Development of Hybrid Biobased Ointment Matrix: The Effect of *Ocimum Lamiifolium* on its Antimicrobial Property

By

Meseret Ewunetu Kibret

A Thesis Submitted to The Department of Chemical Engineering

School of Mechanical, Chemical, and Materials Engineering

Office of Graduate Studies

Adama Science and Technology University

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APPROVAL OF THE BOARD OF EXAMINERS

We, the undersigned, members of the Board of Examiners of the final open defense by Meseret Ewunetu Kibret, have read and evaluated her thesis entitled "Development of Hybrid Biobased Ointment Matrix: The Effect of *Ocimum Lamiifolium* on its Antimicrobial Property" and examined the candidate. This is, therefore, to certify that the thesis has been accepted in partial fulfillment of the requirement of the degree of Masters in Chemical Engineering.

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DECLARATION

I hereby declare that this MSc thesis is my original work and has not been presented for a degree in any other university, and all sources of material used for this thesis have been duly acknowledged.

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This MSc thesis has been submitted for examination with my approval as a thesis by

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May, God bless you all!

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LIST OF ABBREVIATIONS AND ACRONYMS

LA	**Lactic Acid**
OL	***Ocimum Lamiifolium***
ELN	**Elastin**
COL	**Collagen**
HC	**Hydrolyzed Collagen**
AHA	**Alpha Hydroxy Acid**
ECM	**Extracellular Matrix**
ELP	**Elastin like Polypeptide**
OLLA	**Oligomer of L-lactic acid**
PLA	**Poly Lactic acid**
SNC	**Silk Nano Crystal**
E. coli	**Escherichia coli**
S. aureus	**Staphylococcus aureus**
FTIR	**Fourier Transmission Infrared Spectroscopy**
TGA	**Thermogravimetric Analysis**
GCMS	**Gas Chromatography-Mass spectroscopy**
NMR	**Nuclear Magnetic Resonance**

LIST OF NOTATIONS AND SYMBOLS

KD	**Kilo Dalton**
mL	**Milliliter**
%	**Precent**
°C	**Degree centigrade**
±	**Plus, or Minus**
α	**Alpha**
β	**Beta**
sec	**Seconds**
hrs.	**Hours**
mg	**Milli gram**
S_1	**5%ELN/COL-OLLA**
S_2	**10%ELN/COL-OLLA**
S_3	**15%ELN/COL-OLLA**
S_4	**20%ELN/COL-OLLA**
F_1	**1%OL- 20%ELN/COL-OLLA**
F_2	**3%OL- 20%ELN/COL-OLLA**
F_3	**5%OL- 20%ELN/COL-OLLA**
F_4	**10%OL- 20%ELN/COL-OLLA**

ABSTRACT

An ointment is a homogeneous, viscous, semi-solid preparation intended for external use on the skin or mucous membranes. Numerous researches were done on the formulation of herbal ointment taking petroleum derivatives as ointment bases to carry active components of plant extract. However, these petroleum derivatives ointments have a negative impact on the skin due to a compatibility problem. Chronically it clogged the skin pores which subsequently affects the release of moisture. To mitigate this problem, a novel approach has been followed to synthesize a completely green and biocompatible ointment base from Elastin and Collagen which are naturally existed in our skin and lactic acid commonly known as a part of body fluid. The synthesis of L-lactic acid oligomer (OLLA) and lactic acid modified elastin/collagen (OLLA-m-ELN/COL) copolymer was conducted by an in-situ polycondensation reaction. Different grades of OLLA-m-ELN/COL were prepared by varying ELN/COL loading that was used for carrying plant extract. The six-mass loss in the ELN/COL matrix was not seen in the synthesized ointment base. The reason for showing similar degradation characteristics might be due to secondary week interaction that happened between the carboxyl group of lactic acid oligomer and the amide group of ELN/COL matrix. This phenomenon is supported by the formation of a broad shoulder like a peak at 1745 cm^{-1} with the addition of elastin/collagen composition within the lactic acid oligomer. By mimicking the traditional medicine preparation technique and delivery approaches used for skin-related diseases, Ocimum lamiifolium (ደማከሴ) was chosen for its antimicrobial effect. Formulations of antimicrobial ointment were prepared at different concentrations of plant extract by fusion method. These topical formulations and the synthesized ointment bases were tested for pH, viscosity, spreadability, organoleptic characteristics, and homogeneity, and antimicrobial activity. The synthesized ointment base showed an inhibition zone of 14mm and 12 mm respectively for S. aureus and E. coli. As compared to a positive control (which is a general antibiotic, Ampicillin), this thesis shows the highest zone of inhibition against S. aureus (25 mm) and E. coli (23 mm) for the dispersion of 10% OL in ELN/COL-OLLA bioconjugate matrix. Therefore, this research work concludes that the synthesized bioconjugate can be used as a base matrix for the formulation of different herbal biocompatible ointments targeting skin care applications.

Keywords: *Elastin, Collagen, Lactic acid, Ointment base, Ocimum lamiifolium*

CHAPTER ONE

1 INTRODUCTION

1.1 Background of the Study

In the 21st century, biomaterials are widely used throughout medicine, biotechnology, and dentistry. Before 50 years ago the term biomaterials did not exist as we think today even the word "biomaterial" was not used. No manufacturers of medical devices (except for external prosthetics such as fracture fixation devices, glass eyes, limbs, and dental devices) had some experience of biocompatibility, no formalized regulatory approval processes, and no academic biomaterials courses [1]. Currently, the term "Biomaterials" has consecutively been used to describe materials that are used for implant in the human body or describe materials derived from biological sources that are used for therapies [2].

Biomaterials may be synthetic or natural and used in medical applications to support, enhance, or replace damaged tissue or a biological function. The field has grown significantly in the past decade due to discoveries in tissue engineering, regenerative medicine, drug delivery, cosmetics, etc. For the choice of biomaterial, one of the most requirements is its biocompatibility with the human body [3].

Researchers in recent days are eager to find green, sustainable, and renewable biomaterials with a regulated biodegradability and bio-absorbability. As described above, biomaterials may be prepared from any substance that has the potential to communicate with biological processes and is aimed at determining, treating, and regenerating or replacing any part of the body. They have an excellent performance to be utilized in versatile advanced applications, particularly whenever biocompatibility issues are highly required to be addressed. The application of polymeric materials for medical and other purposes is growing very fast. Polymers have various applications in such varied bio-medical fields as tissue engineering, medical device, and artificial organ implantation, prosthesis, ophthalmology, dentistry, bone repair, and many other medical fields.

Delivery systems based on polymer allow the controlled slow release of drugs into the body. It also studied the application of synthetic polymers to gene therapy. Polymeric materials

were also used widely as biosensors, in research equipment, and for bio-regulation. It is suitable for biomedical use, at least on its surface, must be "biocompatible". Biomaterials having bioresorbable properties have been extensively researched and applied in many advanced biomedical applications (internal or body surface i.e., skin) that need cell biocompatibility.

Skin is one of the key organs in the body that eventually injures life, often induced by chemical or physical injuries and microbial infections. Following the burn, pathogens, and organ injury capable of entry into the body, and subsequently, it is susceptible to infection. [4]. To minimize such happening researchers, find wound healing processes for skin treatment and herbal preparations can be more effective and safer than conventional medicines because of their non-toxic nature and able them to be administered over long periods. Herbal medicine, also known as phytomedicine or botanical medicine, refers to the medicinal use of seeds, roots, beers, bark, leaves, or flowers of any plant [5][6].

Recently, the World Health Organization (WHO) estimates 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare [7]. Drugs for skin treatments are prepared in various dosages and forms, including **ointments**, powders, liquids, and pills. Along with other dosage forms, herbal drugs are also formulated in the form of creams and ointment [5]. Drug delivery through the skin has long been a promising concept due to the ease of access, large surface area, substantial accessibility to the circulatory and lymphatic networks, and non-invasive nature of the therapy [6].

An ointment is a homogeneous, viscous, semi-solid preparation intended for external use on the skin or mucous membranes, and commonly they are greasy with a high viscosity. They serve as protective, therapeutic, or prophylactic and used as emollients for the application of active ingredients to the skin where a degree of occlusion is needed. On several body surfaces, ointments are used topically. This involves the eye (an eye ointment), stomach, nose, mucous membrane, etc. Ointments can be classified into two groups, namely medicated and unmedicated ointment. Medicated ointments contain a medicament dissolved, emulsified, or suspended in the base [5] and non-medicated ointments are used as such for emollient or lubricating effects, and the preparation of medicated ointments [8]. Since ointments are semi-solid constituents, they commonly act as viscoelastic materials when shear stress is applied. They usually contain drugs and are meant to be applied to the

body or mucous membrane externally and are commonly referred to as an ointment base. They are very moisturizer and ideal for dry skin [9].

All ointments consist of a base that serves mainly as a drug carrier. The nature of the base regulates its efficiency as well. Therefore, the choice of base for the ointment is a very significant feature of its formulation. It is important to become familiar with the skin structure concerning drug absorption, for scientific understanding of the percutaneous absorption of ointment bases [8]. Ointment bases are almost anhydrous, and usually contain one or more suspension, solution, or dispersion medicines.

In the form of an ointment, herbal medications are often formulated. Different ointment bases were mixed and formulated by adding active ingredients (plant extract) by trituration into the ointment bases at the most efficient ratio. The quality of the ointment is measured in terms of irritancy, pH, spreadability, diffusion, and durability which can be investigated after the completion of ointment formulation.

The screening of plants used in typical medicine is the first step towards this objective. Most of the research was done by fusion method and formulation of different ointment bases was done at 60-70 ℃ for producing herbal ointment, which has an antimicrobial or antifungal effect in vitro. Afterwards, the plant extract was dispersed in petroleum derivatives based ointment [9]–[14].

According to Goggle Scholar reports using keywords of; ointment; ointment for skin; ointment base formulation and ointment base; more than 52,500 research articles on different ointments were published by researchers in the year of 2010-2020. Since 2020, more than 8,430 research articles were published and from those articles, more than 4,640 research articles were published only for skincare ointment in 2020. Perhaps this area was researched for many years; researchers are still inspired to get suitable, non-toxic, and biocompatible ointments formulation using plant extract.

