662.5114	9.9
19	663.5038	11.8
20	665.4994	9.3

.ESI-MS of polymerized VOME



#	m/z	I %
1	279.2316	8.1
2	293.2462	4.1
3	311.2574	6.0
4	317.2448	10.1
5	319.2569	4.9
6	337.2344	4.8
7	351.2510	100.0
8	352.2540	18.1
9	353.2503	5.2
10	367.2269	2.5
11	639.5199	4.3
12	643.4916	2.8
13	647.4852	7.7
14	648.4894	2.9
15	661.5033	38.9
16	662.5062	15.2
17	663.5055	5.2
18	957.7285	3.4
19	971.7474	6.4
20	972.7501	3.8

Generate Molecular Formula Parameters

Charge	Tolerance	sigma limit	H/C Ratio	Electron Conf.	Nitrogen Rule	Chrom.BackGround	Calibration
+1	6 ppm	0.08	3 - 0	both	false	false	TRUE

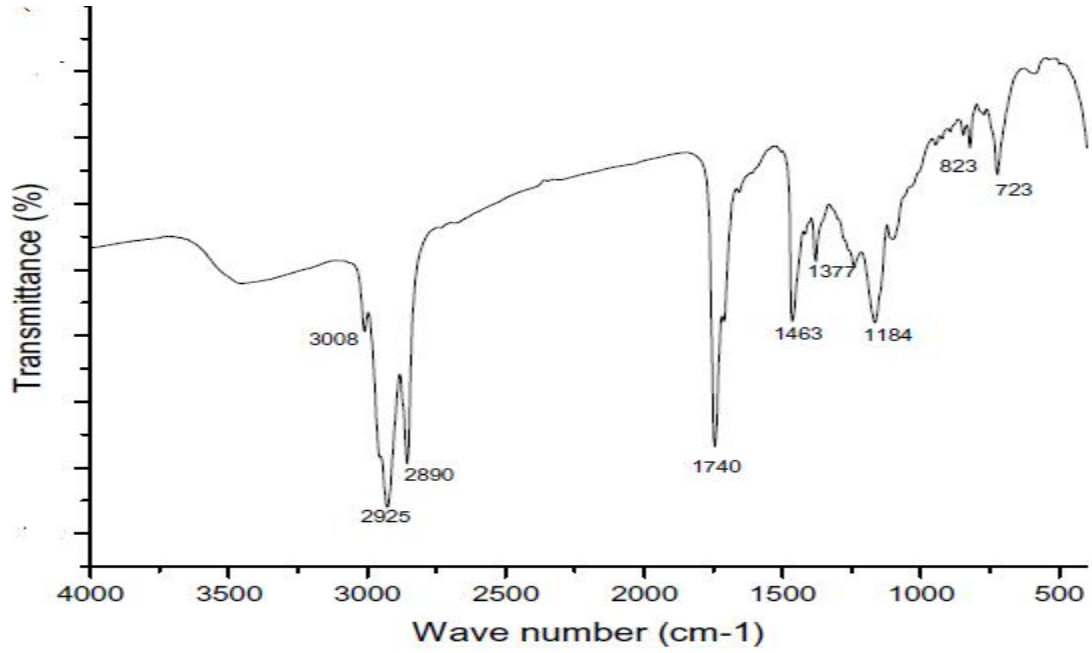
Expected Formula C57 H104 O10 **Adduct(s):** H, Na, NH4, radical

#	meas. m/z	theo. m/z	|Err|[ppm]	Sigma	Formula	Adduct	Adduct Mass
1	971.7474	971.7522	4.90	0.0323	C57H104NaO10	M+Na	22.9898

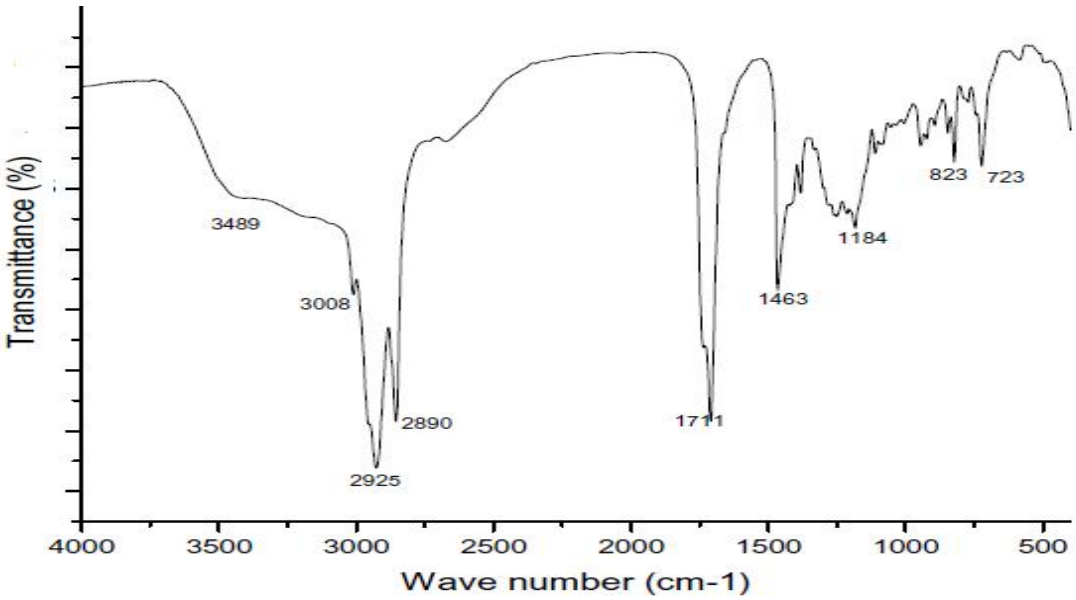
Note: Sigma fits < 0.05 indicates high probability of correct MF

