Development of Three Armed GLY-LAC Bio-Conjugate for the Efficacy of Ointment Base Matrix and Polymer Plasticizer

By

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A Thesis Submitted to the Department of Chemical Engineering

School of Mechanical, Chemical and Materials Engineering

Office of Graduate Studies

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APPROVAL OF THE BOARD OF EXAMINERS

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DECLARATION

I declare that the work which is being presented in the thesis entitled "Development of Three Armed GLY-LAC Bio-Conjugate for the Efficacy of Ointment Base Matrix and Polymer Plasticizer" Submitted to Adama Science and Technology University, school of Mechanical, Chemical and Materials Engineering in partial fulfillment of the requirements for the award of the degree of master of science in process Engineering is entirely my own work. This thesis was carried out from January 2020 to March 2021, under supervision of MelakuTesfaye (Ph.D.) (Advisor) from Adama Science and Technology University, school of Mechanical, Chemical and Materials Engineering lecture. All references, including citation of published and unpublished sources, have been appropriately acknowledged in the work. I further declare that the work has not been submitted for the purpose of academic examination, either in its original or similar form, anywhere else.

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LIST OF ABBREVIATIONS

ASTM	American Society for Testing and Materials
EPA	Environmental Protection Agency's
LA	Lactic Acid
E.coli	Escherichia coli
S.aureus	Staphylococcus aureus
PLA	Poly (lactic acid)
MOs	Mineral Oils
TGs	Triglycerides
FTIR	Fourier Transmission Infrared Spectroscopy
^{1}HNMR	Hydrogen Nuclear Magnetic resonance
^{13}C-NMR	Carbon Nuclear Magnetic resonance
LAOLG	L-lactic acid Oligomer
TGA	Thermogravimetric analysis

LIST OF NOTATIONS

mL	Milliliter
%	Percent
hr	Hour
mg	Milligram
Pa.s	Pascal Second
Cp	Centri poise
AV	Acid Value
g	Gram
CM	*Croton Macrostachiyus*
De	Degree of Esterification
LAOLG	Lactic Acid Oligomer
GLY-LAC 1:3	1 to 3 Glycerol to lactic acid ratio
GLY-LAC 1:6	1 to 6 Glycerol to lactic acid ratio
GLY-LAC 1:9	1 to 9 Glycerol to lactic acid ratio
GLY-LAC 1:12	1 to 12 Glycerol to lactic acid ratio

ABSTRACT

Biomaterials have attracted increasing research interest in recent times because of their renewability, biodegradability and non-toxicity. The biodiesel industry produces a large quantity of glycerol as a byproduct that can be used to synthesize biomaterials. Glycerol and glycerol-based products are interesting for their wide applications. Conjugation of glycerol with other biomolecules has been used to diversify its properties. In this research,a Polycondensation reaction between glycerol and lactic acid molecule have been used to developed three armed bio-based conjugate. Different conditions such as; reaction temperature, mixing ratio and reaction time have been tested to obtain a visco-elastic biomaterial. The mechanism of reaction during this process is well studied by different characterization techniques; instrumental and experimental. The three-armed glycerol-lactic acid (GLY-LAC) conjugateswere synthesized in different mixing ratios. An FTIR and NMR analysis shows the growth of lactic acid on the glycerol core with an active three-armedOH functional group terminal. Depending on the preliminary investigation, reaction temperature, mixing ratio and reaction time highly affects the chain length that growth on the backbone of glycerol, whichis the reason for the change in the viscosity. The acid value and degree of esterification were estimated in order to analyzethe poly-condensation reaction. It is observed that by controlling the reaction condition, it is possible to manipulate the viscosity.The synthesized three armed conjugate having hydroxyl group terminalwith the ability of viscosity adjustment can be used in different applications such as ointment base matrix and polymer plasticizer. In this research work,extract of C. Macrostachyus (CM) were used as an active component to show the application of GLY-LAC conjugate as a carrier for simple ointment application. Additionally, the conjugate was tested forits application as PLA plasticizer.

In the first investigation, 1 wt%, 3 wt%, 5 wt% and 10 wt% of CM were dispersed in GLY-LAC conjugate matrix and anti-microbial properties using two different bacterial species (E. coli and S. aureus) were analyzed. Relatively higher anti-bacterial activity and viscosity were observedfor ointment-base matrix synthesized at 190 °C, 8-hour reaction time with mixing ratio of 1:12 glycerol to lactic acid GLY-LAC 1:12.The result shows that, maximum inhibition zone of 24 mm was obtained duringthe dispersion of 10 wt% CM (3 mg/ml) in GLY-LAC 1:12 conjugate for both E. coli and S. aureus. Most interesting, the GLY-LAC 1:12 conjugate without the addition of CM

shows high antimicrobial activity with inhibition zone of 21 mm and 22 mm respectively for E. coli and S. aureus.Furthermore, end user performance of the ointment base matrix was testedusing thermal stability analysis, spreadability, PH and other organolepetic characteristics.

In the second investigation, 10 wt%, 15 wt%, 20 wt% and 25 wt% of GLY-LAC 1:3 and GLY-LAC 1:12 were dispersed in PLA matrix using chloroform as asolvent followed by film casting. Maximum elongation at break of 220 %was obtained for 20 % GLY-LAC 1:3 incorporated in PLA matrix, which can be considered as a remarkable improvement as compared to the elongation of neat PLA matrix (4.5 %).Even though the plasticization performance ofGLY-LAC 1:3 is higher than the other mixing ratios, highermigration rate from the polymer matrix was also observed. As the mixing ratio of glycerol to lactic acid increased from 1:3 to 1:12,the elongation at break reduces by 55 % (164 %elongation at break of GLY-LAC 1:12) which is due to the increment in chain length. However, the chain length enlargement helps to reduce the migration rate. For the addition of 20 % plasticizer within the PLA matrix, as a mixing ratio increases from 1:3 to 1:12 the migration rate(at 400 min) reduces from 3.56 % and 4.25 % to 2.87 % and 3.17 % respectively at 125 °C and 150 °C.

CHAPTER ONE

1. INTRODUCTION

1.1 Back Ground of the Study

With the growing modernization of the material world of today, synthetic polymers have begun to replace natural materials in almost every location [1]. Nonetheless, due to their easy handling and good properties balance, these synthetic polymers are dependent on a wide variety of petroleum-based raw materials [2]. But the issues facing the rapid depletion of petroleum resources and increased awareness of the environmental impact on the disposal of petroleum waste and the emission of unsafe greenhouse gases inspire researchers to create bio-based and biodegradable polymers [3].For this case,the productionofpolymers from renewable resources has been one of the fastest-growing materials sectors in recent years. The ability of these materials to replace fossil-fuel-based polymers hasfuelled their rapid advancement;Biopolymers are complex molecular assemblies with a specified 3D structure. This is an essential property that makes biopolymers active molecules *in vivo*. Hence, their specified shape and structure are keys to their function. For example, haemoglobincouldnot acts as an oxygen carrier in the blood unless it isfolded in a native quaternary structure [4].

Almost all materials produced from biological sources such as vegetable oil, sugars, fats, resins, protein and amino acid can also be called biopolymers [5]. Due to their high content of functional groups that can be used for cross-linking,biopolymer have received more attention in the field of material science. Such biopolymers are polymeric macromolecules which contains monomeric unit that is bonded covalently thatgrows to become a large molecule.The potential for biocompatibility, chemical modification, gel-forming, and muco-adhesiveness areother important attractive properties of biopolymers[6]. From these properties biocompatibility of biopolymers is typically an incorporated feature and related to the structure of polymers and sources of monomers. Non-biocompatible materials cannot easily be converted into biocompatible materials; this makes biopolymers attractive for applications in pharmaceutical and medical fields.

In the polymerization process,the ability of repeated units to form covalent bonds with adjacent molecules is known as its functionality. The repeating units/monomers may have several features, which can affect the ability to interact with neighbouring molecules through different mechanisms. Each repeated unit with a configuration of two or more may produce a multitude of chemical reactions with adjacent molecules [7]. This creates a significant number of reactions that could be difficult to follow.In their polymerization method, ring-opening polymerization (ROP) and condensation polymerization are the two main mechanisms used in polymer synthesizing.

In condensation polymerization , the reaction between the repeating unit and the growing chain occurs by releasing a small molecule likewater or hydrochloric acid as a by-product [8]. This is the reason why such type of reaction is sometimes called step-growth polymerization. During condensation polymerization, any two molecular species, such as monomers, dimers, trimmers and oligomers, may react with each other in order to produce a larger molecule [9]. Polyesters, polyamides, polycarbonates proteins and polysaccharides are some examples for step-growth polymerization.

The molecular weight of polymers can determine various physical properties such as melting point, viscosity, tensile strength, toughness, thermal behaviour and chemical resistance. Universally, higher molecular weight polymers are associated with complexphysical properties, whereas lower molecular weights are associated with simplerproperties [10].Molecular weight has a great impact on the material's final application. Therefore understanding this material property means a lot to the proper material application. Since molecular weight of a given material is equal to the average weight of molecules that makes up a polymer, the chain length growth is directly related with the molecular weight increment.

Structural Complexity of Macromolecules: Pioneering scientific work by Staudinger [11], Flory [12][13], Stock Mayer[14] and others on both experimental design and statistical modelling of branched polymers established the fundamental understanding of cross-linked architectures [15] from the primary interest of modelling natural polymers such as starch. Even in the last two decades the control of hyper branched polymer (HBPs) configuration has been also one of the major aim of the researchers [16].Staudinger articulated a clear, direct, and yet somewhat subjective perspective on how big a molecule can be called macromolecular in his famous Nobel

lecture [17].“The only difference between macromolecules and the smallmolecules of low molecular substances is one of structural size. If it is desired to lay down a boundary between macromolecular and low molecular compounds - there are of course transitions linking the two groups - the substances with a molecular weight greater than ten thousand, i.e. the molecules of which consist of one thousand and more atoms, may be classified as macromolecular. Beyond roughly this size, characteristic macromolecular properties occur. So far no upper limits have been given for the size of the macromolecules.”

The probability of variations in the molecular parameters of a macromolecule (for example , chemical structure, composition, sequence, topology, conformation, etc.) increases astronomically as the size increases[18]. This means in general, it is the effects of size that distinguish macromolecules from small molecules or their counterparts of oligomeric. It is the impact of size again, that cause a major complexity and collective behaviors in macromolecules. It is also the size effects that bring specific emerging features of macromolecules that differ from small molecules counterparts[18].

Although the most prevalent configuration for macromolecules is linear architecture, it's definitely not the only one.Macromolecules are a polymer that could have also a branched or cross-linked structure subjected to theintermolecular linkages between the individual chains.Occasionally in the case of polymer designing in order to put special physical properties, it is sometimes advantageous to change the architecture of the polymer from linear to branched [19]. Changing the polymer architecture to achieve multi-arm or hyper-branched would alter its morphological and physicomechanical properties [20][16].

Biodiesel Derived Crude Glycerol: Biodiesels an alternative form of fuel which is mostly derived from the transesterification of vegetable or animal fat [21]. The conventional way to produce biodiesel involves the transesterification of triacylglycerols into fatty acid alkyl esters in the presence of an alcohol such as methanol or ethanol and a catalyst such as an alkali or acid to produce alkyl esters of fatty acid (i.e. biodiesel) and glycerol as the main by-product[22].

Chemistry of Glycerol: Glycerol is a polyol molecule with many applications in the renewable industry, Its non-toxic, biodegradable, colorless and highly stable, with a high density of 1.26 g

/cm^3, a pH of 8 to 10, a melting point of 18.2 °C, and a boiling point of 290 °C under normal atmospheric conditions. Due to its chemistry glycerol is a very useful industrial chemical that can be applicable in polymer additive, skin care application and pharmaceutical formulations as, moisturizer, emollient, thickener, dispersant and lubricant [23]. Though glycerol producing plants are closing these days, glycerol sourced from petroleum waste is in demand as a raw material including for drug production such as epichlorohydrin[24]. Traditional glycerol applications includes incorporation as an intermediate compound into the food industry, pharmaceutical and personal care products manufacturing, anti-freezers, botanical extracts, e-cigarette liquids, explosives and many other processes [25].

Lactic Acid: Lactic acid (LA) is a chemical platform that can be biotechnologically produced from agricultural residues, waste, and by-products and used for further production of biodegradable, biocompatible LA polymers [26]. LA has also been widely used in the food, cosmetic and pharmaceutical industries as a flavor, acidulant and preservative as it prevents the growth of many pathogenic microorganisms [27]. It is used in large quantities as a descaling solution but also serves as an effective exfoliant in skincare [27].

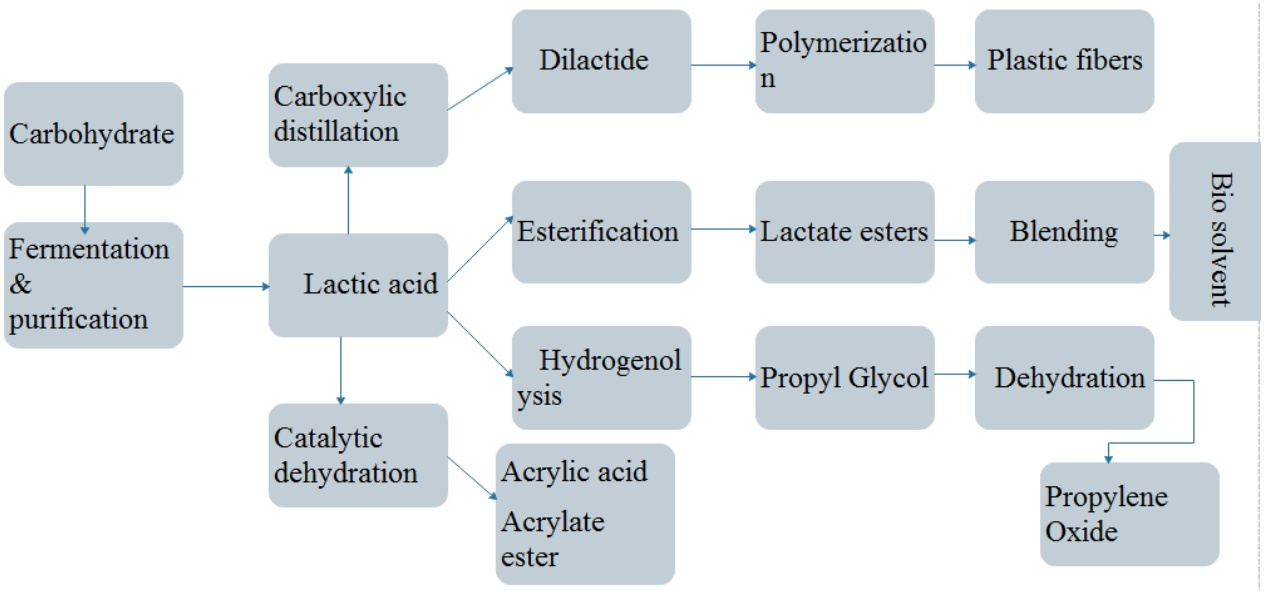


Figure 1-1:- lactic acid based products and their application [28].

1.2 Statement of the Problem

It is increasingly becoming clear that petroleum based raw materials for the polymer industry are danger to the environment and for the long term benefit to our planet [29]. This inspired, among others, the 1990’ Environmental Protection Agency's (EPA) American Pollution Prevention Act that forced companies to use bio-based starting materials, apply environmental friendly production method, and reduce by-product formation so can use raw materials efficiently [5]. Beyond the trend to reduce emissions, increasing the efficiency of processes and minimizing the production of waste, there is a strong interest in developing bio-based polymer materials (bio-polymers) that are biodegradable, biocompatible and non-toxic. A new niche of material science now exists to study these materials and their application in medicine, pharmaceuticals, cosmetic and other industries [30].

Glycerol is a major side-product of the biodiesel industry i.e. for every 9 tons of biodiesel made, 1 ton of glycerol is generated during the trans-esterification process of biodiesel production [24]. The global annual production of glycerol was estimated at 4.2 million tons in 2020, while glycerol demand was estimated at less than 3.5 million tons [31]. In this sense, the high surplus accumulation of glycerol had been a major burden on the biofuel industry and might inhibit the widespread market adoption of biofuels [32]. As many researchers agreed, the credit from the glycerol sales used to offset production costs of biodiesel. Due to this fact, glycerol is no longer a high-priced commodity. This trend leads to close the glycerol plants and open up industries that use glycerol as a starting material in different application. Therefore exploring new approaches to the biotransformation of glycerol into value-added compounds is of great importance, both for the growth of the biodiesel industry and from the environmental protection perspective.

1.3 Research questions (RQ)

RQ1. Is it possible to grow lactic acid oligomer on the three OH functional groups of glycerol to synthesize a three-armed glycerol based bio-conjugate without using any catalyst and any solvent?

RQ2. What would be the effect of temperature, reaction time and mixing ratio on the viscosity of the product.

RQ3. What would be the effect of molar ratio on the chemical and physical properties of the final product.

RQ4. Is it possible to use the synthesized product as an ointment base?

RQ5. Can it be possible to formulate herbal ointment out of the synthesized ointment base?

RQ6. Can it be possible to use the prepared conjugates as a poly-lactic acid (PLA) additive for plasticizing purpose?

1.4 Objectives

1.4.1 General Objectives:

The general objective of this research work includes the development of three-armed GLY-LAC bio-conjugate that tbe used for a targeted application as an ointment base matrix and plasticizer.

1.4.2 Specific Objectives

In order to achieve the aforementioned general objective, the following specific objectives are articulated as follow;

- Synthesize and characterize three-armed GLY-LAC bio-conjugate from glycerol and lactic acid monomer.
- Investigate the possibility of the synthesized three-armed GLY-LAC bio-conjugate to be used as an Ointment base matrix using extract of *C. Macrostachyus* leave as an active component.
- Investigate the plasticizing effect of the synthesized three-armed GLY-LAC bio-conjugate and study the mechanical and migration properties.

1.5 Significance of the Study

Due to the dramatic growth of biodiesel industries today, Conversion of glycerol into valuable products has been a very interested area in recent years.Glycerol is bio-based, compatible, non-toxic and a less cost industrial fluid. However, most of the conversion methods of glycerol are catalytic way; proceeded in the presence of catalyst, solvents and initiator.Due to the cost of purification and the presence of such additive the final product of it will be expensive, environmental harmful and non-convenience for human. On the other hand, after the production, for a material which are mainly based on their viscosity/Molecular weight it is possible to adjust the viscosity of a given product by adding different thickening agents, but this confers different drawbacks that are time-consuming dissolution and negative impact on the linearity of the material structure [33].

If we see most of production our daily activities, starting from the Cosmetics/the daily grooming agent to the plastics they contain a toxic chemical. This toxicity may not be raised from the raw materials but it may be comes from the method of the production or from the additives that are used to modify the product for a specific application.

PLA derived from renewable resources, such as cornstarch, tapioca roots, or sugarcane and it has been extensively studied for a number of applications such as packaging, orthopedics, drug delivery, sutures, and scaffolds and other biomedical applications. Due to this, as much as possible this Polymer has to be free from any dangerous chemical. In this research by using a green synthesizing method, a three-armed glycerol based bio-conjugate is developed, for an Ointment base matrix and for PLA plasticizing.

1.6 Scope of the Study

The following points are used to explain the scopes of this research. This research is mainly focused on the-

- Preparation of the three-armed GLY-LAC bio-conjugates, from the technical grades glycerol and lactic acidby differentiated the reaction conditions such as reaction temperature, mixing ratio and reaction time.
- Study of the physical (viscosity, color and smell) and chemical properties (chemical structures, chain length and thermal stability) of the prepared conjugates
- Extracting of the crude oil of croton macrostachiyus from the leave part
- Applying the crude oil of croton macrostachiyus (CM) to the prepared three-armed conjugate to use as anti-bacterial ointment
- Characterizing the organolepetic properties, PHs, spreadablities, and viscosities of the formulated anti-bacterial ointment
- Investigate the plasticizing and migration effect of the prepared conjugate in the PLA polymer matrix.

CHAPTER TWO

2. LITERACHURE REVIEW

2.1 Introduction

2.1.1 Valorization of Glycerol

Due to its chemistry, reactivity, non-toxicity, compatibility and low cost property, glycerol is a very important industrial fluid. The tri-functional property in its structure is responsible for the wide range of reactivity[34].For example glycerol can be converted to Organic acids such as succinic acid, fumaric acid, L-malic acid, L-lactic acid, and D-lactic acid via a biological catalysis way[29]. Sometimes, Glycerol may not necessarily convert but they can be used as a raw material for a number of polymer syntheses (with or without slight chemical change). Depending on the synthesizing method chosen the produced product may have linear, branched or dendritic structures. Researchers now a days has focused on synthesizing 1,2-Linear Poly(Glycerol Ether), 1,2-Linear Poly(Glycerol Carbonate), 1,3-Linear Poly(Glycerol Carbonate) and polyesters via a polycondensation reactions between glycerol and other linear polymers [35].

2.1.2 Catalytic Conversion of Glycerol

Glycerol can be converted to a valuable product via a different types of selective oxidation methods, and almost all derivatives of glycerol oxygenate have a valuable practical importance. However, the equal reactivity of the three OH's in the glycerol molecule makes it difficult to peak oxidation. Glyceric acid and tartronic acid were produced in the oxidation of primary hydroxyl group in the presence of Pt/C at 50 °C[36]. Whereas the secondary hydroxyl group oxidation of glycerol yields a also commercially valuable compound dihydroxyacetone (DHA)in the presence of catalytic TEMPO [36]. On the other hand, the oxidation of all three classes of hydroxyl produces a highly functionalized ketomalonic (or mesoxalic) acid in the presence of CeBiPt/C catalyst either in acidic or basic conditions[37].

Glycerol monomers are polymerized at higher temperature and they may be etherified to fuel oxygenates, due to this property they cannot be directly applied to the fuel. By putting this problem under consideration, Alkyl ethers can be quickly synthesized by reacting isobutylene with glycerol in the presence of an acid catalyst[38]. The yield is maximized by reacting in a two-phase reaction cycle, one phase being a glycerol-rich polar phase (containing an acid catalyst) and the other phase being an olefin-rich hydrocarbon phase from which the product ethers can be easily isolated [39]. In the case of glycerol hydrogenolysis (i.e. dehydration folowded by hydrogenation), a copper chromate catalyst ($CuCr_2O_4$) at 200°C and less than 10 bar (compared to around 260°C and more than 150 bar for other systems) coupled with reactive distillation is used to produce Propylene Glycol. Propylene Glycol is an antifreeze material (70% propylene glycol and 30% glycerol) that can be made, processed and directly marketed by existing biodiesel plants [24].

On the other way from both an industrial and an innovation point of view, the major achievement of the new glycerol chemistry is the reforming process by using a platinum-based catalyst in a single reactor to convert glycerol in the aqueous phase into hydrogen and carbon monoxide (synthesis gas or syngas) [24]. This synthetic gas formation is important for the future of bio refineries as Fischer Tropsch and methanol syntheses can use syngas as a source of fuels and chemicals. The Glycerol dehydration which can be done by the passage of a glycerol-water gas mixture at 250 to 340 °C over an acidic solid catalyst with a Hammett acidity feature of less than -2 results in the total transformation of glycerol into acrolein. Acrolein is a flexible solvent used in the manufacture of acrylic acid esters, super absorber polymers, and detergents by the chemical industry. Glycerol can also be used as a feedstock for the fermentation of 1,3-propanediol.

Glycerol carbonate (4-hydroxymethyl-1,3-dioxolan-2-one) is a relatively new chemical industry material with a great potential as a novel part of gas-separation membranes, a solvent for several material forms, and a biolubricant due to its adhesion to metallic surfaces and resistance to oxidation, hydrolysis and stress. It can be prepared in a lipase-catalyzed reaction directly and in high yield from renewable glycerol and dimethyl carbonate [24].

Epichlorohydrinis by itself had commercially synthesized from glycerol by a catalytic reaction with HCl followed by dehydrochlorination with NaOH. The glycerol-based process (Epicerol)

involves the direct synthesis of dichloropropanol from glycerin and hydrochloric acid, an intermediate product. Natural glycerol is therefore used as a replacement for the propylene feedstock used in the conventional production process of epichlorohydrin, which includes the formation of allyl chloride by propylene reaction with Cl_2 [24].

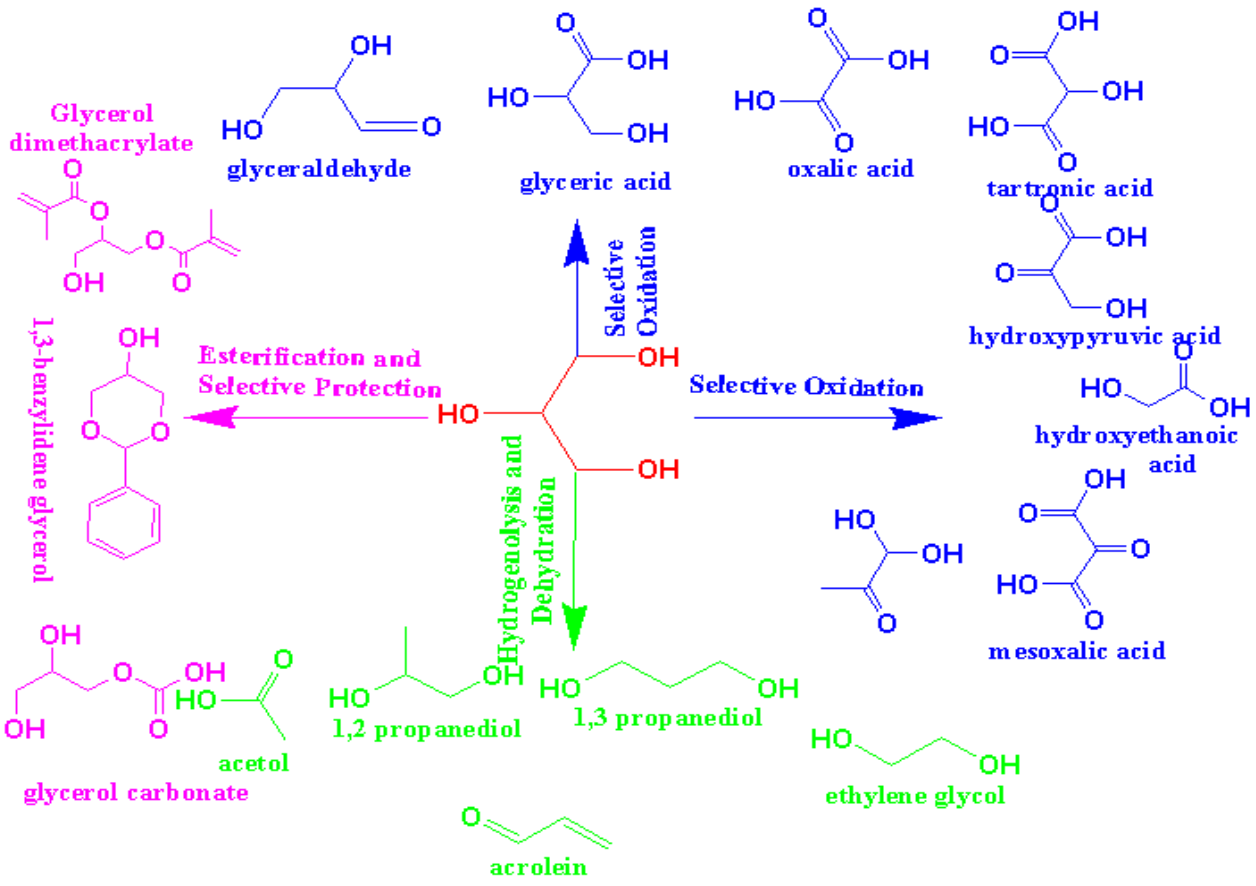


Figure 2-1:- Chemo selective conversion of glycerol into value-added compounds via different catalytic pathways.