Based on their physical structure, there are five groups or types of ointment bases which are used for the formulation of ointments. These are oleaginous base, absorption base, oil emulsion base water, water emulsion base oil, and water-soluble (water-miscible) bases.

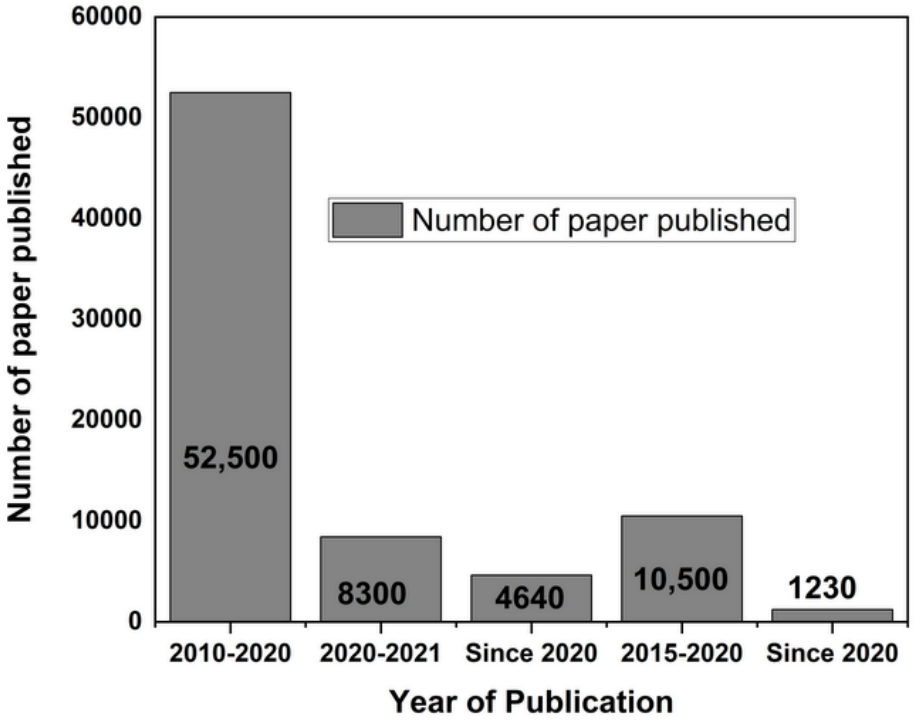

Figure 1-1: Number of research articles published for Ointment Formulations.

The researchers are eager to find the perfect ointment base formulations. Figure 1-1 show that more than 10,500 researches were done on ointment base from 2015-2020. Since 2020, articles on ointment base were more than 1,230. This shows that this area still has space for researchers to find compatible ointment formulation. Most researches were done by mixing different grade levels of ointment bases together using fusion methods; from my knowledge, almost, - nothing was done on the synthesizing ointment bases from ELN/COL and lactic acid bioconjugate. The synthesized ointment base had a composition that entirely bio-based and biocompatible with living systems, which have an ointment characteristic (homogeneity, spreadability, viscosity, pH, and drug release) that are already studied in petroleum derivatives ointment base formulations.

Most of the ointment base contain petroleum derivatives like mineral oil, soft white paraffin, or yellow soft paraffin, hard paraffin, and cetostearyl alcohol [8], [15] [16]. Although they tend to keep skin moisture and use on skin overexposure to sunlight, they have a negative impact in terms of compatibility with living systems. According to the Journal of women's health, the increase in mineral oil saturated hydrocarbon concentration inhuman fat tissue with age shows the increased accumulation of mineral oil over time [17]. It is toxic and does not metabolize if its gates to the system and like mineral oil, when we often use petroleum

jelly and paraffin kinds, also clogged skin pores [17]. Therefore, finding biobased and biocompatible ointment base on our living system still has space for the researcher to find a solution.

Based on this motivation, this research was done for synthesizing a green, biobased, and biocompatible ointment base from elastin (which gives the skin its flexibility), collagen (which is the protein in the body that holds everything together), and lactic acid. Lactic acid is an organic acid, compatible with our body and mostly, used as a carrier, since it is a body fluid. The synthesized ointment base from this research differs from other researched ointment is due to biocompatibility and origin for synthesizing ointment which is green and completely biobased that are used in ointment formulating [9]–[14]. The components (ELN/COL-lactic acid) for producing ointment base is green, biobased, and biocompatible with living system and may be used for replacing petroleum derivatives ointment bases due to their chronical effect [17]–[19]. Additionally, the synthesized ointment base for this research used materials that already exist in our body, and its extraction was made from the broiler skin having ELN/COL matrix, **'Skin for Skin'**. Even though most of the researchers stuck to the formulation of ointment, - this research as is the first on synthesizing a biobased and biocompatible bioconjugates used as an ointment base from body protein and fluid that could be used for carrying natural products for their antimicrobial activities.

1.2 Statement of the Problem

Biomaterials are used to engineer or regenerate tissues with the bioresorbable property that have been extensively applied in many advanced biomedical applications (internal or body surface i.e., skin) that need cell biocompatibility. Biomaterials can be prepared from poultry byproducts. Poultry by-products have a great economic potential that need to be exploited. Poultry skin could be utilized to produce elastin and collagen, which is often incorporated in the production of functional food or medicine due to its antioxidative properties [20]. In current Ethiopian scenario broiler skin considered as a byproduct. Considering one local market in Adama city, minimum 500 broiler skins per day have been disposed or landfilled to the environment. In average one broiler skin had 115 g, monthly, 57,500g broiler skin have been disposed to environment for 500 broiler skin. Therefore, utilizing this enormous waste for synthesizing elastin and collagen that have a potential to be incorporated in different pharmaceutical application would be done in this thesis research thesis.

Even though ointment production is extensively researched area, most of the articles focused on formulation of ointments from different grade conventional base matrix using hot mixing (Fusion method). Only few researches have been done so far in the direction of herbal ointment formulation. Still, the lack of biobased ointment matrix is the biggest challenge to substitute petroleum derivatives. Biocompatible formulation is highly essential to carry active components of a plant extract with the efficacy of antimicrobial, antifungal, anti-inflammatory properties with additional skin care and wound healing ability. However, petroleum-based ointments have known to have a negative impact particularly on the skin pores. Different studies indicate that frequent utilization of petroleum-based ointments results clogging of the skin pores which is distinguished as a main factor for skin dryness [17]–[19], [21]–[24]. For example, petroleum jelly, a topical ointment derived from fossil fuels is extensively used from dry skin to noise bleeds to a chest cold. Due to the hydrophobic nature of the base, it has a carcinogenic effect to be deposited in the pores of the skin and does not have the nature to nourish the skin. Moreover, extensively use of petroleum jelly have a tendency to reduce the release of moisture from the skin [25].

In general, in keeping our skin healthy and mist; petroleum derivatives base ointments destroys the skin in the long run and promotes ELN/COL breaks down. The reason for the breakdown of protein is due to its non-compatibility and do not have essential components to nourish the skin. When the amount of ELN/COL with in the skin reduced, the skin used to start cracking. The skin's ability to breathe naturally and get hydration from the atmosphere is dependent on the stability of the proteins with in the skin which can be highly affected by essential components delivered by the ointment base.

To mitigate the above problem, this thesis proposes antimicrobial ointment using a completely green and biocompatible ointment base that is already existed naturally in our skin (Elastin and Collagen). The ointment base is prepared by the in-situ oligomerization of lactic acid and ELN/COL. By mimicking the traditional medicine preparation and delivery approaches used for skin-related diseases, *Ocimum lamiifolium (ደማከሲ)* is chosen for its antimicrobial effect and the synthesized ointment base is used to carry *Ocimum lamiifolium* extract. The bioconjugate, ELN/COL-lactic acid, used for skin moisturizing and *Ocimum lamiifolium* used for bacterial infection in skin treatment. Therefore, exploring this application to develop ointment that can replaces petroleum-based matrix for the skin care utilization can be considered as a current research gap.

To do so, an extensive experimental attempt and characterization were made to answer the following research questions.

RQ1. Is it possible to extract elastin/collagen from local broiler skin?

RQ2. Can it be possible to synthesize, and develop a completely green and biocompatible ointment base having lubricant characteristics through in-situ oligomerization of lactic acid in the presence of ELN/COL?

RQ3. Is it possible to form macromolecular level interaction between the carboxylic group of lactic acid oligomer and amide groups of ELN/COL to regulate important characteristics of the matrix to be used as an ointment base?

RQ4. Can it be possible to use the synthesized ointment base to utilize indigenou knowledge regarding *Ocimum lamiifolium* and produce antimicrobial ointmen which replaces petroleum-based matrix?

1.3 Objectives of the Study

1.3.1 General Objective

The general objective of this research is to synthesize a green, sustainable, and biocompatible ointment base from Elastin/ Collagen - Lactic acid for the formulation of *Ocimum lamiifolium* based antimicrobial ointment.

1.3.2 Specific Objectives

The following specific objectives are addressed to achieve the general objective.

- To extract and characterize ELN/COL from local broiler skin.
- To synthesize, formulate, and characterize ELN/COL - Lactic acid bioconjugate as an ointment base through in-situ oligomerization.
- To extract and formulate herbal based ointment using *Ocimum lamiifolium* and synthesized ointment base.
- To test the antimicrobial characteristics of the formulated herbal Ointment.

Based on the above research questions and objectives; The choice of the systems, methodology, and characterization techniques are framed as follows:

Elastin/Collagen, Lactic acid, and *Ocimum lamiifolium* are selected as a base and focal point due to:

- ELN/COL-driven poly-peptide is one of the promising biomaterials which has anti-aging property and can interact with biocompatible monomers such as lactic acid.
- Due to their compatibility with a living system since they are serving as a part of body fluid and body protein.
- The monomer and pre-polymer (oligomer) produced using lactic acid can be used as a carrier for many biocompatible products and due to this, it can be used to develop a bio-based template for a selected natural product.
- OH, a group of lactic acid can have a possibility to bind with an amide group of ELN/COL to form macromolecular interaction so that it can help to regulate the viscosity.
- *Ocimum lamiifolium* also known as "queen of all herbs", culturally well-known in different countries [26]–[32] used for the treatment of skin diseases, bronchitis, bronchial asthma, malaria, diarrhea, and dysentery. Therefore, by mimicking cultural medicine towards *Ocimum lamiifolium*, it can be possible to develop antimicrobial skin care ointments.