ESI-MS spectrum of polymerized VOME in super acid

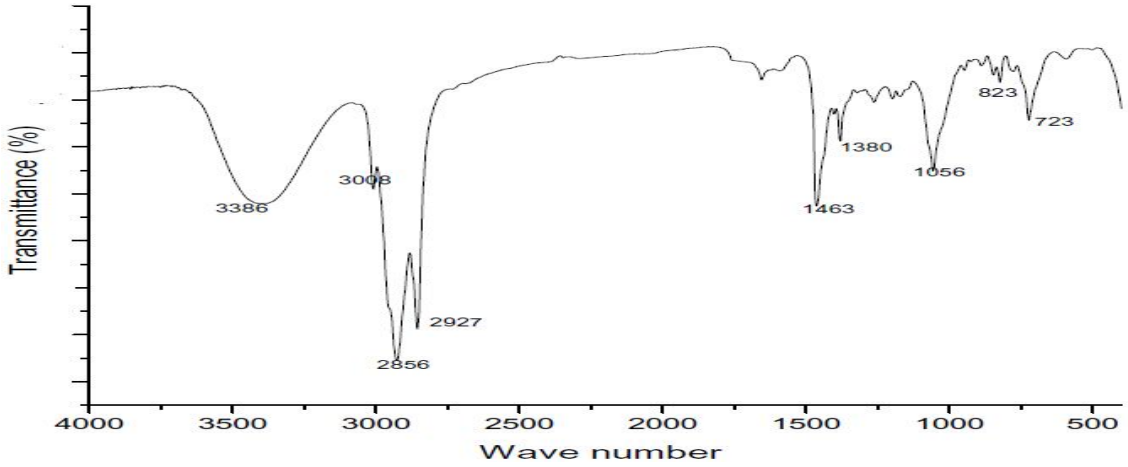
Appendix 3: FTIR Spectra



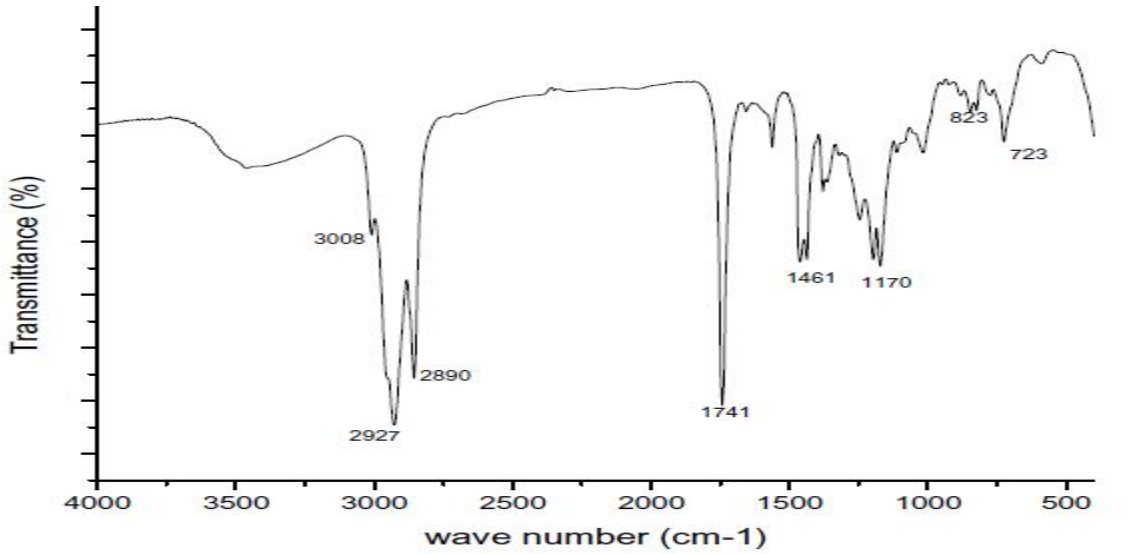
FTIR spectrum of vernonia oil



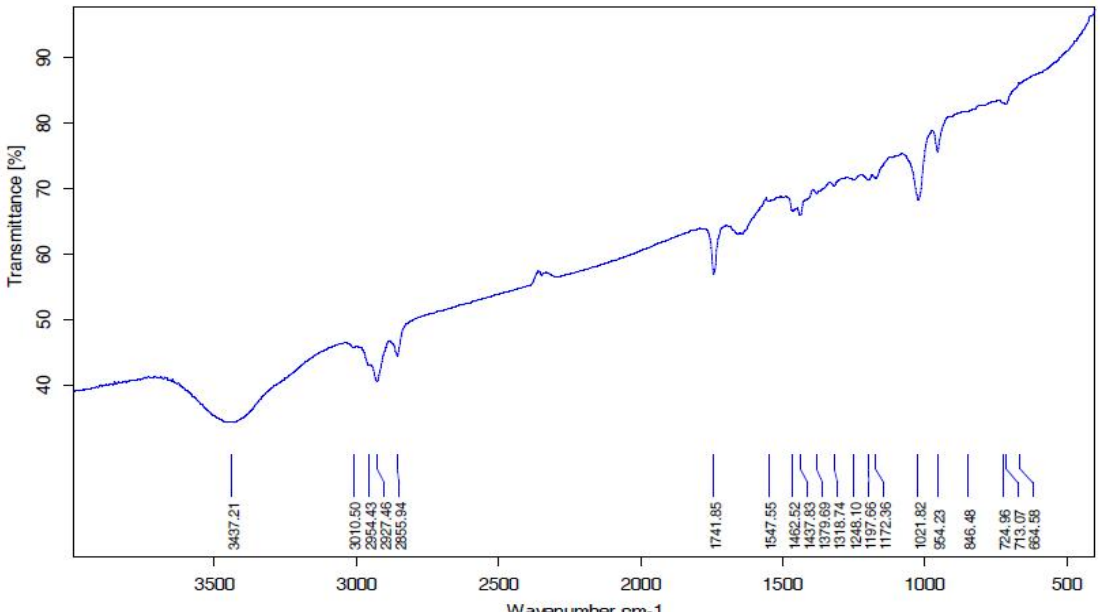
FTIR spectrum of Vernolic acid



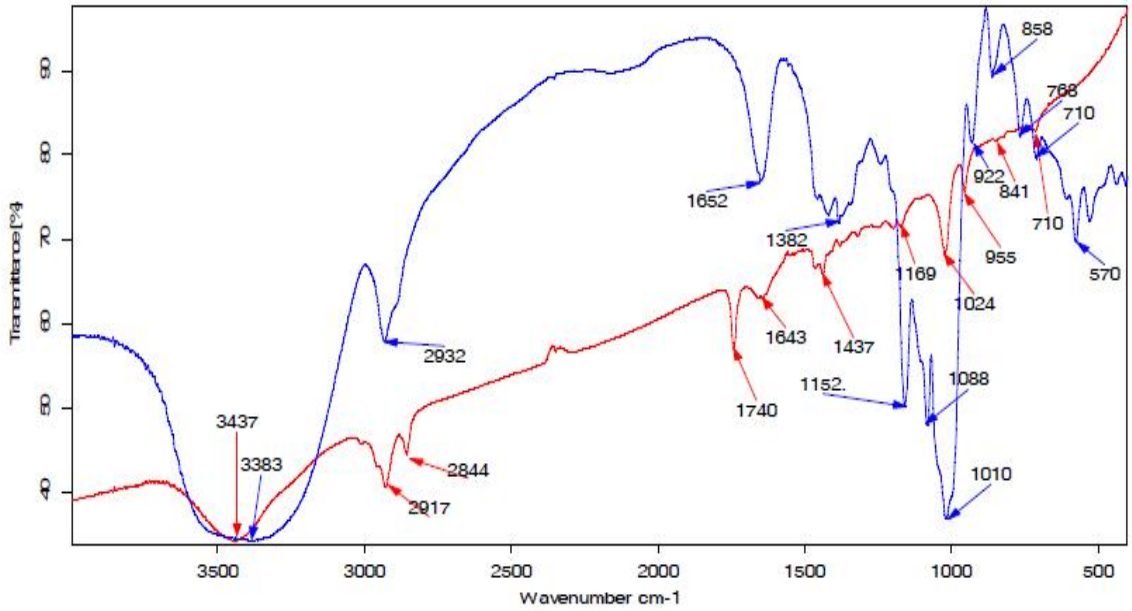
FTIR spectrum of vernanol



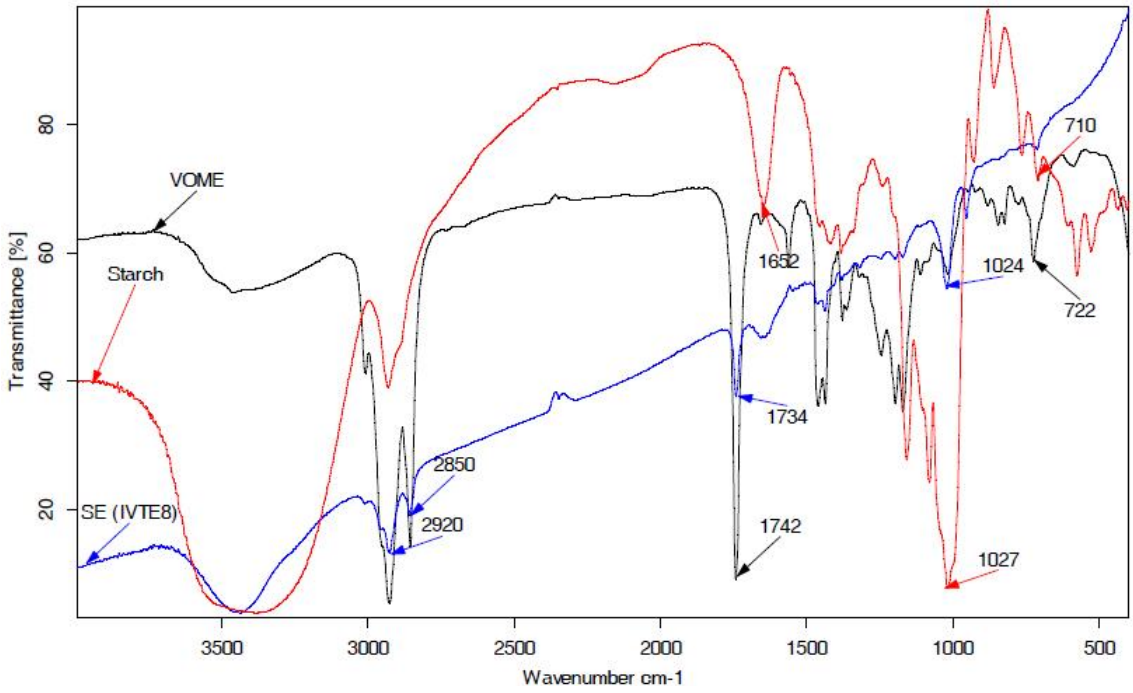
FTIR spectrum of VOME



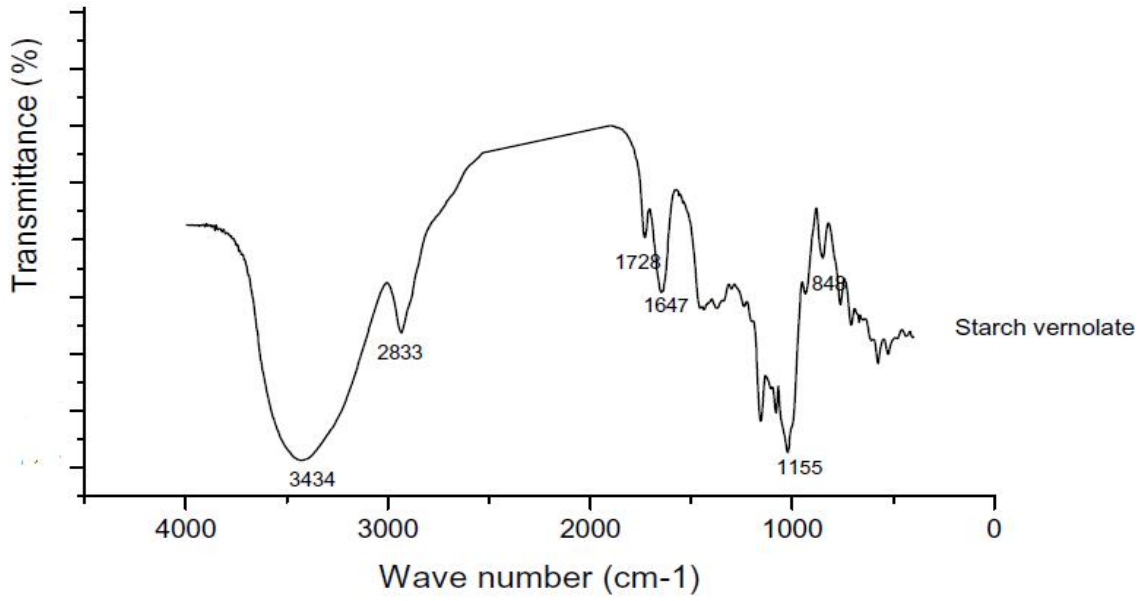
FTIR spectrum of starch vernolate (IVTE8)



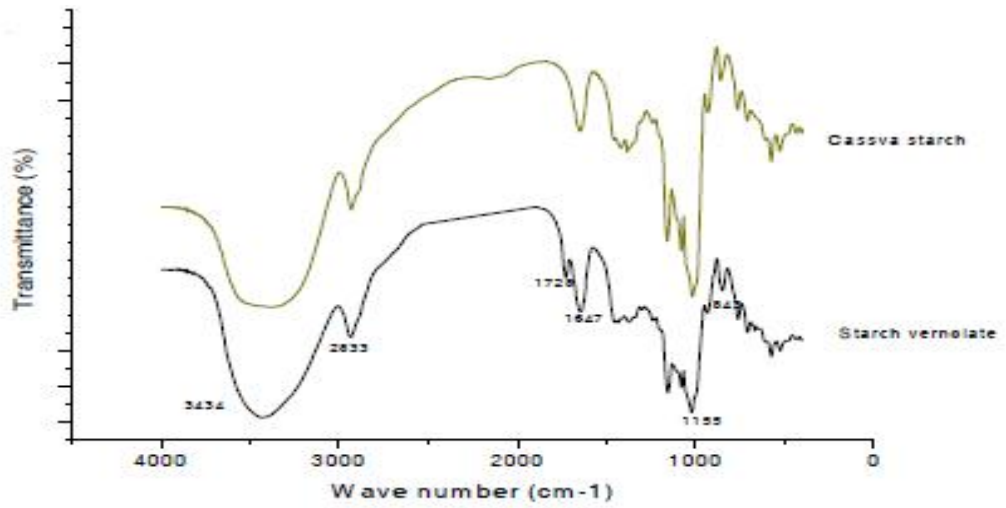
Comparison of FTIR of cassava starch (top) with starch vernolate (bottom) (IVTE8)



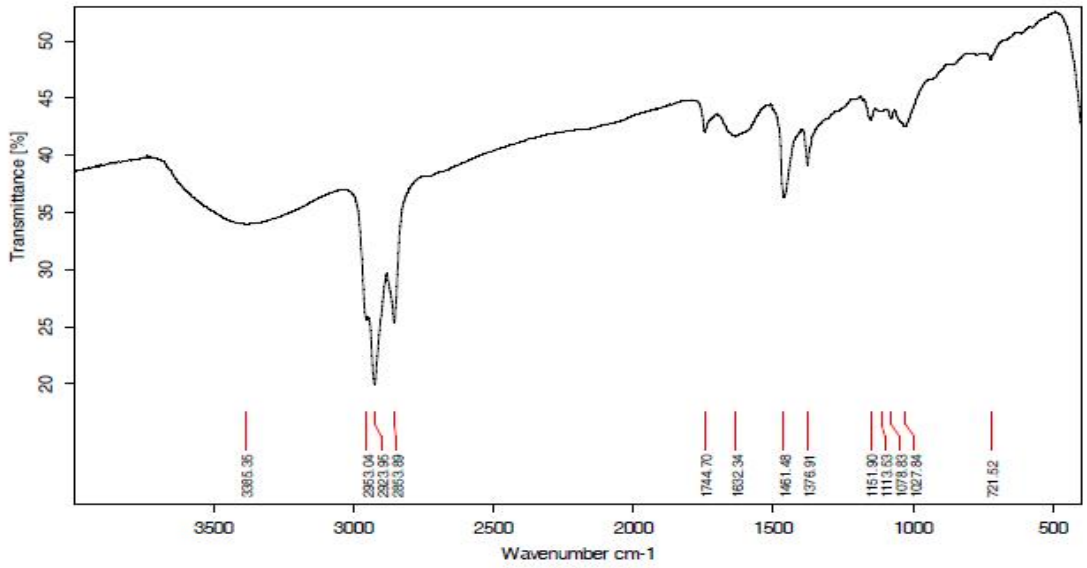
Comparison of FTIR spectrum of VOME (top), Starch (Middle) and Starch vernolate (bottom)(IVTE8)



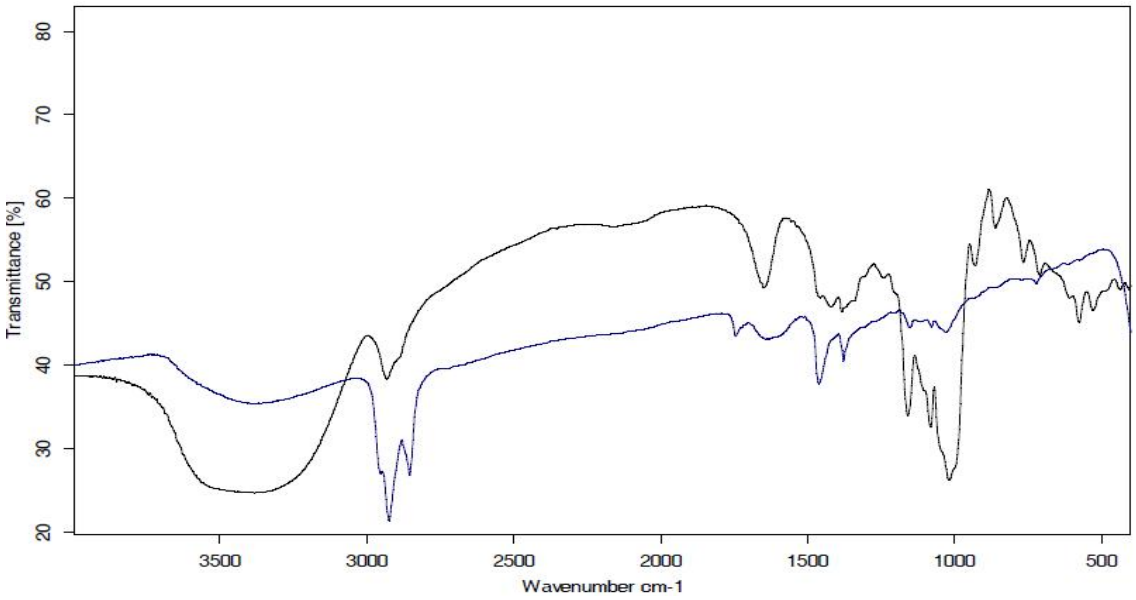
FTIR spectrum of starch vernolate (YCTE62)



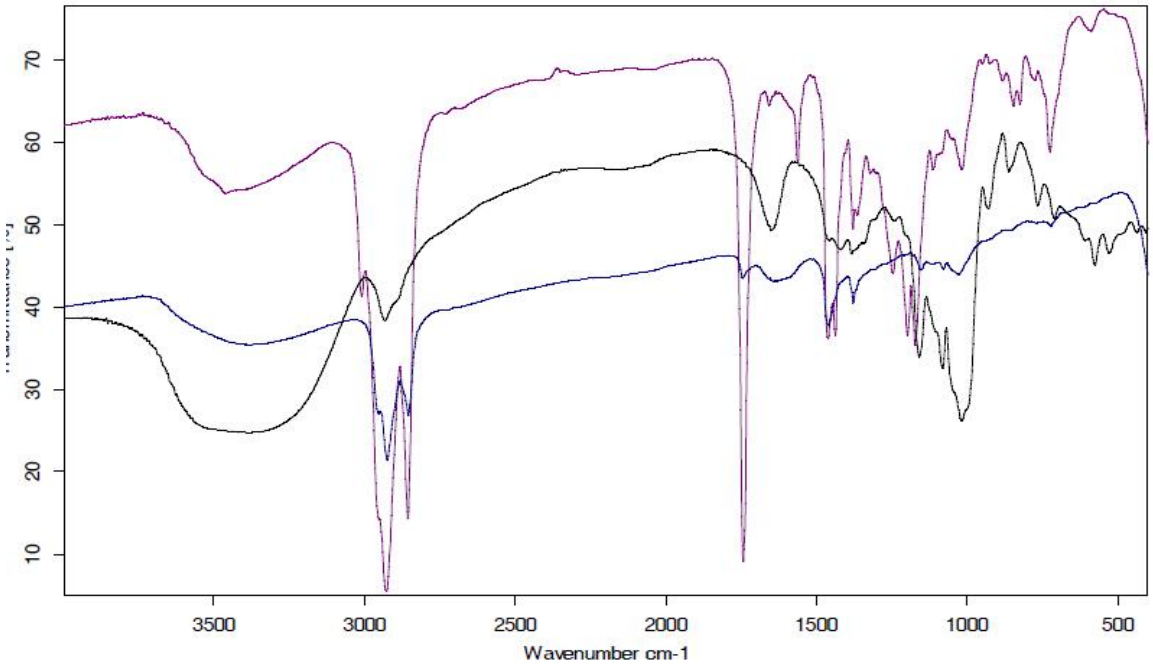
Comparison of FTIR spectrum of starch (top) and starch vernolate (bottom) (YCTE 62)