Different types of multivalent metal salt were screened as catalyst for esterification reaction between alcohol and the carboxylic acid. Their basic yet versatile chemistry of catalytic produced esters can used in such diverse applications in the manufacture of lubricants, plasticizers, paints, solvents, flavors, pharmaceuticals, cosmetics, and liquid fuels [40]. Most of them are produced via a method of condensation, which takes an important industrial significance.

Chemical compounds derived from the arrangement of monoesters are seldom used as pure lubricants, more as solvents or dispersants. Monoesters are formed from alcohols of a mono carboxylic acid and a mono functional group in the presence of solid acid catalyst. Diesters by them self are produced from a dicarboxylic acid, usually adipaic or sebacaic acid and two alcohol molecules, or diesters can also be produced from the alcohol reaction with two groups of hydroxyl and a mono functional carboxylic acid. For example, the reaction product of neo pentylglycole (3,3dimethyl propane – 1, 4 diol) with oleic acid is important for lubrication technologies used as a friction reducer and for minimal lubrication systems [41].

Tri-Esters are the other group of compound that mainly created by the reaction of trivalent alcohols with monocarboxylic acids, they are multi-purpose and different in types [41]. Esters derived from polyols can also be called tri-ester and this group of compounds is generally more branched than those derived from mono and di–alcohols. Due to their branched structure they may be quite large molecules [42]. Polyols are multifunctional alcohols with branched structures where every arm ends with a group of –OH. These free –OH groups were used to build a variety of polymer structures starting from cross-linked to linear to star-shaped or multi-armed[43].

2.2 Skin Care Products

Cosmetics and products for skin care are part of daily grooming. The skin, the body's largest organ, separates and protects the internal environment from the external environment. Skin due to the form of active ingredients found within, it is essential for good health to protect and preserve the skin. Here the point is the use of cosmetics and beauty products will not improve or cure the skin; such items are only intended to cover and beautify. There are items for skin care that go beyond coloring and skin decoration. Due to the type of functional ingredients they contain, cosmeceuticals that are cosmetic products with medicinal or drug-like benefits may affect the

skin's biological function. Therefore a wide panel of skin care products for therapeutic purposes is thus developed [44].

Cosmeceuticals; are cosmetic-pharmaceutical hybrids products aimed at enhancing beauty through ingredients that provide additional function or benefit related to health. They are used topically as cosmetics, but they contain ingredients that influence the biological function of the skin [45]. Extracts from grape seeds, aloe Vera, mushrooms, olive oil, green tea, licorice, Arabica coffee, and extracts from coffee berry some examples of cosmeceutical ingredients[46]. However, The main desirable characteristics of cosmeceutical agents are their efficacy healthiness, stable formulation, novelty, patent protection, skin metabolism, and economical manufacturing [47].Different types of topical dermatological products are available for skin care, ranging from solids to liquids and the rest of them are semi-solids, including creams, gels, lotions and ointments.

Ointments are typically formulated from polyols, Fats, vegetable oils, lanolin, Vaseline, paraffin, beeswax, glycols, and alcohols [48]. All ointments consist of a base that serves primarily as a drug carrier or vehicle. As many researches on the ointment formulation indicate that the performance of the ointment had controlled by the nature of the base, hence, a very important aspect of formulation is the selection of the ointment base[49]. For this reason, so many researchers have been focused on the preparation of different types Ointment base.

2.2.2 Ointment Base (Vehicles)

Traditionally, ointment base was used as a general class name for all external-use semisolids and for the subclass, oleaginous semisolids. For instance, USP 31 Chapter 1151 classified ointments generally as "semi-solid preparations for external use" for the skin or mucus membranes [50].However, more precisely, pharmaceutical manufacturers use the word ointment to indicate that a drug incorporated into an oleaginous ointment base. They serve as a medicinal agent vehicle for topical application to treat, protect and emulsify the skin[51]. For example, the term Hydrocortisone ointment suggests that hydrocortisone incorporated into a semisolid base of the oil form.

The ointment base should have a desirable properties of chemical and physical stability under normal condition of use and storage, compatible with the skin, free from any objectionable odor, non-toxic, non-sensitizing, non-irritating, easy to apply and non-greasy and remain in contact with the skin until removal is desired [52]. Another requirement for successful pharmacotherapy with absorptive vehicle-based ointments is the absence of rapid loss of volatile components (i.e. water) from the matrix.

There are four general classes of ointment bases, the oleaginous or hydrocarbon type, absorption, water-removable and water-soluble. The main examples for the Oleaginous or hydrocarbon type Ointment base are the white wax and white petrolatum. Petrolatum and white petrolatum are a mixture of purified semisolid saturated hydrocarbons extracted from petroleum. Petrolatum is a translucent yellowish colored compound. White petrolatum a soft ointment base on the other hand has undergone additional treatment to decolorize. Both are tasteless, odorless and greasy to the touch. They have a 38 °C to 60 C ° melting point range, smooth texture, stable and practically insoluble in water, hot or cold alcohol, acetone, and glycerin. The petrolatum bases are faces a difficulty of incompatibilities. Because the purified forms are more exposed to oxidation, to reduce the incompatibility addition of small amounts of antioxidants is needed. Petrolatum does not mix with aqueous or hydro alcoholic solutions. White petrolatum is an, all-purpose, It has an incorporates powders easily, and spreads evenly on the skin. It is used both by itself and as a major component of combination ointment bases. If a stiffer base is desired, a portion of White Wax may be added.

Glogowskiet.al's research focuses on the morphological parameters (viscosity, viscous elasticity and rate of volatile component loss) of salicylic and boric acid-containing magisterial ointments. By using two different preparation techniques and by varying the composition of the formulae they were tried to adjust the viscosity and thermal properties of the ointment base. It was shown that conventionally prepared salicyl-containing formulae were characterized by better viscous elasticity than the preparations made with an aid of an unguator. Moreover, the ointments based on an absorptive vehicle (containing lanolin) were distinguished by similar viscous elasticity irrespective of the preparation technique. The volatile (water) component loss rate from ointments containing a 3% boric acid solution is no more than 2.3 % of their mass when it

exposed to 37 °C. but the main constituents of this base are White petrolatum, yellow petrolatum, eucerin and anhydrous lanolin. To make the viscosity of the ointment more stable 3 % solution of Boric acid was added [49]. From all formulations, F4 (composed of anhydrous lanolin, yellow petrolatum and zinc oxide) and F6 (composed of anhydrous lanolin, yellow petrolatum) have a higher viscosity. The higher viscosity that can achieve in this study was 1,033,760Cp.

The other researchers who studied about formulation and evaluation of levofloxacin anti-bacterial ointmentareS. K. Jakeer Hassan et.al., Under this investigation, the levofloxacin ointment was formulated from two different bases; Oleaginous and Emulsion bases. The Oleaginous base synthesized from white bees wax and petrolatum whereas emulsion base contains the methyl paraben, propyl paraben, sodium lauryl sulfate, propylene glycol, stearyl alcohol white, white petrolatum and purified water. Fusion method was used to formulate the desired ointment. The physical parameters of the formulated ointment were examined visually. The ointment formulated from oleaginous base have a light yellowish color, where the ointment formulated from emulsion base is creamy in color. The parallel plate method was used to determine the spreadability, and it was6 and 7 for the oleaginous based and emulsion based ointment respectively. The higher viscosity that achieve in this experiment is 96,600 Cp with a pH of 5.1. Viscosity of the formulated ointment was measured by brook field viscometer using spindle number **'4 '.** both the oleaginous and emulsion based ointment have 0.28 g loss on drying [51].

Avish D Maru, Swaroop R Lahotiwererereplaced the white bees' wax of ointment by sunflower wax and formulate. In this formulation, 3% of sunflower wax was mixed with 97 % of white petrolatum by fusion method. The formulated ointment is used for topical and systemic delivery of active salicylic acid ingredient. However, the main ingredients of the formulation are White petrolatum, White beeswax, Sunflower wax, Wool fat, hard paraffin and Cetostearyl alcohol. The components of the base were put together in a melting pan in the fusion method and allowed to melt together at 70 °C. The ingredients were gently stirred after melting, holding the temperature at 70 °C for around 5 minutes and then cooled to 40 °C with continuous stirring.

Ointments were then blended until a smooth consistency was obtained and stored at room temperature (25 °C) and used for further analysis. The formulation of an ointment containing 2% salicylic acid was carried out by incorporating 2 g of the drug into the optimized F3 formulation by grinding on an ointment slab with a spatula. An optimized formulation, whichwas coded as F3 was chosen, based on the good viscosity, strength, and spreadability of the formulated ointment. The PH of the formulation was found between 6.80±0.152 and 7.02±0.174, the viscosity is found in the range of 2314±6.13 to 2851±9.93 Cps, with a spreadability of102.91±4.12. The organolepetic results showed that the ointments had a fine, attractive appearance and a smooth texture, and they were all homogeneous without any signs of phase separation. The color was white with an aromatic odor [53]. The summary of important research articles is represented in the table 2-1.

Table 2-1Literature review summary of the formulated ointments

Ointment Base Type	**Formulations**	**Method**	**Advantage**	**Disadvantage**
		Hydrocarbon Oleaginous Ointment Base		
White Ointment[54]	✓ 50 g White Wax ✓ 950gWhite Petrolatum	Fusion Method	✓ Inexpensive ✓ Nonreactive ✓ Nonirritating ✓ Good emollient, protective, and occlusive properties	✓ greasy nature ✓ are not removed easily with washing (may be removedusing mineral oil) ✓ Do not absorb or mix with aqueous solutions.(aqueous skin secretions do not readily dissipate)

Absorption Bases				
Hydrophilic Petrolatum (Anhydrous) [54].	✓ 30g Cholesterol ✓ 30g Stearyl Alcohol ✓ 80 g White Wax ✓ 860g White Petrolatum	Fusion Method	✓ Protectant ✓ Can absorb water solutions ✓ Vehicle for aqueous solids drugs ✓ Emollient ✓ Occlusive ✓ Drug release poor but betterfor hydrophobic drugs.	✓ greasy nature ✓ sticky and have a mildly unpleasant odor ✓ Not easily removed with washing ✓ sensitive to hydrolysis ✓ Requires preservative to microbial growth
Water-Soluble Base				
Polyethylene Glycol Ointment (Carbowax)[55]	✓ 400 g Polyethylene Glycol 3350 ✓ 600 g Polyethylene Glycol 400	Fusion Method	✓ greaseless ✓ Available in various Mwt range of 200 to 8,000, With a properties of clear viscous liquids, soft solid and waxy solid ✓ suppository bases ✓ solvents ✓ viscosity-increasing agents ✓ plasticizers, ✓ lubricants in tablet and capsule	✓ Can be irritating ✓ No emollient properties ✓ they faces compatibility problems and stability problems associatedwith water, and needs preservative

			making	
Magisterial ointment[49]	✓ anhydrous lanolin ✓ yellow petrolatum ✓ zinc oxide ✓ Precipitated sulfur ✓ Boric acid solution (3%) ✓ Salicylic acid	Conventional and unguator preparation method	✓ Can adjust the viscosity ✓ Can adjust thermal property ✓ Have viscosity of 1,033,760 Cp.	✓ Uses additive ✓ 3% solution of Boric acid Stabilizer ✓ The main constituents of this base are contain petroleum based.
Levofloxacin [51]	✓ white bees wax ✓ petrolatum ✓ methyl paraben ✓ propyl paraben ✓ sodium lauryl sulfate ✓ propylene glycol ✓ stearyl alcohol white ✓ white petrolatum ✓ Purified water	Fusion method	96,600 Cp	Uses additive ✓ Colorants ✓ Stabilizers ✓ Petroleum based
Sunflower wax based ointment [53]	✓ White petrolatum ✓ White beeswax ✓ Sunflower wax ✓ Wool fat ✓ hard paraffin ✓ Cetestearyl alcohol	Fusion method	Can adjust the viscosity ViscosityIn the range of 2314±6.13 to 2851±9.93 Cps	Petroleum based

2.3 Plasticizers

Plasticizers are a class of non-volatile compound which have a lower molecular weight and used in many polymer industries as an additive to improve the strength and ductility of a given polymer by lowering the glass transition temperature [56]. Plasticizers have a non-volatility, higher boiling point and low molecular weight properties and it improves the processability, flexibility and stretching ability of the polymer. They are also used to decrease the temperature of the manufacturing, glass transition (Tg) and the sticking in molds, and increase the wetting, durability, Elongation [57]. A given plasticizer had been achieved its target by modifying the mechanical properties making the films more ductile, reducing the product's melt viscosity and Tg without altering the plasticized material 's fundamental chemical character [58]. These additives have two functions to assist in the production of polymers and to change the final product properties. A plasticizer having low molecular weight and small number of polar groups is expected to have higher flexibility and plasticization[59].

The extent of plasticization of polymer is highly dependent on superstructure of plasticizer such as molecular weight, chemical composition and functional group [59][60]. There are so many parameters that can be listed out to be a sign of effective plasticizing performance, Film thickness, moisture content, solubility, water absorption and density of plasticized film [61]. But from those criteria’s the following three parameters are a pillarsfor choosing a plasticizer: its compatibility with the polymer, its effectiveness in plasticizing the polymer, and its permanence in the polymer matrix [62].

Since migration is one of the main problems of plasticizers, they should have low vapor pressure and low rate of diffusion in polymer, in order to keep their permanence in the matrix polymer. But different strategies can be used to reduce the migration of plasticizers among them, increasing of the molecular weight of the plasticizer, the addition of the mineral nano particles and hardly work on the compatibility of the polymer with the plasticizer are some known solutions. As many researches indicate that when the Mw of plasticizers increase the migration from the polymer matrix will decreased. On the Other hand the plasticizers may lose their miscibility with the polymer matrix as the molecular weight increases to a certain value [63]. The other disadvantages

of higher Mwt plasticizer is they needs an elevated temperature to mix with the polymer matrix, which can also be called waste of energy [64].Polarity, dielectric constant, solubility and hydrogen bonding are some factors that can affect compatibility [65], [66].

There are five steps in the incorporation of plasticizers in the polymer material.The first step is adsorption step where the polymer material/ resin is mixed with plasticizer; the second step is adhesion step in which the plasticizer penetrates the resin particles and swells them; the third one is the absorption step at which the polar groups in the polymer material are liberated from each other; the fourth step is intermolecular plasticizing in which the polar groups of plasticizers interact with polar groups on the polymer, the last and fifth step is the intermolecular plasticizing step which helps the composition of the material to re-established, with complete retention of plasticizer.

The first two steps are called physical plasticization step and the rate at which step 2 occurs depends on the viscosity of the plasticizer, degree of branching, size of the resin pore and free volume, and size of the particle[64]. Since the rate at which their process occurs depends on the chemical property of the molecule, polarity, free volume, molecular composition and molecular weight, the next two steps are classified under the chemical plasticization step.

The lubrication theory, gel theory, free volume theory and thermodynamic or mechanistic theory are theories of the plasticizer action. The lubrication theory is based on the assumption that once the plasticizers is incorporated to the polymer, the plasticizer molecule shield and lubricate the chain of the polymer and prevent the reformation of the rigid network of the polymer. In the gel theory, plasticizer breaks the resin-resin attachment by masking the center of attachment from each other and prevents their reformation. The free volume theory is all about the measure of internal space available in a polymer for the movement of the polymer chain that imparts flexibility to the polymer matrix. This process can facilitate the movement of plasticizer molecule so as to the flexibility. As indicated on the free volume theory the role of plasticizer is increasing the free volume of polymers and prevents the interaction between neighboring polymer chains. The free volume increment is arises from the motion of the chain ends, side chains or the main chains. Plasticizer having a lower molecular weight than the polymer has the ability to impart higher free volume per volume of the material because there is an increase in the proportion of

end groups and the plasticizer has a lower glass transition temperature than the polymer. From the observation of migration the thermodynamics theory states that plasticizer molecules are not bound permanently to the polymer, rather they exist in a dynamic equilibrium between salvation and desolvation of the polymer chains by plasticizer.

It was in 1920 that phatalic acid esters first found applications as plasticizers and remained the largest class of plasticizers for packaging plastic polymers in the 21st century. From those phatalic esters Di(2-Ethylhexyl) phthalate (DEHP) which also known as dioctyl phthalate (DOP), was one of the most widely used plasticizers since the 1930s [67]. However, this dioctyl phatalate plasticizers are petroleum derived, non-biodegradable, toxic and dangerous for human health, can even leads to cancer. Since packaging Plastics are huge sources of landfill waste and is eaten regularly by numerous sea and land animals, leading to fatal consequences, due to these bio-based plasticizers are very important today.

Specially the **Poly (lactic acid)**Polyesters materials synthesized from renewable resources under environmental friendly reaction conditions[68] and should be free from any toxic catalyst and additive at the time of production and plasticization since it used as a food packaging and biomedical application.

2.3.1 Poly (Lactic Acid) PLA

Poly lactic acids are hydrolytically degradable, good transparent, bio based, biodegradable and renewable polymer that could also act as an alternative plastic to replace conventional petroleum based plastic (PET) [69][70]. PLA is derived from renewable resources, such as cornstarch, tapioca roots, or sugarcane and has been extensively used in a number of applications such as packaging, orthopedics, drug delivery, sutures, scaffolds and other biomedical applications. Nevertheless PLA have faces some structural drawbacks like brittleness and low crystal forming ability.

In order to meet the performance requirements, PLA can be synthesized by various methods such as poly-condensation reaction, ring opening polymerization (ROP) and solid state polymerization using different catalysts [71]. To improve its toughening property, PLA has been mixed with

poly(ethylene oxide)[72],poly(ε-caprolactone), polyethylene[73], polyurethane[74],poly(butylene succinate)[75], poly(butylene adipate-co-terephthalate), polyamide[76], natural rubber[77] and bio-based polyesters[78], [79]. Plasticizers are preferentially used among the numerous toughening agents to reduce the brittleness of PLA. For example, a number of citrate esters [65]have been used to modify PLA, including triethyl citrate (TEC), tributyl citrate (TBC), acetyltriethyl citrate (ATEC), and acetyltributyl citrate (ATBC)[80]. triacetin, glycerol [81], oligomeric lactic acid [82] , malonate oligomers, poly(ethylene glycol), poly(propylene glycol), and epoxidized vegetable oils are some other typical examples of PLA plasticizers.

In their finding Gildas Coativy, et.al reports that the polycondensation of biodiesel waste glycerol with di acid such as sebacic acid leads to occur a though polyester material, which cannot even melt, with PLA. To overcome the very high toughness problem of this material many researchers have been done by focusing on its viscosity. Gu.et.al in their own work tries to stop the gel formation by quenching the reaction before the gel formation (3pa.s) [83]. The other solution for this problem is the addition of mono acids to the reaction, Waig Fang et.al can convert the multifunctional alcohol to diol by adding fatty acids then polymerize with diacids to avoid the gelation[84]. In this work glycerol and sebasic acid was reacted at 180 °C for 3.45 hr± 15 min to obtain a yellowish translucent material. The final product in this case was soluble in THF, which is the indication of non-crosslinkablity. This indicates that if the property of trifunctional glycerol does not manage wisely it forms a highly cross-linked and rigid material that may difficult to understand.

Fatimat Oluwatoyin Bakare et.al [85]was also investigate the direct polycondensation reaction of glycerol and lactic acid to synthesize deferent types of Glycerol based oligomers. They were also got the effect of LA chain length on the viscosity of the resin. Different viscosities 1.09, 5000, and 600,000 for the LA chain length of 3, 7 and 10 were reported. Nima Esmaelli et.al was studied the synthesizing and characterization of methacrylate branching PLA by ROP. One of a great investigation from this paper is the effect of hydroxyl group number of the core molecule on the properties of the final product[86]. Three different core molecules were selected for this study, Ethyl glycol, glycerol and erythritol with two, three and four hydroxyl group. The reaction was performed by ROP method, for 1 to 6 mixing glycerol to lactic acid ratio in the presence of 0.1 wt

% (II) -2- ethylhexanoate with 50 g toluene. The temperature was started from 150 ℃ for 2 hr, and raised to 170 ℃ for 1 hr then finally to 190 ℃ for 1 hr. from their NMR data the author reported that the absence of peaks at 63-64 ppm is the Indication for the absence of unreacted, EG,GLY and ER. From all samples the glycerol based product is more viscose (43.8 pa.s) with a four lactic acid unit in its chain. Thermal stability of glycerol-based product is reported as 5 % wt loss at 260 ℃ and 1 wt % loss at 600 ℃.

In their study on anaerobic adhesive production, Nasrin, Moini et.al have been first dilutedLactic acid and Glycerol with toluene then reacted via condensation reaction, in the presence of 0.15 wt% PISA catalyst under toluene reflux. The obtained chain length from this experiment was four. In addition, different viscosity in the range (from 20Cp to 510 cp) was obtained.

As Biela et al. reported their study, in an atmosphere of dry nitrogen, the lactide to THMP mole-to-mole ratio was 20:1, and dry chlorobenzenewas added. For homogenization, before cooling to 120 ° C the reactants were heated to 150 ° C and after 5 min, Bi(OAc)3 was applied with a ratio of 0.02 mole to THMP mole [87], [88]. The same core molecule, THMP, was used in another related study at 120 ° C with $Sn(Oct)_2$ as the initiator in which lactide was polymerized with the polyol[89]. Star-shaped LA polymer (S-LA polymers) with three core molecules of hydroxyl groups has been also synthesized by lactide and glycerol reactions through ROP method using pressurized ampoules in the presence of $Sn(Oct)_2$ or tetraphenyltin as catalysts with at temperature of 130°C in the duration of 4 days [90]. Bakere et al. were synthesized an S-LA thermoset resin through a two-stage process: a direct condensation reaction of LA with glycerol and end functionalization of the oligomer with methacrylic anhydride (MAAH). The condensation reaction was performed in the presence of toluene (reflux from the apparatus as an auxiliary solvent for water removal) containing 0.1wt% of methanesulphonic acid (catalyst) in Azeotropic distillation apparatus. The temperature was set for two hours at 145°C, for another two hours at 165°C and one more hour temperature of at 195°C. For the end-functionalization stage, 3.3 moles MAAH per mole of glycerol was added under a nitrogen atmosphere in the presence of 0.1 wt% of hydroquinone as the stabilizer. Thereafter the resin was purified from the remaining water/toluene and the resulting MAA by a rotary evaporator at 13 mbar at 60 ° C with a duration time of 2 hours [85]. When the mole-to-mole ratio of lactide to glycerol was below

20:1 the obtained polymer became amorphous. And there obtain a semi crystalline thermoplastic when the mole to mole ratio of lactide to glycerol is higher than 20:1. The molecular weight of the final polymer is usually proportionally decreased to the glycerol content in the polymer [90].

In more recent research, tri (hydroxymethyl) benzene, used as the core molecule using an$Sn(Oct)_2$ catalyst, for producing S-LA oligomers with 10 lactide monomer units arms. Under the nitrogen atmosphere, the polymerization reaction was conducted at 125°C for 8 h. The cold methanol precipitation was used to isolate the polymers from the reactants [91].

The use of a central molecule with adjustable functional groups in the center of the S-LA offers the unique opportunity to design and control the resin's properties. More hydroxyl groups of the central molecule are assumed to have a stronger expanded network for the product. On the other hand, increasing the number of core molecule hydroxyl groups in the S-shaped architecture results in lower conversion rates, during the polycondensation process [85][92]. Ultimately, the core molecule's unsaturated hydroxyl groups increase the hydrophilicity of the resin developed, making the resin more hydrophilic compatible. The advantage of these material systems over other systems is that by modifying the chemical structure or altering the crosslinking length, these resins may be optimized for certain functionality. In addition, the properties of polymers can be further modified by modifying the structure and portion of the block units of the copolymer chain. By using longer arms that will serve as plasticizers, the size of the voids in the network can be increased and the hydrophilic monomers in copolymers can be adapted to the hydrophilicity of the polymer network. As many researchers agreed that the length of the arm in the star-shaped oligomer is directly proportional to the total number of dipole-dipole interaction so as to the resin viscosity [93], [94].

Generally, catalytic conversion of glycerol to other industrial chemical is used widely, however green way of synthesizing glycerol based macromolecules with branched structure can have the possibility to be used as a bio based macromolecule targeting different engineering and medical applications. Additionally, it can also be applied as a plasticizer in order to enhance the flexibility of different polymer matrix. In the recent years, development of fully bio based materials has the core research directions. Among this materials, development of fully biodegradable and compatible structurally controlled viscoelastic materials are need in the development base matrix

for skin care products as well for polymer additives. This research work focuses on the development of three armed glycerol based product using lactic acid as growing chain for the intended use of ointment base matrix and poly(lactic acid) plasticizer.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Chapter Overview

Starting from the preparation of GLY-LAC bio-conjugate up to the application of targeted purpose there was a number of materials and consumables, Different-synthesizing methods and characterization techniques were involved during the experimental work. Therefore introducing the materials and methods of the research work and material characterizations will be the role of this chapter. In first section of the chapter the materials and consumables of the experimental work was explained briefly. In the second section the whole methods of the research were described step by step and the last but not the least section of this chapter is very important part of the research which is the material characterization section.

3.2 Materials and consumables

L-lactic acid (88% aqueous solution), glycerol (99%), film grade Luminy LX175 PLA, *Croton macrostachyus* (CM). distilled water, n-hexane, chloroform and methanol was purchased from the local market. Hydrochloric acid, potassium hydroxide and phenophtaline were used from the laboratory. This chemicals and materials are listed below the table in well-organized way.

Table 3-1Summary of Materials and Consumables for the Experimental Work

Final Products	Chemicals	Equipments/Materials
Three armed GLY-LAC based bio-conjugate preparation	L-lactic acid (88% aqueous solution) and glycerol.	500 ml round bottom flask, measuring cylinder, Temperature controlled reactor, horizontal condenser, chiller, toss and ice bath
Free acid value determination test (Titration).	Methanol, chloroform,distilled water, potassium hydroxide and phenophtaline.	Measuring balance, measuring sylinder, erlimiyer flask, dropper and pepete.
Crude oil extraction from croton macrostachiyus (CM)	Ethanol, hexane and dried leaf powder of croton macrostachiyus (CM)	Erlimiyer flask, round bottom flask, filter paper, rotary evaporator chiller and Condenser.
Formulation of anti-microbial ointiment	crotonmacrostachiyus (CM) extracted crude oil and GLY-LACbioconjugate.	Petridishes, spatula and hot palte.
Preparation of GLY-LAC plasticized PLA film.	Film grade PLA, chloroform, GLY-LAC Bioconjugate and wiper.	15 cm glass pertidishes, cardboard covers and hot plate with magnetic stireer.