1.4 Significance of the Study

Traditionally using leaf extracts as a traditional medicine for healing different diseases is a habit. In Ethiopia, using cultural medicine based on different types of leaf extract is known trend and culture even to date. One of the known and most researched traditional medicine for different types of diseases is *Ocimum lamiifolium (OL)*. Therefore, by mimicking this culture towards its use for skin-related disease; this thesis provides the development of a biodegradable, biocompatible, and sustainable green template (ELN/COL and lactic acid) or ointment base as a carrier for *Ocimum lamiifolium* for its efficacy of antimicrobial ointment for skin treatment.

This thesis has major significance in assuring the development of an alternative, biocompatible, and bio-lubricant ointment base from poultry byproducts (broiler skin) and

introducing process technologies for our nation. As it is a pioneer in terms of synthesis and development of biocompatible ointment bases for the formulation of herbal ointment, it will also motivate researchers to study the area of biobased materials. The other major significance of this study is replacing petroleum derivatives ointment with biobased ointment. This ointment has antimicrobial activity on its carrier or template using culturally used natural plant leaf extract that is used for skincare treatment. On the other hand, this product in response to realizing alternative efficient, cost-effective, convenient, and safe to meet our need regarding antimicrobial ointment for skincare treatment.

1.5 Scope of the Study

The scope of the thesis work within the given time and resource includes: -

- Extracting and characterizing ELN/COL and synthesizing ointment base from elastin/collagen - oligomer of L-lactic acid (ELN/COL-OLLA).
- Showing the reaction mechanism of the base when they possess the in-situ oligomerization process by FTIR and solubility of ELN/COL within lactic acid monomer at room temperature.
- Developing antimicrobial ointment using green, sustainable, and biocompatible ointment base (ELN/COL-OLLA) and extract of *Ocimum lamiifolium*.
- Determining the synthesized ointment quality and investigating antimicrobial property of the produced herbal ointment

1.6 Limitations of the Study

The thesis work does not cover the following due to time and resource limitations.

- Instead of performing HPLC (high-performance liquid chromatography) analysis for quantitative determination of amino acids, simple ***Biuret test*** was used for qualitative analysis of peptide bond and support by functional group investigation through **FTIR.**

1.7 Organization of the Thesis

The remaining part of the thesis is organized as follows:

Chapter 2: Discuss the relevant literature and related works regarding the structure of ELN /COL - lactic acid and their derivatives with a different polymer; types ointment base and its formulation; and natural product with their efficacy.

Chapter 3: Features the materials and methodology for the research, including extraction and characterization of ELN/COL as well as *Ocimum lamiifolium*; synthesis, formulation, and characterizing ointment base as well as its antimicrobial test analysis.

Chapter 4: Explains the result obtained from the desired proposed work and discusses the characterization results followed by postulating the reaction mechanism that happens from herbal ointment formulation using a different instrument. This chapter also discusses the physio-chemical characteristics of raw materials and ointment. It also compares with other related work to have the best judgment.

Chapter 5: Concludes on every proposed specific objective and recommend different possible direction for further studies.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Introduction

Polymeric biomaterials (biopolymers) can be used within physiological environments over long or short periods, - without causing any deleterious local or systemic effects. These polymers can be either synthetic or natural in origin, and their mechanical properties are similar to tissues. These properties allow their use for the replacement of various types of tissues. Biopolymers can contain various polymeric chemical compounds that give rise to a wide range of mechanical properties [33].

Biosynthetically derived polysaccharides, proteins, chemically synthesized aliphatic polyesters, and polyphosphoester are the primary sources of biopolymers. It can be broadly classified as:

i. Synthetic biopolymers: These are mainly synthesized from the biomass originated monomers through a biological and chemical transformation with a tendency to be processed in commercially viable technique such as extrusion process [34]. Monomers originated from the biomass can be utilized to produce biodegradable as well as non-degradable polymers. Polylactic acid (PLA) is the first largely produced synthetic biodegradable polymer.

   Chemically synthesized biopolymers conjugated with biosynthetic biopolymers have become attractive alternatives for biomedical applications since they have less risk to trigger immune responses in the body and can more easily be modified.

ii. Naturally existing biopolymers: These extracted biopolymers can be used directly with some chemical modifications such as polysaccharides and proteins from plant and animal sources (including cellulose, lignin starch, chitin, silk, elastin, collagen, gum, pectin, casein, etc.). In addition to this, polymers that are synthesized naturally by microorganisms and plants such as poly (3-hydroxyalkanote), poly (glutamic acid), and bacterial cellulose are also categorized under naturally existing biopolymers [35].

Natural polymers derived from microbial, plant, or animal sources have been extensively investigated for applications in regenerative medicine as they possess some structural similarities with the natural supporting structures of the body, such as connective tissues and extracellular matrix (ECM). They are non-toxic, biocompatible, and versatile and adaptable to technological needs, due different functional groups' presence in their structure [36].

Many studies have been carried out on investigating the potential applications of natural polymers in regenerative medicine and exploring the relationship between the polymer nature and its physical form (such as hydrogels, microspheres, microcapsules, sponges, foams, and fibers) [37][38]. An efficient way to tune the biopolymer-based biomaterial properties, towards a wide range of biomedical applications, is their combination with other components (organic or inorganic) to prepare a new range of composites with unique properties. This approach allows the development of materials with specific features in terms of cell compatibility, mechanical strength, biodegradability, gelation property, and possessing properties that are different from those of the original components. Moreover, composite materials allow a flexible design, since their structure and properties can be optimized and tailored to specific applications [39].

2.1.1 Application of Biopolymers in the Pharmaceutical formulation

Biopolymers are preferred because of their low toxicity, durability, sustainable nature, and biodegradability. Biopolymers are among the most abundant compounds in the world and are also materials that occur naturally. Because of their biodegradability and resorbability, medical and pharmaceutical industries turn to natural materials. Biopolymers have been found to have very promising industrial applications in various forms [40]**.** Several forms of biopolymers are known and used in medical and pharmaceutical applications; the function that the biopolymer plays differ depending on the drug release mechanism and its nature.

Numerous types of biopolymers are used and known for pharmaceutical and medical applications; the role the biopolymer plays vary depending on the drug release mechanism and its form. In many years the biopolymers have been used as excipients by the oral route in traditional, immediate-release formulations, playing a role in the manufacturing process, and shielding the medication from degradation during storage. Excipients like biopolymers have been used to formulate medicines and to enhance their efficacy [40].

Biopolymer reduces the toxicity of drugs and exhibit prolonged release of the active ingredient kinetics and reduces the amount of daily intake during the day to control and maintain a circulating level of active principle in the blood and give better efficiency. Today they are playing an increasingly important role in developing various delayed-release mechanisms and manipulating drugs. In recent years, biopolymers have been well studied and used to produce pharmaceutical drug formulations [40].

Biodegradable biopolymers from renewable resources can be classified into three categories:

- Polymers derived directly from plant resources such as polysaccharides (cellulose, starch, chitin, and chitosan) and animals such as **elastin and collagen** a type of body protein.
- Polymers of bacterial origin such as poly hydroxybutyrate (PHB).
- Polymers which are obtained indirectly by the polymerization of monomers themselves. Typically derived from plant resources such as **lactic acid**, these polymers result from the sugars fermentation or reactive monomeric compounds derived from vegetable oils.

Protein-based biomaterials can be obtained from living organisms, chemical or physical processing. They can be reprocessed after being dissolved by solvents or enzymes or obtained from decellularized structures. The main types of biopolymers which are derived from protein compounds are elastin, collagen, silk, polyhydroxy alkenoates, and other biopolymers.

2.2 Elastin and its Derivatives

Elastin is predominant elastomeric proteins or polymer in the normal extracellular matrix (ECM) and it is the major abundant protein in our body next to collagen and provides resilience, elasticity to organs and tissue. This structural protein is an integral component of elastic fibers, supplying numerous tissues and organs with elasticity, including blood vessels, skin, and lungs. Elastin occurs in the skin, elastic ligaments, lungs, and bladder. The presence of elastic fibers in blood vessels, for instance, makes it possible for the vessel to stretch and relax more than a billion times during its lifetime. It is composed of elastic fibers (a yellowish fiber composed chiefly of elastin and occurring in networks or sheets), giving elasticity to

tissues in the body found primarily in connective tissue in combination with collagen and polysaccharides [41].

Elastin is one of the most stable proteins in the body, with a half-life of 70 years. Elastic fibers are highly crosslinked in native tissues and are extremely insoluble. This persistent insolubility prevents the processing of elastin-based biomaterials from intact elastic tissues. Therefore, various forms of soluble elastin including animal-derived hydrolyzed soluble elastin [42]–[44], elastin-like polypeptides (ELPs) [36], [45]–[47] and recombinant human tropoelastin (RhTE) [48]–[50] have been synthesized and utilized to engineer synthetic elastin-based tissue constructs. These elastin-derived molecules have the potential to self-assemble or coacervate under physiological conditions like natural elastin protein and have been used to generate biomimetic elastic biomaterials for the regeneration of various elastic tissues.

Deficiencies in the elastin gene may lead to diseases such as cutilaxa, Williams–Beuren syndrome, and supra- valvular aortic stenosis (SVAS). The loss of elastin in the skin decreases the flexibility of the skin and reduces wound healing. Elastin and elastin-based materials can be obtained by various processes such as electrospinning, wet spinning, freeze-drying and cross-linking, and these can be used in solvent casting to obtain particles, fibers, gels, tubes, sheets, and films [51].

2.2.1 Biochemistry and Synthesis of Elastin

Elastin is encoded by a single-copy gene, ELN, and is synthesized as a soluble precursor protein by multiple cells, including fibroblasts, smooth muscle cells, and endothelial cells, which mature to give 60-70 kDa tropoelastin on secretion. Tropoelastin is an elastin precursor protein and is composed of hydrophilic domains (lysine, valine, and proline) and hydrophobic (glycine, valine, and proline). The hydrophobic domains are involved in coacervation, and for cross-linking the hydrophilic domains are used. In the skin, tropoelastin is the main source of dermal fibroblast cells. The alternating hydrophobic and hydrophilic domains are mainly tropoelastins [52].