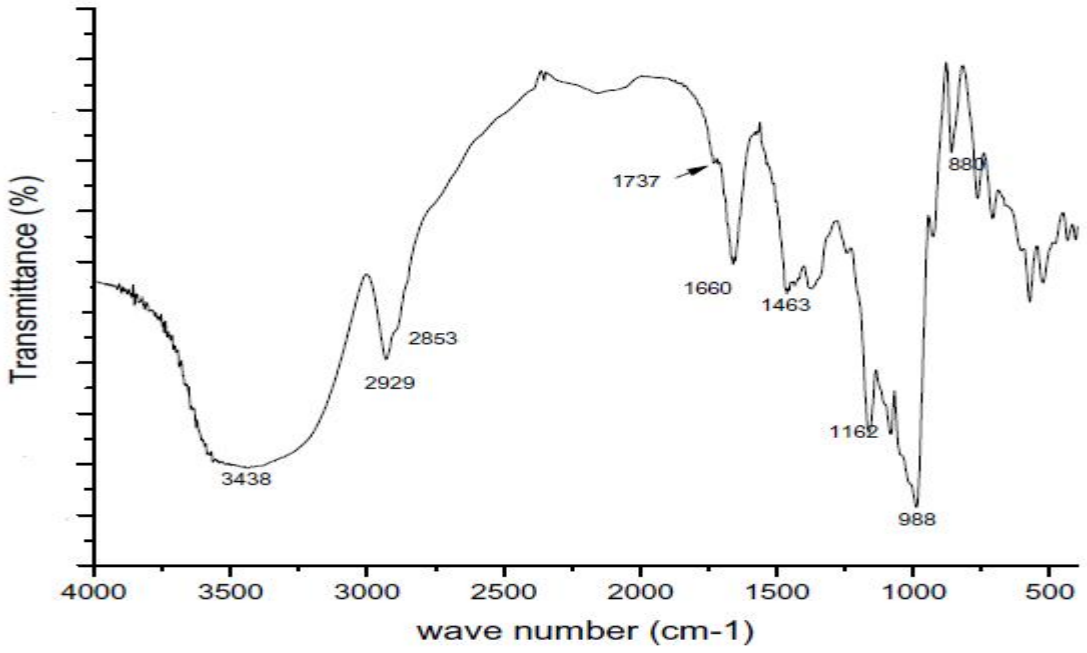
FTIR spectrum of starch vernolate (SE3)



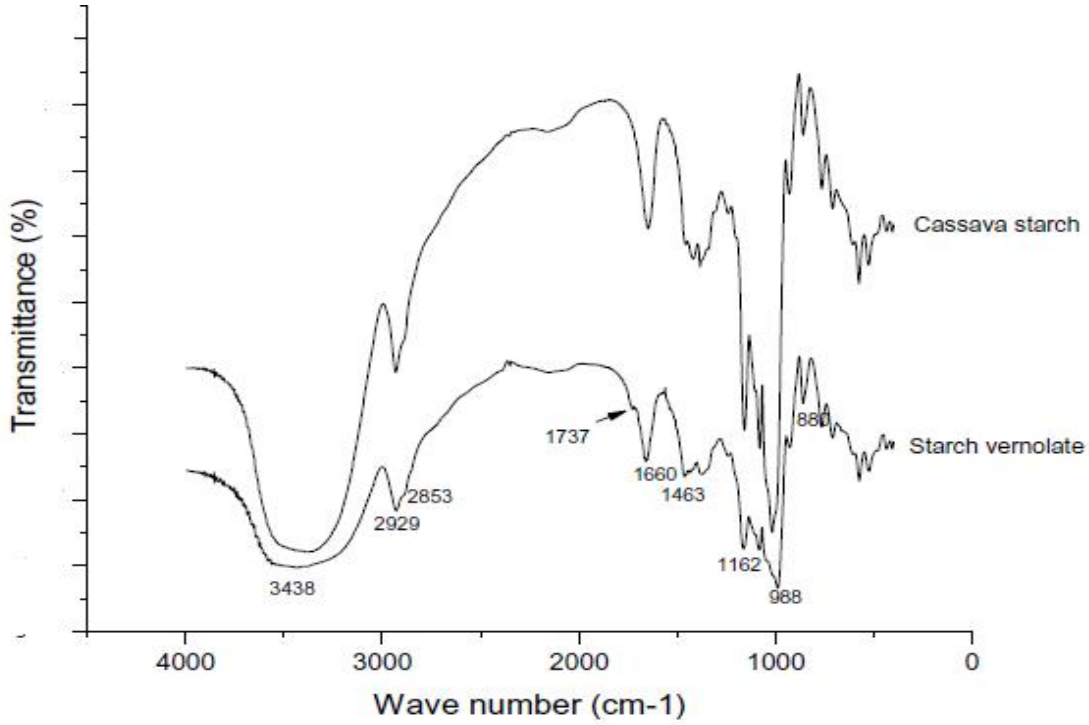
Comparison of FTIR starch (top) and starch ester (bottom) (SE3)



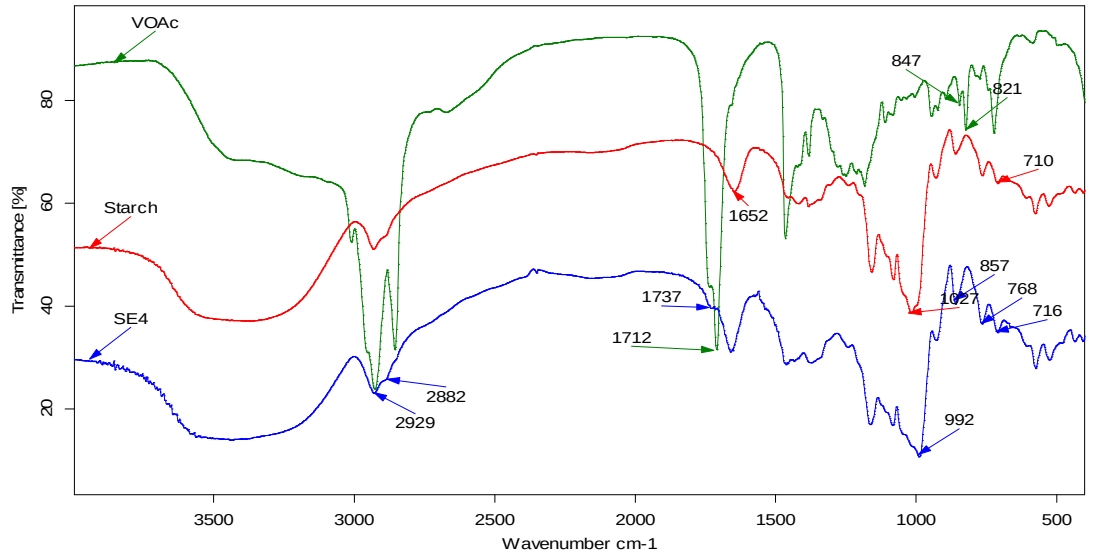
Comparison of FTIR VOME (top) starch (middle) and starch ester (bottom) (SE3)



FTIR spectrum of starch vernolate (SE4)



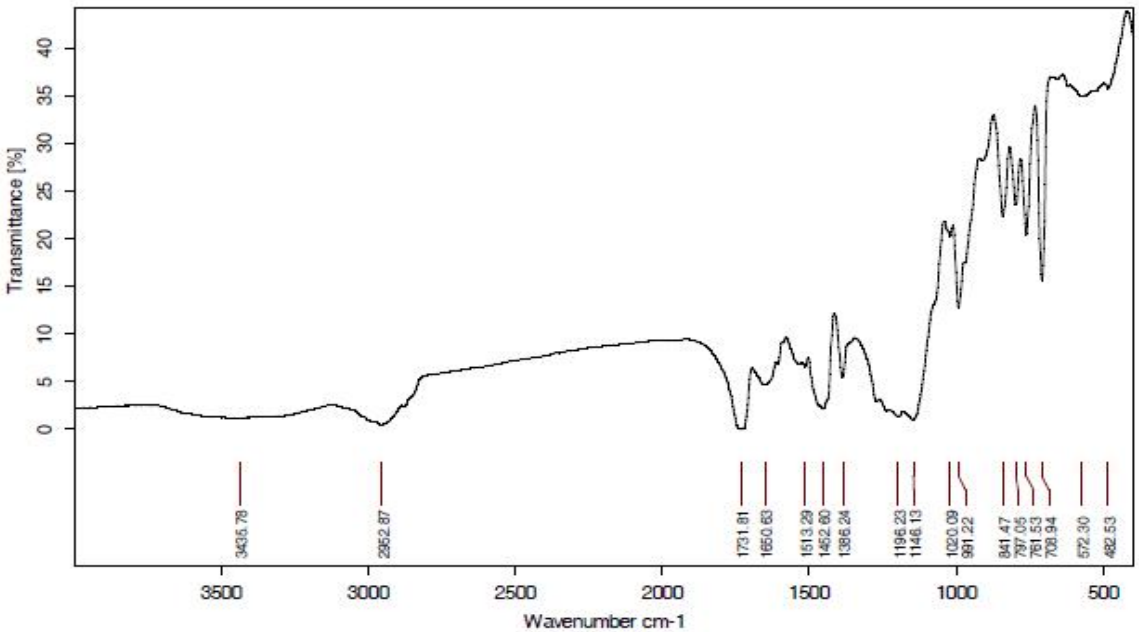
*Comparison of starch (top) with starch vernolate (bottom) (**SE4)***



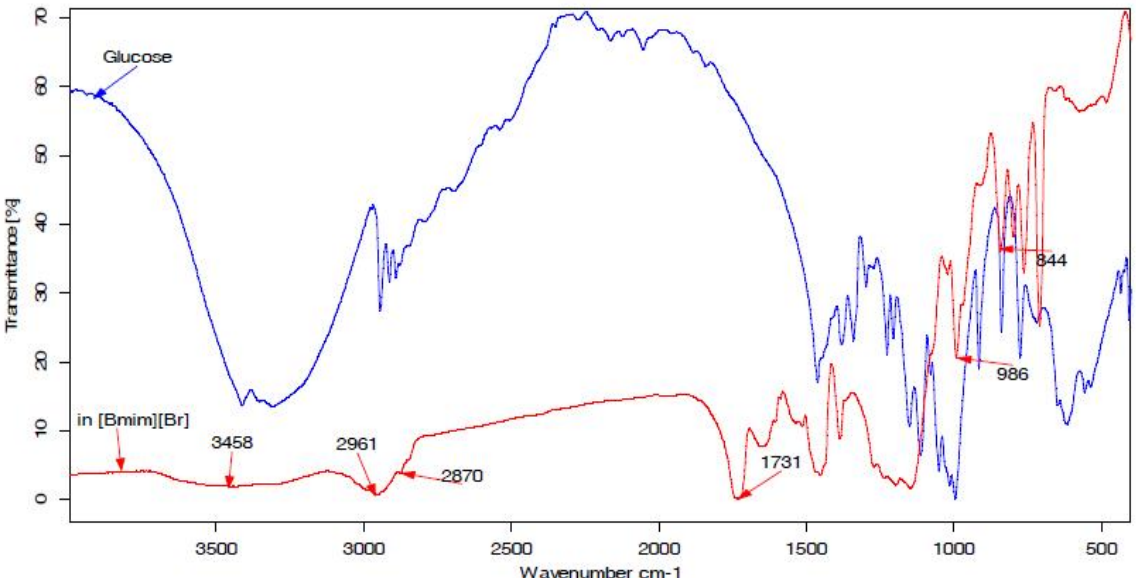
C:\TED\Cassava Starch (in KBr).0	Cassava Starch (in KBr)	Sample form	17/06/2011
C:\TED\Starch ester UK4.1	Starch ester UK4	Sample form	20/06/2011
C:\TED\VOAc (New).1	VOAc (New)	Sample form	08/09/2011

Page 1 of 1

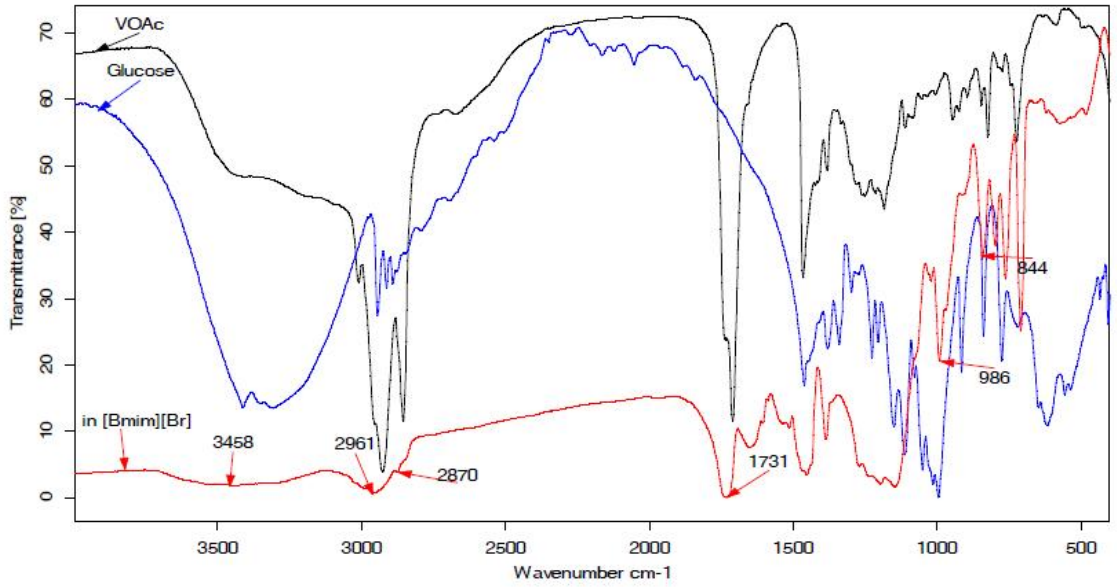
Comparison of starch (---) with starch vernolate (----)(SE4)