3.3 Methods

3.3.1 Synthesis of Lactic Acid Oligomer

A 500mL one-necked round bottom flask was placed in the reactor equipped with a horizontal condenser. 300ml (252g)of L-lactic acid was charged into the flask and temperature was raised to 190 °C in order to remove water through the connected condenser for an optimized time (1, 3, 5 and 8 hr). Different molecular weight oligomers was prepared at three different temperatures (150, 170 and 190°C) and the molecular weight will be estimated using intrinsic viscosity. The formed LA oligomer may have contains a free LA monomers, dimmers, trimers, tetramers and pre-polymers.

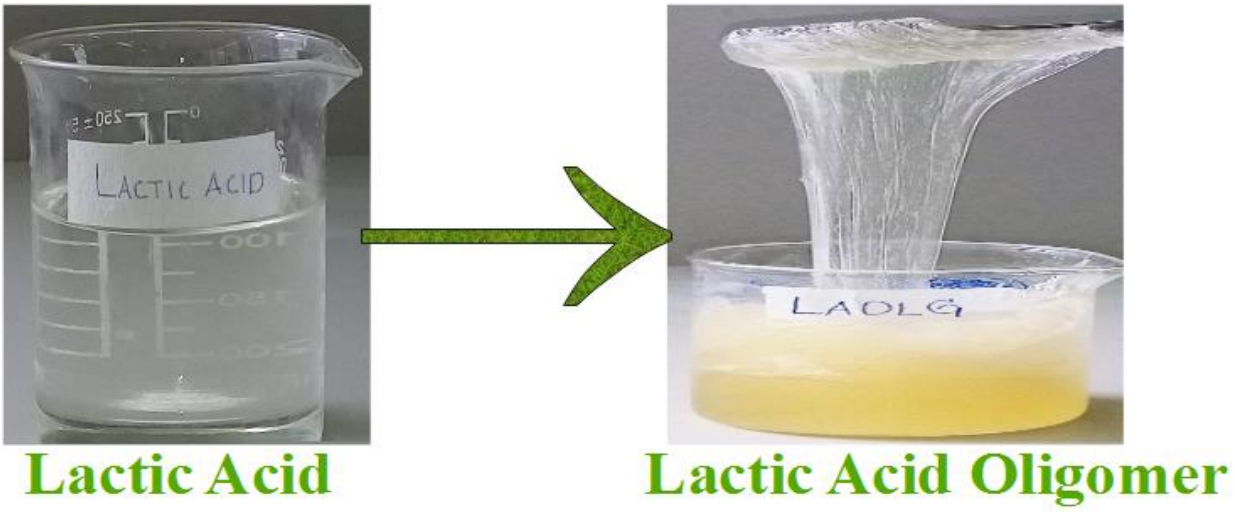


Figure 3-1Image of lactic acid monomer and oligomer

3.3.2 Synthesis and characterization of the Three Armed GLY-LAC Bio-conjugates

The esterification of Glycerol (99 %) and Lactic acid (88 %) via a condensation reaction was performed in a Temperature controlled heating oven with four different molar contents of lactic acid and glycerol. The two reactants were added into one necked round bottom flask (RBF) equipped with horizontal condenser for water segregation and was placed in temperature-

controlled oven at a temperature of 190 ℃ and reaction time of 8 hr. The reaction condition was selected based on the preliminary investigation.

Table 3-2Reaction conditions for the GLY-LAC bio-conjugate synthesis

Temperature (°C)	Time (hr)	Molar ratio	Product
190	8	3	GLY-LAC 1:3
190	8	6	GLY-LAC 1:6
190	8	9	GLY-LAC 1:9
190	8	12	GLY-LAC 1:12

In the esterification of the three-hydroxyl groups in Glycerol molecule, there may involves three moles of lactic acid. For the commercially available 99 % pure glycerol,1mole is equal to 74 ml of liquid glycerol. Whereas the commercially available 88 % 3 moles of lactic acid is equal to 3 (84.879 ml) = 254.636 m. Therefore, in the case of GLY-LAC 1:3 bio-conjugate synthesis 74 ml of glycerol was reacted with 255 ml of lactic acid at a temperature of 190 °C for an 8 hr retention time. The water that escaped from the reaction mixture was collected by using a water container.

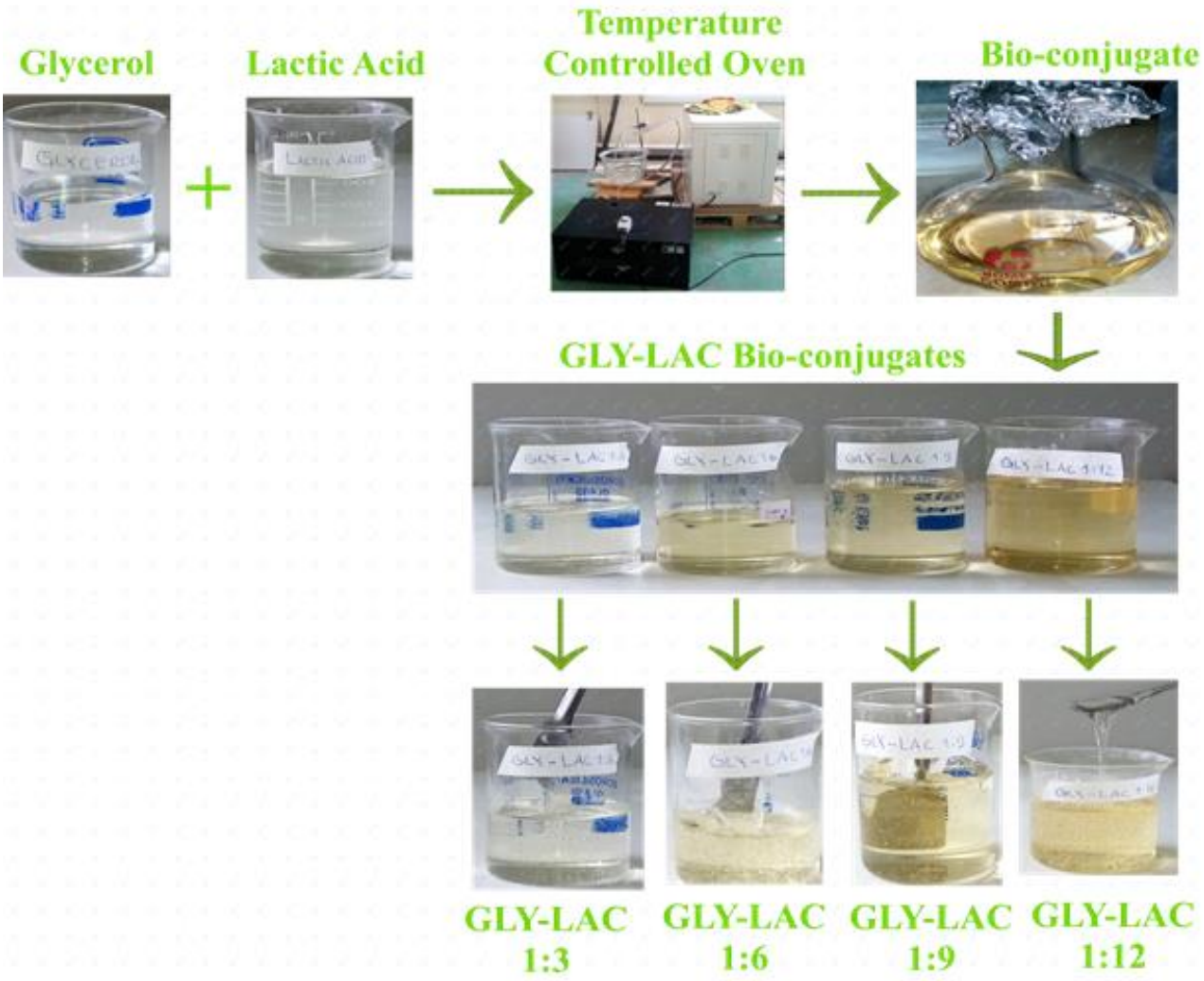


Figure 3-2 Development of the Three-armed GLY-LAC Bio-conjugates

In order to get a three-armed GLY-LAC bio-conjugate with a one, two, three and four lactic acid chain length, (i.e. GLY-LAC 1:3, GLY-LAC 1:6, GLY-LAC 1:9 and GLY-LAC 1:12 respectively)different molar ratios was used as described in the table above. 74 ml of glycerol was reacted with 510 ml of lactic acid to synthesize the GLY-LAC 1:6 bio-conjugate with similar method of production with the other conjugates. By doing, similar experimental methods except the mixing ratios the rest of the other conjugates were prepared. The reaction conversion was analyzed using carboxylic acid titration, and degree of esterification and supported by NMR, and

FTIR analysis. Moreover, the viscosity and thermal degradation properties of conjugate were investigated using Brookfield viscometer and TGA. The experimental protocol is described as follow.

Carboxylic Group Titration: The titration in this experiment were done according to ASTM D974-12 method [95]. In this case, l g of sample has been measured and dissolved in a 20 ml of chloroform. The solution then titrated with 0.1 N KOH solution in the presence of 10 droplets of penophtaline (indicator) solution [96]. The acid value was calculated according to equation 3.1.

$$\boldsymbol{AV = \frac{(V - Vo).\ NKOH.\ 56.1}{m}.100} \qquad \textbf{(1)}$$

Where; AV is acid value (mg/g sample) of the conjugate, Vo is volume of 0.1 N_{KOH} solution used to titrate the blank solution, V is volume of 0.1 NKOH solution used to titrate the sample, N_{KOH} – KOH titer used for titration (0.1 N) and m is mass of the sample (1 g).

Esterification Degree Determination: In this research work, degree of esterification was determined by using two different methods, The ^{13}C –NMR characterization method and simple calculation from the water collected during the reaction and theoretical amount of water expected from the reaction.

$$\textbf{DE}\ (\%) = \frac{\textbf{amount of water collected}}{\textbf{Theoretical amount of water formed}} * \textbf{100} \qquad \textbf{(2)}$$

NMR Analysis: The idealized chemical structure and the expected LA chain length of the conjugates was investigated by ^{1}H NMR and ^{13}C-NMR characterizations. The samples was dissolved in deuterated chloroform ($CDCl_3$), filtered with a 0.25 μm filter, and transferred to 5 mm diameter NMR tube. For the case, the samples was completely dissolved in chloroform before the analysis.

Fourier Transform Infrared Spectroscopy (FTIR): The existence of the expected functional groups and the expected interactions between the alcohol glycerol and the carboxylic acid in the synthesized product were further confirmed by using FTIR spectroscopic machine. The (ATR)attenuated total reflectance FTIR with model name of FTIR-6600 type A and serial number A013861790 in the range of 4000 to 400 cm^{-1} with 4 cm^{-1} resolution and 64 scan rates was used.

Intrinsic Viscosity Evaluation: The bio-conjugates viscosity was determined by two different types of viscometers; CAP-2000 Brookfield and VK 2000 viscometer types based on modified method of *Akanksha et.al*.;[97] and KREBS operating method respectively. The sample to be tested was transferred to a clean and dry 250 ml beaker, and the viscosity of the sample was determined by using spindle N° 5 at speed of rotated 25 rpm, near to 100% torque and a

Figure 3-3 Measurement of the Conjugate Viscosity

Thermal Stability Analysis: A thermal stability analysis, like the T_5, T_{10}, T_{50} and the degradation temperature of the synthesized conjugates was measured by thermal gravimetric analysis (TGA) instrument. The protocol under this characterization was done by weighting 8-10 mg Sample and heated from 25 °C to 800 °C under heating rates of 20 °C/min under nitrogen (N_2) atmosphere. Then the result was recorded automatically as sample weight loss (wt %) versus temperature (°C).

Organoleptic Properties: Both the ointment base blank formulations (i.e., formulations without the CM extract) and the herbal ointment (formulation with CM extract) were tested for physical appearance by visual observation.

3.4 Extraction of crude oil from *Croton Macrostachiyus (CM)*/(ብሳና)

The leaves part of the plant croton macrostachyus (CM)was collected from Oromiya regional state, Adama zone, Liben woreda. The extraction of crude oil from the leave of *C. macrostachyus (CM)*was performed at room temperature by Solvent extraction method. In this method, the fresh leave part of the plant was first dried in an open-air under shaded area. Then it was grinded into a powder form using a grinder. The air-dried leaves powdered material (300g) was then soaked within 1-liter n-hexane for96 hours at room temperature. A Buchner funnel and Rotary evaporator was used to separate the soaked extract from the plant residue and the crude extract from the solvent respectively and obtained 3g of deep green crude extract. The extracted oil was analyzed using GC-MS.

Determination of Major Component using GC-MS: The Agilent Technologies 7890B GC System, equipped with an Agilent capillary column (30 m * 0.25 mm inner diameter, *0.25 µm film thickness; maximum temperature, 325 °C), was used to analyze various crude organic extracts of C. Macrostachiyus leaves (MS). At a steady flow rate of 1.0 mL/min, ultra-high purity helium was used as the carrier gas.

Temperatures of 290 °C were used for the injection, ion source, and transfer lines. The energy of ionization was 70 eV. The voltage of the electron multiplier was obtained using auto tune. The oven temperature was programmed to rise at a rate of 3 °C/min from 60 °C (hold for 2 minutes) to 280 °C. The crude samples were filtered after being diluted with a sufficient solvent (1/100, v/v). The diluted crude extracts (1L) were taken particle-free. All of the data was gathered by collecting full-scan mass spectra in the range of 40-550 amu. The crude extract constituents' percentage composition was expressed as a percentage by peak area. The GC retention time was used to identify and characterize chemical compounds in various crude extracts. The mass spectra was compared to those of available standards using a computer program.

3.5 Formulationsof Anti-MicrobialOintmentfrom GLY-LA/CM extract

In this research work a base matrix having a lower viscosity (GLY-LAC 1:3)and higher viscosity (GLY-LAC 1:12)were selected to show the possible formulation of the herbal ointment. The only

method that we were used in this research is hot mixing/fusion method. In this method, the crude extract of *C. macrostachiyus* (CM) was mixed with the prepared conjugate in different mixing ration (1%, 3%, 5% and 10%).The added crude oil and the selected ointment base were stirred continuously for 5 minute on a hot plate, at a temperature of 70 °C until a homogenous dispersion in the base matrix was obtained.

Organoleptic Characteristics: The organoleptic properties of the synthesized ointment base and the formulated herbal ointment were examined by sensory analysis. The odor and color evaluation was performed by smelling and visualizing method respectively. Whereas, the consistency of smoothness, lubricity and greasiness properties of the conjugate were evaluated by touching with hands. The film forming ability and washablity of the ointment base matrix was tested by applying the base on the skin.

pH: The pH of the prepared ointment base matrix and the formulated herbal ointment was investigated by using a digital PH meter. The tested solution was prepared by dissolving 1 g of GLY-LAC bio-conjugate product in 100ml of distilled water and set aside for 2hrs. For the solution, PH was measured in triplicates and the average value was calculated.

Solubility: Solubility test was examined by dissolving the ointment base in water, alcohol, petroleum ether and chloroform.

Speredablity Evaluation: Speredablity evaluation for the ointment base matrix and the formulated herbal ointment was determined by following the method stated by *Panda* [98]. *This was* a set up that was assembled in our laboratory. In order to compact the sample to a uniform thickness, the four ointment bases were placed between two glass petri-dishes slides and a weight of 1000 g was placed on the slide for 5 minutes. Then a weight of 463 g (M) was attached to the upper slide of the glass Petri dish. The time needed to move the slides over a distance of 9 cm in seconds has now been taken as the Spreadability measure [98]. The Eq.3 formula below was used to calculate Spreadability.

$$S = ML/T \quad (3)$$

Where S is spreadablity, M is weight of the attached object to the upper slide of glass, L is the length of slide and T is the time taken to separate the two slides.

3.5.1 Antimicrobial Test for the Formulated Herbal Ointment

The Agar disk diffusion method is simple, practical and has been well standardized. This testing method is performed by applying microbial inoculums of approximately 1.5×10^{8}CFU/ml to the surface of a Mueller-Hinton agar plate. Commercially prepared with fixed concentration, paper antibiotic disks will be placed on the inoculated agar surface. Due to its cheap, flexible and allows visibility of growth properties the disc diffusion method was employed to test the antimicrobial effect of the CM extract, the ointment base matrix and the herbal ointment. The anti-bacterial potential of the formulated herbal ointment was studied with different concentrations of 0%, 1%, 3%, 5% and 10% for both Ointment base matrixes. To do this the ointments with different concentration of CM extract was prepared first, and from the prepared ointment 1mg/ml and 3mg/ml was again prepared to determine the zones of inhibition.

3.5.2 Testing for anti-bacterial activity

With an office paper puncher, 6 mm diameter paper discs were cut from whatman-No.1 filter paper and put in a beaker filled with aluminum foil and sterilized in an oven at 180 o C for 1 hour. The discs were then pipetted with 1mg/ml solution of CM extract, 1%, 3%, 5% and 10% GLY-LAC 1:3and GLY-LAC 1:12 based herbal ointment. And with a different experiment the discs were pipette with 3mg/ml solution of CM extract, 1%, 5% and 10% GLY-LAC 1:3 and GLY-LAC 1:12 based herbal ointment. Plates were incubated for 24 hr at 35°C prior to the determination of results. The zones of growth inhibition around each of the antibiotic disks against the E. coli and S.auraus bacteria was measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each product will be interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks.

3.6 Preparation of GLY-LAC Plasticized PLA films

After the preparation of different conjugates having diverse properties, the best plasticizer was chosen by studying the effect of different formulations of GLY-LAC. Next to this step, the selected two samples (V_{min} and V_{max}) products were added to the PLA matrix with different weight percent. In order to gets a uniform plastic film by solution casting method, the polymer (3g) was pre-dissolved by chloroform (90ml) for at least 15-20 min using magnetic stirrer at rotational speed of 20 rpm. Then the GLY-LAC plasticized material was prepared at PLA/ GLY-LAC ratios of 100:0 (0%), 90:10 (10%), 85:15 (15%), 80:20 (20%), and 75:25 (25%). After all the samples of the mixed solution was casted into a 15 cm glass Petri-dishes and leave it for 24 hour. The prepared plastic film was secured in the dissector, to avoid the effect of moisture.

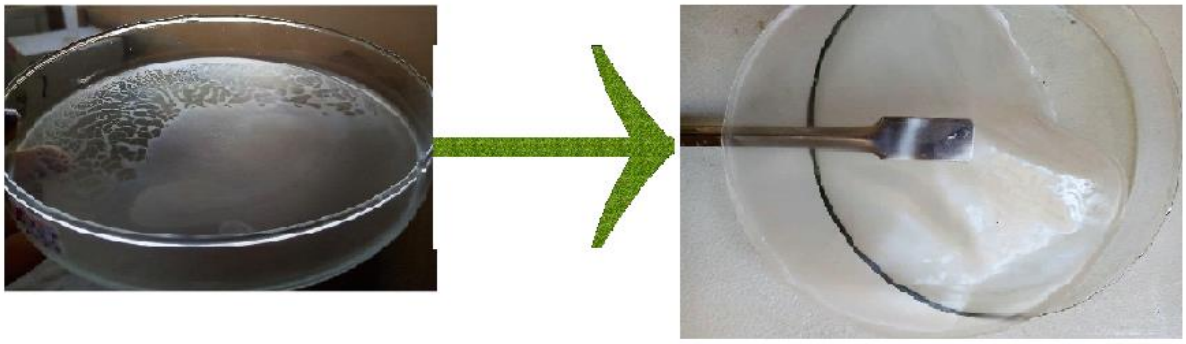


Figure 3-4 preparation of GLY-LAC plasticized PLA film by solution casting method

Tensile strength and Elongation at Break Test: Before preparing the sample for the test, the plastic films were conditioned overnight, under a conditioning oven at 50 °C and 60 humidity. After this step sample was prepared in 80 cm length by 20 cm width by using ruler, pen (marker) and scissor. This test was performed By the Ethiopian Conformity Assessment Enterprise, following the ASTM D638 tensile strength measuring procedure using universal tensile strength testing machine. The elongation at break and tensile strength was done simultaneously.

Plasticizer Migration Tests: The rectangular samples (10 * 10^3* 1 mm^3) contained on watch crystals were put into two vacuum ovens at 125 and 150 °C to test the migration rates of the

plasticizers. The samples were taken out at regular intervals, wiped to clean the plasticizer that had moved to the surface, and then weighed on an analytical balance. The migration rates (ϖ_s) were calculated by the following equation:

$$\varpi = [(m_1-m_2)/m_1] *100\% \textbf{(4)}$$

Where m1 and m2 are the mass of the sample before and after they were putted in to the vacuum oven, respectively.

CHAPTER FOUR

4. RESULT AND DISCUSSION

4.1 Synthesis and Characterization of Three Armed GLY-LAC Bioconjugates

4.1.1The Reaction Mechanism

The reaction in this case was conducted between two renewable sourced starting materials, which are the glycerol and lactic acid. In this reaction the three reactive 'OH' groups of glycerol is expected to react with three molecules of lactic acid via a condensation reaction. In this case, there may be a lot of possible ester bond formations, this bond may be formed by either the reaction between the hydroxyl group of glycerol with carboxylic group of lactic acid or by the reaction between the hydroxyl groups of one lactic acid with the carboxylic group of the other lactic acid monomer. However, the reaction between the two-hydroxyl groups of glycerol is negligible here, because it needs higher temperature to occur. In the poly-condensation reaction, the ratio of reacted to initial carboxylic groups is related to the degree of completion of the condensation reaction. Therefore, the degree of reaction completion is estimated by the free acid value titration method.

The temperature of a reaction has a direct relationship with the rate of reaction, this is because as the temperature increased the average kinetic energy of a molecule in the reactant increased and so many possible molecular collisions have been occurred. The effect of reaction temperature on the unreacted lactic acid value of the conjugates has seen on the figure 4-1 below. As the temperature increased from 150 ℃ to 190 ℃ the acid value significantly decreases from 235 to 99, which can be correlated with the consumption of lactic acid to produce larger macromolecular structure. Since the decomposition temperature of lactic acid is related to the temperature and time of exposure to heat selection of reaction condition must be performed carefully. Previous studies confirms that lactic acid decomposes at 290 ℃ when it is exposed to heat for 30 min. based on the literature and preliminary investigations, a temperature of 190 ℃ and 8 hrs of reaction time was selected to facilitate the reaction.

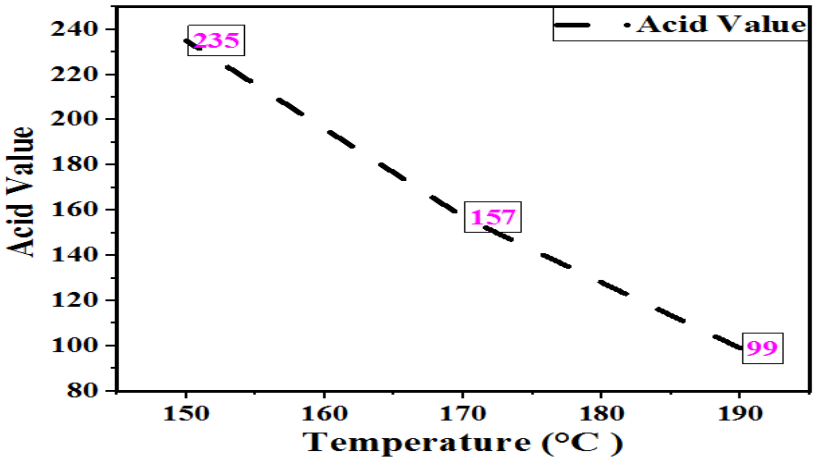


Figure 4-1 Effect of Temperature on the Acid Value

The inadequate reaction time in the reactor results in unreacted reactants and on the other hand, excessive reaction times lead to transesterification reactions that eventually degrade the oligomer structure [92]. Therefore, a sufficient reaction time of condensation is a critical factor. In the in the figure 4-2, it can easily observed that the amount of unreacted lactic acid monomers decreased with the increasing of reaction time.

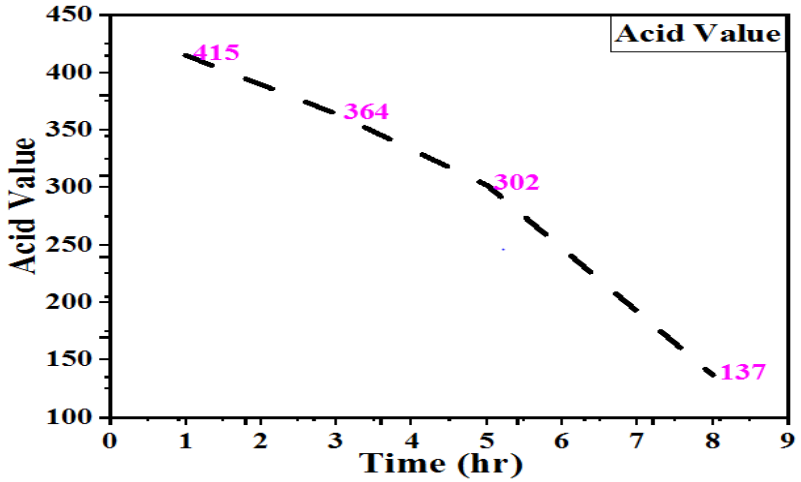


Figure 4-2 Effect of reaction time on the acid value

The acid value decreases from 415 to 137 as the reaction time increases from 1 hr to 8 hr. This may be due to the consumption of lactic acid during the reaction as time increases. Therefore, in order to get the desired big macromolecular structured conjugate a sufficient reaction time up to 8 hr is required.

From the figure 4-3,it has been indicated that for a different reaction time at a constant temperature of 190 °C, the AV is increased with the molar ratio increment. The unreacted lactic acid values for the 8 hr sample were increase from 76 to 157 as the molar ratio increases from 1:3 to 1:12. On the other hand for the 1:12 molar ratios sample the AV were increased from157 to 486 as the time increases from1hrto8 hr. even though the unreacted lactic acid value increases with the molar ratio increment, there also seen that the increment of water escaped from the reaction was also increased. However, the increment in water that escaped from the reaction is also observed when the temperature and time increased. Measuring of the amount of water that escaped from the reaction is used to estimate the degree of esterification of the reaction.