Coacervation requires the agglomeration of protein molecules since it helps tropoelastin monomers to align and form cross-links, which is a significant step in forming elastin fibers. Elastin may be used in different types of biomaterials, including hydrolyzed soluble elastin,

insoluble elastin fibers, recombinant tropoelastin (fragments), synthetic peptide sequence repetitions, elastin block copolymers, or in combination with other biopolymers.

2.2.2 Sequence and Structure of Elastin

Elastin is the intrinsically disordered polymeric protein that gives the extracellular matrix of most vertebrates the exceptional properties of extension and elastic recoil. By a colloidal phase separation process called coacervation, the monomeric precursor of elastin, tropoelastin, as well as polypeptides containing smaller subsets of the sequence of tropoelastin, will self-assemble. Sean E. Reichheld et al., suggests that the interaction of hydrophobic domains found in the tropoelastin sequence facilitates self-assembly, while polymerization is done by covalent lysine side chains joining [53]. Tropoelastin, the elastin monomer, is synthesized in the cell and transferred to the extracellular matrix, where it is assembled into a cross-linked polymeric substance in conjunction with other matrix proteins [54].

The increased protein concentration and decreased hydration will facilitate a change to the secondary structure in the cross-linking domains during the self-assembly process, as phase separation takes place. This could be fulfilled by the formation of stabilizing hydrogen bonds, either alpha-helix or β-sheet, so that at least initially after coacervation, equilibrium may occur between these two structures. Indeed, for the joining of lysine side chains, transient β-sheets formed in the coacervate can help to align and coordinate cross-linking areas. However, it appears that cross-linking domains in mature coacervates are primarily in β-strand conformations, likely due to higher charge repulsions in an alpha-helical conformation between closely spaced lysine side chains [53].

Elastin form primary and secondary (β sheet or β strand) like silk protein. Urry and Venkatachalam attributed the elasticity to a reduction of entropy in the segments linking the β -turns as the coils stretch by developing a model of elastin-like peptides (ELP) with the repeat (VPGVG)n that formed continuous coils of β-turns [55].

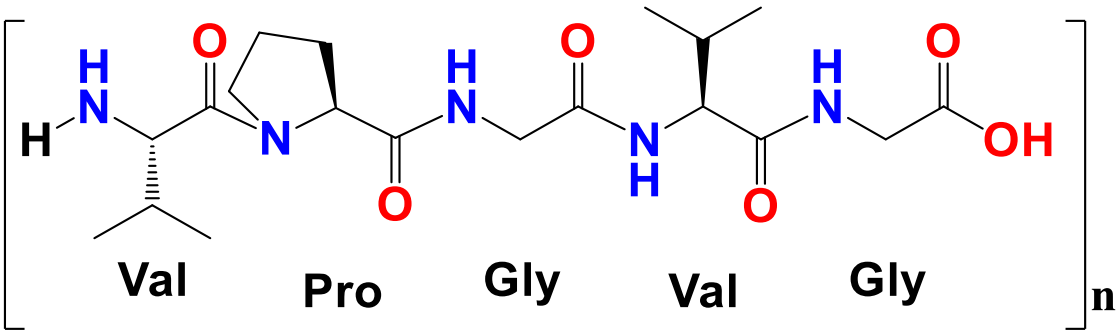

Figure 2-1: Amino acid repeating units (VPGVG) of Elastin.

2.2.3 Extraction of Hydrolyzed Elastin from an Animal source

Elastin has been investigated in the fields of biomedical, tissue engineering, molecular therapy, and cosmetic [51], [56], [57]. Commercially, elastin is distributed in powder form that is extracted from animal origin such as bovine and porcine. The protein accumulates in the animal parts postnatally. This will make the extraction more intricate, hence the final product expensive [58]. Therefore, the primary concern for elastin extraction is finding a cheaper and easier alternative with high throughput materials. Hydrolyzed soluble elastin has been extracted from waste poultry skin, having antioxidant and antihypertensive elastin peptides [59] [44]. A similar protocol was also used by Lansing et al., - for extracting elastin from broiler skin using a hot alkali process (NaOH) for hydrolyzing the skin. From his work, elastin having some amount of collagen was successfully extracted with 68.25 ± 0.05 crude protein [60].

Hugo E. Ramírez-Guerra et al., - also study the structural properties and physicochemical of recovered elastin from Jumbo Squid (Dosidicus Gigas) by-Products [63]. He reports the secondary structures of squid elastin analyzed by Fourier transform infrared spectroscopy (FTIR) were ~45% β-sheets, ~15% α-helices due to hydroxyl proline of collagen, ~10% β-turns, and ~30% undefined structures. In addition, squid elastin experienced glass transition at 82.01 ± 0.01ºC and denaturation temperature exist at 110.45 ± 0.64ºC [61].

A similar report was done on elastin extraction using tannery waste by Zerihun Yoseph et al.; - 90% yield obtained through thermo-chemical treatment method using alkali pre-treatments in elastin extraction from raw trimming wastes. The physical characterization using differential scanning calorimetry (DSC) showed the melting point at 104 ℃. Elastin

has shown a higher denaturation temperature above 275 ℃, and thermal gravimetric shows six mass loss steps [43].

2.2.4 Elastin in Skin Tissue

Skin is the major indicator of age, health, and vitality. Solar exposure, acne, repetitive movements, and gravity lead to erosion reflect an individual's age. Both intrinsic and extrinsic factors contribute to tissue aging throughout the body. Examples of extrinsic factors are cigarette smoke, solar exposure, alcohol consumption, and considerable weight gain and loss. With age, dynamic and static facial wrinkles become visible. Dynamic wrinkles result from muscle contraction. Static wrinkles, however, appear in the resting face due to loss of skin elastin, collagen, and hyaluronic acid caused by aging [62].

Elastin, which contains an elastic dermis and is an essential part of healing skin wounds, is an essential functional component of the ECM. The essential signaling factors and cells that contribute to the physiological cycle have been better elucidated by recent scientific advances and have led to wound care technology developments. Elastin-related biomaterials, especially tropoelastin-based ones, are particularly promising as tropoelastin is assembled to form elastin [52].

Elastin is a vital component of the ECM that allows soft connective tissues the property of elastic recoil after deformation and regulates the cellular response by biomechanical transduction to maintain tissue homeostasis, and is naturally contained in the skin and gives the skin its springy, elastic texture. The limited ability of most adult cells to synthesize and assemble elastin precursors into mature cross-connected structures has prevented the discovery of functional tissue-engineered structures showing the structure and biomechanics of natural native elastic tissues in the body [63].

Hydrolyzed elastin is a natural protein obtained from fish and other animal skin that can be used to replenish the skin. Elastin hydrolyzed is a humectant. Humectants are beneficial in any daily skincare because they capture moisture and keep it against the skin. Elastin is a skin conditioner that helps your skin stay elastic as you age and this protein has been used as a skin booster. Once applied topically, it promotes cell growth, and the renewing effect of elastin, when used over time, helps the skin appear cleaner and fresher [64].

Asako Inoue et al., investigate the moisturizing and whitening effects of elastin by examining its usefulness as a cosmetic material using water-soluble hot alkali pig aorta (HAPA) [65]. Ingestion of water-soluble elastin prepared from several animal sources has been reported to have a beneficial impact on human health and beauty. The consumption of water-soluble elastin, for example, improved skin quality, such as improved skin texture and moisture.

2.3 Collagen and its Derivatives

The main component of connective tissue is collagen, which is the most abundant protein in animals and makes up 25% of the total protein mass. It is contained in the fibrous tissue and the ECM. Biodegradable sutures, called 'catgut,' a collagen biomaterial derived from sheep's intestines, were the first recorded collagen biomaterial. The word collagen does not refer to a single protein, but a protein family with a 300 kDa mass.

In all animals, collagen is an abundant structural protein. Collagen is a major structural protein that forms molecular cables that stabilize the tendons and protect the skin and inner organs with durable sheets. By adding mineral crystals to collagen, bones and teeth are produced. Collagen provides our bodies with structure, protects the softer tissues, and maintains them connecting them with the skeleton [66].

At present, 29 forms of collagen have been reported [67]. A right-handed bundle of three parallel, left-handed helices of polyproline II-type comprises this fibrous structural protein. A great deal of progress has been made in elucidating the collagen triple helix structure and the physicochemical basis for its stability [66]. The most common triplets (10.5%) in collagen are proline (Pro), hydroxyproline (Hyp), and glycine (Gly) [68]. Individual triple helices of collagen, known as tropocollagen (TC), assemble in animals in a complex hierarchical way that eventually contributes to the macroscopic fibers and networks observed in tissue, bone, and basement membranes.

The development of collagen and other ECM components (elastin and proteoglycans) is high when the level of mechanical stress on fibroblasts is appropriate. As this stress is decreased, such as with aging, the development of matrix proteins decreases, and matrix-degrading expression increases enzymes [69].

In skin tissues, both collagen and elastin are essential structural proteins that are widely expressed. During photoaging, skin relaxation is manifested by a decrease collagen and elastin's content. Collagen accounts for 75% of the dermal composition and is the dermis primary part. Although elastin accounts for just 2% of the dermal composition, it plays a vital role in promoting skin homeostasis. Collagen and elastin fibers may display signs of aging, such as cross-linking and breakage when the body ages. The development of collagen and elastin may also decrease, leading to increased skin stiffness and decreased skin elasticity, and to more frequent wrinkle formation, which is a typical sign of skin aging [70].

2.3.1 Structure of Collagen

Collagen is a protein composed of three polypeptide chains, each containing a repeated sequence of Gly-X-Y amino acids, where X and Y are normally proline and hydroxyproline, respectively. The 29 collagen forms are identical because they all contain three chains of polypeptides in a characteristic triple-helix structure. The variance is due to their composition, where the variations are confined to the helix length and the number and function of the triple-helix carbohydrates attached. Forms I, II, III, V, and XI of collagen have fibrillary quaternary structures [33]. Their structure is arranged as follows: amino acids form the primary structure; the secondary structure is the local chain configuration of the Gly-X-Y peptide; the triple-helix molecule is the tertiary structure, and the arrangement of triple-helix molecules is the quaternary structure to shape microfibrils.