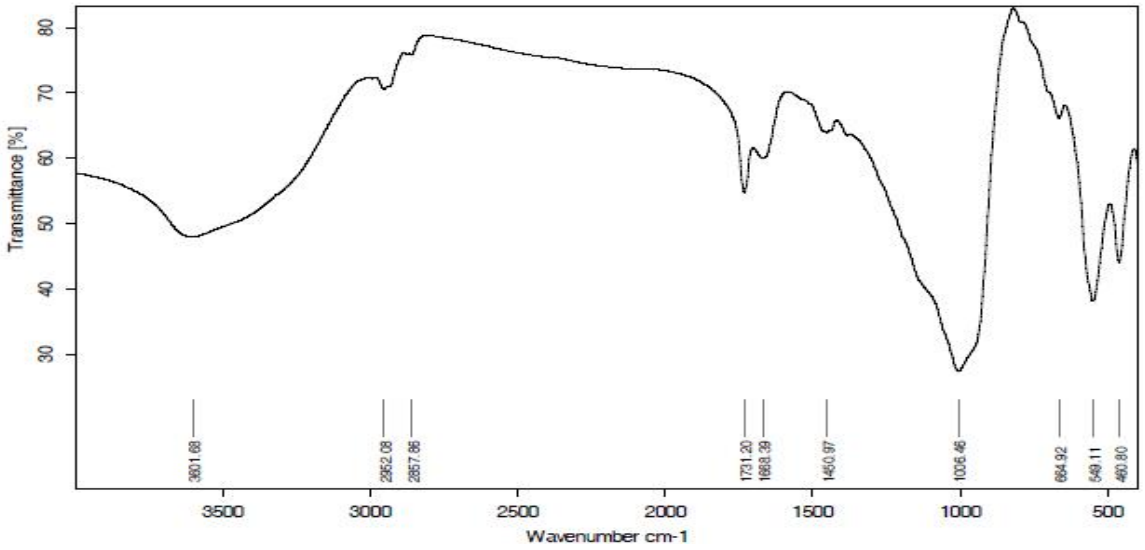
FTIR spectrum of glucose vernolate (GE in ILs)



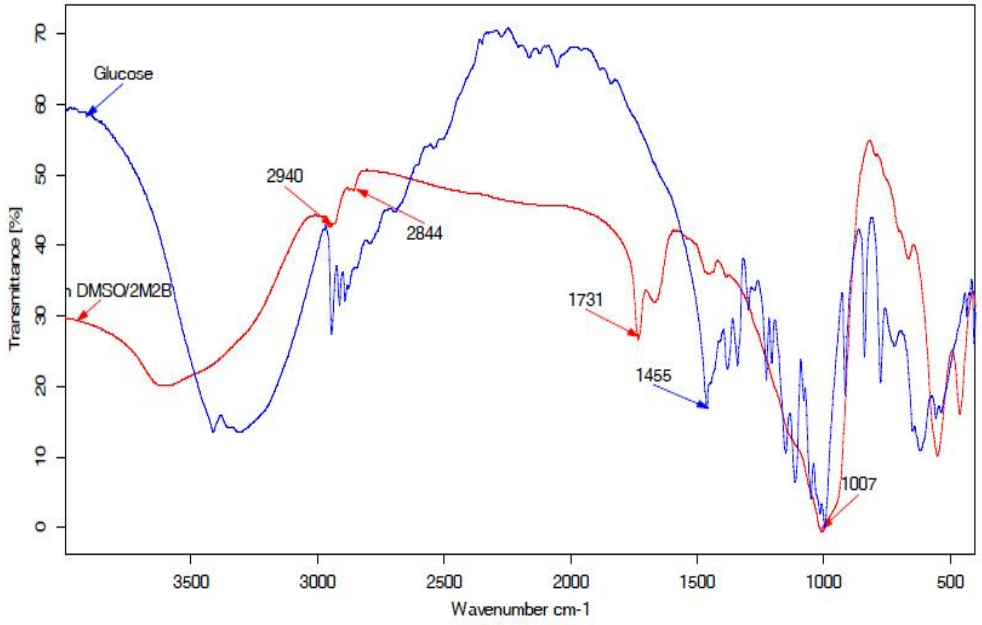
Comparison of glucose (top), glucose vernolate (bottom)(GE in ILS)



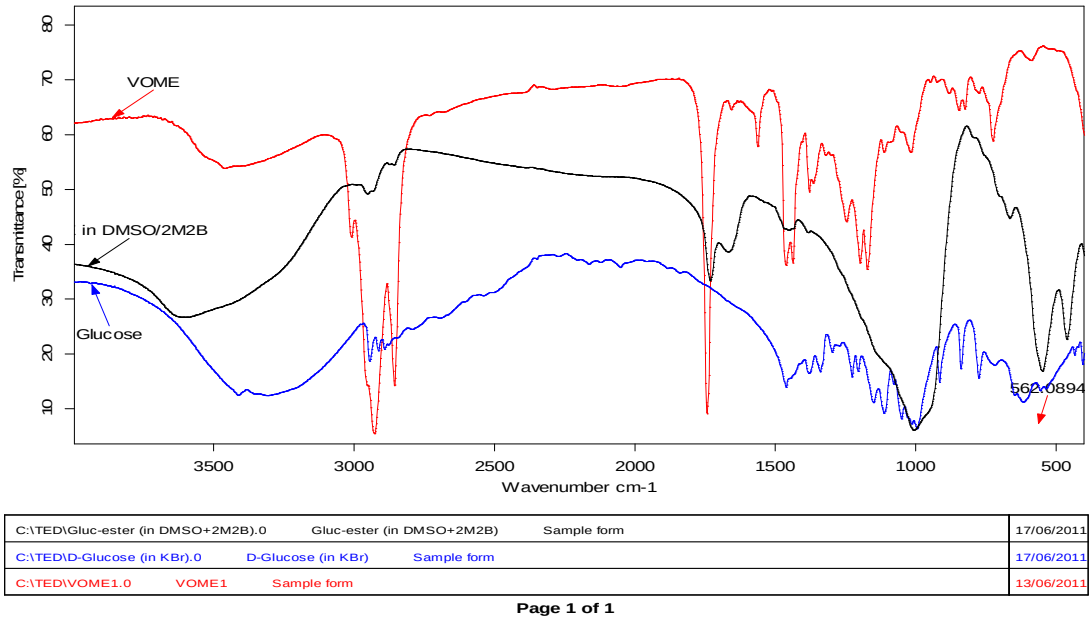
Comparison of glucose (middle), VOAc (top) and glucose vernolate (bottom) (GE in ILS)



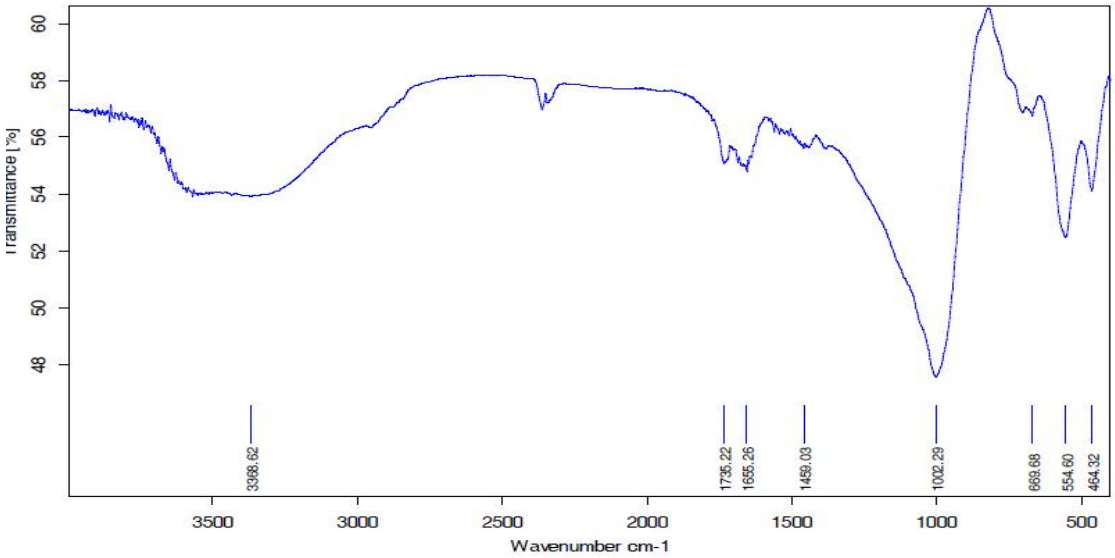
FTIR spectrum of glucose vernolate in 2M2B /DMSO



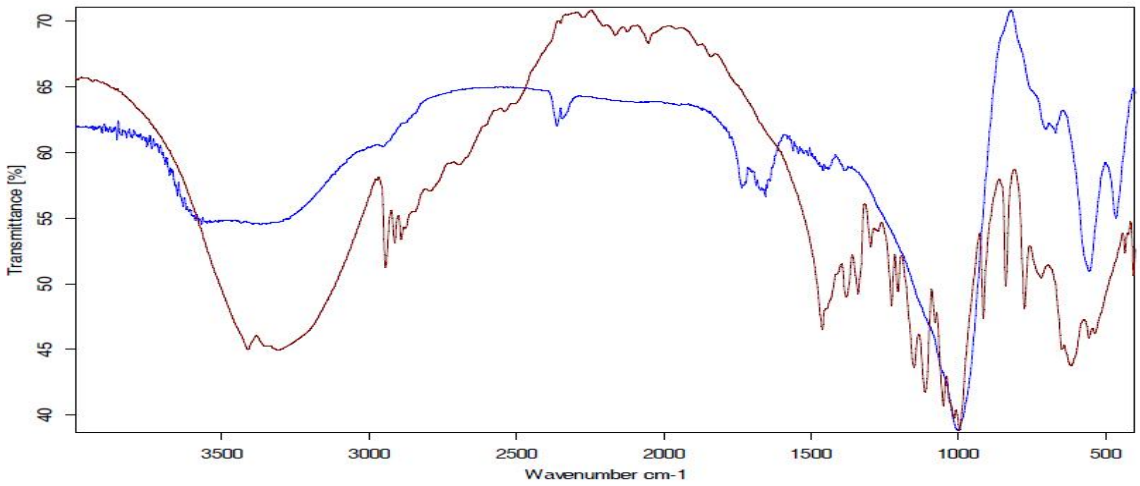
Comparison of FTIR of glucose and glucose vernolate in 2M2B /DMSO



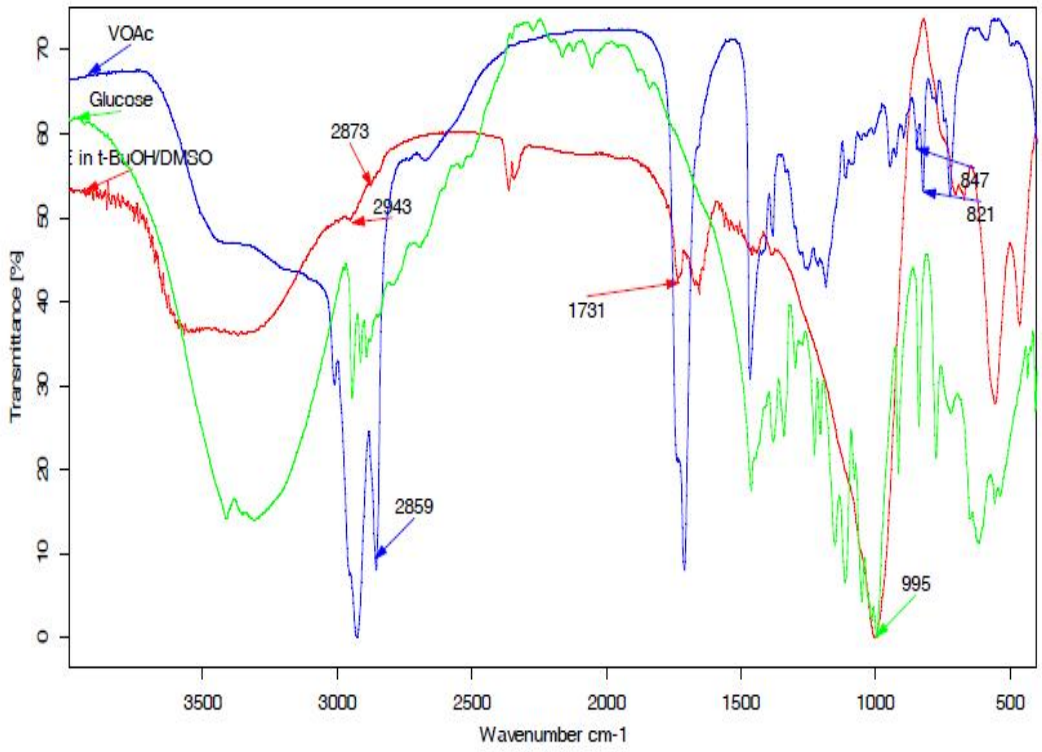
FTIR of VOME (top), glucose vernolate (middle and glucose (bottom) in 2M2B /DMSO