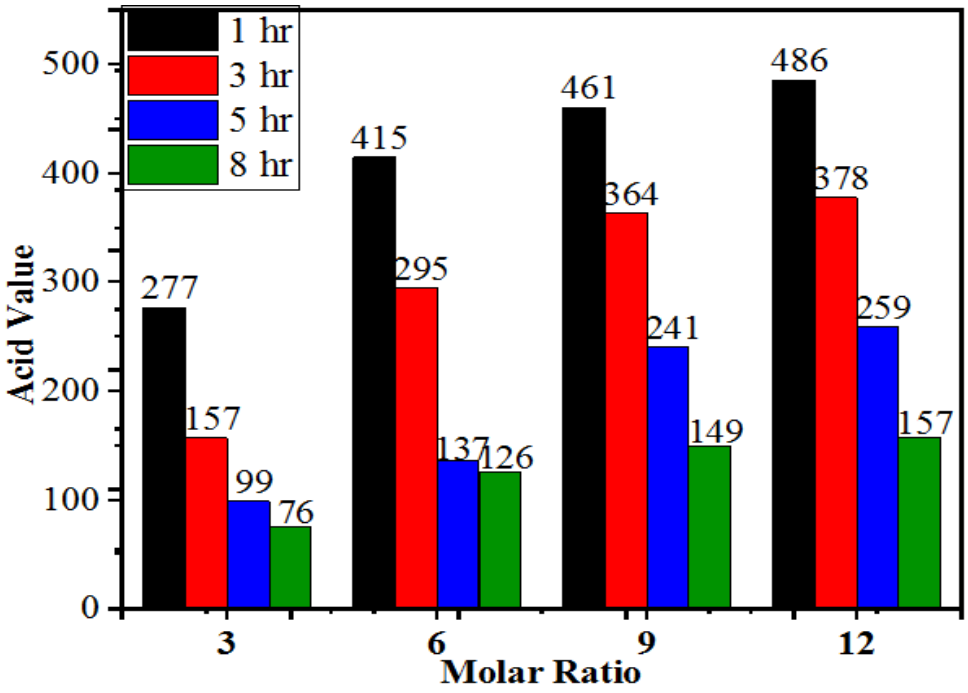
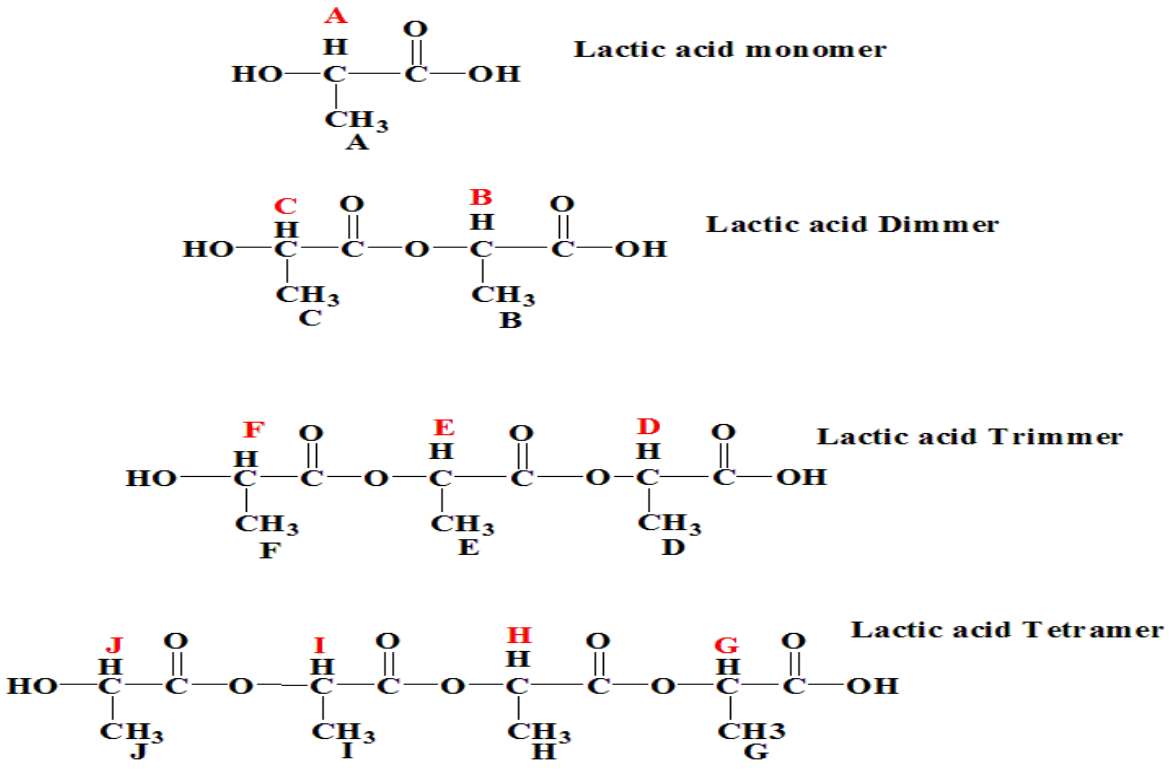


Figure 4-3 Effect of molar ratio on the acid value

Since the reaction is taken place between glycerol and lactic acid, the -COOH groups of lactic acid was reacted, and the length of the glycerol arm is increased. Therefore in order to know the exact behavior of the conjugate the reaction mechanism should be further supported by the chemical structure analysis, the chain length analysis, the possible functional group formation, the effect of chain length on the thermal stability and the viscosity of the synthesized bio-conjugate using ^{1}H-NMR, ^{13}C-NMR, FTIR, TGA and Viscometer.

I. Chemical Structure Analysis

LAOLG Bio-conjugate: As indicated in Scheme. *1*and further confirmed by H-NMR spectra of LAOLG (figure 4-4), there are four distinct positions of the methine and methyl groups; this are in the free LA monomer, terminal position near COOH group or to the OH end groups and in the middle chain.



Scheme.1 Reaction Scheme for the synthesis of Lactic Acid Oligomer (LAOLG)

The methine group in the free lactic acid monomer has a singlet peak at chemical shift of 4.03 ppm, methane group in the terminal position, beside the carboxylic and hydroxyl groups can be found at around 4.92 ppm and 4.19 ppm respectively. The other position for this group is between the two terminals, which also called in the middle chain, having a singlet peak at ~ 5.1 ppm. On the other hand the methyl group of lactic acid can be found in the unreacted monomer and also in the terminal position (close to carboxylic or hydroxyl group) of lactic acid oligomer, and in between the chain with a chemical shifting of 1.23 ppm,1.38 ppm, 1.27 ppm and 1.4 ppm respectively. The two terminals of methyl and methine group positions of lactic acid oligomer is formed due to the esterification of OH and COOH groups of lactic acid.

As the chain length of the LAOLG increases the chemical shifting of the chemicals became greater or moved to the left for all hydrogen types. As clearly indicated in table 4-1, for LAOLG the shifting for the COOH free H in CH of dimmer, trimmer, and tetramer would increase to 4.92, 4.98, and 4.99, respectively and for the OH free Hydrogen in CH group the shifting will changes to 4.19, 4.20 and 4.20 for dimmer, trimmer and tetramer respectively [99].

Table 4-1 ^{1}H NMR Chemical Shifts (in ppm) of CH and CH_3 Protons in A-T units of L-Lactic Acid Oligomers

Hydrogen type	Monomer	Dimmer		Trimmer		Tetramer		Polymer
	A	B	C	D	F	G	J	
CH_3	1.27	1.38	1.27	1.44	1.40	1.40	1.27	1.46
CH	4.03	4.92	4.19	4.20	4.98	4.99	4.20	5.1

For the hydrogen in the middle chain CH, chemical shifting will be ~ 5.1 ppm. For the CH_3 also the three hydrogen's have different chemical shifts for the monomers, dimmers, trimmers and tetramers. For the terminal lactic acid CH_3 with COOH free chemical shifting will be changes to

(1.27, 1.40, 1.40 ppm) and for OH free (1.38, 1.44 and 1.40 ppm) respectively for the dimmers, trimmers and tetramers.

Therefore, this lactic acid oligomer is an oligomer, which has contained a developed structure to tetramer and polymer macromolecule. The other information that we have gotten from the H-NMR spectra of LAOLG is the absence of spectral peak at ~ 1.23 and ~ 4.03 which indicate the absence of unreacted lactic acid monomer [99]. This result was also similar with the acid base titration result of the oligomer. Since the LAOLG is synthesized by similar method to that of the GLY-LAC bio-conjugates by comparing the ^{1}H-NMR spectra of the three-armed bio-conjugates with the LAOLG, it can be perfectly defining the ongoing reaction mechanism during polycondensation reaction.

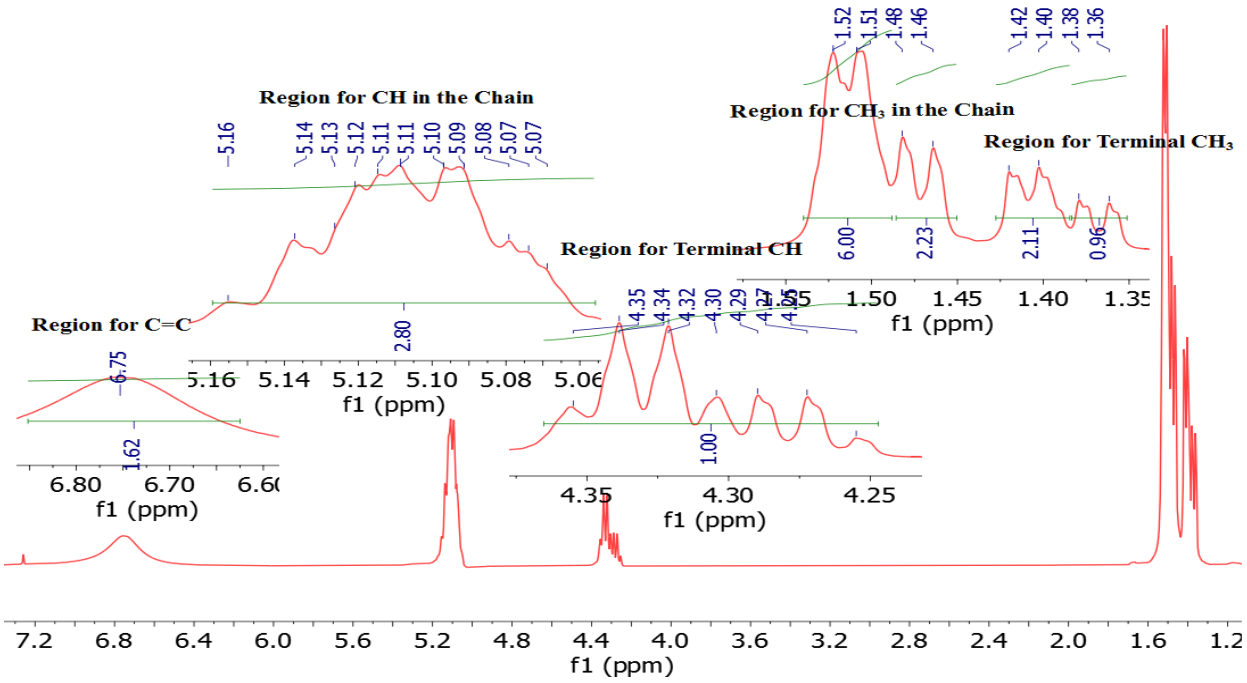
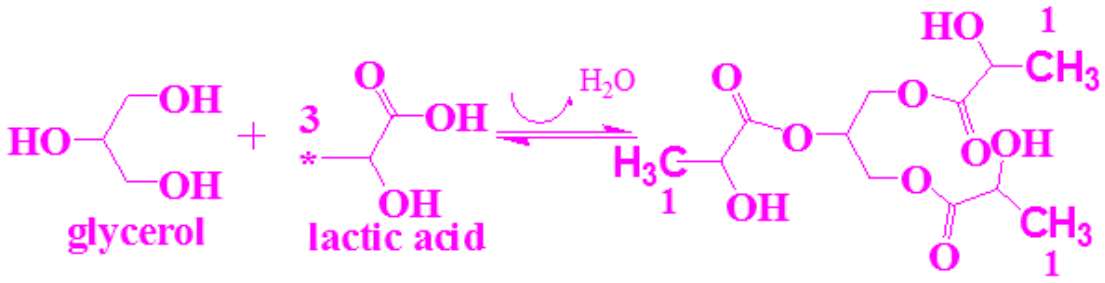


Figure 4-4^{1}H-NMR Spectra for LAOLG Bio-conjugate

GLY-LAC 1:3 Bio-conjugate: The methyl group region of its H-NMR, the GLY-LAC 1:3 Bio-conjugate**(H-NMR spectra on Appendix 2)** have four different doublet peaks at around 1.23 ppm, 1.27 ppm, 1.32 ppm and 1.34 ppm. There are also peaks between the 3.3 and 3.9 ppm, two of them are smaller peaks at ~ 3.4-3.6 ppm. There is also another intentense and broad peak

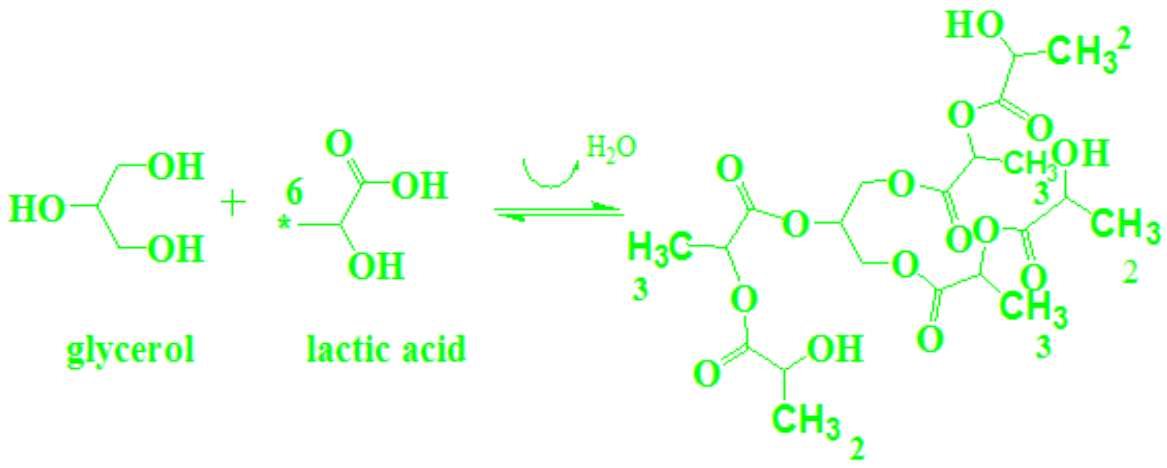
observed at ~ 4.12 ppm and ~4.9 ppm respectively. The presence of doublet peaks between 1.15 and 1.23 ppm is responsible for the unreacted methyl group of lactic acid. In addition, the doublet peaks found between the 1.27 and 1.34 ppm is stands for the existence of OH free (COOH esterified) methyl group in the chain. The next three small peaks are found on the region of alcohol functional group, between 3.3 ppm to 3.9 ppm. In our case the two smaller peaks at ~ 3.34 – 3.51 ppm are stands for the terminal OH groups of the chains. The area for this is around 1.59, which is larger than the other conjugates; this indicates that the GLY-LAC 1:3 bio-conjugates contains more OH end chain group than the other. This is because of the shorter chain length of the GLY-LAC 1:3 bio-conjugate relative to the other (***Scheme.2***). The intense peak at 3.97 ppm can be attributed to the two-methylene groups of the core glycerol. In addition, the other intentense peak at ~ 4.12 ppm is for the methine group of lactic acid in the chain having OH free or COOH esterified end. However, this peaks are not similar with the LAOLG peaks, therefore this in chain methine group of lactic acid may not be for the oligomer. But it can be the glycerol core lactic acid oligomer of the conjugate.



Scheme.2 Reaction Scheme for the synthesis of the three armed GLY-LAC 1:3 Bio-conjugate

Further, this is confirmed by the broad peak at ~4.9 ppm, which is formed by the effect of the attached lactic acid moieties on the methylen group of glycerol moieties. The unreacted lactic acid monomer amount present in GLY-LAC 1:6bio-conjugate are little more than the GLY-LAC 1:3 conjugated product. This is confirmed by the comparable peak area of 1.82 for GLY-LAC 1:6 bio-conjugate and 1.48 for GLY-LAC 1:3 bio-conjugate.

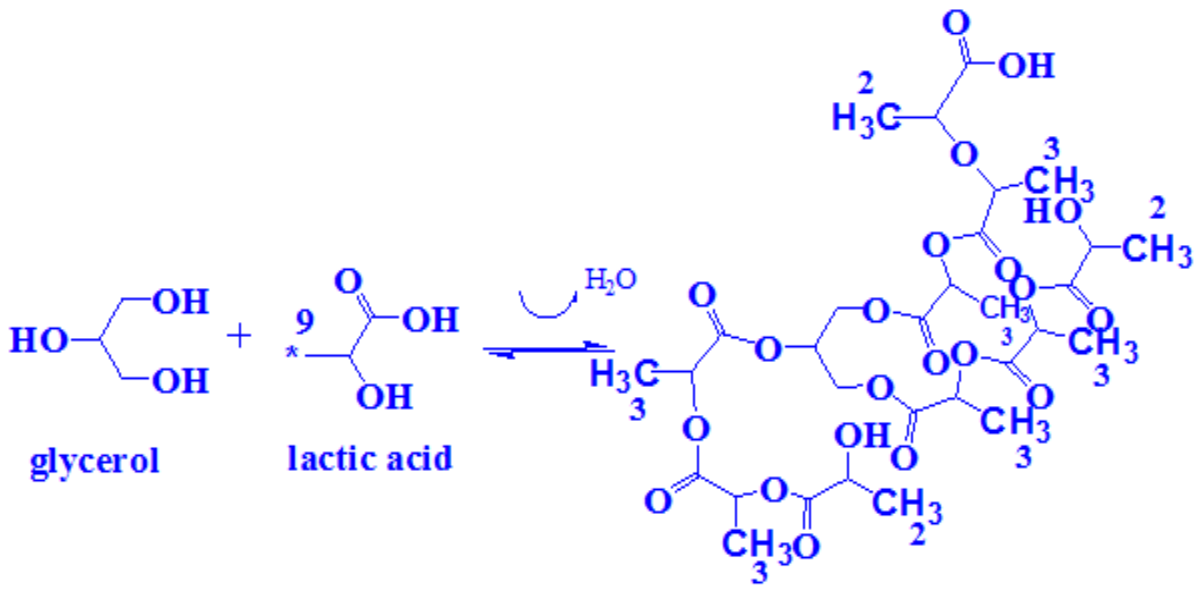
GLY-LAC 1:6 Bio-conjugate: As shown on the H-NMR spectral **(H-NMR spectra on Appendix 3)**the peaks between 1.41 to 1.48 ppm are stands for the CH_3 group in the chains. The next small peaks found between 3.50 & 3.67 ppm are indicate the presence of the OH ended group of the conjugate. Specially, the weak and singlet peak at 3.87 ppm is for the indication of OH end functionalization of the chains. The two different peaks around 4.06 - 4.32 ppm are for the glycerol methylen and lactic acid methine group. The subsequent peak ~ 5.0 ppm is giving an explanation for the effect of chain length on the methylene group of glycerol. The broad peak at 5.25 ppm is for the effect of chain length on the methine group of the core glycerol. The other singlet peak at 5.64 ppm is for the double bond formation during the reaction, which might be comes from oxidation reaction with the external oxygen. The possible schematic diagram for reaction mechanism of GLY-LAC 1:6 Bio-conjugate is indicated in ***Scheme 3.***



Scheme 3 Reaction Scheme for the synthesis of the three armed GLY-LAC 1:6 Bio-conjugate

GLY-LAC 1:9 Bio-conjugate: In the H-NMR spectral of GLY-LAC 1:9 conjugate **(H-NMR spectra on Appendix 4)** the peak observed at 1.26 & 1.33 ppm is for methyl group in the unreacted lactic acid monomers. The next two peaks are for the methyl group of the conjugate at the OH free end. The peak in the range 3.49 - 3.64 ppm is for the unreacted glycerol moieties. Peak around 3.84 ppm is an indication of effective OH end functionalization of the chain. The other weak and singlet peak at 3.87 ppm is for the indication of OH end functionalization of the

chain. The two different peaks around 4.06 - 4.32 ppm are for the glycerol methylen and lactic acid methine group. The subsequent peak ~ 5.0 ppm is give an explanation for the effect of chain length on the methylene group of glycerol. The broad peak at 5.25 ppm is for the effect of chain length on the methine group of the core glycerol. The other singlet peak at 5.64 ppm is for the double bond formation during the reaction, which might be due to hydrolytic decomposition reaction.The possible schematic diagram for reaction mechanism of GLY-LAC 1:6 Bio-conjugateis indicated in ***Scheme 4.***

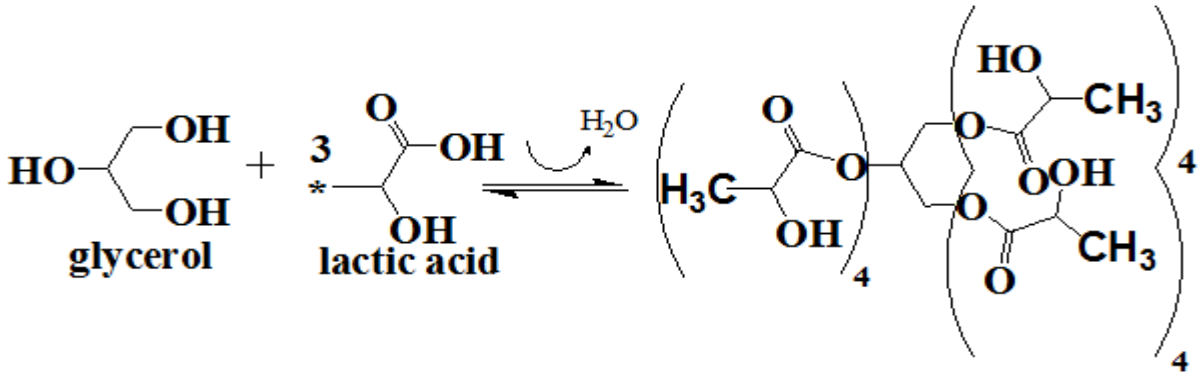


Scheme 4 Reaction Scheme for the synthesis of the three armed GLY-LAC 1:9 Bio-conjugate

GLY-LAC 1:12 Bio-conjugate: The H-NMR spectra of GLY-LAC 1:12 bio-conjugate **(H-NMR spectra on Appendix 5)**shows a much more amount of unreacted lactic acid monomer at the region of both $CH_3$and CH (1.23 & 4.03). The presence of two different peaks at around 1.27 & 1.29 ppm and 1.32 & 1.34 ppm indicates the existence of OH free (COOH esterified) terminal methyl group. This means no other chains rather than one is formed on the core molecule. The next three small peaks are found on the region of alcohol functional group, between 3.3 ppm to 3.9 ppm. In our case the two smaller peaks at ~ 3.4 – 3.6 ppm are stands for the unreacted

glycerol moieties in the conjugate. The area for this is around 1.59, which is larger than the other conjugates, this indicates that the GLY-LAC 1:3 bio-conjugates contains unreacted glycerol monomer than the other. The single weak peak at around 3.70 ppm can be attributed to the terminal hydroxyl group providing evidence of successful hydroxyl functionalizing of the branches.

The intense peak at 3.97 ppm can be attributed to the methylene group of the core glycerol. And the other intentense peak at ~ 4.12 ppm is for the methine group of lactic acid in the terminal (OH free) position. This peak is also be used as an evidence for the one lactic acid attachment of the GLY-LAC1:3 bio-conjugate. The other broad peak at ~4.9ppmis formed by the effect of the attached lactic acid moieties on the methylen group of glycerol moieties. The possible schematic diagram for reaction mechanism of GLY-LAC 1:6 Bio-conjugate is indicated in ***Scheme 4.***



Scheme 5 Reaction Scheme for the synthesis of the three armed GLY-LAC 1:12 Bio-conjugate

II. **Chain Length Analysis:**

LAOLG Bio-conjugate: Different types of carbonyl bonds were expected for the conjugates, including a) main-chain carbonyls, b) LA carbonyl groups adjacent to the core molecule (-O-CH2) branches, and c) LA end-group carbonyls for unreacted LA (figure 4.5). The observed CH groups could be from a) the core molecule structure, b) adjacent to the reacted LA carbonyl groups and the oxygen atom, or c) adjacent to the hydroxyl end group. LA methyl groups are the

other expected groups in the range of 16–22 ppm. Here one COOH esterified (OH free) type terminal CH_3 or CH groups is observed.

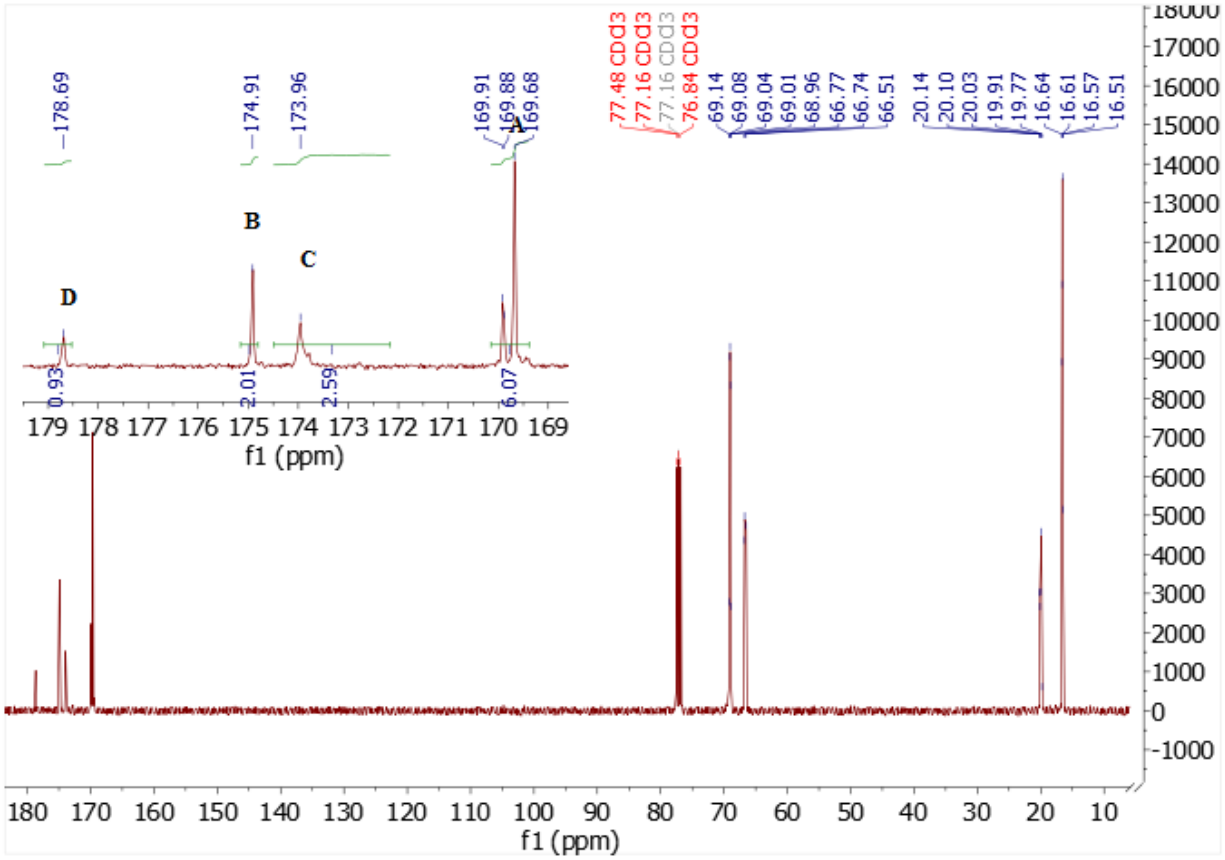


Figure 4-5 ^{13}C-NMR Spectra for LAOLG Bio-conjugate

GLY-LAC 1:3 Bio-conjugate: From the ^{13}C-NMR spectral data (figure 4.6)it can be calculated the total amount of lactic acid unit present in the product, percentage of lactic acid unit react with glycerol and in the free oligomer, and the chain length of the glycerol branches and oligomer branches. The carbonyl group area of the conjugate in the ^{13}C-NMR spectra below is found between 160 and 180 ppm. Carbonyls in the lactic acid branch and in the lactic acid oligomer show two signals at ~169.5-170.5 ppm (area marked by A). The carbonyl adjacent to (-O-CH_2) is giving a peak at ~169.8 ppm.