Collagen type I occurs predominantly in the skin, arteries, and tendons, and the prevalent collagen in cartilage is type II, and collagen type III occurs in the walls of the blood vessels. Type IV collagen, on the other hand, is a membrane component that separates the epithelial tissue from the mesodermal tissues. It is also non-helical, and fibrils do not grow. The most abundant type of collagen type I in animal skin is the type most widely used in medicine [33].

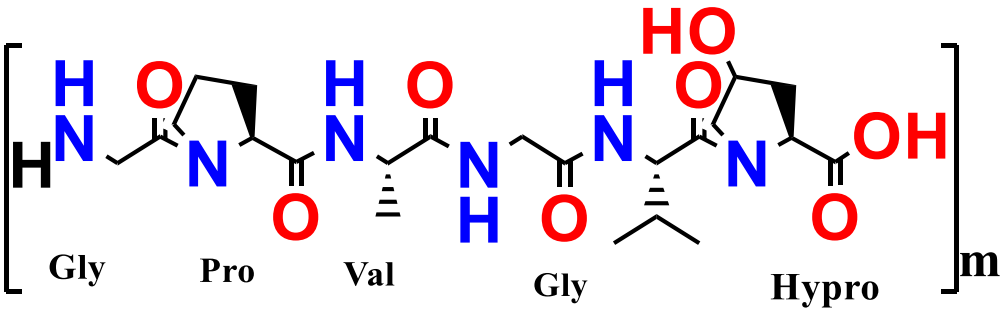

Figure 2-2: Repeating amino acid (GPVGVHYP) unit of Collagen.

Ieva Goldberga et al., researching the structure of collagen and solid-state NMR Spectroscopy feature relationships. Detailed NMR and molecular modeling work have shown that in contrast to the assumption that these triplets confer structural rigidity, the abundant Gly-Pro-Hyp triplets in collagen triple helices provide well-defined versatility because the proline is conformationally metastable. Within the fibril structure, the alignment of the Gly-Pro-Hyp triplets means that the molecular flexibility of the Gly-Pro-Hyp produces fibril flexibility. Gly-Pro-Hyp fibrillar bands are strongly associated with collagen ligand binding sites, leading to the inference that Gly-Pro-Hyp triplets' fibril alignment is important for the safety of collagen-ligand binding against external tissue stresses [71].

2.3.2 Collagen for Skin tissue

The role of locally active agents, including wound healing drugs and microbicides, has been played by collagen-based formulations, such as films, pellets, scaffolds, and a combination of liposomes [72]. Injectable microspheres based on gelatin, a degraded form of collagen, implantable collagen-synthetic polymer hydrogels, and interpenetrating collagen networks, are other available forms. The main biomedical applications of collagen are; burn/wound cover dressings, resorbable surgical suture, osteogenic and bone filling products, hemostatic agents, and therapeutic enzyme immobilization [67]. Collagen, which provides a natural scaffold or substrate for new tissue growth, is a critical component of the wound healing process. Thus, in all phases of wound healing, including hemostasis, inflammation, proliferation, and remodeling, collagen plays an important role.

Hydrolyzed elastin and collagen are primary ingredients for the cosmetics and pharmaceutical industry due to their antioxidant, moisturizing, antiaging, antihypertensive activity, rapture property for damaged skin, and anti-wrinkles. Similarly, there are also

cosmetics products made from lactic acid which is biocompatible like ELN/COL. Lactic acid is mostly used for skin moisturizing and it is a tool for restoring, smoothing, and correcting facial deformities.

Collagen, which provides a natural scaffold or substrate for new tissue growth, is a key component of the wound healing process. Thus, in all phases of wound healing, including hemostasis, inflammation, proliferation, and remodeling, collagen plays an important role.

In terms of ease of application and being safe, nonimmunogenic, nonpyrogenic, hypoallergenic, and pain-free collagen formulations for wound healing have general advantages over traditional dressings [73]. By assembling and accumulating freshly formed fibers and granulation tissue in the wound bed, collagen promotes wound healing, thereby providing the optimal physiological interface between the wound surface and the environment [74]. For viruses and bacteria, they are impermeable, especially for the two most common bacteria involved in wound processes; Pseudomonas aeruginosa (Gram-negative) and Staphylococcus aureus (Gram-positive) [75].

A common ingredient and considered an antioxidant in the cosmetic industry is hydrolyzed collagen (HC) that can be obtained from broiler skin, pigs, and fish. This low molecular weight protein has been widely utilized because of its excellent biocompatibility, easy biodegradability, and low antigenicity. It is a healthy biomaterial for cosmetics with good skin moisturizing properties. The antioxidant properties of HC depend on the size of the molecule; the lower the molecular weight of peptides, the greater the ability to stabilize radicals by contributing an electron or hydrogen. The antioxidant potential of HC is primarily due to the presence in the peptide of hydrophobic amino acids [76].

By having support nets all along with the cellular structures, collagen is responsible for the body tissues' stability and strength. The fiber gets weakened over time and gives the skin the unwanted wrinkle appearance as one of the many effects. Accurate research has shown that new ones may replace damaged fibers when the hydrolyzed protein (elastin and collagen) is absorbed by the subject [77]. This led to the stimulation of collagen output that helped the recovery and improved tissue appearance. Therefore, the cosmetics industry has made great efforts to incorporate this biomolecule into many available items.

It has also been shown that bioactivities such ass, antioxidant properties, lipid-lowering activity, antihypertensive activity, as well as reparative properties in damaged skin have been shown by collagen hydrolysate [77]. It has also been observed that this unique collagen presentation has a double action in the skin, which provides the first instance of the building block for the formation of elastin and collagen and acts as ligands or binding receptors in fibroblasts and hyaluronic acid [78]. The cosmetics industry focuses on collagen enhancement to improve the skin's appearance, especially in the areas of the face and neck. Collagen used in the pharmaceutical, beverage, food, and healthcare industries is driving the increase in collagen's worldwide use, with an estimated USD 3.7 billion in the current global market and estimated growth of over 6.6 billion by 2025 [79].

Due to antioxidants, moisturizing, antiaging, anti-hypertensive activity, rapture property for damaged skin, and anti-wrinkles, hydrolyzed elastin and collagen are major ingredients for the cosmetics and pharmaceutical industry. There are also cosmetic products made from lactic acid due to its biocompatibility with the living system. For skin moisturizing, lactic acid is often used and is a tool for restoring, smoothing, and fixing facial deformities.

2.4 Lactic acid and its Application

Lactic acid (LA) is a biomolecule that provides various industrial applications. As it is seen from Figure 2-4 the application of lactic acid versatile in different industries such as; cosmetics industry, pharmaceutical industry, chemical feed Stock, and food industry, and it is one of the large-scale fermentation chemicals. The widely used feedstocks are carbohydrates derived from various sources, depending on local availability, such as sugarcane or tapioca starch and maize starch. Carbohydrates are hydrolyzed into monosaccharides, then fermented into lactic acid by microorganisms in the absence of oxygen. The building block for polylactic acid (PLA) is a lactic acid monomer. PLA biocompatible and biodegradable that has been used in many applications. Figure 2-3 showed polymerization of the reverse reaction of lactic acid monomer. Bio-based lactic acid is optically active, and bioengineered microorganisms can be targeted to generate either L or D lactic acid [80].

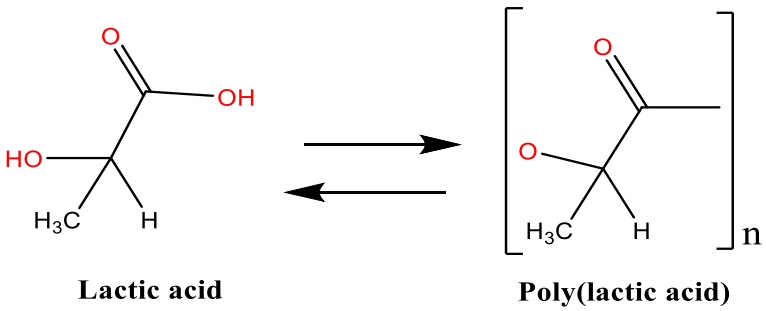

Figure 2-3:Polymerization of Lactic acid.

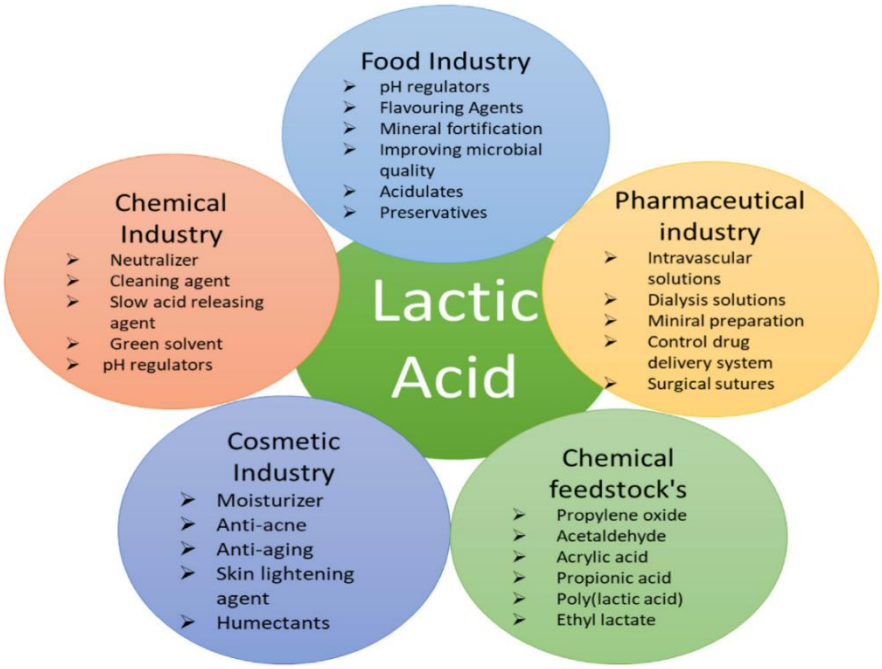

Figure 2-4: Industrial application of Lactic acid Adapted from [80]**.**

2.4.1 Lactic acid for Skin treatment

Lactic Acid is an organic alpha hydroxy acid (AHA) extracted from milk or fruit sugars, this exfoliating component has larger molecules than glycolic, which means that it will not reach the skin surface; it contributes to a lighter exfoliation degree and water-soluble like glycolic acid but resides on the skin's outer layers more. It unglues dead cells to expose clearer, evener skin while staying on the outer layers of the skin, and supplying extra moisture to the outer layers of the skin. Although helping to moisturize the skin to create smooth skin, balance the pH level of person skin, helps to keep the skin feeling hydrated and less dry, and it is known to accelerate cell turnover [81].