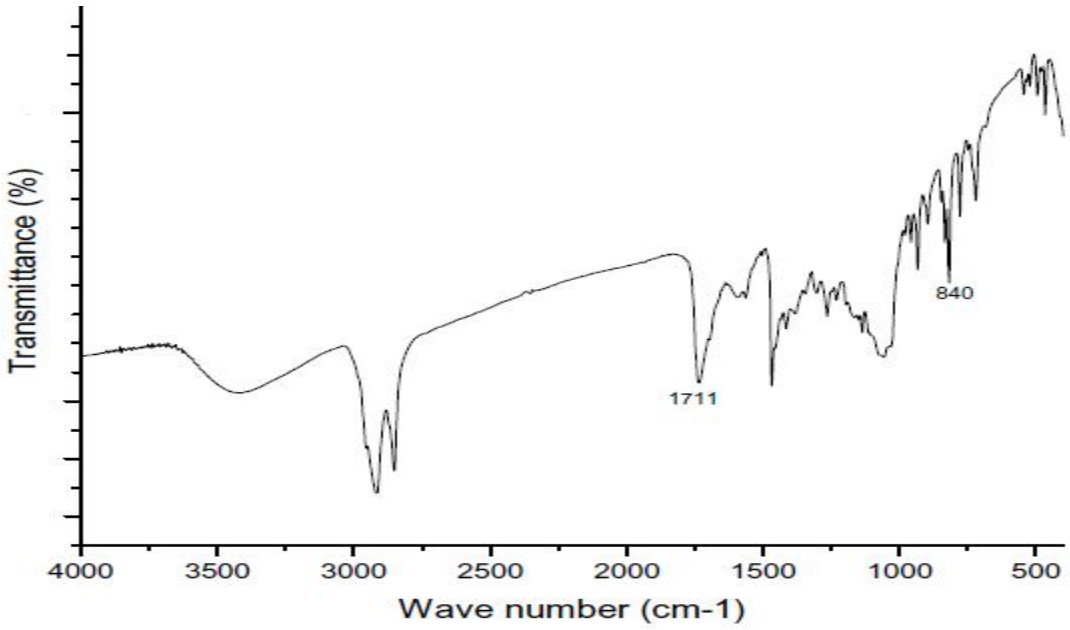
FTIR of Glucose vernolate in DMSO/t-butanol



Comparison of glucose (----) and glucose vernolate (----) in DMSO/t-butanol

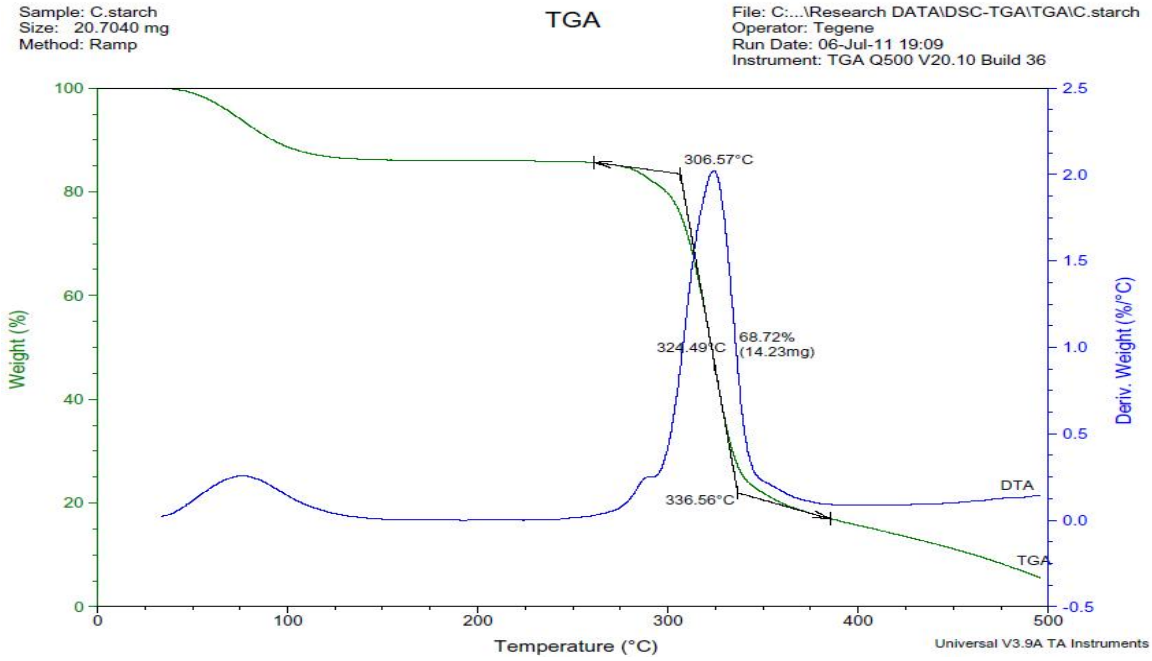


Comparison of FTIR spectrum of glucose (green), glucose vernolate (red) and VOAc (blue) in DMSO/t-butanol

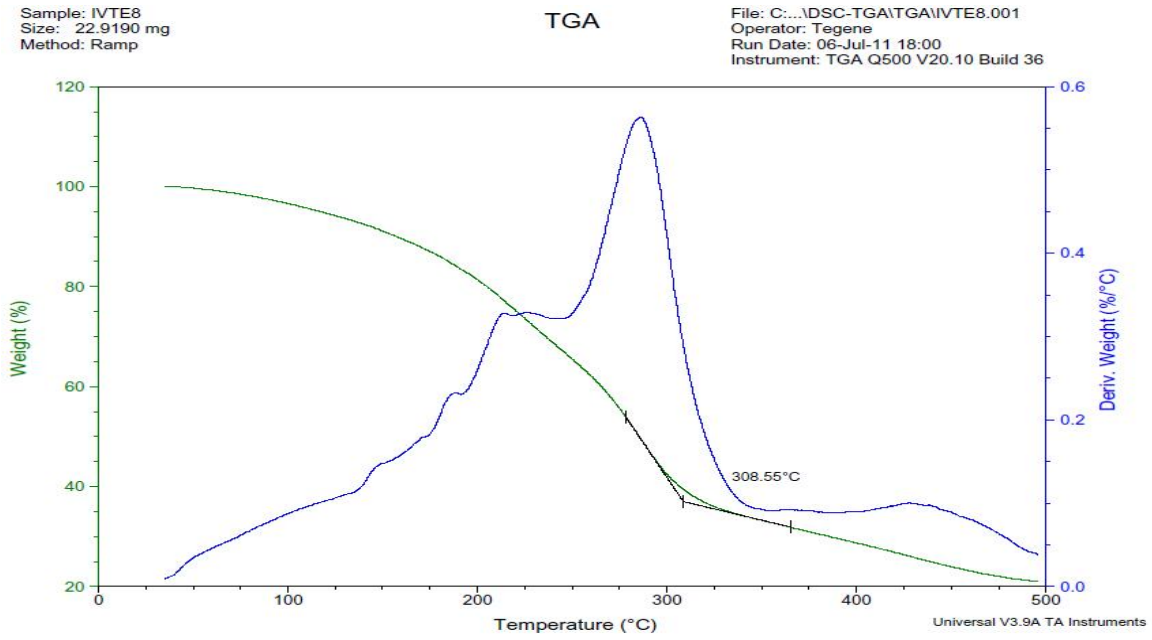


FTIR spectrum of epoxidized vernolic acid

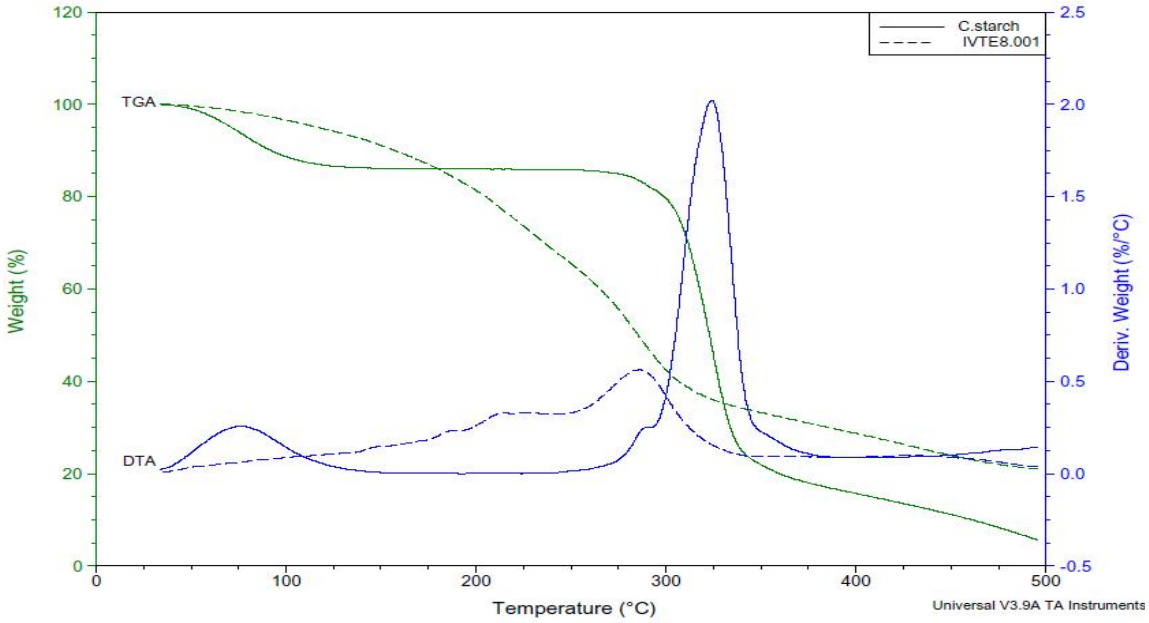
Appendix 4: DSC and TGA Thermogram



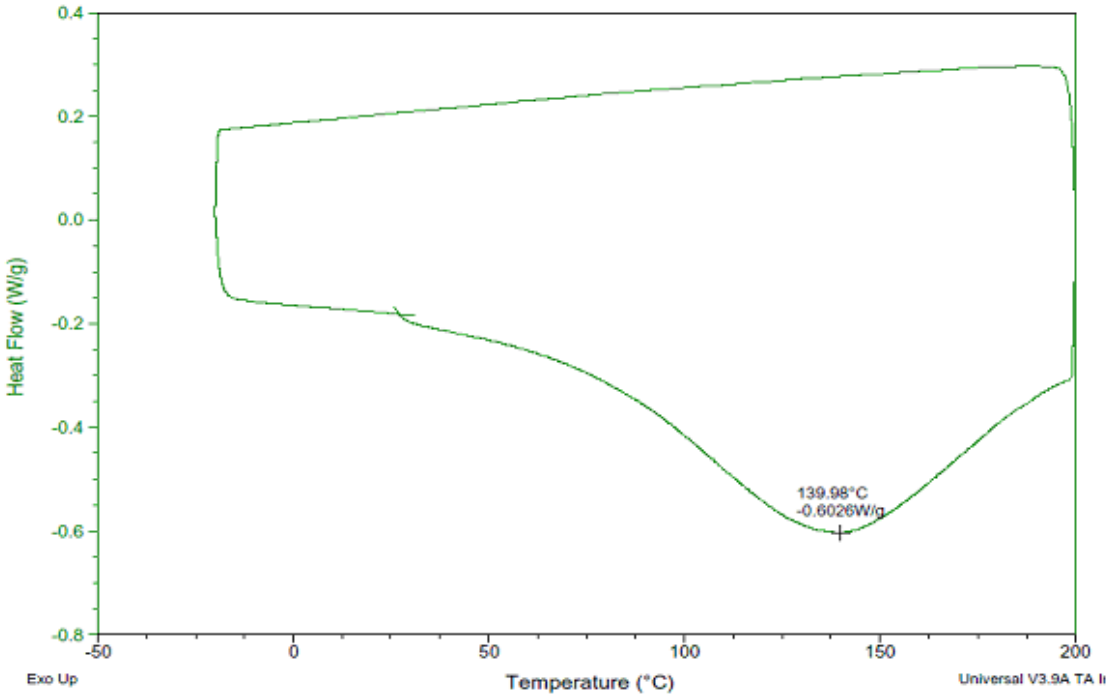
TGA thermogram of cassava starch (IVTE8)



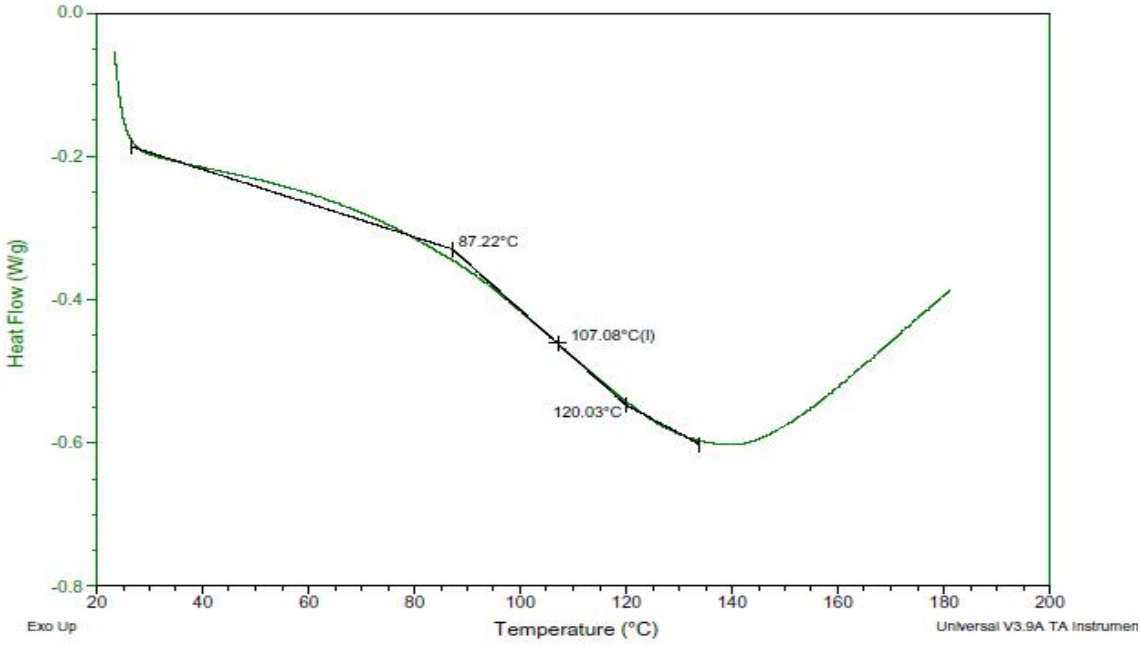
TGA thermogram of starch vernolate (IVTE8)