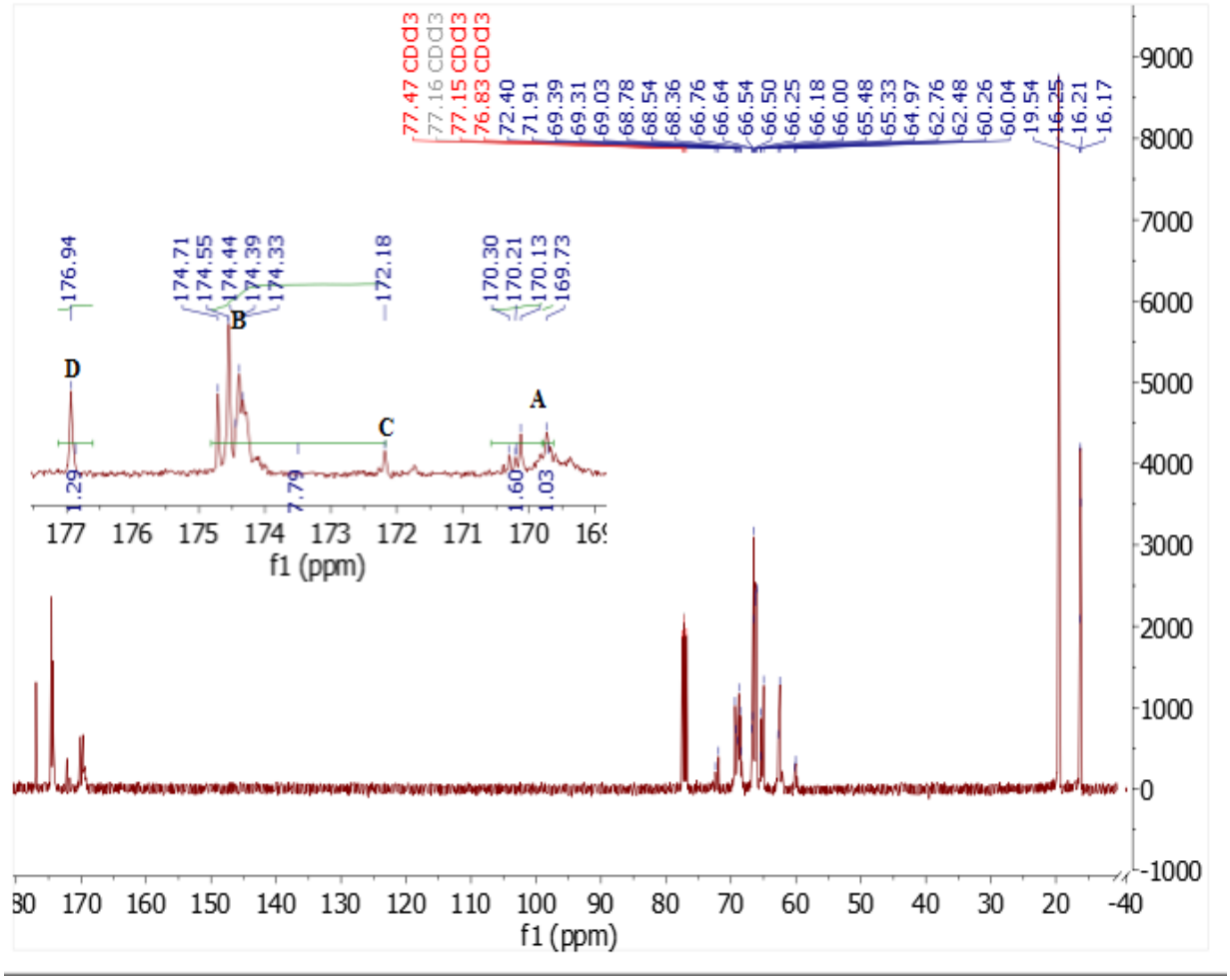


Figure 4-6 ^{13}C-NMR Spectra for GLY-LAC 1:3 Bio-conjugate

The lactic acid end group carbonyl signal is observed at 174.3-174.95 ppm (area marked by B), but it cannot distinguish between the LA oligomers reacted and not reacted with glycerol. This signal is from the carbonyl group adjacent to the ester oxygen for glycerol based and lactic acid oligomer. On the other hand the carbonyl group adjacent to the hydroxyl group shows two smaller peaks at 172 and 173.9 ppm (area marked by C). However, the lactic acid monomer carbonyl group can be seen at ~ 177.6 ppm (area marked by D).

From the carbonyl peak area we can calculate the percentage of lactic acid reacted with glycerol and percentage of lactic acid reacted to the free oligomer. We can also get the chain length of

glycerol-lactic acid branches in the conjugate. The sum of all peak areas in the carbonyl area is equivalent to the sum total of lactic acid amount present in the conjugate. On the other hand the amount of lactic acid monomer reacted in the free oligomer and in glycerol branches is equivalent to the sum of peak areas for the two main peaks found at 169.5-170.5 ppm (A) and 174.3-174.95 ppm (B), subtracted with the smaller peak area found at 172-173.9 ppm (C). The presence of two small peaks at point C is the evidence that the reaction with glycerol has occurred.

GLY-LAC 1:6, 1:9 and 1:12 Bio-conjugate: As it can be observed from ^{13}C-NMR spectra (figure 4-7 for GLY-LAC 1:12 Bio-conjugate and **Appendix 6 and 7** of GLY-LAC 1:6, and1:9 Bio-conjugate) the carbonyl area expansion of all samples there exists a different amount of lactic acid monomer unit which is reacted neither with the free oligomer nor to the glycerol. All quantitative values from carbonyl area were calculated from the identified peak integral ratio to the chain units. For example the percentage of lactic acid unit reacted with glycerol is obtained from the peak area ratio of hydroxyl group terminated end group carbonyls to the carbonyls in the branches. The percentage of lactic acid unit reacted with free LA oligomer is also calculated by similar method, peak area ratio of carboxylic group terminated end group carbonyls to the carbonyls in branches. The chain length of the glycerol branches is calculated by dividing the peak area corresponding to the amount of lactic acid present in the braches by the end group lactic acids. The main presumption here is that there is only one glycerol unit in every conjugated pre-polymeric chain. And the use of di-functional substance (lactic acid) allows changes in the balance between the hydroxyl and acid groups, so instead of having equal numbers of functional groups, the added di functional compound moves the balance toward the desired composition. Somewhere in the polymerization process the lactic acid monomer or the lactic acid oligomer then joined to the three armed conjugate chain by the reaction of the OH & COOH groups. Then if this assumption is correct the resulting conjugate chain should contain mainly one kind of end group then the molecular weight of the conjugate will determined by the amount of di functional substance (lactic acid).

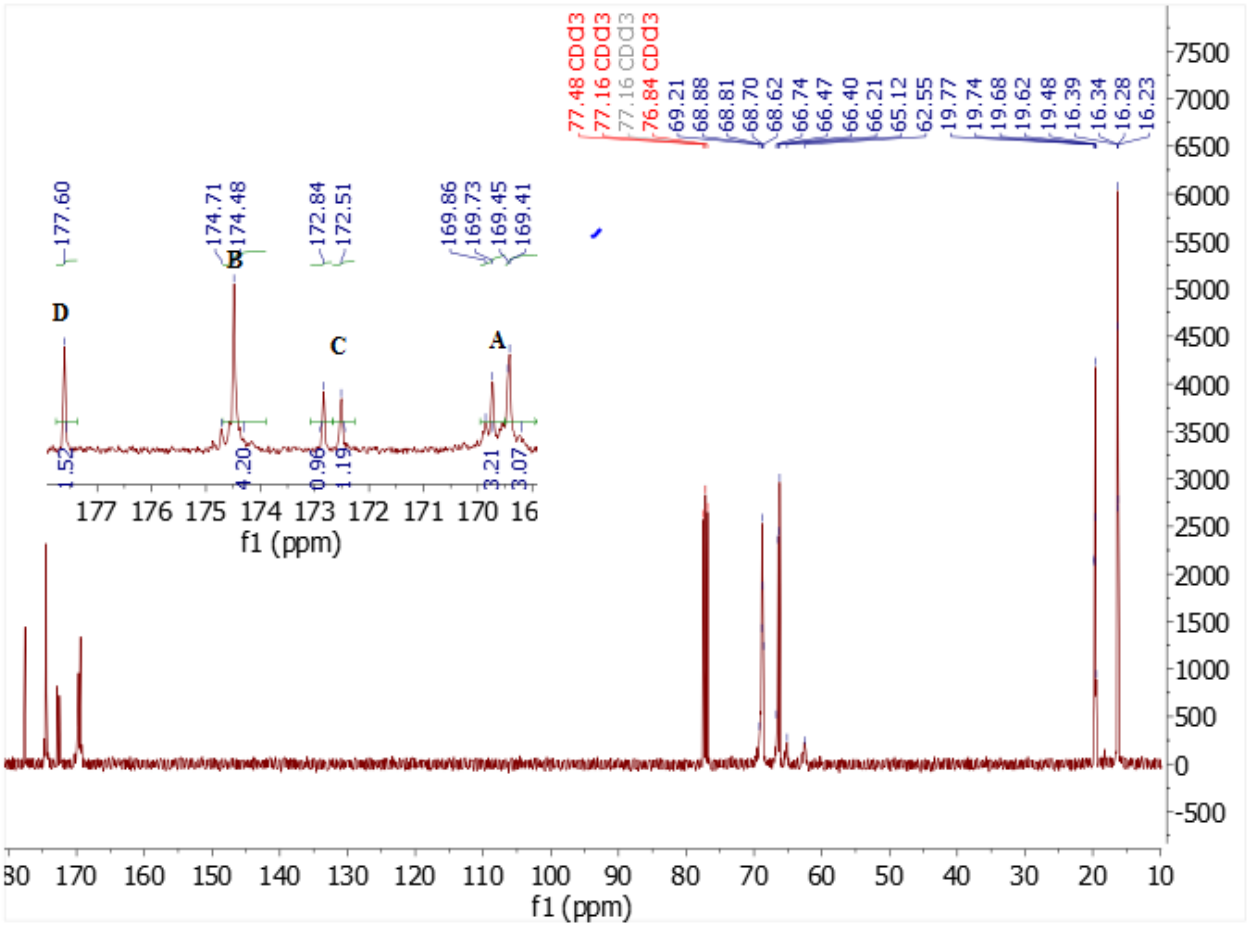


Figure 4-7 ^{13}C-NMR Spectra for GLY-LAC 1:12 Bio-conjugate

The peak integral of the C-NMR were also used to calculate the degree of polymerization of the conjugate, from the ratio of end group carbons to the chain carbon. The results are generalized and shown in table 4-3. This was also supported by the H-NMR result of the samples.

Table 4-2General ^{13}C-NMR Result of the Four Bio-conjugates

	GLY-LAC 1:3	GLY-LAC 1:6	GLY-LAC 1:9	GLY-LAC 1:12
%of LA reacted with glycerol	73 %	40.8 %	21.21 %	23.5%
% of LA reacted with LA	3.4 %	17.6 %	12.6 %	27.2 %
Chain length of the attachment on glycerol	1	2	3	~3

The three armed Glycerol based bio-conjugate, resulted from the condensation reaction of lactic acid and glycerol have reactive end groups, either the terminal LA's hydroxyl group or the core molecules hydroxyl group. This end group has a capability of caring a positively charged particle. This property makes it to act as a drug carrier ointment base matrix. Yet, this end groups are not reactive enough for a satisfactory cross-linking or further esterification. The absence of peaks in the range of 63-64 in all samples is the indication of absence of unreacted glycerol in the product. Depending on the mole amount of lactic acid that involved in the reaction for the optimized 8 hr time at a temperature of 190 ℃ the number of lactic acid unit that attached to the glycerol arm is varied. Here in this paper the lactic acid chain length on the glycerol arm is 1, 2, 3 and 4 for the molar ratios of 3, 6, 9 and 12 respectively. This result was verified by the NMR results.

III. Functional group analysis using FTIR

Figure 4-8below displays the Fourier transform infrared (FTIR)result for the synthesized GLY-LAC bio-conjugate. The IR analysis is only used as a qualitative tool to check the differences in end group of the different chain in the conjugate. All samples exhibits a characteristics transmittance peak of an ester functional group at 1746 cm -1and ether functional group at 1090 cm-1 and –CH2 group at 2940 cm-1, -CH3 at 2989 cm-1. A broad transmittance peak is observed at 3100-3700 cm -1.A broad peak found at about 3464 cm−1 is an evidence for the presence of OH end groups in the synthesizedconjugates[100]. This peak is almost similar with the peak for LAOLG, which is the evidence for not only the OH end of glycerol or lactic acid monomer.In another case,a transmittance peak is observedat 995 cm-1that isfor the C-O stretching of primary hydroxyl group in glycerol,and this peak is observed in all GLY-LAC bio-conjugates but not found on the LAOLG spectra.The other peakfound at 1044 cm-1 is for C-O stretching in secondary OH group of glycerol and this peak is also found on the LAOLG spectra.

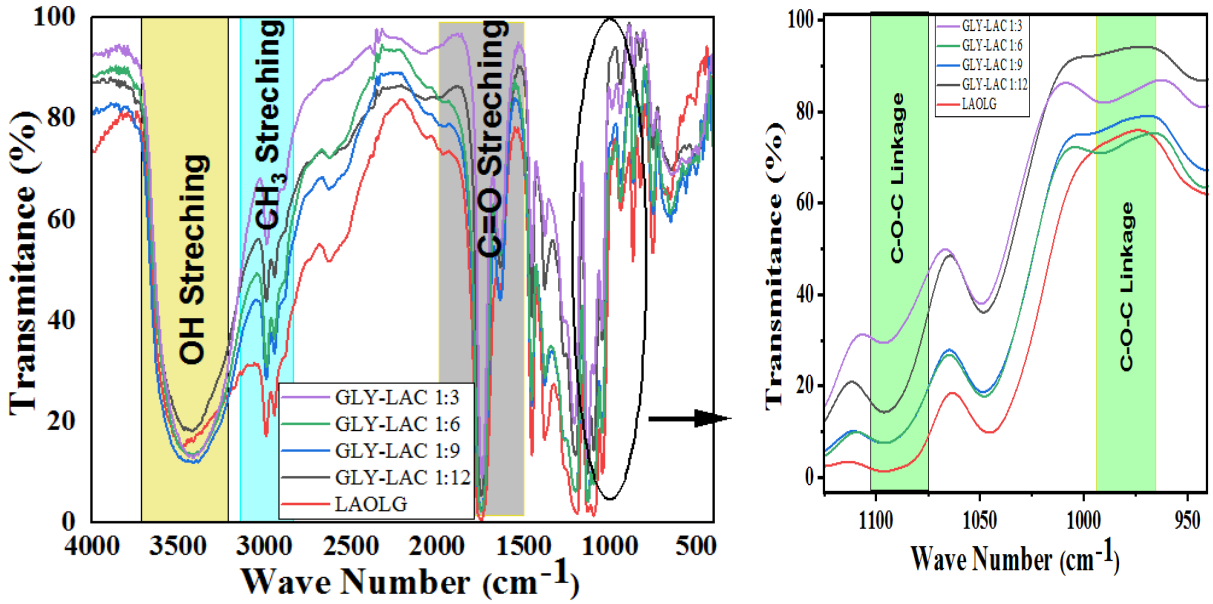


Figure 4-8 FTIR graph of the synthesized GLY-LAC Bio-conjugates and LAOLG

The peaks at 1746 cm $^{-1}$ and sholder between 2500 cm-1 and 3000 cm-1 are assigned to the carbonyl and hydroxyl funcional groups of carboxylic acid respectively. The small peaks at around 2940cm^{-1}are coming from the stretching of –$CH_3$and –CH_2– groups in the chain. The transmittance peak found at 1641 cm^{-1} indicates the carbonyl stretching of the remaining free lactic acid after the condensation reaction.

Finally, it can be concluded that the product is made of a glycerol core and lactic acid chain unit, where the lactic acid is attached to the primary and secondary hydroxyl groups of glycerol with the presence of a reactive OH end functional group at the end of the chain.

4.1.2 The effect of reaction conditions on viscosity and thermal stability

I. Viscosity: In the polycondensation of glycerol with lactic the monomer first produces oligomers of glycerol and lactic acid, and then the oligomers continued to react with other monomers and other oligomers to produce a higher molecular weight species [16]. In the course of the reaction, the lower molecular weight oligomers tend to react among themselves to produce higher molecular weight particles. Therefore theinsufficient reactiontime and temperature is may be leads the formation of lower molecular weight molecule. On the other hand the imbalance of reactive hydroxyl or carboxyl group to perform further reaction between the two reactants may lead a low molecular weight product at the end of the experiment; this is what we call Deviations from the stoichiometric ratio of reactants [101], [100], [102].

Effect of reaction time and molar ratio on viscosity: As indicated in figure 4-9 and table 4-4 a clear effect of reaction time and mixing ratio is observed. The intrinsic viscosity of the conjugate is increased with the molar ratio; this is due to the availability of enough amounts of reactive groups in the system. The more the concentration of the reactant is the more the reaction rate so the more product formation. By holding the mole of glycerol constant and adding different amount of lactic acid, we get four different viscosity levels. As the amount of glycerol core increased the viscosity became lower, but the viscosity will increased with the lactic acid amount. This tells us the viscosity of the conjugates is dependent on the chain length of the glycerol arm. For a constant reaction temperature of 190 °C, different four reaction time and molar ratios have a significance change in the viscosity.

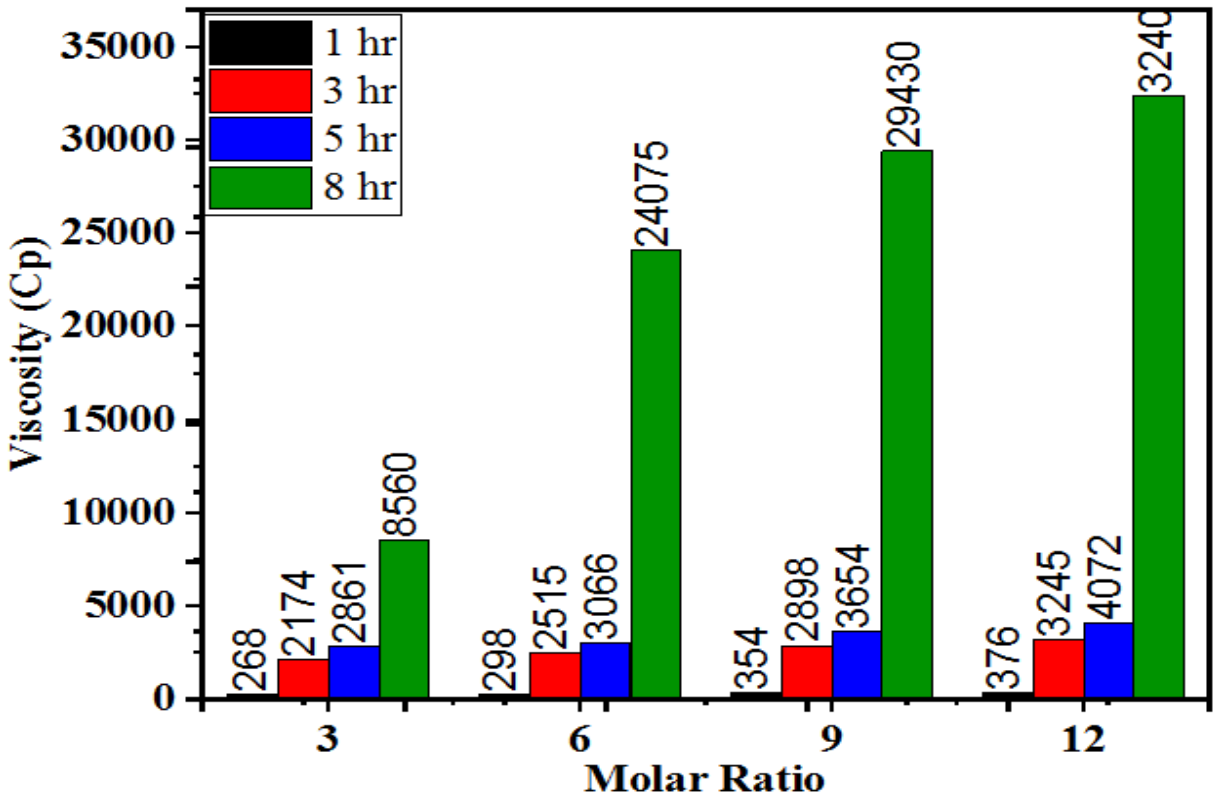


Figure 4-9 Effect of mixing ratio and time on viscosity of the conjugate

Therefore, in this research molar ratio of the reactants is used to adjust the viscosity of the synthesized three-armed glycerol based bio-conjugate. Reaction time on the other hand is an important parameter of this research that affects the viscosity of the produced conjugate. As time is increased to 8hr from 1hr the viscosity of the sample will become increased. However, beyond this optimized time the conjugate changes from a property of lubricant to adhesive.

This is because of the insufficient time of reaction to conjugate/attach the excess lactic acid monomers on the glycerol core, especially for 9 and 12 molar ratios. Due to the higher unreacted lactic acid monomers amount, the final viscosity of the products is became lowered. Therefore, for fixed molar ratios the reaction temperature and reaction time are important parameters.

Table 4-3Effect of molar ratio on the nature of the reaction at 190 ºC and 8 hrs

molar ratio	Acid value	Viscosity
3	75.73	268
6	137.44	298
9	148.66	354
12	157.08	376

Effect of reaction temperature on viscosity: As we have been discussed before the reaction temperature have a great role in the rate of the reaction.

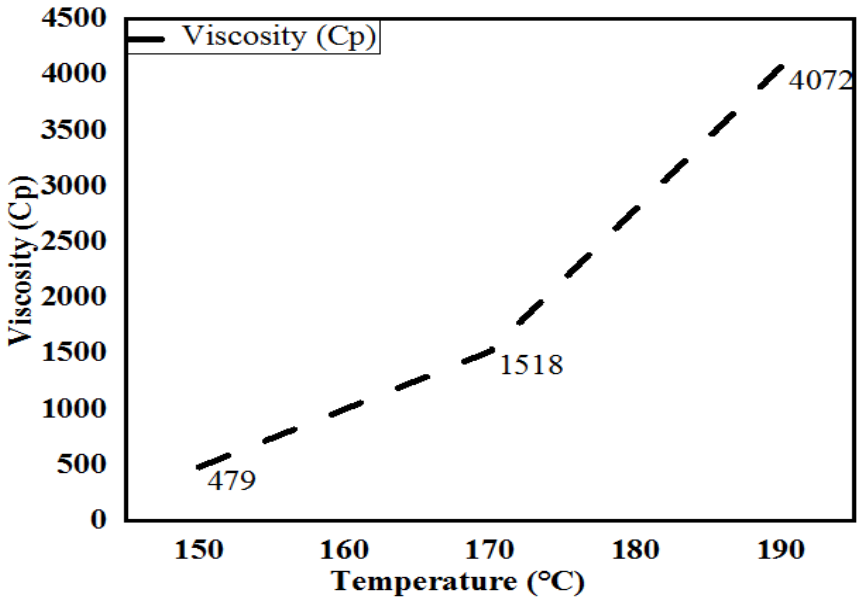


Figure 4-7 Effect of Reaction Temperature on the Viscosity of the Bio-conjuagte

As the rate of the reaction is increased there will a lot of ester bond formation in a given time, this makes the final product to have a desired viscosity. The temperature effect on the property of the desired conjugate is shown in the figure 4-10. As it can be seen from the graph, as the temperature of the reaction increased from 150°c to 170 °C then to 190 °C the viscosity of the conjugate is increased respectively to 479 Cp, 1518 Cp and 4072 Cp.

The glycerol and lactic acid molecules that involved in this reaction do not reacted at room temperature. This means the energy level of the reactants should have to rise up to some point by heating them. By doing this the movement of the molecules in the mixture is became faster. These phenomena can alter the nature of the reaction. The effect of reaction temperature on the acid value, esterification degree and intrinsic viscosity is shown in table 4-5.

Table 4-4 Effect of reaction temperature on the nature of the reaction for 1:3 molar ratio and 8 hrs

Temperature (°C)	Acid value	Esterification degree	Viscosity (Cp)
190	99	29	4072
170	157	14	1518
150	235	3	479

II. Thermal Stability Analysis: The Thermal Gravimetric Analyzer (TGA) was used to investigate the thermal stability of the conjugate, by recording the temperature versus percentage weight loss of the samples. This was performed to determine whether the chain length of the conjugate affects the thermal resistance or not. TGA thermo grams of the one stage exhibited a weight loss in all the samples. The initial decomposition of the conjugate was observed at around 150 °C, which is due to the presence of water.

From the TGA graph (figure 4-11), the GLY-LAC 1:3 bio-conjugate lost its 10 wt %at about 245 °C (T_{10}) and it had lost its 50 wt % at about 319 °C (T_{50}). The T_{90} temperature of GLY-LAC 1:3 is 351 °C, which is the conjugate lost 90.0 wt%. The Tmax temperature, which is a maximum amount of degradation, occurred at 506 °C.