Applying lactic acid in skin regularly, improves signs of aging, stimulates collagen and elastin renewal, and secures the skin from UV light. Lactic acids reduce the epidermal calcium ion concentration and remove calcium ions from the cell adhesions by chelation. This results in a loss of calcium ions from the cadherins of the desmosomes and ultimately disrupts their adherence, which results in skin shedding or flaking. Hence, they stimulate new skin growth, resulting in a rejuvenated, and fresher complexion [81].

2.4.2 Lactic acid Grafted Biopolymers

Lactic acid grafted gum: Growing of lactic acid oligomer on gum backbone takes place due to the presence of free amino groups of gum and hydroxyl group of lactic acid through Maillard-type reaction [82]. Neelima Tripati et al., observed that the percentage grafting and grafting efficiency decrease from 1287 to 174 % and 65 to 35%, while the mixing ratio is increased from 5 to 20% [82]. According to their study, the reaction is highly facilitated by microwave energy. When heating is done on microwave hydrophilic gum was grafted by lactic acid oligomer through in-situ polycondensation reaction so that the grafted gum dispersed uniformly in OLLA (oligomer L lactic acid) due to the formation of a chemical bond between the amino group of amphiphile gum and the hydroxyl group of OLLA chain.

Lactic acid grafted chitosan: Growing of lactic acid oligomer on chitosan backbone takes place due to the presence of free amino function and the hydroxyl group of chitosan and the hydroxyl group of lactic acid without a catalyst. Hassan R Ramay et al., developed lactic acid-grafted chitosan for high drug loading and prolonged drug release. By using bovine serum protein, A drug encapsulation efficiency was 92% and a release rate of 28% from chitosan nanoparticles over four weeks was confirmed and lactic acid-grafted chitosan nanoparticles demonstrated a drug encapsulation efficiency of 96% and a protein release rate of 15% over four weeks [83]. The drug release rate increased and drug encapsulation efficiency decreased because of the increased protein concentration. The chitosan amended with lactic acid oligomer prepared from neutral pH solution, proposing an additional advantage of allowing drugs to be uniformly incorporated in the matrix structure with insignificant or no denaturation of chitosan [83].

Lactic acid grafted cellulose: Lactic acid oligomer-grafted-untreated bacterial cellulose (PLA/OLLA-g-BC) bio-nano composites were successfully prepared by Rahul Patwa et al.,

using solvent casting technique and demonstrated the synthesis of (OLLA-*g*-BC) by in-situ condensation polymerization. During the synthesis of OLLA-g-BC, hydrophilic BC was converted into hydrophobic due to structural grafting of OLLA chains with BC molecules increased and compatibilization between hydrophobic polylactic acid (PLA) and hydrophilic BC, thus enhancing various properties of PLA based bio nanocomposites [84]. The degradation temperatures for bio-nano composites films were above PLA processing temperature showing that bio-nano composite processing can be industrially viable.

Lactic acid grafted silk: Growing of lactic acid oligomer on silk backbone takes place due to the presence of free amino function of silk and hydroxyl group of lactic acid. Melaku Tesfaye et al., - demonstrate novel manufacture by reactive extrusion method of branched cum cross-linked polylactic acid (PLA) with graft chain topology nano silk fibroin. This result gave the basic information to predict the formation of radicals on the SNC macromolecular structure that promotes grafting on PLA backbone chains [85]. The grafting of silk nanocrystals (SNCs) on PLA macromolecules offers a remarkable improvement in the thermal properties and rheological. Melaku Tesfaye et al., observed the gel percentage of SNC −PLA increases from 10% (0.5 wt % DCP) to 60% (1.5 wt. % DCP) and there is also a significant interaction between SNC and PLA macromolecules apart from the polymer-to-polymer cross-linking interaction, which leads to the long-chain branching. This result gave the fundamental information to predict the formation of radicals on the SNC macromolecular structure that promotes grafting on PLA backbone chains [85].

Generally, from Melaku Tesfaye et al., study it can be concluded that lactic acid can grow in a silk backbone that is a type of protein having an active amide group similar to elastin and collagen. Lactic acid may also be used as a carrier for producing a biopolymer by creating an interaction between the ELN/COL amide group and the carboxyl group of lactic acid to produce a biobased and biocompatible ointment base.

2.5 Topical Ointment

Topical medicine is a medication on or in the body that is applied to a specific area. Topical administration most commonly includes application to body surfaces such as the skin or mucous membranes to treat illnesses across a wide variety of classes, including creams, foams, gels, lotions, and ointments [86], [87]. Several topical drugs are percutaneous, which

means that they are applied directly to the skin. Topical pharmaceutical products can also be inhalational, such as medicinal products for the treatment of asthma or added to the surface of tissues other than the skin, such as eye drops, ear drops (for the ear), and medicines which are applied to the tooth surface.

In the form of semisolid consistency, many medicines intended for topical application to intact or broken skin or mucous membranes have been presented, variously designated as ointments, creams, salves, pastes, etc., and used primarily as skin protective or emollient. Their typical property is the capacity to adhere to the application surface for a suitable period before it is washed [88].

Ointments are used topically for several purposes, for example, as protectants, emollients, antiseptics, keratolytic, antipruritic, and astringents. Ointment bases are almost always anhydrous, and usually contain one or more suspension, solution, or dispersion medicines. Drug delivery through the skin has long been a promising concept due to the ease of access, large surface area, substantial accessibility to the circulatory and lymphatic networks, and non-invasive nature of the therapy [89].

2.5.1 Types of Ointments

Based on the end product, an ointment is classified into two groups which are: Medicated ointments; These contain the medicament either dissolved or dispersed in the vehicle as fine powders or the form of a micronized powder, e.g. (Gentamicin ointment) and Non medicated ointments; These are used as vehicles for the preparation of medicated ointments or can be used for their physical effects, e.g. Soft Paraffin.

The major properties for an ideal ointment are non-sensitizing, non-irritating, pharmaceutically elegant, efficient release of medicament at the site of application, and water -washable. This property should be included for ointment base formulation [8].

2.5.2 Types of Ointment Base

Ointment bases are generally classified into four groups these are [8]:

- Oleaginous bases: Also termed as hydrocarbon bases and used to emollient effect, occlusive, and dressings and protect against the escape of moisture from the skin.

- Absorption bases: There are two absorption bases; those which allow the incorporation of aqueous solutions resulting in the creation of water-in-oil emulsions (e.g. hydrophilic petrolatum) and those which are water-in-oil emulsions and require additional amounts of aqueous solutions (e.g. Lanolin) to be incorporated.
- Water removable bases: This type of bases are oil-in-water emulsions resembling creams in appearance. Due to the aqueous external phase of the emulsion, they are easily washed out of the skin and are often called 'water washable' bases. They may be diluted with water or aqueous solutions e.g. Hydrophilic ointment.
- Water-soluble bases: Do not contain oleaginous components and these are fully water-washable and are sometimes branded 'greaseless'. Since they significantly weaken with water addition, these bases do not easily absorb significant volumes of aqueous solutions. We are often used for solids integration. For. Polyethylene glycol ointment.

Preparation of Ointments

Ointments can be prepared either by mechanical incorporation (can be achieved using mortar and pestle, ointment slab and spatula, and an ointment mil) or by fusion methods, which is a method that all or some of the components of an ointment are combined at 7-75°C to melted together and cooled with constant stirring until congealed. From the two methods, most researches were done using fusion methods for ointment and in this method, the non-melted materials are applied to the congealing mixture as it is cooled and stirred formulation [10], [12], [16], [88]–[93].

All ointments consist of a base that serves mainly as a drug carrier. The nature of the base regulates its efficiency as well. Therefore, the choice of the base of the ointment is a very significant feature of its formulation. It is important to become familiar and compatible with the skin structure concerning drug absorption for scientific understanding of the percutaneous absorption of ointment bases [8]. Most of the ointment bases are derived from petroleum derivatives, and the production of herbal ointments was done simply by using selected petroleum bases and plant extract by fusion method for the formulation of different types of herbal ointment.

2.6 Herbal Medicine

Herbal medicine, also known as phytomedicine or botanical medicine, refers to the medicinal use of seeds, roots, beers, bark, leaves, or flowers of any plant. Herbalism has long been practiced outside traditional medicine, as up-to-date analyzes and studies indicate its importance in the treatment and prevention of diseases. Recently, the World Health Organization estimated that 80% of people worldwide rely on herbal medicines for some aspect of their primary healthcare [94]. Plant drugs are frequently considered to be less toxic and freer from side effects from the synthetic ones.

The development and use of traditional medicine (THM) have a very long historical background that corresponds to the stone age. Traditional medicine is seen as a synthesis of the science and experience used in disease detection, prevention, and elimination. In the continent of Africa, the practice of traditional healing and magic is much older than some of the other traditional medicinal science and seems to be much more prevalent compared to conventional medicine [6].

The World Health Organization (WHO) defines traditional medicine as health approaches, practices, beliefs, and knowledge incorporating animal, plant, and mineral-based medicines, manual techniques, spiritual therapies, and exercises, applied singularly or in combination to treat, diagnose and prevent illnesses and maintain well-being [7].

Healers (traditional doctors) obtain their drugs mainly from natural substances and in descending order of frequency, these constitute plants, animals, and minerals. Drugs are prepared in various dosages and forms, including **ointments**, powders, liquids, and pills.

Plant medications are the mainstay of treatment in the underdeveloped region with unaffordability, unsettled to the side effects, unavailability, and of the conventional therapy. Among the traditional plants that have been used as an alternative therapy for many diseases especially for skin, *Ocimum lamiifolium* is mostly used and studied plant. However, it has received limited medical and scientific evaluation to assess its efficacy [95]. In our country, it is common to use different parts of *Ocimum lamiifolium* (leaves, stem, flower, root, seeds, and even whole plant) to treat different diseases. Mimicking the traditional approach towards skin-related diseases, *Ocimum lamiifolium* was successfully extracted and optimized for the

antimicrobial activity that is used to produce antimicrobial ointment using biocompatible materials (lactic acid and elastin/collagen) for the efficacy of skin treatment.