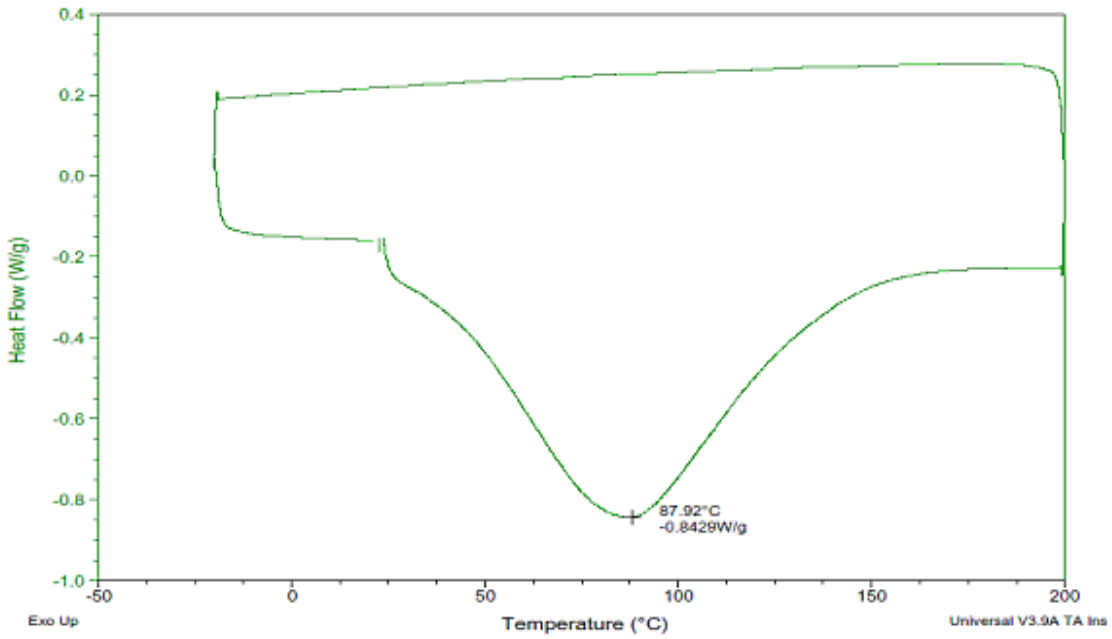
Comparison of TGA and DTA thermogram of starch and starch vernolate (IVTE8)



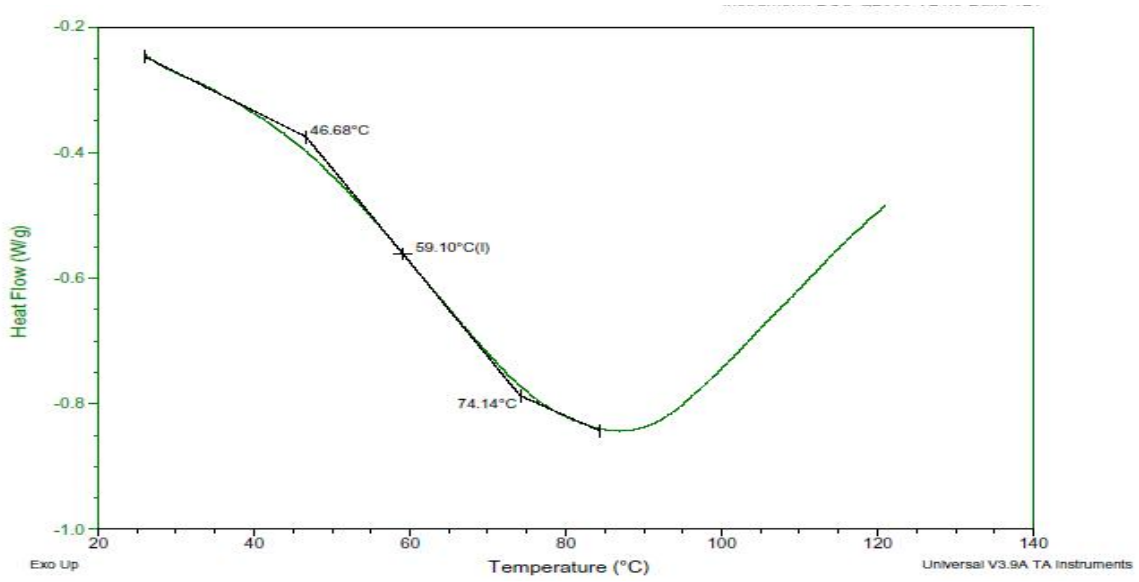
TGA thermogram of cassava starch (YCTE62



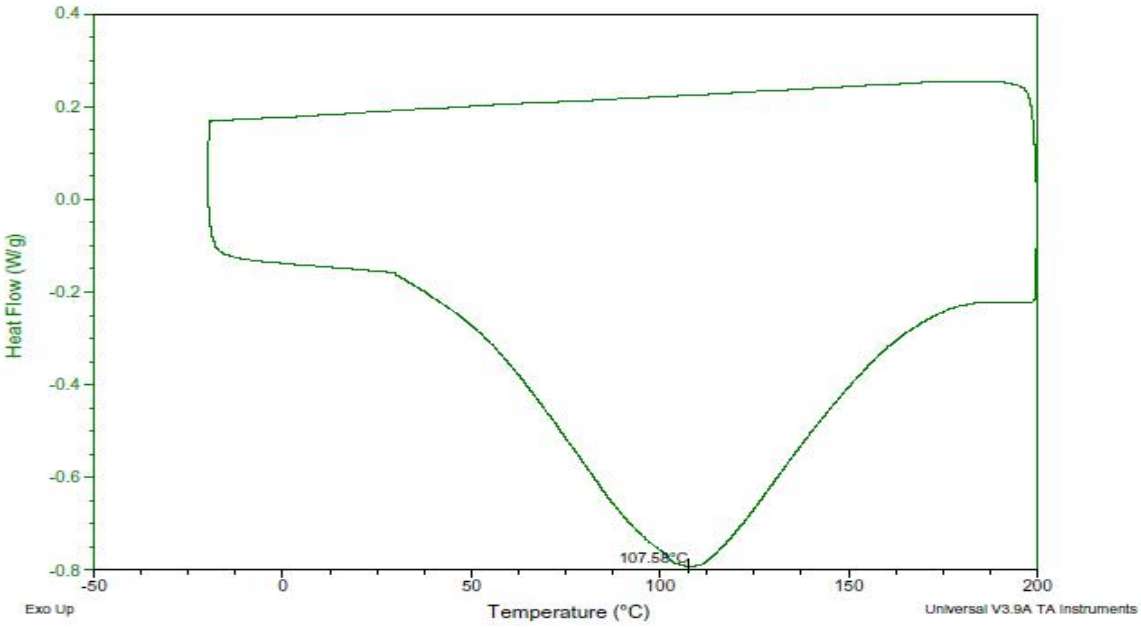
DSC thermogram of cassava starch (YCTE62)



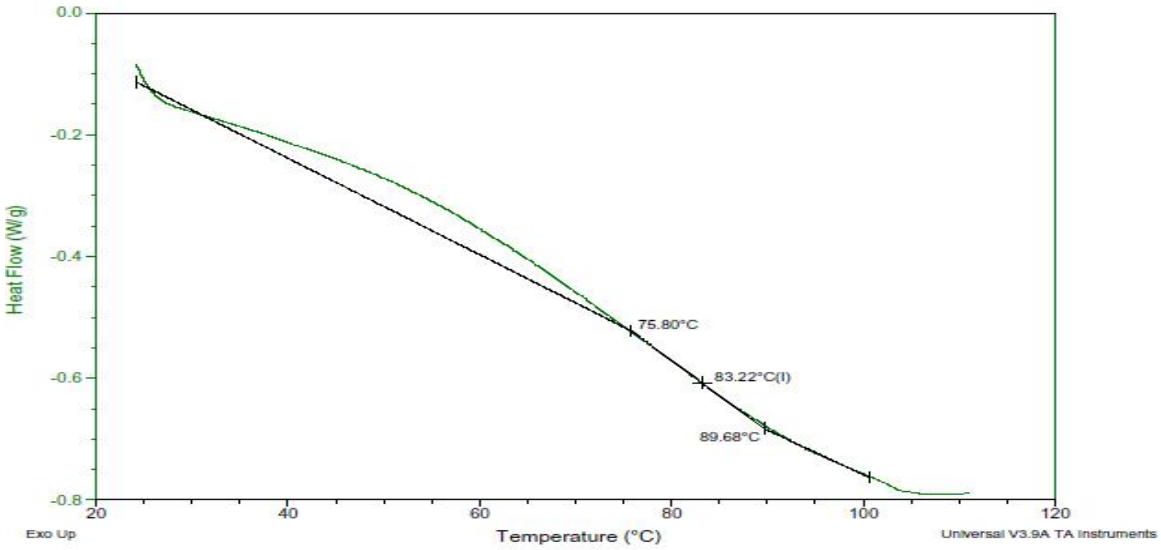
DSC thermogram of starch vernolate (YCTE62)



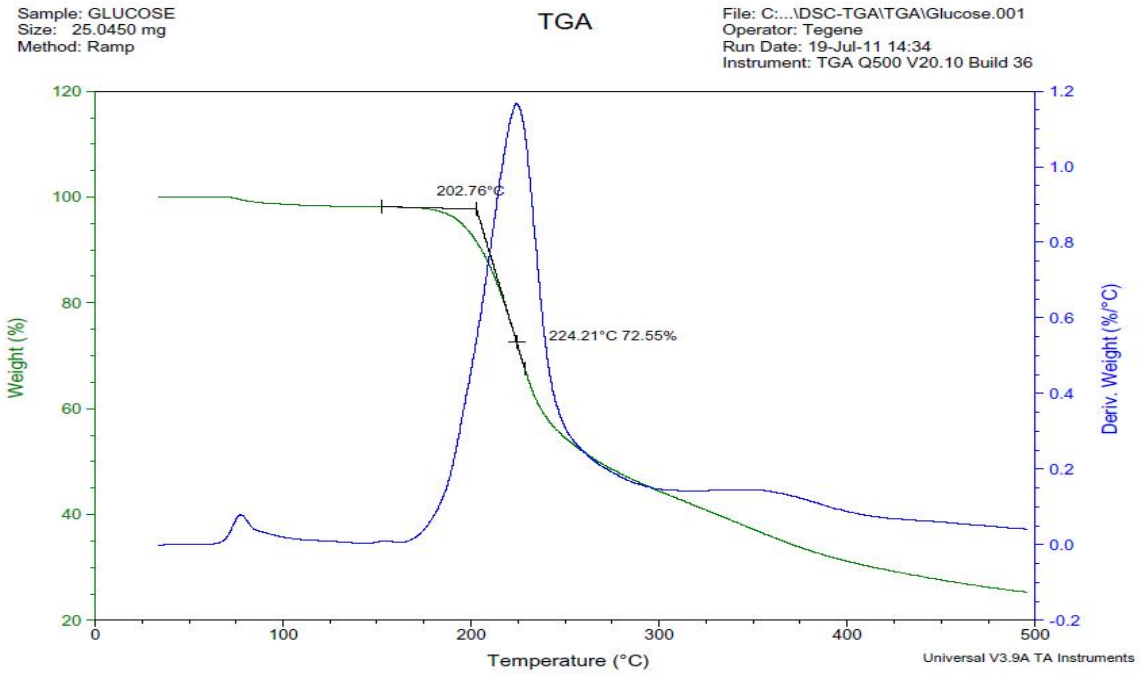
DSC of starch vernolate (YCTE 62)



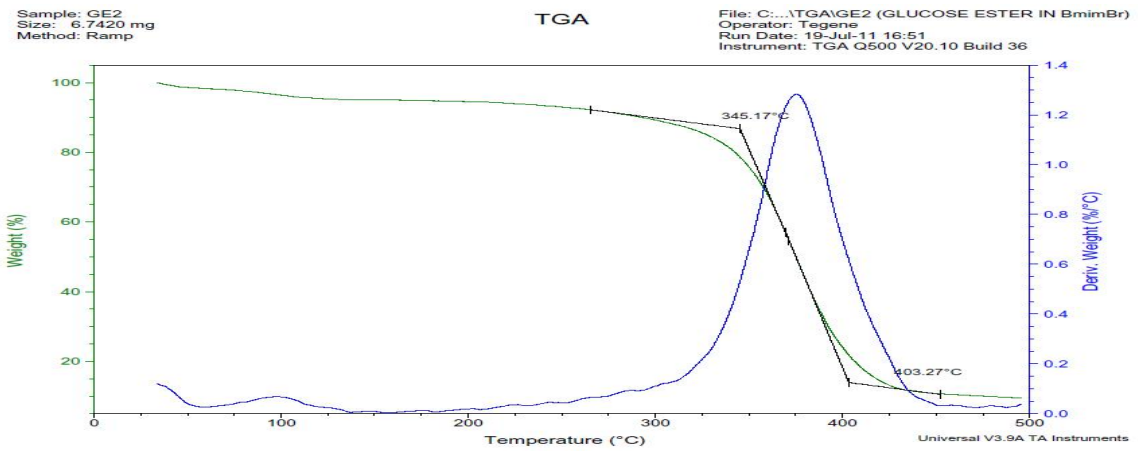
DSC thermogram of starch vernolate (SE4)