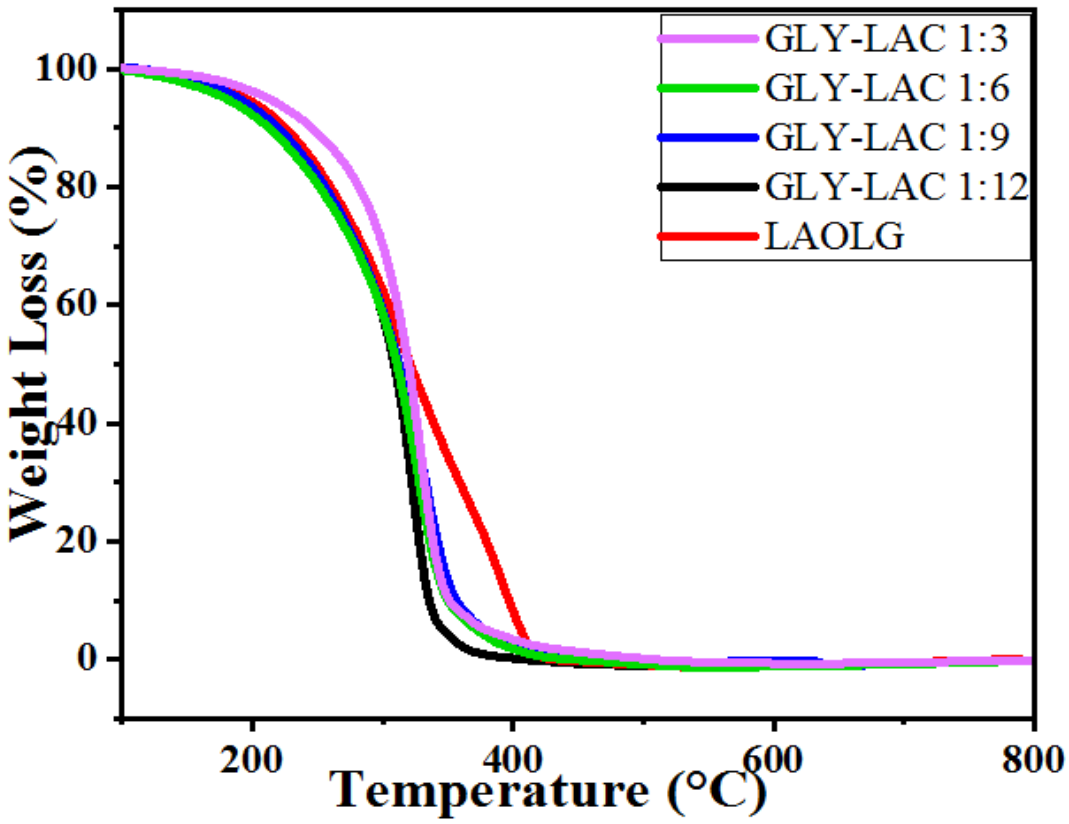


Figure 4-8 TGA Graph of the synthesized LAOLG and GLY-LAC Bio-conjugates

As indicated in table 4-6, the GLY-LAC 1:6 bio-conjugate is also stable up to 200 °C. At about 211 °C the conjugate lost its 10wt %, and at about 310 °C, it had lost its50wt %. In the GLY-LAC 1:9 bio-conjugate the T_{10}, $T_{50}$and T_{90} are 218 °C, 308 °C and 351 °C respectively. For the GLY-LAC 1:12 bio-conjugate T_{10}, T_{50} and $T_{90}$was observed225 °C, 310°C and 335 °C respectively.

Table 4-5 Thermal stability analysis table for the selected weight loss area

	T_{10}	T_{50}	T_{90}	T_{max}
LAOLG	225 °C	319.5 °C	397 °C	506 °C
GLY-LAC 1:3	245.5 °C	319.5 °C	351 °C	506 °C
GLY-LAC 1:6	211 °C	310 °C	351 °C	443 °C
GLY-LAC 1:9	218 °C	308 °C	351 °C	505 °C
GLY-LAC 1:12	225 °C	310 °C	335 °C	444 °C

In the GLY-LAC 1:12 bio-conjugate the chain of the arms is increased with the formation of ester bond which can be increase the probability of intramolecular transesterification occurring [103], [104] through lactide formation at lower temperature (around 260 °C) or other cylicoiligomer at higher temperature (around 320°C) [103]. This may facilitate the degradation of the intermolecular bond of the longer chain conjugates. On the other, hand the presence of greater amount of unreacted lactic acid unit relatively in the GLY-LAC 1:12 conjugate makes it a less thermally stable material. This is due to the involvement of the lactic acid monomers in the thermal degradation rate when it exposed to heat.

4.2 Application of GLY-LAC Bio-conjugate as an Ointment Base

4.2.1Physical evaluation of the base

Spreadability: Spreadability is a broad term to explain the capability of media being spread. The nature of spreadability of an ointment is the net result of a combination of rheological contributions, viscosity is one of them. The spreadability of ointments in our case can be divided into two groups: low and high. It is observed that Spreadability is inversely proportional to the lactic acid concentration. The ointment became thicker as the amount of lactic acid increased, due to this spreadability decreased. The spreadability of all synthesized ointments has been calculated and it has been observed that GLY-LAC 1:3 based ointment has higher spreadability.

Table 4-6Physical properties of the synthesized Ointment base

	Bio-conjugated Base	Viscosity (Cp)	Spreadablity	PH
S1	GLY-LAC 1:3	8560	8.88	5.01
S2	GLY-LAC 1:6	24,075	6.75	4.62
S3	GLY-LAC 1:9	29,430	5.05	4.01
S4	GLY-LAC 1:12	32,400	3.98	3.82

Sensory Analysis: Almost all organoleptic properties of the synthesized ointment base shows desirable properties of transparent slightly yellowish color, free from any objectionable odor, smooth and no greediness texture, homogenous without phase separation and a very good immediate skin feel properties.

pH: The pH values for the synthesized GLY-LAC conjugated base ranges from 3.82 to 5.01. Even though the pH of the skin is ranges from 4 to 6, the acidic PH values of the bases is comes from the lactic acid, which also called body fluids. When pH adjustment is desired, we can adjust the PH values.

4.2.2 Qualitative Analysis of the *C. macrostachyus* extract

Phytochemical constituent of *C. macrostachyus*: The genus Croton is rich in terpenoids (diterpenoids and triterpenoids), alkaloids, flavonoids, lignoids, proanthcyanidins and volatile oils containing monoterpenoids, sesquiterpenoids and some shikimate-derived compounds.

Previous studies showed the existence of crotin(a chalcone), lupeol (a triperpene), crotepoxide (a cyclohexanediepoxide), proteins, fatty acids, saponins, resins and alkaloids in the CM extraction. Terpenoids exhibit various important pharmacological activities, like anti-viral and anti-bacterial activities.

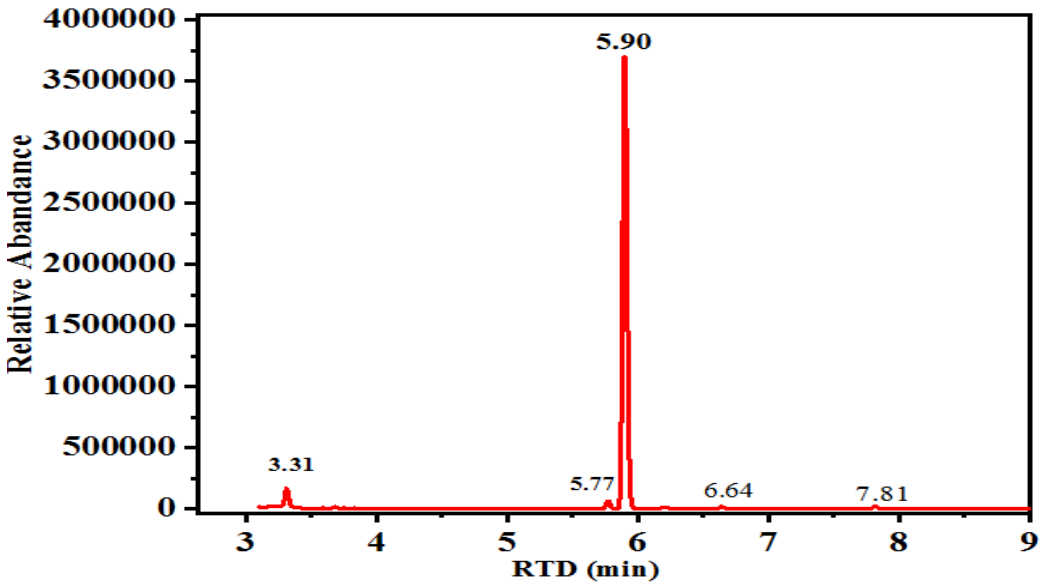


Figure 4-9 GC-MS chromatogram of the leaf extract of C. Macrostachyus

Secondary metabolites of medicinal plants have different mechanism of actions against bacteria agents. The mechanism can be through inhibition of bacterial enzymes, affecting cell division, bacterial membrane disruption and affecting virulence genes. However, the mode action of alkaloid is through inhibition.

4.2.3 Formulation of Herbal Ointment

In the herbal ointment formulation, *C. Macrostachyus* crude oil extract with different concentrations of 1%, 3%, 5%, and 10% is used to incorporate in the selected GLY-LAC bio-conjugates (table 4-8). In this case, the minimum viscosity V_{min} that is the GLY-LAC 1:3 bio-conjugate and the maximum viscosity V_{max} that is GLY-LAC 1:12 is selected as an Ointment base matrix.

Table 4-7 Physical properties of the formulated Herbal Ointment

Formulation	Herbal Ointment	Spreadablity	PH
F1	1%GLY-LAC1:3/CM	8.30	5.00±0.5
F2	3%GLY-LAC 1:3/CM	7.82	5.68± 0.9
F3	5%GLY-LAC 1:3/CM	7.10	5.92 ± 1.1
F4	10%GLY-LAC 1:3/CM	6.82	6.23 ±0.4
F5	1%GLY-LAC **1:12**/CM	3.72	4.0 ± 0.9
F6	3%GLY-LAC **1:12**/CM	3.44	4.32 ± 0.8
F7	5 %GLY-LAC **1:12**/CM	3.23	4.50 ± 1.1
F8	10%GLY-LAC **1:12**/CM	3.01	4.67 ± 1.2

The selected ointment base S1 and S4 have a viscosity of 8560 Cp and 32,400 Cp with a spreadability of 8.88 and 3.98 respectively. However, the spreadability for the formulated herbal ointment have a decreased amount of 8.30, 7.82, 7.10 and 6.82 for the F1, F2, F3 and F4

respectively. The effect of adding CM active component on the spreadability and pH is summarized in the Table 4-8.

4.2.4Anti-Bacterial Properties

With two, separate concentrations (l mg/ml and 3 mg/ml); the antibacterial potential of the formulated ointment against *Escherichia coli* (gram-negative) and *Staphylococcus aureus* (gram-positive) bacterias investigated. The mechanism of action against the bacteria's in this case is through inhibition of bacterial enzyme.

Anti-Bacterial Activities of GLY-LAC 1:3 Based Herbal Ointment: the synthesized GLY-LAC 1:3 conjugate have anti-bacterial activities for both E.coli and S.auraus bacteria's. In its application as an ointment base matrix, the efficacy of the herbal ointment out of this conjugate is enhanced with the incorporation of 1 wt%, 3 wt%, 5 wt% and 10 wt% of the active component from *C. Macrostachyus.*

Before adding the extracted active component of CM, the 1mg/ml sample concentration of the Vmin Ointment base (GLY-LAC 1:3) have no any activity (6mm inhibition zone) for both E.coli and S.aureus bacteria's (see figure 4.13 (a) and (b). However, after adding 1%, 3%, 5% and 10 % CM extract the inhibition zone increases to 8 mm, 10 mm, 13 mm and 16 mm for the E.coli and 7 mm 10 mm, 12 mm and 15 mm for S.auraus bacteria respectively.

a) **b)**

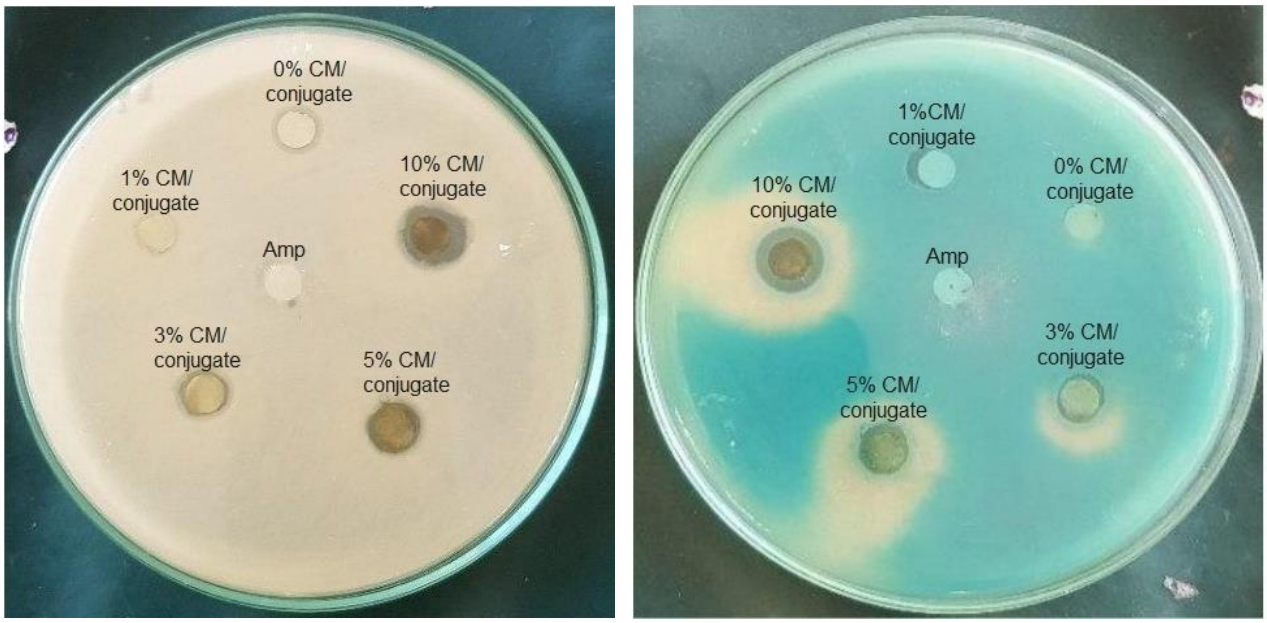


Figure 4-10 Inhibition zone for the 1mg/ml sample concentration of GLY-LAC 1:3 based herbal Ointment against (a) E.coli and (b) S.auraus bacteria

Table 4-8 Zone of bacterial growth inhibition (mm) of 1mg/ml concentration GLY-LAC 1:3 based herbal ointment against E-coli and S.auraus bacteria

Formulated ointment (1mg/ml)	Zone of Inhibition (mm)	
	E. coli ATCC25922	*S. aureus* ATCC25923
GLY-LAC 1:3 Ointment Base	6	6
C. Macrostachyus Crude Oil	6	6
F1	7	8
F2	10	10
F3	12	13
F4	15	16
Ampicillin	9	9

As compared to the inhibition zone of 9 mm for Amphicilin, the 1 mg/ml sample concentration of F2, F3, and F4 have a better ant-bacterial activity for both bacteria.

For the 3 mg/ml concentration, of the Vmin conjugate, anti-bacterial properties of the synthesized ointment base and the formulated herbal ointment shows significant activities for both bacteria's. The inhibition zone for the blank ointment base without the addition of CM extract is 15 mm and 18 mm respectively for E.coli and S.auraus bacteria. As it can clearly observed in figure 4-14 a) and b) and stated in table 4-10 below, after adding 1 %, 5 % and 10 % CM active component the inhibition zone increased to 12 mm, 18 mm and 19 mm respectively for E.coli and 19 mm, 21 mm and 22 mm respectively for S.auraus.

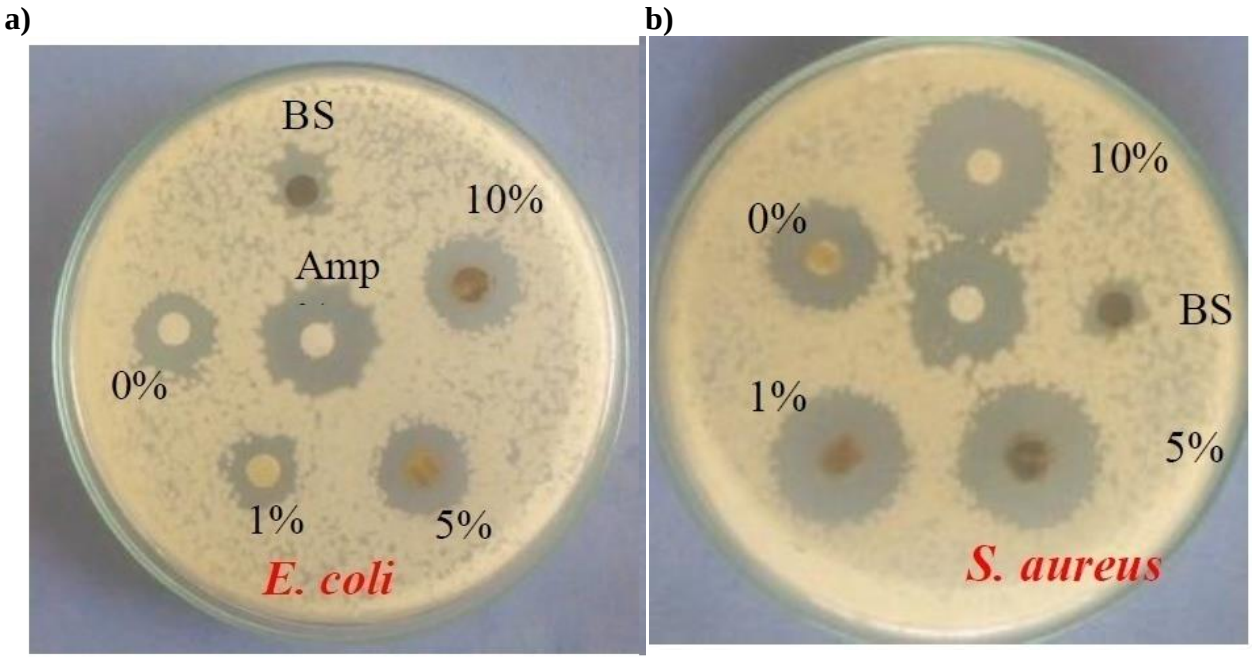


Figure 4-11 Inhibition zone for the 3 mg/ml sample concentration of GLY-LAC 1:3 based herbal Ointment against (a) E.coli and (b) S.auraus bacteria

Table 4-9 Zone of bacterial growth inhibition (in mm) of 3mg/ml concentration GLY-LAC 1:3 based herbal ointment against E-coli and S.auraus bacteria

Formulated ointment (3mg/ml)	Zone of Inhibition (mm)	
	E. coli ATCC25922	*S. aureus* ATCC25923
GLY-LAC 1:3 Ointment Base	15	18
C. Macrostachyus Crude Oil	7	8
F1	12	19
F3	18	21
F4	19	22
Ampicillin	20	20

Anti-Bacterial Activities of GLY-LAC 1:12 Based Herbal Ointment: As it can be seen on the figure 4-15 and tabulated in table 4-11 below, the 1 mg/ml sample concentration of blank GLY-LAC 1:12 have inhibition zone of 8 mm and 11 mm respectively for the E.coli and S.auraus bacteria. However, after adding 1%, 3%, 5% and 10 % CM the inhibition zone increases to 10 mm, 15 mm, 17 mm and 19 mm respectively for the E.coli and 12 mm, 20 mm, 21 mm and 22 mm respectively for S.auraus bacteria.

a) **b)**

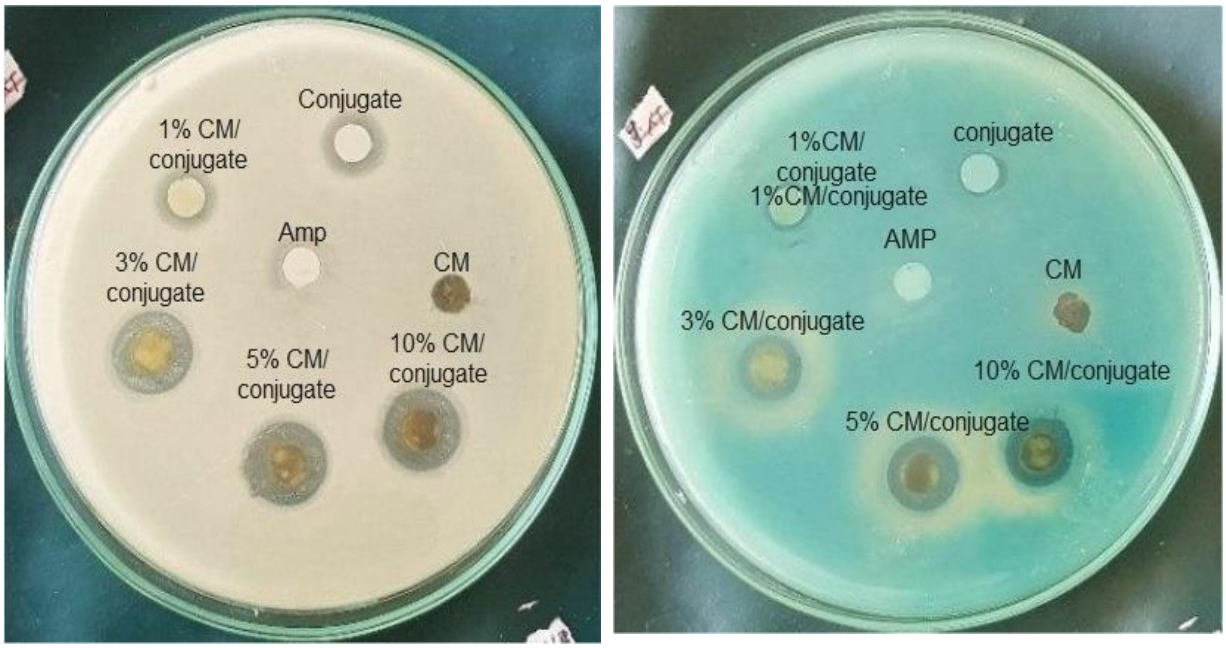


Figure 4-12 Inhibition zone for the 1mg/ml sample concentration of GLY-LAC 1:12based herbal Ointment against (a) E.coli and (b) S.auraus bacteria

Table 4-10 Zone of bacterial growth inhibition (mm) of 1mg/ml concentration GLY-LAC 1:12 based herbal ointment againstEcoliand S.auraus bacteria

Formulated ointment (1mg/ml)	Zone of Inhibition (mm)	
	E. coli ATCC25922	*S. aureus* ATCC25923
GLY-LAC 1:12 Ointment Base	8	11
F1	10	12
F2	15	20
F3	17	21
F4	19	22

However, as the sample concentration increased from 1mg/ml to 3 mg/ml the anti-bacterial activity of the conjugate is dramatically increased as shown on the figure 4-16 and table 4-12. This is because of the increasing of inhibition concentration against the tested bacteria. For the GLY-LAC 1:12 bio-conjugate based herbal ointment, as the amount of CM active component extract incorporation increased the ant-bacterial activity also increased. In figure 4-16 it is observed that 19 mm, 23 mm and 24 mm inhibition zone against E.coli bacteria is for the 3 wt%,

5 wt%, and 10 wt% CM incorporation respectively. Whereas for the S,auraus bacteria 20 mm, 23 mm and 24 mm inhibition zone is observed.

a) **b)**

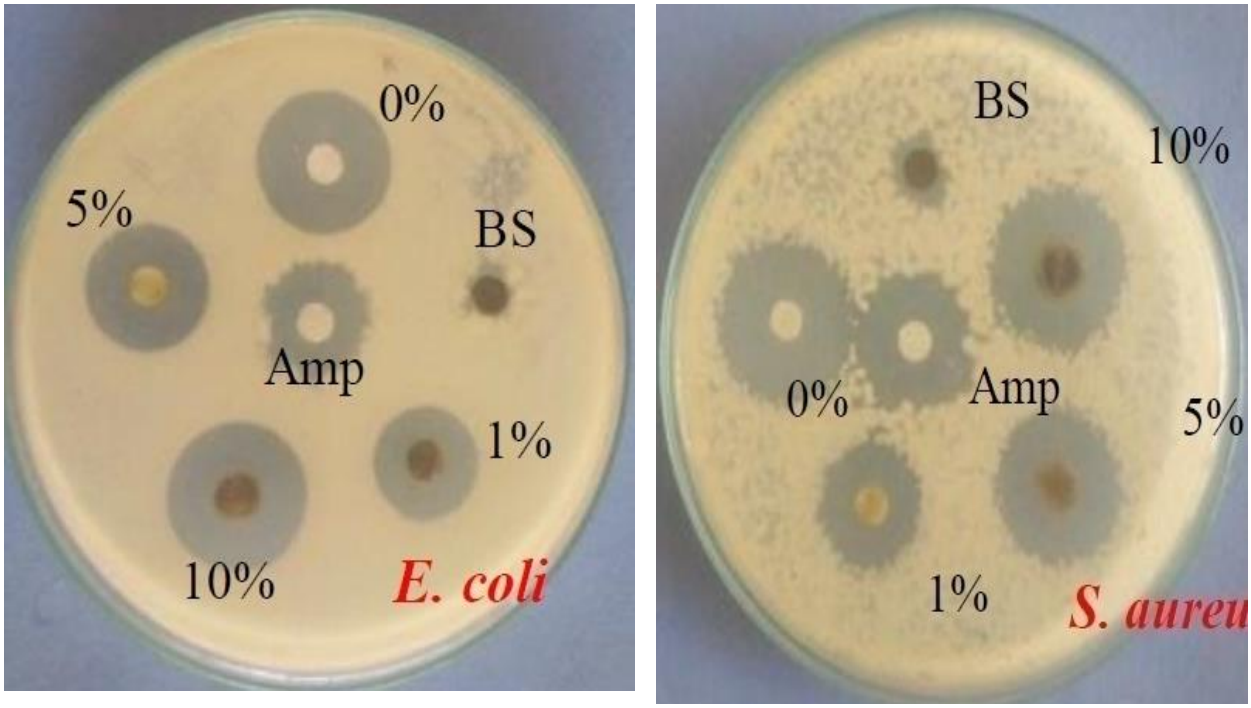


Figure 4-13 Inhibition zone for the 3 mg/ml sample concentration of GLY-LAC 1:12 based herbal Ointment against (a) E.coli and (b) S.auraus bacteria

Table 4-11 Zone of bacterial growth inhibition (mm) of 3mg/ml concentration GLY-LAC 1:12 based herbal ointment against E-coli and S.auraus bacteria

Formulated ointment (3mg/ml)	Zone of Inhibition (mm)	
	E. coli ATCC25922	*S. aureus* ATCC25923
GLY-LAC 1:12 Ointment Base	21	22
C. Macrostachyus Crude Oil	7	8
F5	19	20
F6	23	23
F7	24	24
Ampicillin	19	20

From all combination the GLY-LAC 1:12 based herbal ointment shows a better anti-bacterial property against the two bacteria's. This is because of the longer chain or higher viscosity of the conjugate. 24 mm zone of inhibition is observed for the ointment formulated from GLY-LAC 1:12 and 10 wt% plant extract. The ant-bacterial activities of the formulated herbal ointment are also increased with the concentration of active component loading. The small anti-bacteria effect of CM extract (7 mm and 8 mm for E.coli & S.auraus respectively) is enhanced with the ointment base. Therefore, we can conclude that ointment's antibacterial efficacy was due to a synergistic effect of the two; the conjugate base and the active constituents of the extract, and the activity was significantly enhanced when they were combined together as a herbal ointment.