2.6.1 *Ocimum lamiifolium* Efficacy in a Skin tissue treatment

Ocimum lamiifolium is commonly known as the *"Queen of herb".* It has been reported for several biological beneficial activities and thus the potential for the treatment of skin diseases, bronchitis, bronchial asthma, malaria, diarrhea, dysentery, arthritis, painful eye diseases, chronic fever, and insect bite, etc. [32].

It has been reported that *Ocimum lamiifolium* widely used in food preparation for several biological, beneficial activities and thus the potential for the treatment of various health conditions. However, the biological activities related to skin anti-aging, including anti-collagenase, anti-elastase, and anti-hyaluronidase activities, have not been reported so far [26].

Wantida Chaiyana et al., a study that *Ocimum lamiifolium* extracts used for the skin anti-aging activity. The floating part of *Ocimum lamiifolium* was extracted by fractionated extraction using n-hexane, ethyl acetate, and ethanol, respectively. The anti-aging activity was investigated by in vitro inhibition of collagenase, elastase, and hyaluronidase activities. The ethanolic extract of *Ocimum lamiifolium*, which had the highest yield (6.5%), contained the highest Rosmarinus acid (19.3% w/w) and the highest total phenolic content (50.2 ± 0.6 mg gallic acid/g sample) [32].

Figure 2-5: Image of Ocimum lamiifolium.

2.6.2 Herbal Ointment

Along with other dosage forms, herbal drugs are also formulated in the form of creams and ointments. Medicated ointments contain a medicament dissolved, emulsified, or suspended in the base. Drug delivery through the skin has long been a promising concept due to the ease of access, large surface area, substantial accessibility to the circulatory and lymphatic networks, and non-invasive nature of the therapy [12].

The ointment is used for antibacterial and antifungal activities using different plant extract having a compound which has responsible for antibacterial efficacy, antibacterial activity is the ability of a substance to inhibit or kill bacterial cells. Different types of antibiotics and chemotherapeutic agents are being used in the treatment of one form of disease or the other. Many of these antibiotics were initially derived from microorganisms while the chemotherapeutic agents are plant-based [53].

2.6.3 Formulation and Evaluation of Antimicrobial Herbal ointment

In the form of an ointment, herbal medications are often formulated. The ointment base is prepared, and herbal ointment is formulated by adding the active ingredients of plant extract

by trituration into the base at the most efficient ratio. The ointment quality is measured in terms of irritancy, homogeneity, washability, spreadability, diffusion, pH, and stability.

The screening of plants used in typical medicine is the first step towards this objective. Most of the researches were done on ointment formulations using fusion method. This method is done by the formulation of different ointment bases at 60-70℃ and for producing herbal ointment with an antimicrobial or antifungal effect plant extract in vitro was dispersed in a homogenized base at efficient ratio [9]–[14].

Similar reports were also made on the study, development, optimization, formulation, and evaluation of herbal ointment from local medicinal plants having antimicrobial effect by Himal Paudel Chhetri et al., Amgad. An et al., Bhopal et al., Prabhudutta Panda et al., and Avish D.Maru et al., report their study on ointment characterization,- spreadability, drug release, antimicrobial effect, pH, viscosity, homogeneity, irritancy, and stability. From their research, concluded that the prepared herbal ointments showed no irritant effect for the skin when the ointment was applied to regular and damaged skin. When applied topically on the skin the ointment spread readily and rubbed gently. The product was well diffused around the agar media cup in the media and the product's diffusion was comparable to the identical marketing ointment of reputed brands. Likewise, during a study duration of four weeks, the ointment was found to be mechanically stable at various temperatures i.e. 2 °C, 25 °C, and 37 °C. There were no changes in the irritant, diffusion, and spreadability effect even after the exposure to different temperatures [89], [5], [12], [10] and [96].

Table 2.1 clearly shows that the ingredients used for producing different kinds of ointment formulations are almost derived from petroleum derivatives, which negatively impact and are not biocompatible with the living system. Even though the produced ointment which is listed in Table 2.1; used for topical areas, there has a gap in using- ointment base, which is derived from entirely green, biocompatible, and biodegradable that has a positive impact with living systems.

Nowadays most researches were done on formulating antimicrobial, antifungal, anti-inflammatory, and antioxidant herbal ointments from different plant extract. R. Preethi et al., studied antimicrobial and antioxidant efficacy of some medicinal plants against foodborne pathogens and identified eleven compounds that have antimicrobial, antioxidants,

and anti-carcinogenic effect in vitro. The components identified by R. Preethi et al., were 2-Methoxy-4- vinyl phenol, Megastigmartrienone, 3-O-methyl-d- glucose, Tetradecanoic acid, n-Hexadecanoic acid, Hexadecanoic acid, ethyl ester, Phytol, 9,12,15, -Octadecatrienoic acid, Octadecanoic acid, Eicosanoic acid, and Squalene.

As seen from Table 2.2, different results were reported on ointment characterizations: mainly Physio-chemical property (color, phase separation, odor, homogeneity, and texture), pH, and spreadability, viscosity, stability, drug release, and antimicrobial activity.

Similar reports having petroleum derivatives ointment base were done by Mahendran Sekar et al., The study was on the formulation of antimicrobial ointment from plants, and the antibacterial potential of the formulated ointment was studied with three different concentrations (100, 250, 500 mg/mL). The ointment also showed significant antibacterial activity against all the tested organisms with a zone of inhibition ranges from 8.00 ± 2.00 to 18.67 ± 2.09 mm [15].

From Table 2-1, it is shown that different kinds of ointments were formulated by using simple ointment and other petroleum-based ointment base; however, from my knowledge, there was no synthesizing of ointment base from ELN/COL-OLLA bioconjugate which is biobased and biocompatible ointments. Development of an alternative **ointment base** that can replaces **petroleum matrix** for the skin care utilization is considered as a current research gap. Therefore, searching for a biocompatible ointment base with our living systems is a study for the researcher to find biobased materials that has ingredients responsible for skin biocompatibility. Because of biocompatibility with living systems, biopolymers like lactic acid and ELN/COL matrix could be incorporated for ointment base productions.

Table 2-1: Related work on Ointment formulation.

Types of Ointment	Ingredient	Methods	Use	Precaution
Simple Ointment [93]	Cetostearyl Alcohol, White soft paraffin or yellow soft paraffin, Wool fat, and Hard paraffin	Fusion Method	Employed chiefly as a pharmaceutical aid and forms basis of ointments.	-
Ammoniated Mercury Ointment(White Precipitate Ointment) [8]	Finely powdered Ammoniated Mercury and Simple ointment	Triturated Method	Externally used as an anti-infective agent.	Not be applied to extensively excoriated areas for prolonged periods.
Sulfur ointment [97]	Sublimed Sulphur, finely sifted, Simple Ointment prepared with White soft paraffin	Triturated Method	Mainly used as scabicide, it is applied to the affected area of the skin after washing with soft soap. Also used as a mild antiseptic.	It should not be applied longer than three days otherwise dermatitis may be caused.

Wool alcohols Ointment [98]	Wool alcohols, Hard paraffin Yellow soft paraffin and liquid paraffin	Fusion Method	Used as an emollient and forms an ointment base for many therapeutically active ingredients.	-
Paraffin ointment [97]	White Beeswax, Hard Paraffin, Cetostearyl Alcohol, and White Soft Paraffin or Yellow Soft Paraffin	Fusion Method	Used as an ointment base for therapeutically active substances.	-
Salicylic acid ointment [97]	Salicylic acid, and finely sifted Wool alcohols ointment	Fusion Method	Used as bacteriostatic and fungicide. Generally applied for the treatment of chronic ulcers, dandruff, eczema, psoriasis, hyperhidrosis, and parasitic skin diseases.	Its application to large areas of the body should be avoided and It has also been reported that the continuous application of salicylic acid ointment causes dermatitis occasionally

Table 2-2: Summary of Formulated Herbal Ointment with their Characteristics.

Formulations by Fusion methods	Viscosity (cp)	pH	Spreadability g.cm/s	Drug Release %	Stability	Antimicrobial Effect Zone of inhibition (mm)
Investigation of cream and ointment on antimicrobial activity of Mangifera indica extract [5].	81-87	5.6-6.9	173-206.7	-	Stable for a month	E. coli: 11.8 S.aureus: 12.4
Development, optimization, and evaluation of different herbal formulations for wound healing [12].	5120-6480	6.5-7.1	42-69	-	-	-
Formulation and evaluation of topical dosage form of Pandanus	63.7-116	5.96-6.72	20-26	97.43-98.91	Stable at 4, 25 and 30 ℃	-

fascicularis Lamk. and their wound healing activity [10].						
Formulation and Evaluation of Polyherbal Ointment for Wound Healing and Antimicrobial Activity [88].	5.48	6.9	6.31	-	Stable for 18th days	S.aureus: 5 P. aeruginosa: 8
Formulation and evaluation of ointment containing sunflower wax [96].	2314-2851	6.8-7.02	80-115.91	99.63	Stable at 25 ℃ and 30 ℃ for 3 months	-
Formulation, Evaluation, and Antibacterial Properties of Herbal Ointment Containing Methanolic Extract of Clinacanthus nutans leaves [15].	-	6.4	-	-	Stable at 37 ℃ for 3 months	E. coli: 17.2 S.aureus: 16.67 for 25 mL/mg

CHAPTER THREE

3 MATERIALS AND METHOD

3.1 Chapter Overview

This chapter aims to present the materials required; Sample preparation methods, and proper characterization techniques that need to be followed for various experiments that were performed in this research work. For simplicity, the required materials, experimental protocols, and characterization techniques were categorized into three sections. The former discusses the extraction and characterization techniques of elastin/collagen matrix from local broiler skin;- The second segment describes the synthesis protocol and characterization techniques for the development of elastin/collagen-lactic acid bioconjugate, which is used as an ointment base; The later one describes the protocol and characterization techniques followed for the incorporation of Ocimum lamiifolium in the ointment base for producing antimicrobial herbal ointment. Afterwards, the synthesized ointment base and formulated antimicrobial ointment have been evaluated using physicochemical characterization, thermogravimetric analysis (TGA), Fourier transform infrared (FTIR). Finally, the antibacterial susceptibility test was conducted.