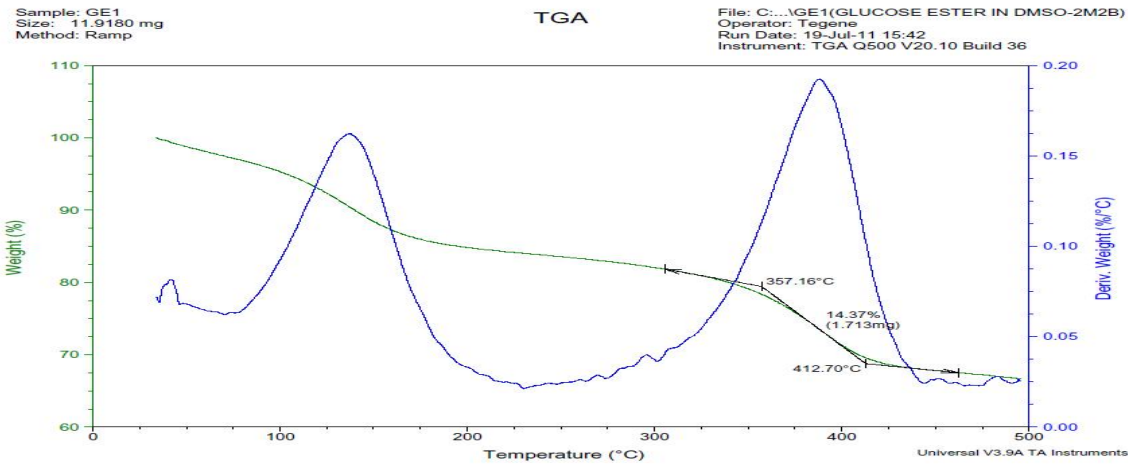
DSC thermogram of starch vernolate (SE4)



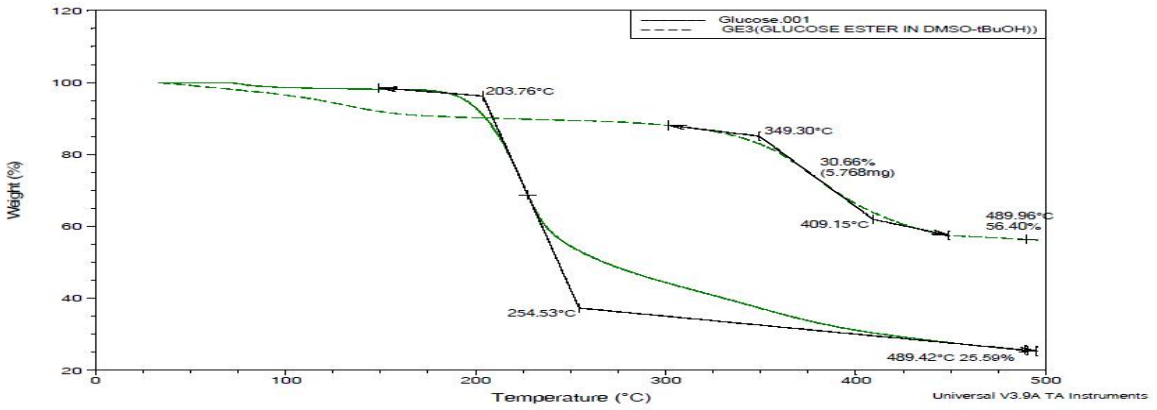
TGA thermogram of glucose in ILs



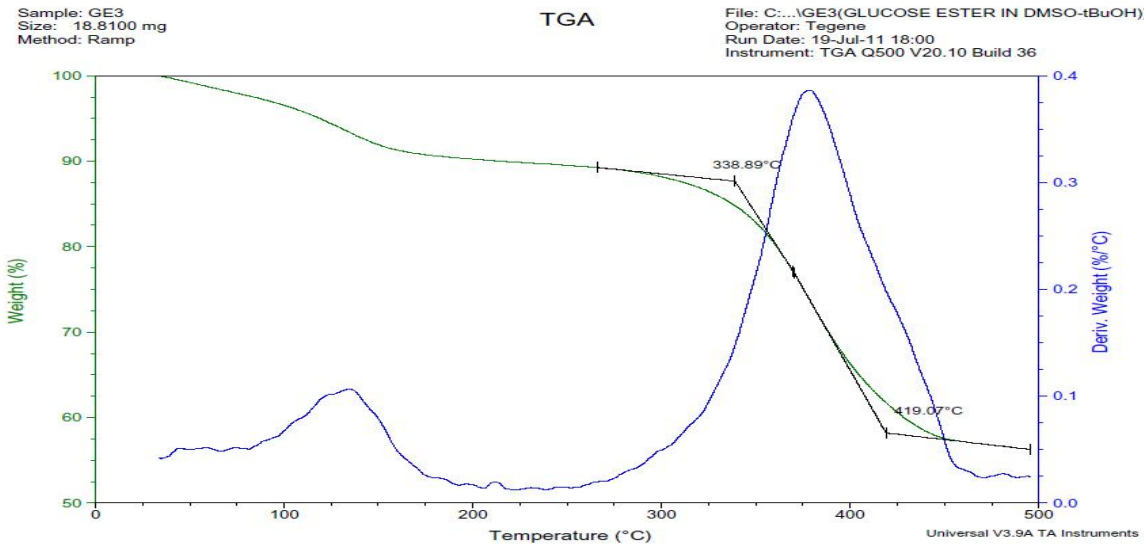
TGA thermogram of glucose vernolate in ILS



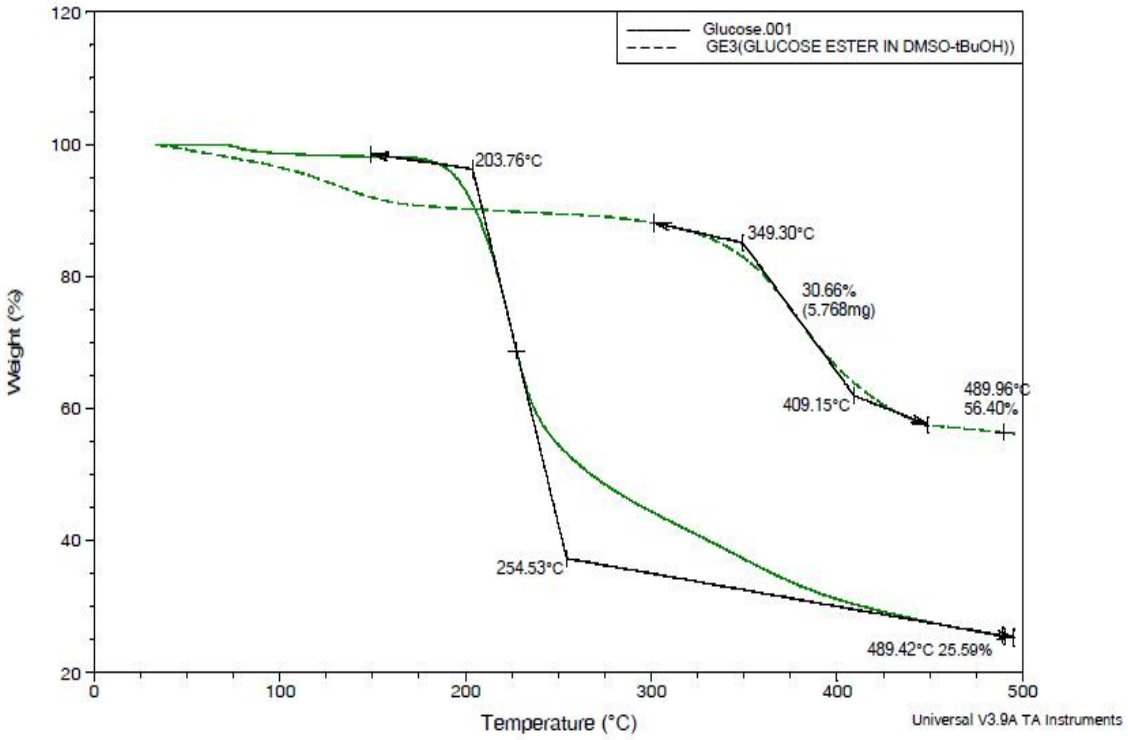
TGA thermogram of glucose vernolate in DMSO/2M2B