4.3 Application of GLY-LAC Bio-conjugate as PLA additive

4.3.1 Mechanical test result

It is known that the neat PLA has a higher tensile strength so that a less flexible property at all. As shown on the figure below the PLA without a plasticizer loading have a higher tensile strength at break of 92.62 N. whereas the GLY-LAC 1:3 plasticizer loading PLA has a lower tensile strength at break than GLY-LAC 1:12 plasticized PLA. Tensile strength of the 10 wt%, 15 wt%, 20 wt% and 25 wt% GLY-LAC 1:3 loading PLA is observed respectively 51.8 N, 34.37 N, 33.02 N and 35.6 N. as the plasticizer loading concentration increased to the polymer matrix, the tensile strength of the plasticized PLA became have a lower tensile strength at break. However, as the plasticizer loading concentration reached at 25 wt% for both types of plasticizers there have seen the increasing of the force at break/tensile strength of the PLA film.

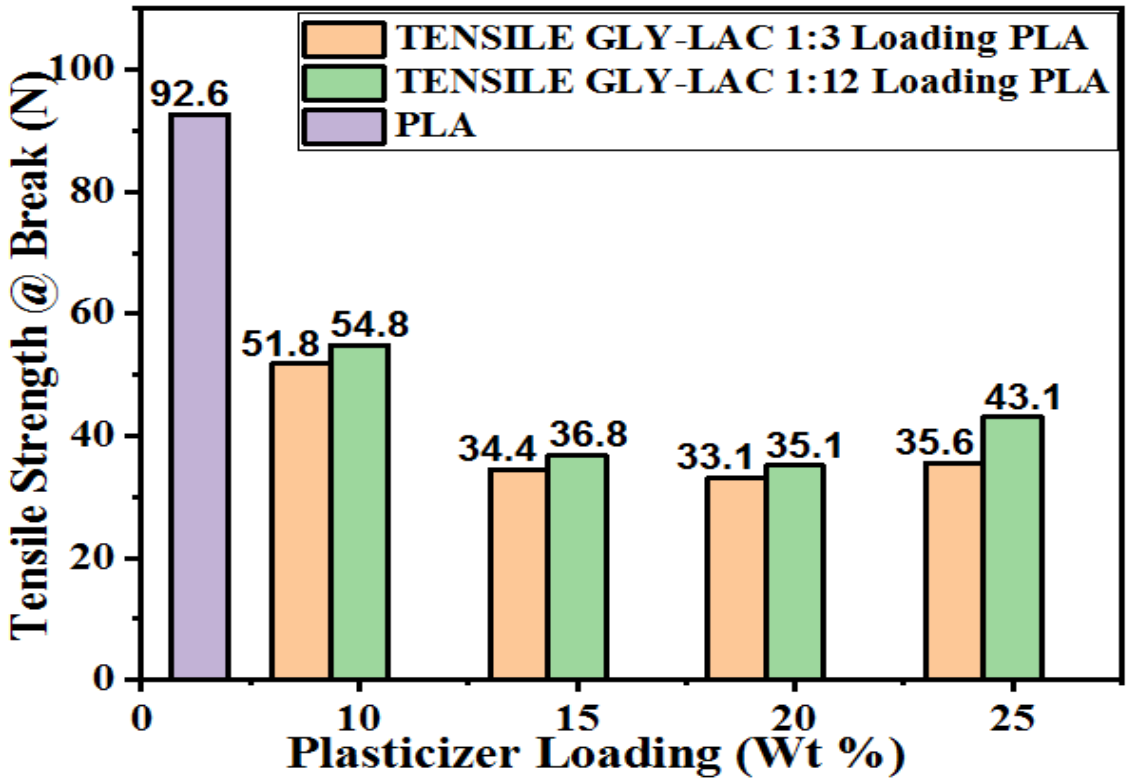


Figure 4-14Effect of plasticizer loading on the tensile strength @ break of PLA plastic film

The tensile strength of the PLA film is increased as the incorporation of GLY-LAC 1:12 bio-conjugate than the GLY-LAC 1:3 does. This is also correlated to the chain length and macro molecular structural size of the plasticizer. As the chain length of the plasticizers increased, its macromolecular structure is also increased due to this the entanglement of the plasticizing material in the polymer matrix is taken place. The entanglement of a molecule in a given polymer matrix can leads to give a stiffness property to the material.

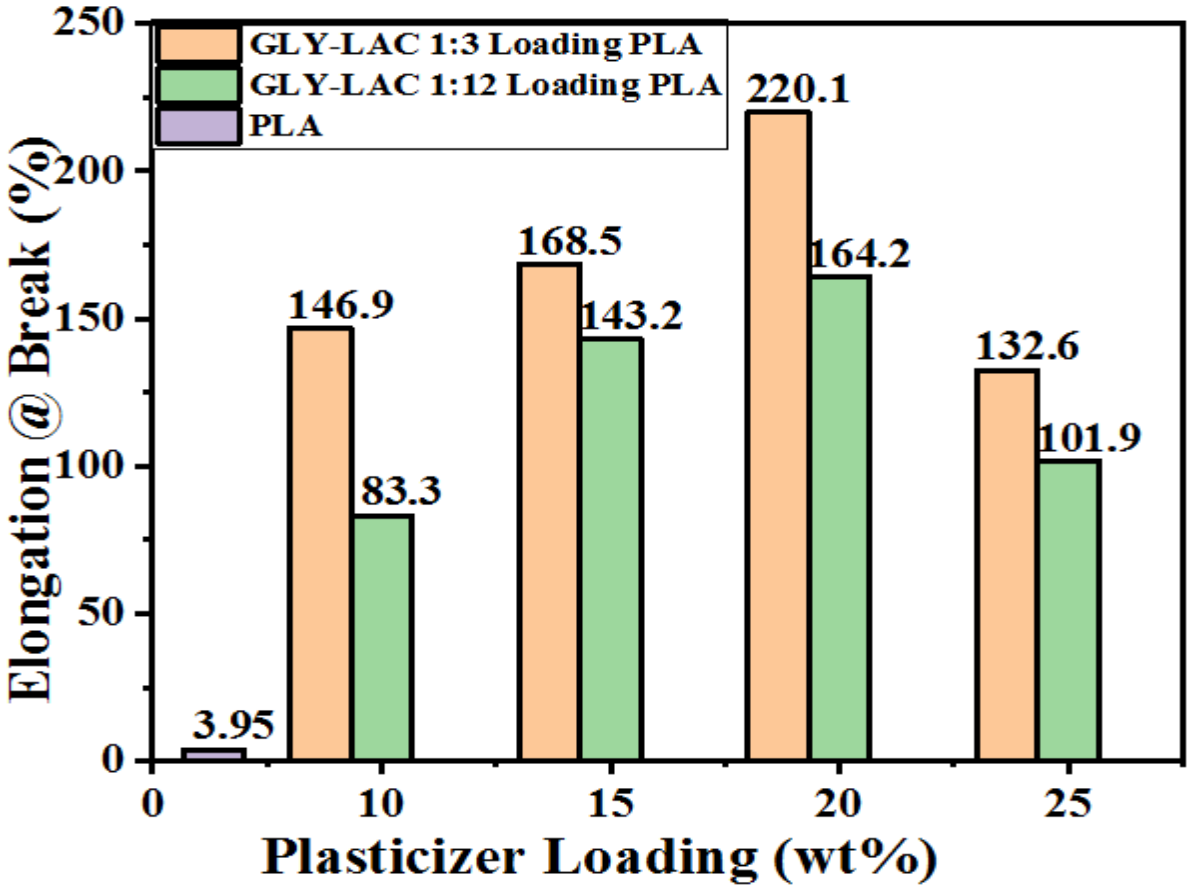


Figure 4-15 Effect of plasticizer loading on the elongation @ break of PLA plastic film

Due to the shorter chain length of the conjugate the probability to immerse in the polymer matrix is higher for the GLY-LAC 1:3 than the GLY-LAC 1:12 conjugate. On the other hand the lower the viscosity and the higher lubricating property of the GLY-LAC 1:3 conjugate makes it possible to easily lubricate the chains of the plasticizing material. The bigger the chain length of the

plasticizer is the bigger the macro molecular structure and the lesser to miscible with the polymer matrix, so reduces its plasticizing effect. As many researchers agreed that in order to be a good plasticizer a molecule should have to be able to move freely in the polymer matrix. Shorter chain length plasticizing molecules have a good mobility throughout the polymer matrix, so that acts as a good plasticizing agent. The PLA with shorter chain length GLY-LAC 1:3 plasticizer of 10 wt%, 15 wt%, 20 wt% and 25 wt% have an elongation at break of 117.5 mm, 134.8 mm, 176 mm and106 mm respectively. The decrease in the elongation at break of the 25 wt % plasticized PLA is due to the excess amount plasticizer loading. This result is similar to previously reported data [63].

4.3.2 Plasticizer Migration Test Result

Migration or leaching property of a plasticizer is highly dependent on the compatibility with the plasticized polymer, either chemically or structurally. The rate of plasticizer migration in the plasticized PLA as a function of time and temperature has been shows in the graph below. The Migration rate of a plasticizer is increased with time. For the same plasticizer loading, Compared to the plasticized PLA at 125 ℃, the plasticized PLA at 150 ℃ had a higher migration rate.See the 4-12 and 4-13 graphs below.

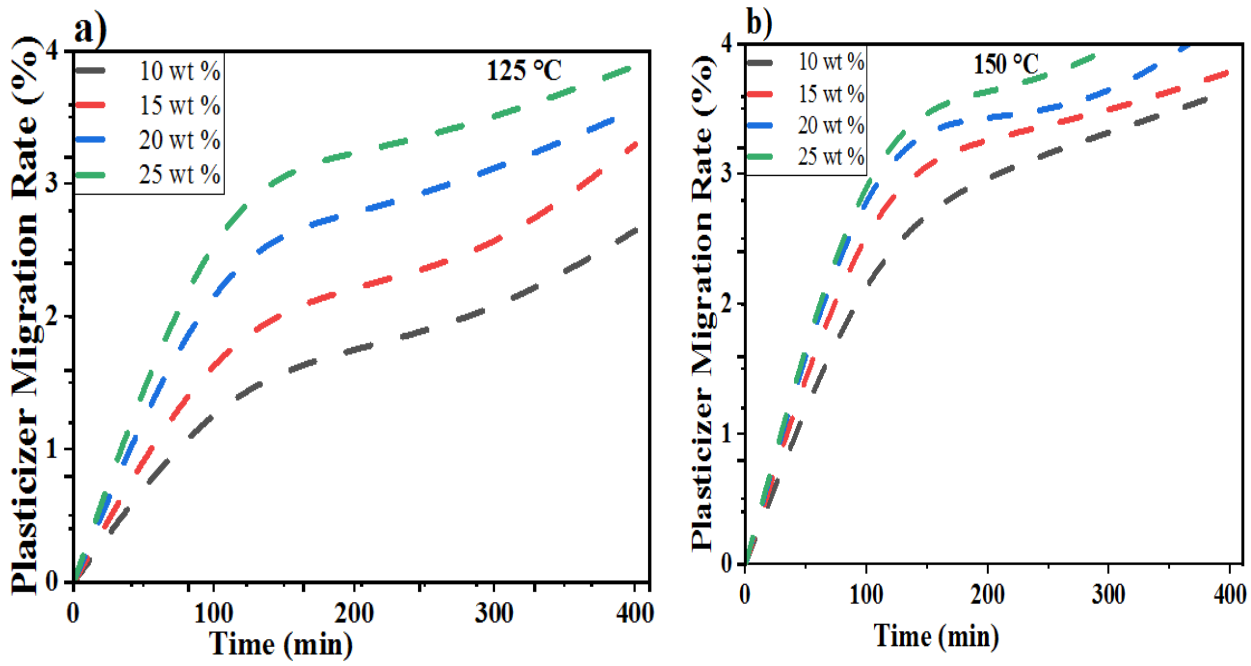


Figure 4-16 Migration rate of GLY-LAC 1:3 plasticized PLA at a) 125 °C and b) 150 °C

Since 150 °C is close to PLA's melting point, the PLA molecules' segmental motion was enhanced at this temperature. Meanwhile, the PLA molecules' interactions with the plasticizer became weakened, and the plasticizer's mobility increased significantly. At 125 ° C and 150 °C, we also found that the migration rate of both GLY-LAC plasticizers increased as its weight percent increased at the same time.For a higher plasticizing effect concentration of 20 wt%GLY-LAC 1:3 loading at a temperature of 125 °C, as the time exposed to heat of 100 min, 200 min,300 min and 400 min, the migration rate in percent is observed 2.54 %, 2.77 %, 3.1 % and 3.5 % respectively.

Due to its macromolecular structure the GLY-LAC 1:12 plasticized PLA have a lower migration rate when compared with the GLY-LAC 1:3 plasticized PLA. This is also the effect of chain length of the plasticizer in the structure of the polymer matrix. As shown below the migration rate of GLY-LAC 1:12 plasticized PLA at 150 °C have a lower migration rate than the GLY-LAC 1:3 plasticized PLA at 125 °C. this shows that how much the plasticizer molecule is entangled in the polymer matrix of PLA.

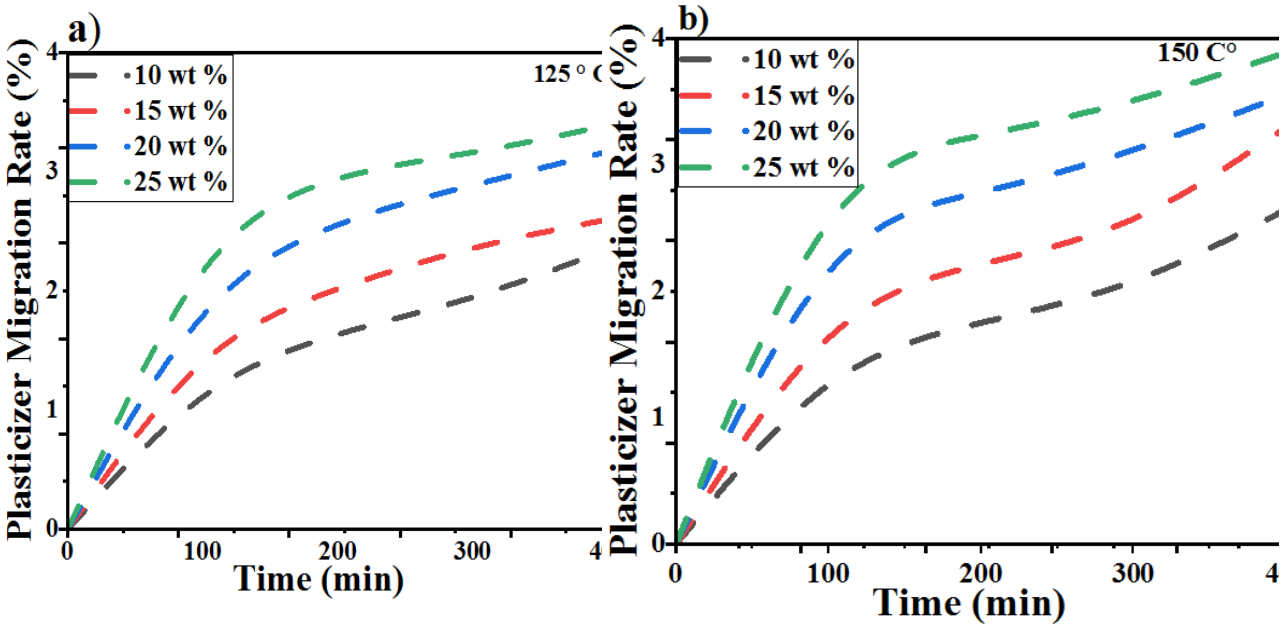


Figure 4-150Migration rate of GLY-LAC 1:12 plasticized PLA at a) 125 °C and b) 150 °C

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

This research work discussed on the synthesizing, characterizing and applications of a bio-based three-armed bio-conjugate with a glycerol core molecule and lactic acid chain unit. The effect of the reaction temperature, mixing ratio and reaction time on the completion of the polycondensation reaction was studiedby using an acid-base titration method and estimation of degree of esterification in the preliminary investigations of this research.

As the polycondensation reaction exposed to a higher reaction temperature of 190 ℃ and a reaction time of 8 hr, the consumption of the reactant is increased and so the unreacted lactic acid value is became decreased. However,at 190 °C and 8 hr reaction time the unreacted lactic acid value is increased from 76 to 157 for the GLY-LAC 1:3 and GLY-LAC 1:12 respectively. This is because of the insufficient reaction time and temperature for the higher mixing ratios of 1:12.Even though the unreacted lactic value for the GLY-LAC 1:12 conjugate is more than the other conjugates, the water escaped from the reaction in this case is larger, which is directly related to the degree of esterification of are action. Due to this the viscosity and chain length of GLY-LAC 1:12 conjugate is higher than the other is. The obtained chain length for GLY-LAC 1:3, GLY-LAC 1:6, GLY-LAC 1:9 and GLY-LAC 1:12 bio-conjugates are 0.37, 1.45, 1.91 and 2.83 with a viscosity of 8560Cp, 24075 Cp, 29430 Cp, 32,400 Cp respectively. In the FTIR result of all conjugates it is observed that, the three OH groups of glycerol is reacted with the COOH group of lactic acid and there is a formation of three armed glycerol and lactic acid based bio-conjugate having a reactive end group.

The ability to adjust the viscosity, the possibility of adding other functional groups at the end of the arm and the possibility of setting secondary interactions (hydrophobic or hydrophilic) makes the developed hydroxyl group terminated, three-armed conjugate to have a capacity of carry an active component of *C. macrostachyus*, which makes it suitable for the formulation of herbal ointment. The synthesized conjugates have an anti-bacterial activity in its blank test and have

enhanced anti-bacterial property after the incorporation of *C. macrostachyus* extract, against the E.coli and S.auraus bacteria's. The higher the viscosity or the longer the lactic acid chain the better the anti-bacterial property of the ointment.

The ability to adjust the macro structural size, the compatibility to adsorb in PLA polymer and the presence of limited amount of polar end groups to intermolecular plasticizing the developed three-armed glycerol based bio-conjugate have been also used as a polymer additive. The shorter the chain of the conjugate means the higher the mobility in the polymer matrix, which is directly related to the plasticizing ability of a plasticizer. On the other hand the longer the chain or the larger the size, miscibility with the polymer matrix is difficult and have lower plasticizing effect. However, the small sized plasticizer faces a leaching problem.

Almost all ointment formulations have done by using fusion method at a temperature of 70 °C and PLA have been melt processed at a temperature of 150 °C. All the synthesized conjugate in this research have enhanced thermal property, which is stable up to 200 °C. This is a suitable thermal stability property of the conjugate to be used as an ointment base matrix and PLA polymer additive.

5.2 Recommendation

In this research work a valorization of glycerol to valuable product via a green synthesizing method was performed to produce a three armed glycerol and lactic acid based bio-conjugate for the targeted application as an ointment base matrix and polymer additive. The biodegradability, biocompatibility, non-toxicity and ability to adjust its chemical and physical property make this material to be used in versatile application.

Some recommended research work opportunities are as follow:

- Since the production, process does not alter the lubricant nature of the glycerol and lactic acid reactants, the developed three armed bio-conjugate can be used as a lubricant base oil.
- Due to the presence of a reactive hydroxyl group terminal at the end of the chain, this promising bio-material can be used as a surfactant; antifoaming agent.

- The other recommended research work opportunity for the developed conjugate is a biodiesel additive to enhance the diesel properties.

- Since the presence of free lactic acid monomers in the conjugate affects the thermal stability, purification of the conjugate from the unreacted lactic acid is crucial. Further study can be explored for this area.

- Due to the green synthesizing method that used in this experiment, the developed product can be used for a versatile application in the medical, cosmetics and drug carrier application.

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APPENDIXES

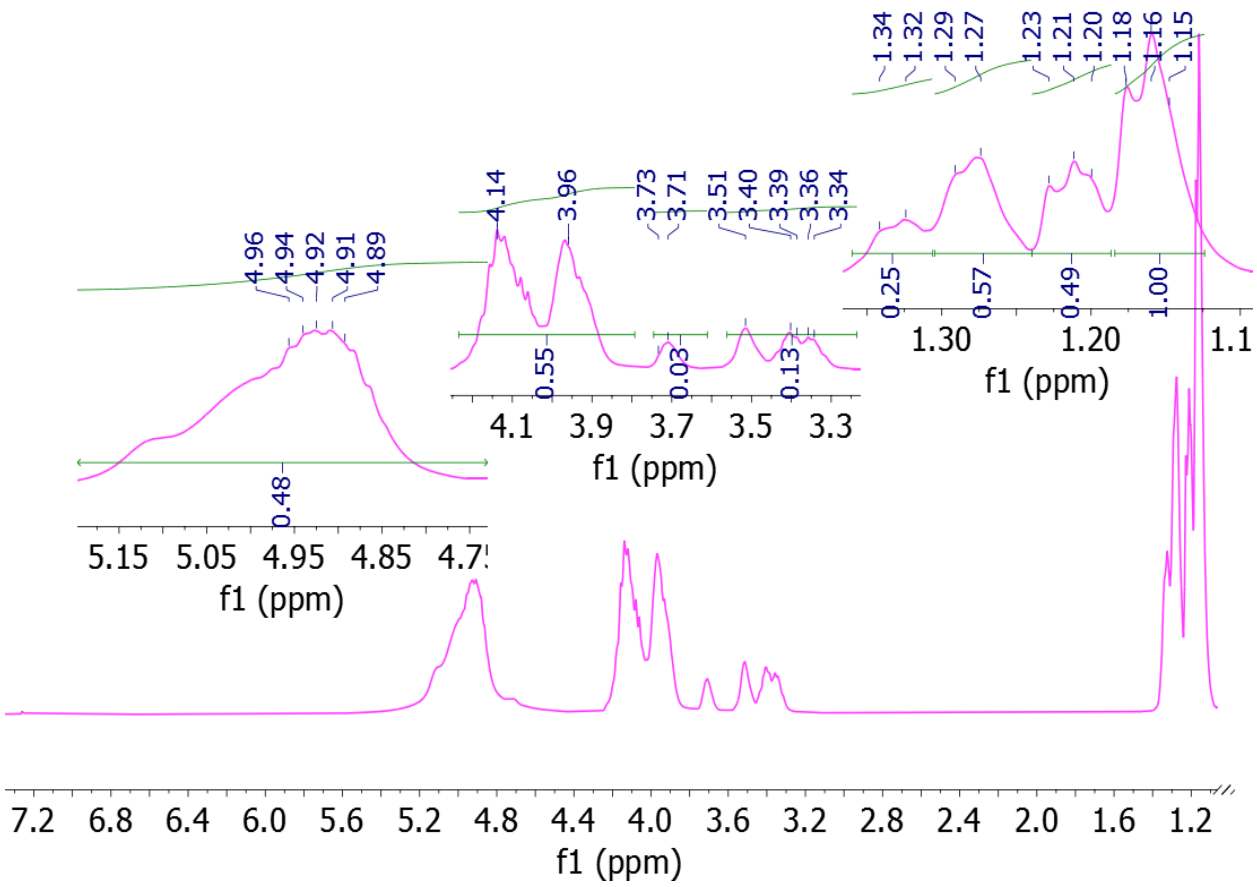
Appendix 1.Assignments of Peaks from C-NMR & H-NMR

Peak label	Chemical shift (ppm) C-NMR	Assignment
A	~ 169 – 170	C=O main chain
B	~ 169.8	C=O Adjacent to (-O-CH_2)
C	~ 173.5 - 174.5	LA end C=O (adjacent to the ester oxygen).
D	~ 171 & 171.5	LA end C=O (adjacent to hydroxyl group).
E	~ 176.5	C=O Terminal unreacted LA
F	~ 68 – 69	--CH , of lactic acid
G	~ 66.4	--CH-OH of lactic acid
H	~ 68 - 70	--CH, Of Core molecule
I	~ 16-22	--CH_3 of lactic acid
J	~ 19.5-20	--CH_3 of lactic acid (Hydroxyl terminated)

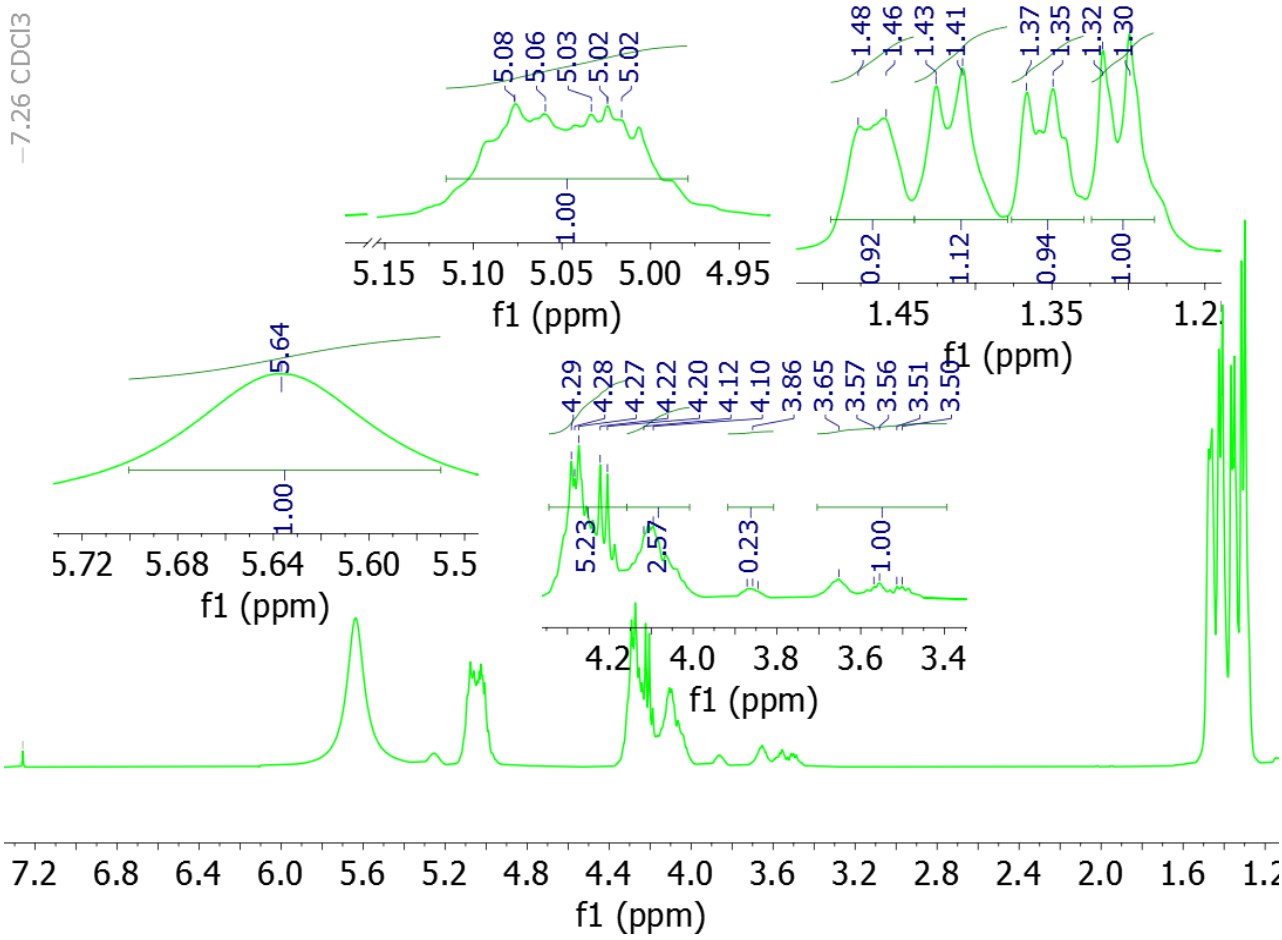
Peak label	Chemical Shift (ppm) H-NMR	Assignment

A	~ 1.44	--CH_3- Terminal LA
B	~ 1.54	--CH_3- in the chain
C	~ 3.5	--OH – chains terminal
D	~ 4.3	--CH_2 – core molecule
E	~ 5.1	--CH in the chain

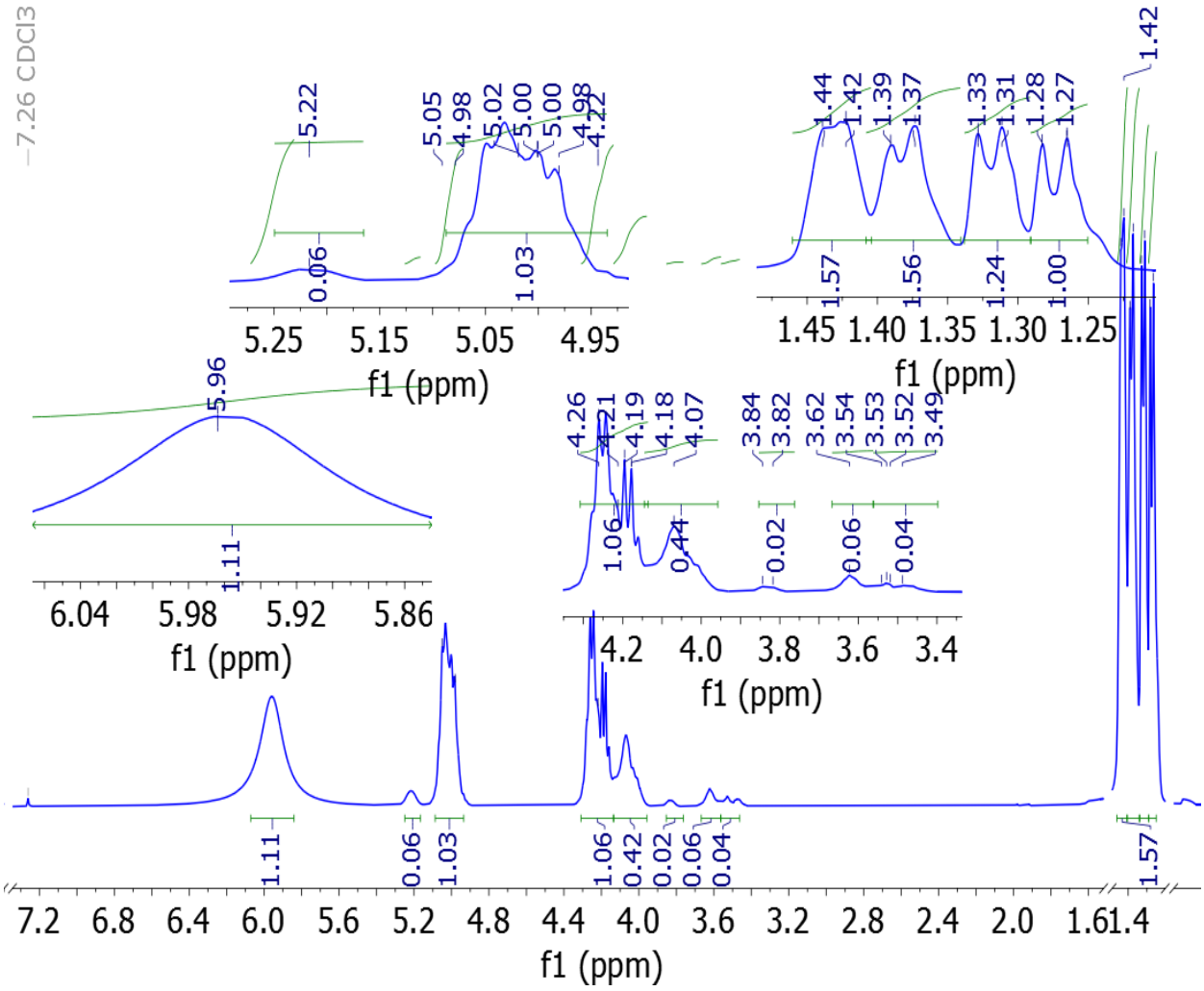
Appendex 2 1H-NMR Spectra for GLY-LAC 1:3 Bio-conjugate



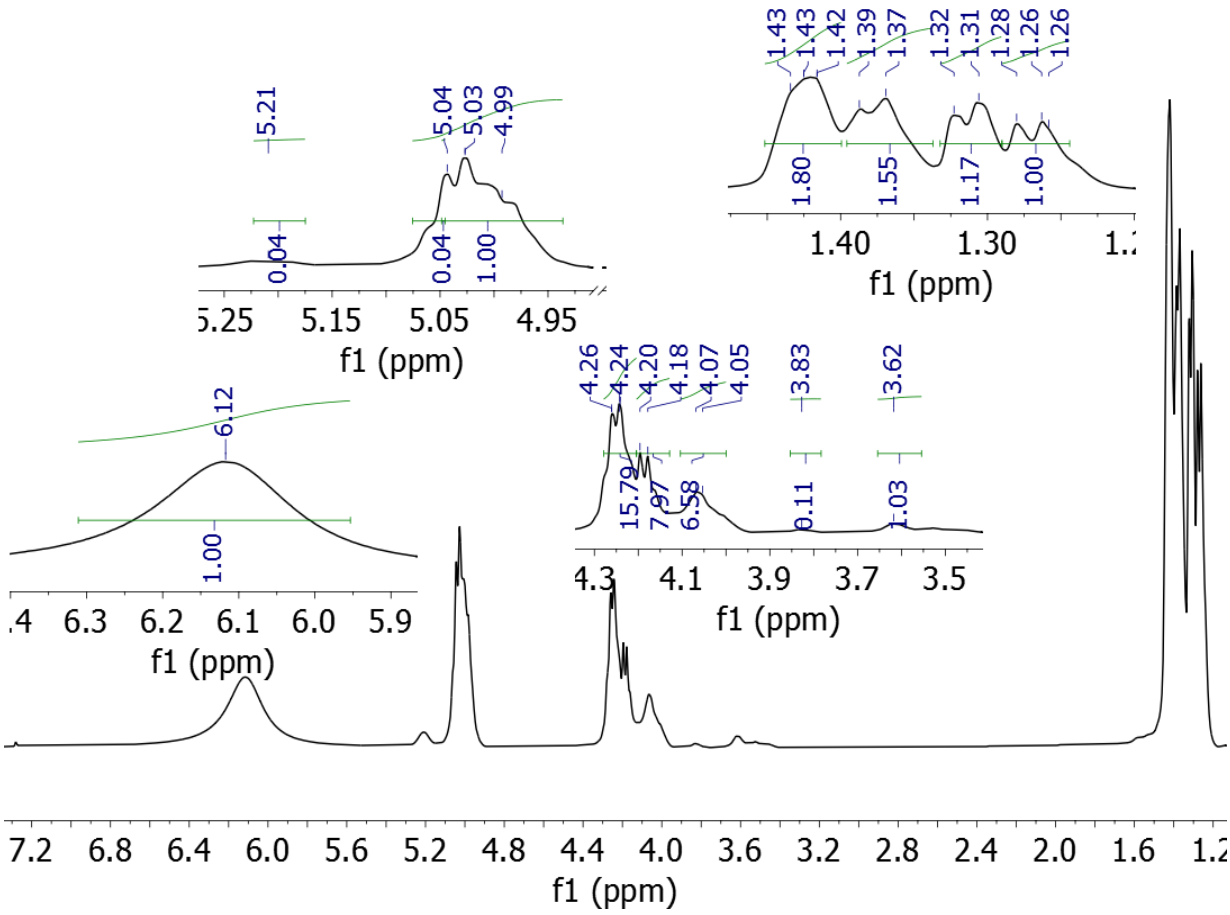
Appendix 31H-NMR Spectra for GLY-LAC 1:6 Bio-conjugate



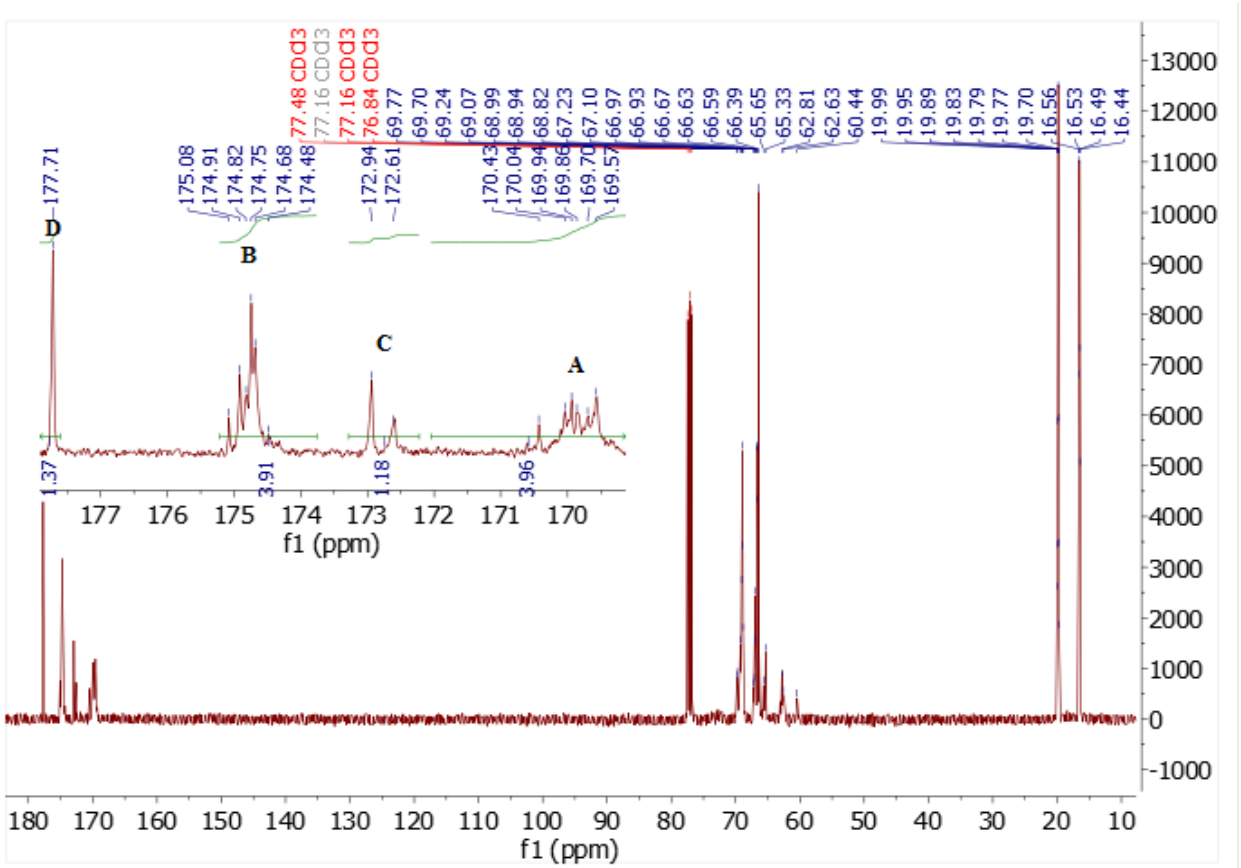
Appendix -4 1H-NMR Spectra for GLY-LAC 1:9 Bio-conjugate



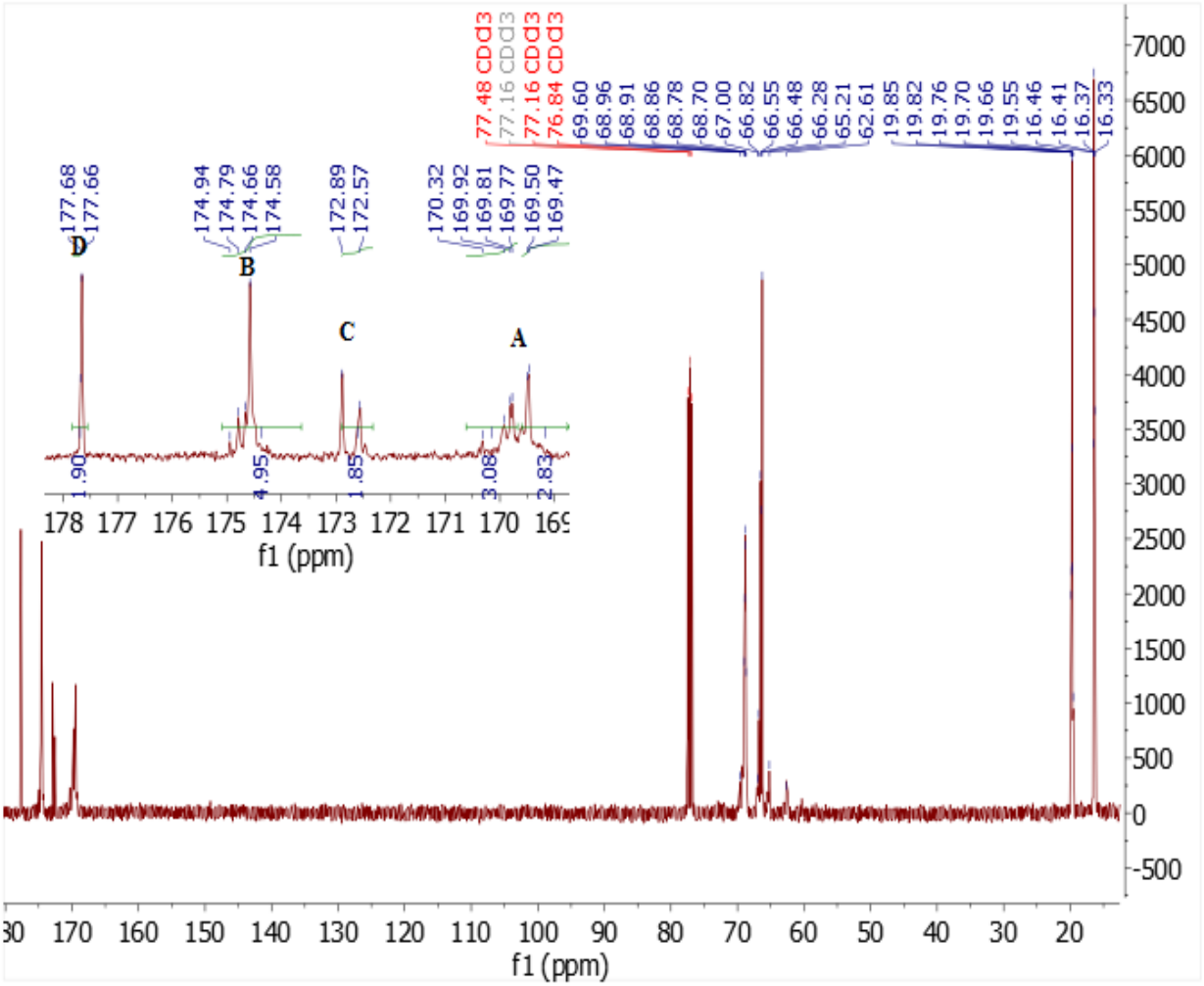
Appendex-5 1H-NMR Spectra for GLY-LAC 1:12 Bio-conjugate



Appendix-6 ^{13}C-NMR Spectra for GLY-LAC 1:6 Bio-conjugate



Appendix-7 ^{13}C-NMR Spectra for GLY-LAC 1:9 Bio-conjugate



Appendix-8 Letter from the Ethiopian conformity assessment enterprise

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Ethiopian Conformity Assessment Enterprise

ቁጥር(No) 2/23/7/284/2021
ቀን (Date) 01 MAR 2021

To: Tsion Seifu
Addis Ababa

On your letter dated Jan 12, 2021 you have requested for the analysis on Plastic Film.

Accordingly, the analysis is completed as per your request and hence you find the report attached here with.

Regards,

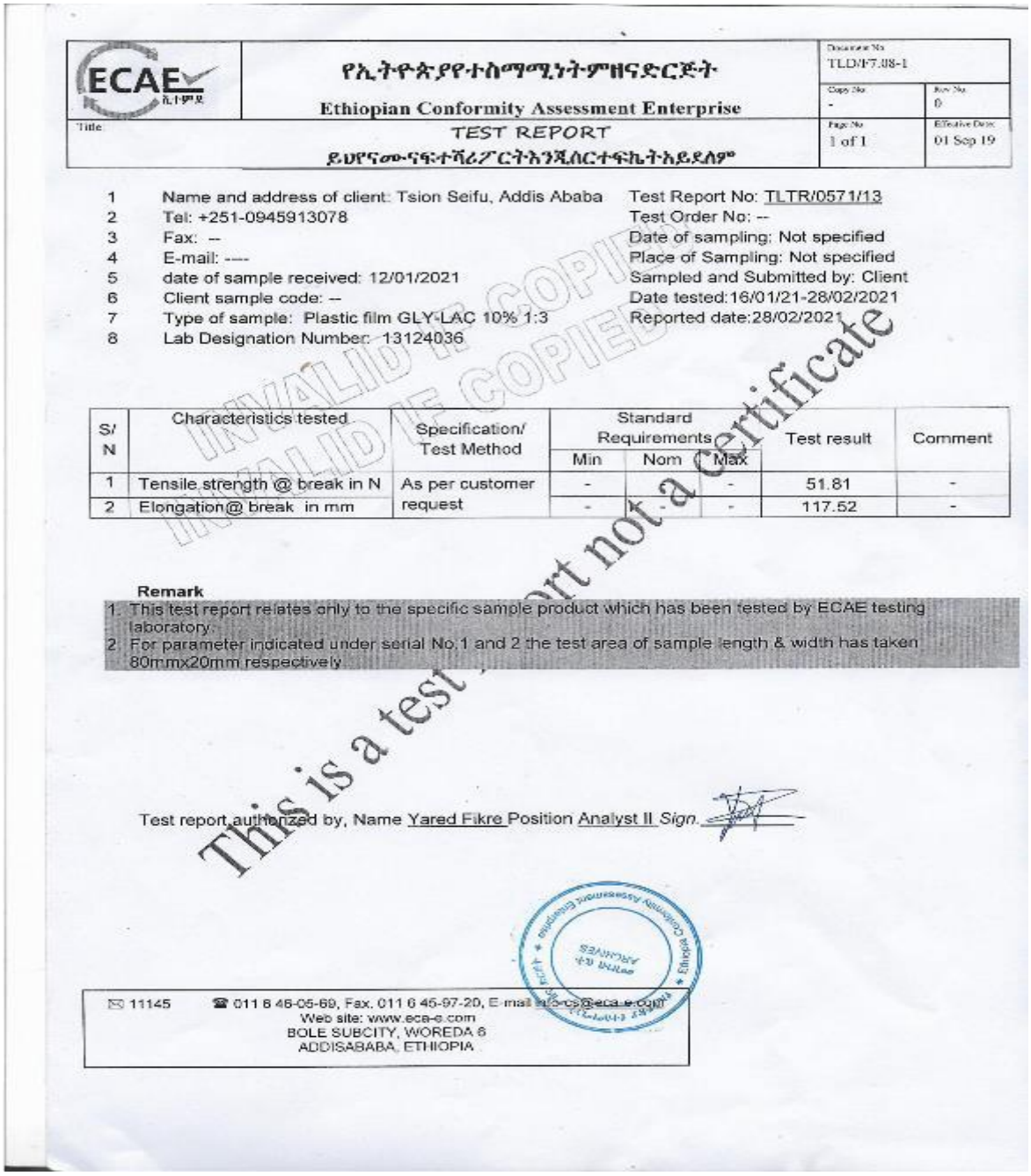
Solomon Mulubrhan
Customer Service, Manager

Enc: 8 Page of test reports TLTR/0571-0578/13

CC. ECAE
- Customer's Service
  Addis Ababa

ወደ ላቀ ብቃት የሚያደርሰው!
Moving you forward!

Appendix-9 Tensile strength @ break and Elongation @ break result for 10 wt% GLY-LAC 1:3 plasticized PLA



ECAE ኢ.ኮ.ም.ድ

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Ethiopian Conformity Assessment Enterprise

Document No.	
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Copy No: -	Rev No: 0
Page No: 1 of 1	Effective Date: 01 Sep 19

Title: TEST REPORT

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code: --
7 Type of sample: Plastic film GLY-LAC 10% 1:3
8 Lab Designation Number: 13124036

Test Report No: TLTR/0571/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements Min	Nom	Max	Test result	Comment
1	Tensile strength @ break in N	As per customer request	-	-	-	51.81	-
2	Elongation@ break in mm		-	-	-	117.52	-

Remark

1. This test report relates only to the specific sample product which has been tested by ECAE testing laboratory.
2. For parameter indicated under serial No.1 and 2 the test area of sample length & width has taken 80mmx20mm respectively

Test report authorized by, Name Yared Fikre Position Analyst II *Sign.*

✉ 11145 ☎ 011 6 46-05-69, Fax. 011 6 45-97-20, E-mail: ecae-cs@eca.e.com
Web site: www.eca-e.com
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ADDISABABA, ETHIOPIA

Appendix-10 Tensile strength @ break and Elongation @ break result for 15 wt% GLY-LAC 1:3 plasticized PLA

ECAE

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Ethiopian Conformity Assessment Enterprise

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code:--
7 Type of sample: Plastic film GLY-LAC 15% 1:3
8 Lab Designation Number: 13124037

Test Report No: TLTR/0572/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements			Test result	Comment
			Min	Nom	Max		
1	Tensile strength @ break in N	As per customer request	-	-	-	34.37	-
2	Elongation@ break in mm		-	-	-	134.83	-

Remark

1. This test report relates only to the specific sample product which has been tested by ECAE testing laboratory
2. For parameter indicated under serial No.1 and 2 the test area of sample length & width has taken 80mmx20mm respectively.

Test report authorized by, Name Yared Fikre Position Analyst II Sign

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Appendix-11 Tensile strength @ break and Elongation @ break result for 20 wt% GLY-LAC 1:3 plasticized PLA

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Ethiopian Conformity Assessment Enterprise

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code: --
7 Type of sample: Plastic film GLY-LAC 20% 1:3
8 Lab Designation Number: 13124038

Test Report No: TLTR/0573/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements			Test result	Comment
			Min	Nom	Max		
1	Tensile strength @ break in N	As per customer request	-	-	-	33.02	-
2	Elongation@ break in mm		-	-	-	176.08	-

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Remark

1. This test report relates only to the specific sample product which has been tested by ECAE testing laboratory.
2. For parameter indicated under serial No.1 and 2 the test area of sample length & width has taken 80mmx20mm respectively.

Test report authorized by, Name Yared Fikre Position Analyst II Sign.

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Web site: www.eca-e.com
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Appendix-12 Tensile strength @ break and Elongation @ break result for 25 wt% GLY-LAC 1:3 plasticized PLA

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Ethiopian Conformity Assessment Enterprise

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ---
5 date of sample received: 12/01/2021
6 Client sample code: --
7 Type of sample: Plastic film GLY-LAC 25% 1:3
8 Lab Designation Number: 13124039

Test Report No: TLTR/0574/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements Min	Nom	Max	Test result	Comment
1	Tensile strength @ break in N	As per customer request	-	-	-	35.60	-
2	Elongation@ break in mm		-	-	-	106.05	-

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Remark

1. This test report relates only to the specific sample product which has been tested by ECAE testing laboratory.
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Test report authorized by, Name Yared Fikre Position Analyst II Sign.

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Appendix-13 Tensile strength @ break and Elongation @ break result for 10 wt% GLY-LAC 1:12 plasticized PLA

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code: --
7 Type of sample: Plastic film GLY-LAC 10% 1:12
8 Lab Designation Number: 13124040

Test Report No: TLTR/0575/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements			Test result	Comment
			Min	Nom	Max		
1	Tensile strength @ break in N	As per customer request	-	-	-	54.77	-
2	Elongation@ break in mm		-	-	-	66.68	-

Remark

1. This test report relates only to the specific sample product which has been tested by ECAE testing laboratory.
2. For parameter indicated under serial No 1 and 2 the test area of sample length & width has taken 80mmx20mm respectively.

Test report authorized by. Name Yared Fikre Position Analyst II Sign.

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Appendix-13 Tensile strength @ break and Elongation @ break result for 15 wt% GLY-LAC 1:12 plasticized PLA

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code: --
7 Type of sample: Plastic film GLY-LAC 15% 1:12
8 Lab Designation Number: 13124041

Test Report No: TLTR/0576/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements			Test result	Comment
			Min	Nom	Max		
1	Tensile strength @ break in N	As per customer request	-	-	-	36.76	-
2	Elongation@ break in mm		-	-	-	114.58	-

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Appendix-14 Tensile strength @ break and Elongation @ break result for 20 wt% GLY-LAC 1:12 plasticized PLA

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code: --
7 Type of sample: Plastic film GLY-LAC 20% 1:12
8 Lab Designation Number: 13124042

Test Report No: TLTR/0577/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements			Test result	Comment
			Min	Nom	Max		
1	Tensile strength @ break in N	As per customer request	-	-	-	34.99	-
2	Elongation@ break in mm		-	-	-	131.38	-

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Test report authorized by, Name Yared Fikre Position Analyst II Sign.

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ADDISABABA, ETHIOPIA

Appendix-15 Tensile strength @ break and Elongation @ break result for 25 wt% GLY-LAC 1:12 plasticized PLA

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1 Name and address of client: Tsion Seifu, Addis Ababa
2 Tel: +251-0945913078
3 Fax: --
4 E-mail: ----
5 date of sample received: 12/01/2021
6 Client sample code:--
7 Type of sample: Plastic film GLY-LAC 25% 1:12
8 Lab Designation Number: 13124043

Test Report No: TLTR/0578/13
Test Order No: --
Date of sampling: Not specified
Place of Sampling: Not specified
Sampled and Submitted by: Client
Date tested:16/01/21-28/02/2021
Reported date:28/02/2021

S/N	Characteristics tested	Specification/ Test Method	Standard Requirements Min	Nom	Max	Test result	Comment
1	Tensile strength @ break in N	As per customer request	-	-	-	43.05	-
2	Elongation@ break in mm		-	-	-	81.49	-

Remark
1. This test report relates only to the specific sample product which has been tested by ECAE testing laboratory.
2. For parameter indicated under serial No.1 and 2 the test area of sample length & width has taken 80mm x20mm respectively.

Test report authorized by, Name Yared Fikre Position Analyst II Sign.

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