Comparison of TGA thermogram of glucose and glucose DMSO/2M2B

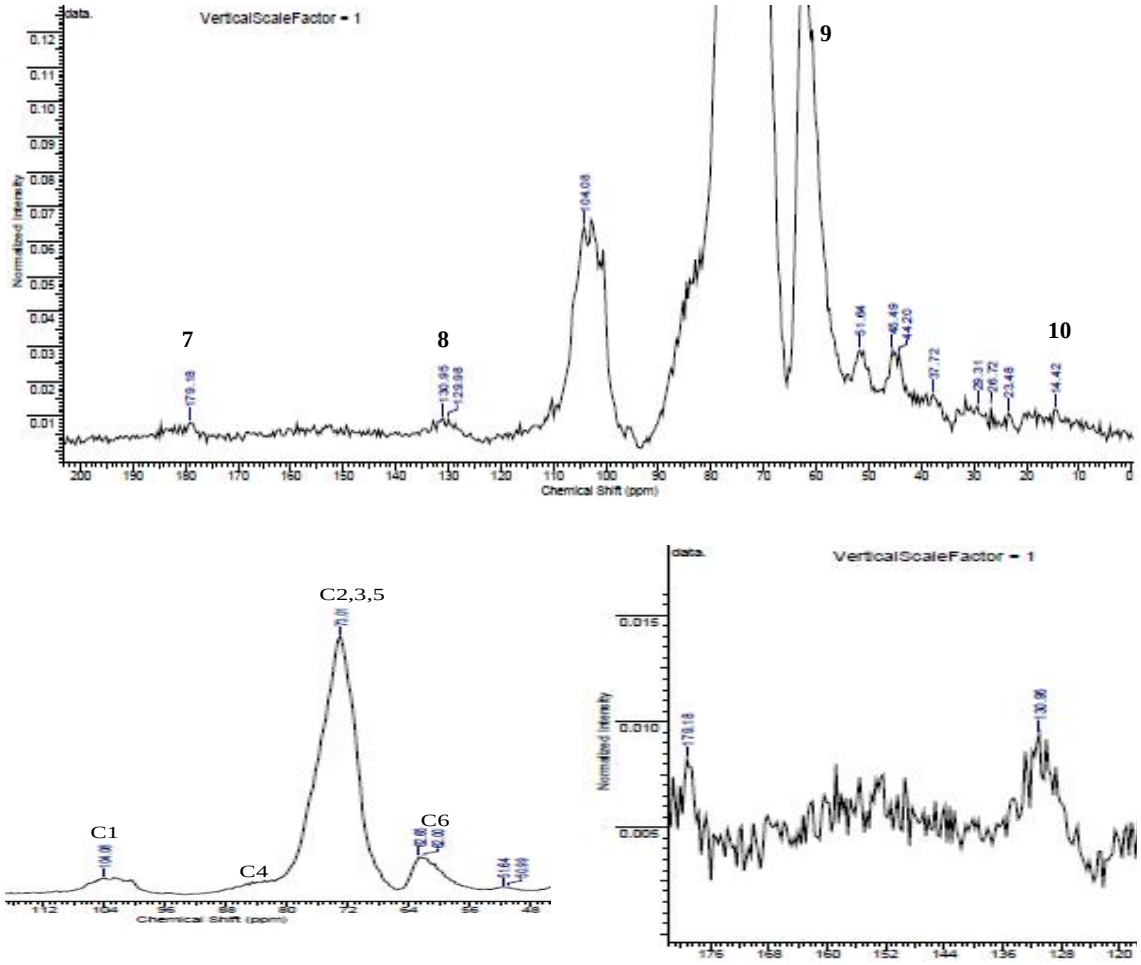


TGA thermogram of glucose vernolate in DMSO/t-butanol

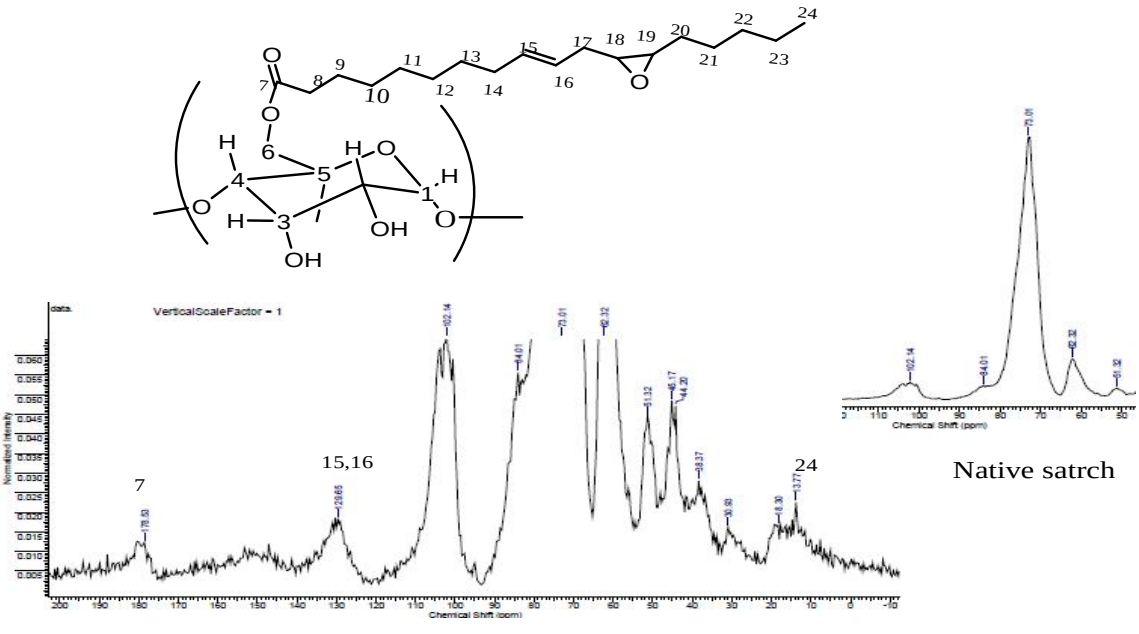


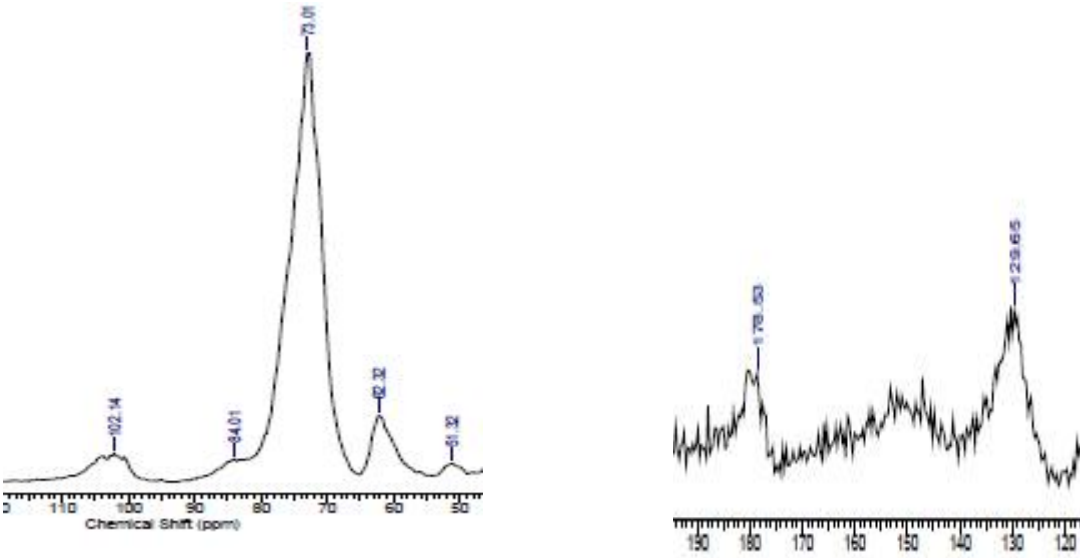
Comparison of TGA of glucose and glucose vernolate in DMSO/t-butanol

Appendix 5: Solid state (CP-MAS ^{13}CNMR) spectra

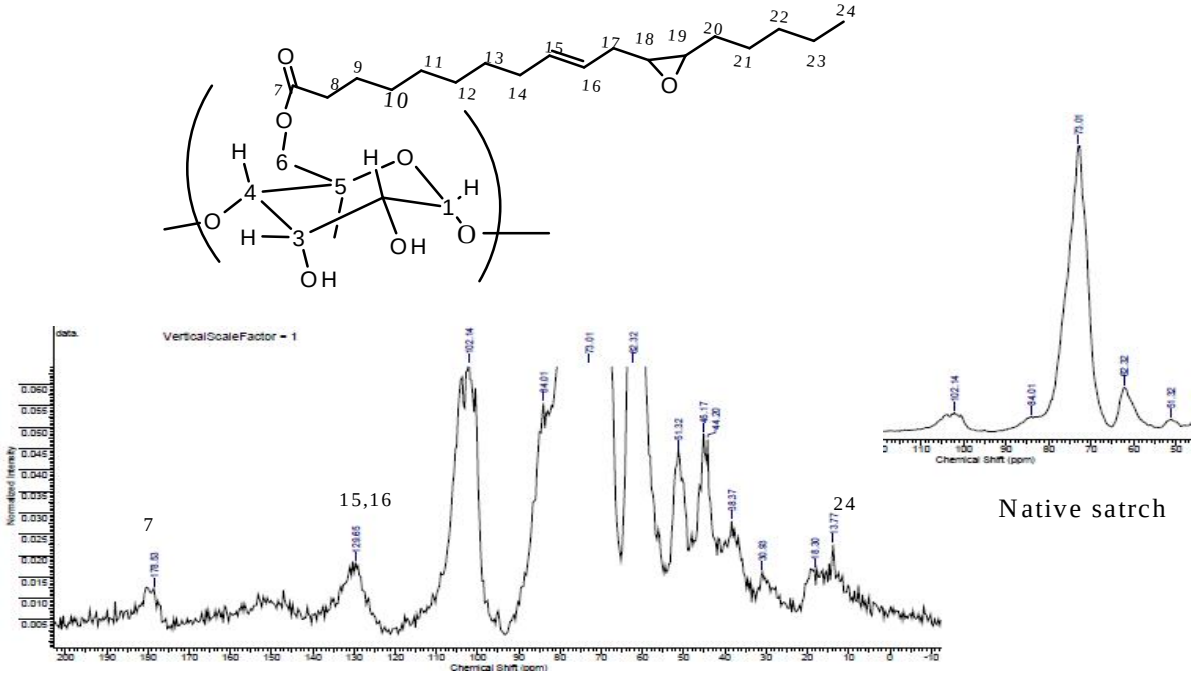


Solid state NMR spectrum of starch vernolate (IVTE8)

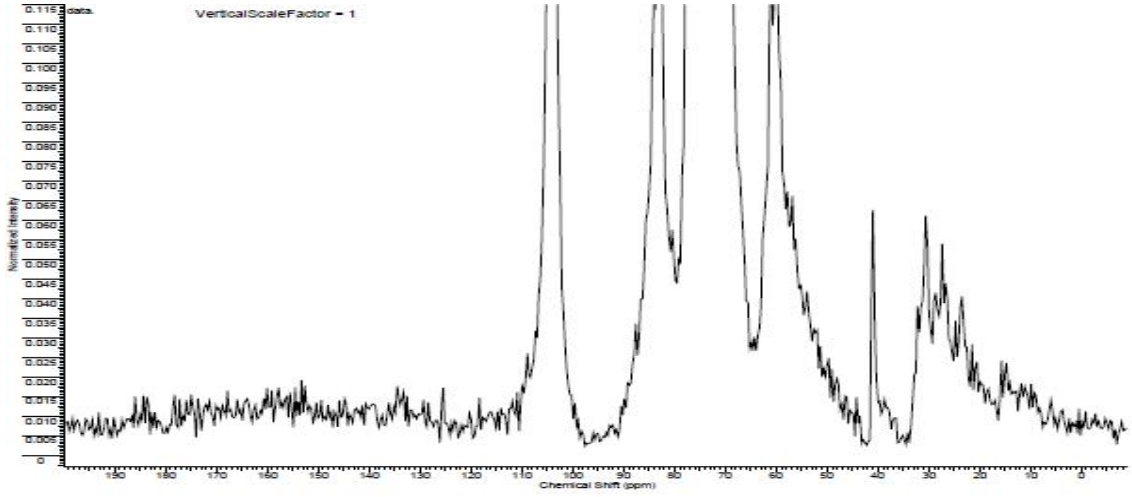




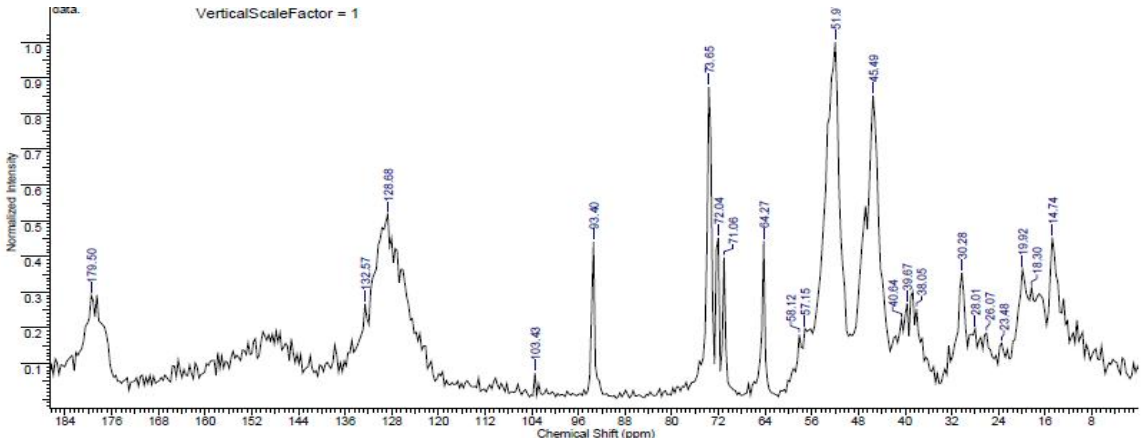
Solid state NMR spectrum of starch vernolate (YCTE62)



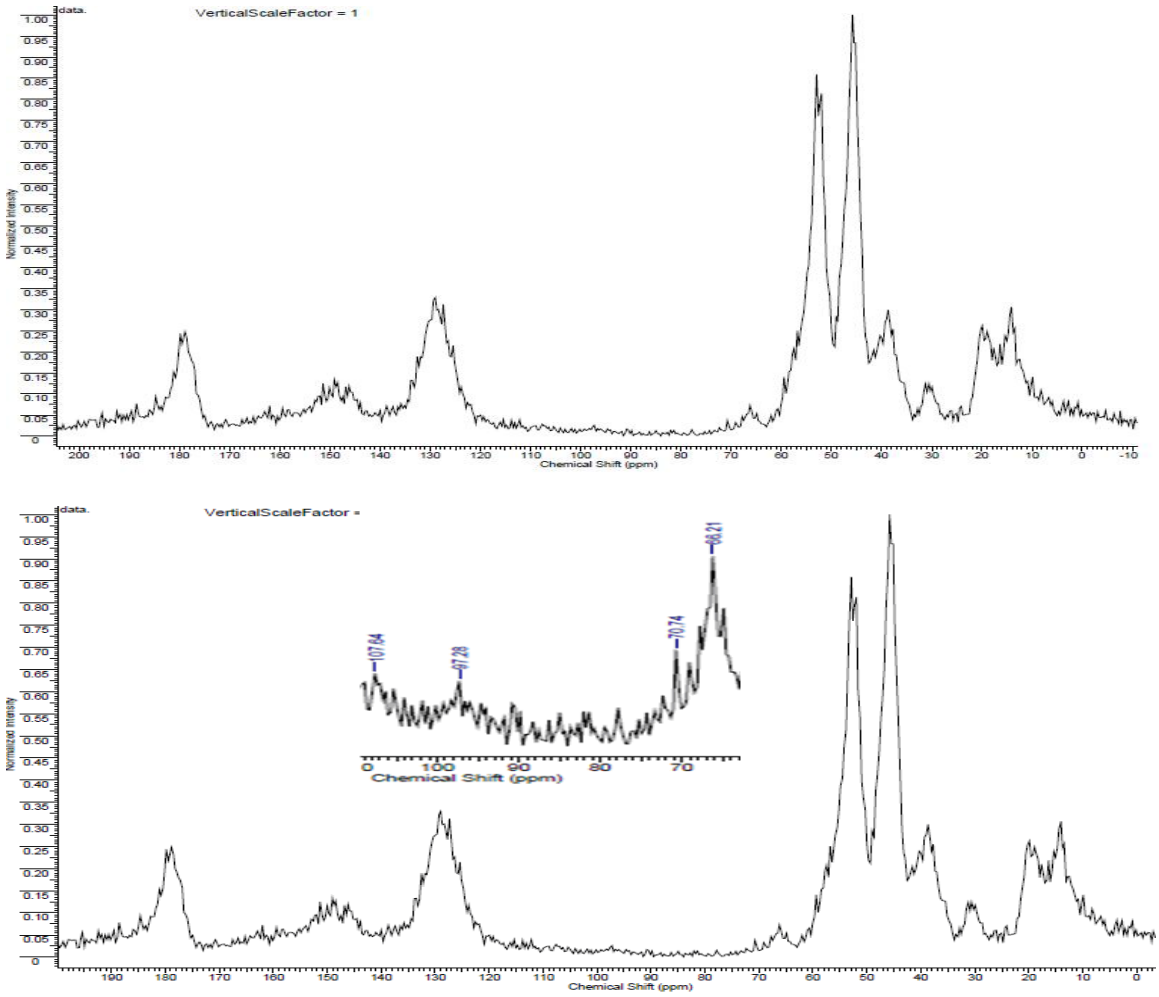
Solid state NMR spectrum of starch vernolate (SE3)



Solid state NMR spectrum of starch vernolate (SE4)



Solid state NMR spectrum of glucose vernolate (GE in ILs)



Solid state NMR spectrum of glucose vernolate in DMSO/2M2B