3.2 Materials and Consumable

In carrying out experiments for the extraction ELN/COL matrix, ointment base development, extraction of *Ocimum lamiifolium*, and formulation of antimicrobial ointment the materials and equipment listed in Table 3-1 were used.

3.2.1 Beneficiation Equipment/Chemicals

The equipment, materials, and chemicals used for the thesis are listed in Table 3.1. Broiler skin was collected from Adama Fronko, (17 Kebele), broilers merchant where the skin withdraws as a byproduct. Per day on average, 500 broilers' skin has been discarded as a byproduct. The chemicals used for all experiments were purchased from local chemical importers.

Table 3-1: List of Materials/Equipment and Chemicals used during the production of Antimicrobial ointments

	Chemicals	Equipment/Materials
Extraction ELN/COL matrix.	NaCl, Acetone, NaOH, and Distilled water.	Broiler Skin, Beaker, Cutter, Measuring cylinder, Water bath, balance, Separatory funnel, and Glove.
Synthesis of ointment base (ELN/COL-lactic acid).	Lactic acid (88% aqueous solution) and extracted ELN/COL matrix.	The reactor, Temperature controller, Chiller, Condenser, Spatula, Ice bath, Spatula, and Sample holder.
Extraction of *Ocimum lamiifolium* (OL).	Hexane, Ethanol, and Dried leaf powder	Soxhlet extractor, Chiller, Condenser, Rota vapor, and Round bottom flask.
Formulation of antimicrobial ointments.	Crude oil (OL), DMSO, and Ethanol, and ointment base.	Petri plate, Inoculation loop, McFarland solution, Incubator, Ruler, Hot plate, Petri dish, and spatula.

3.3 Methodology to Produce Ointment base

The methods for the extraction of ELN/COL matrix; Ointment base development; Extraction of *Ocimum lamiifolium*, and formulation of antimicrobial ointment were discussed in the following subheadings.

3.3.1 Extraction of Elastin/Collagen matrix

Broiler's skin has been cut into small pieces and homogenized in a 10 %vol of 1 M NaCl. After 24 hours extraction, the pellet was washed with demineralized water three times and defatted with 30 %vol acetone for 1 hour three times. The dry skin was suspended in 30 %vol of 0.1 N NaOH and heated for 15 min in a boiling water-bath with constant shaking. After cooling, centrifugation, and removing the remaining fat the residue was mined again for 45min in 0.1 N NaOH at 100 °C. NaOH soluble and insoluble material was washed several times in distilled water and dried in an oven at 80 °C [99]. This method was taken from Lansing et al. with some modifications [60].

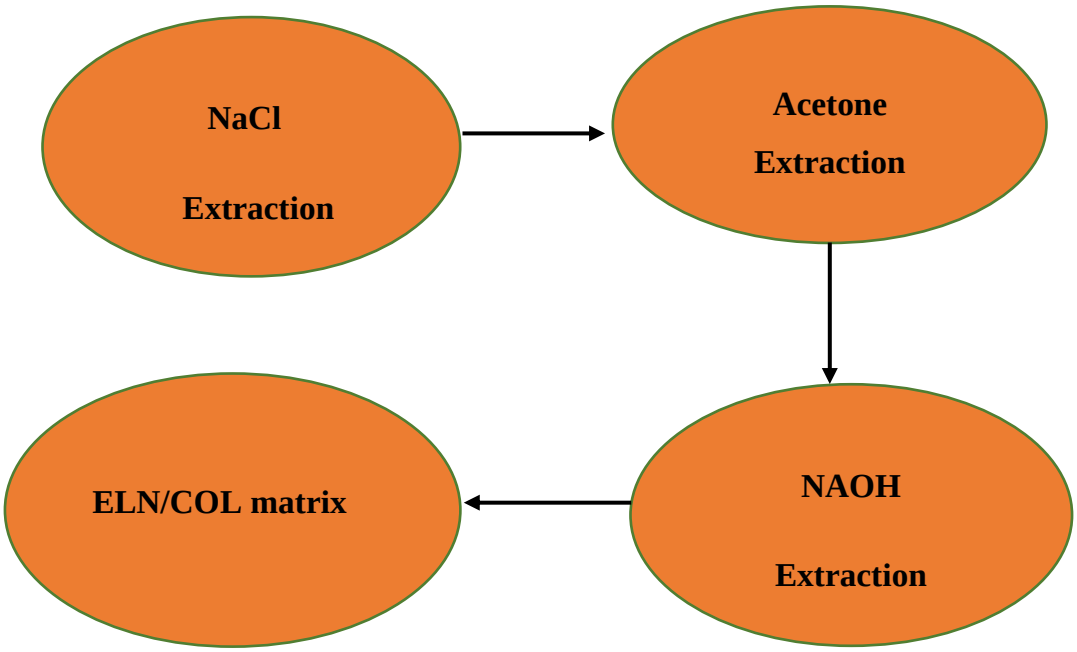

Figure 3-1: Extraction process of ELN/COL matrix

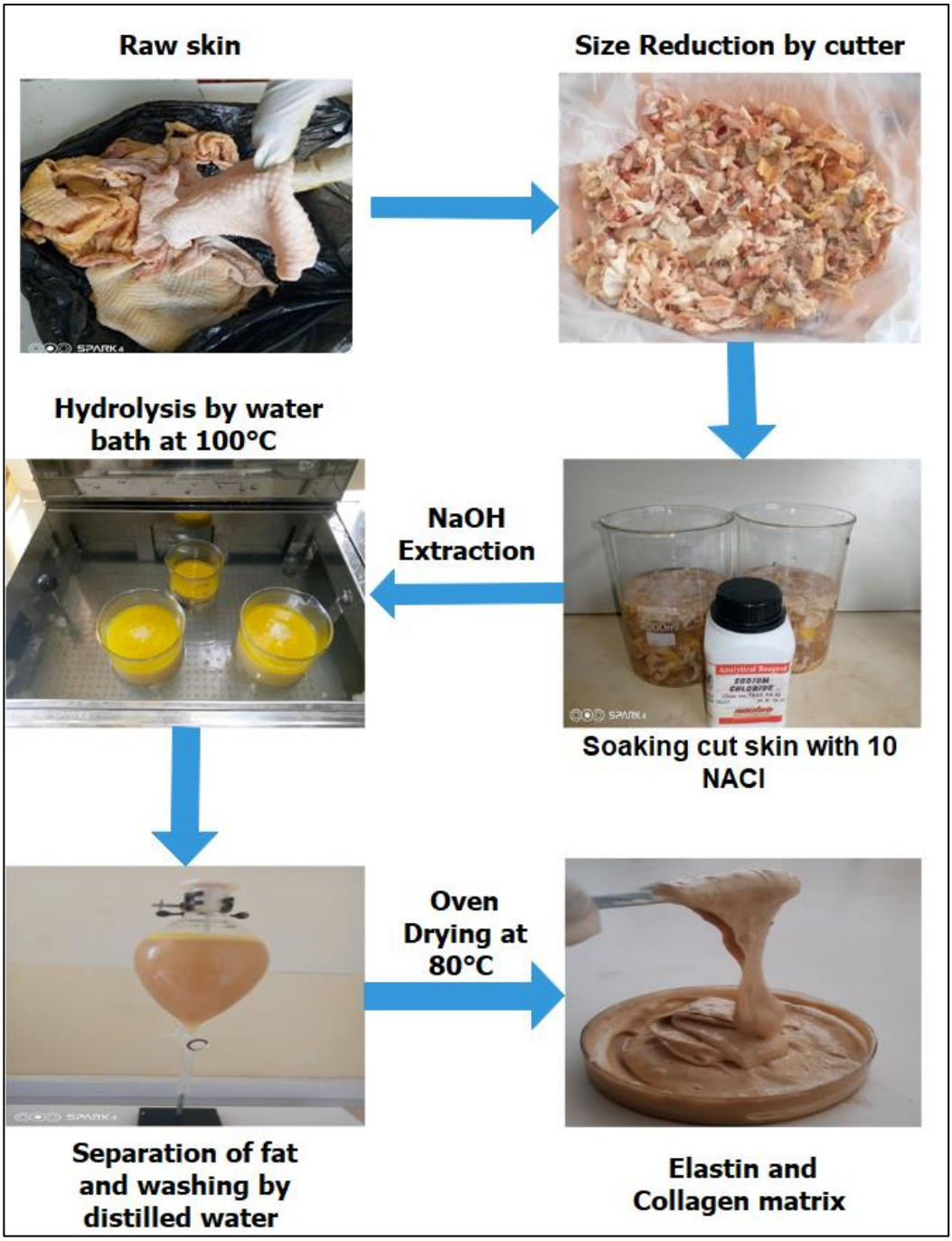

Figure 3-2: Extraction of Elastin and Collagen from broiler skin

3.3.2 Development of Bioconjugate from ELN/COL-Lactic acid monomer for Ointment base

The synthesis of oligomer L-lactic acid (OLLA) and lactic acid modified elastin/collagen (OLLA-*m*-ELN/COL) copolymer was conducted by in-situ polycondensation reaction. Different grades of OLLA-m-ELN/COL were prepared by varying ELN/COL loading (5, 10, 15, 20 wt./wt. %), keeping the other parameters constant such as reaction time, reaction temperature, and amount of lactic acid. Briefly, Lactic acid monomer and ELN/COL matrix were initially taken in a round bottom flask in various proportions and were mixed vigorously using a spatula until a homogeneous mixture appeared at room temperature. The solubilized mixture was placed in the temperature-controlled reactor for the polycondensation reaction at a temperature of 150 °C ± 5 °C for 5 hours. This reaction temperature was chosen using thermal property analysis (TGA) of ELN/COL matrix and amount of byproduct formation of polycondensation reaction in preliminary experiments. The outlet of the flask was connected to a condenser from which the byproducts were continuously passed and collected in the byproduct receiver. The byproduct receiver was kept in the ice bath. During the reaction, both macro interaction and oligomerization took place. As a result, ELN/COL-OLLA was synthesized as an ointment base after the completion of the reaction. The sample was collected and kept in sealed vials for further analysis.

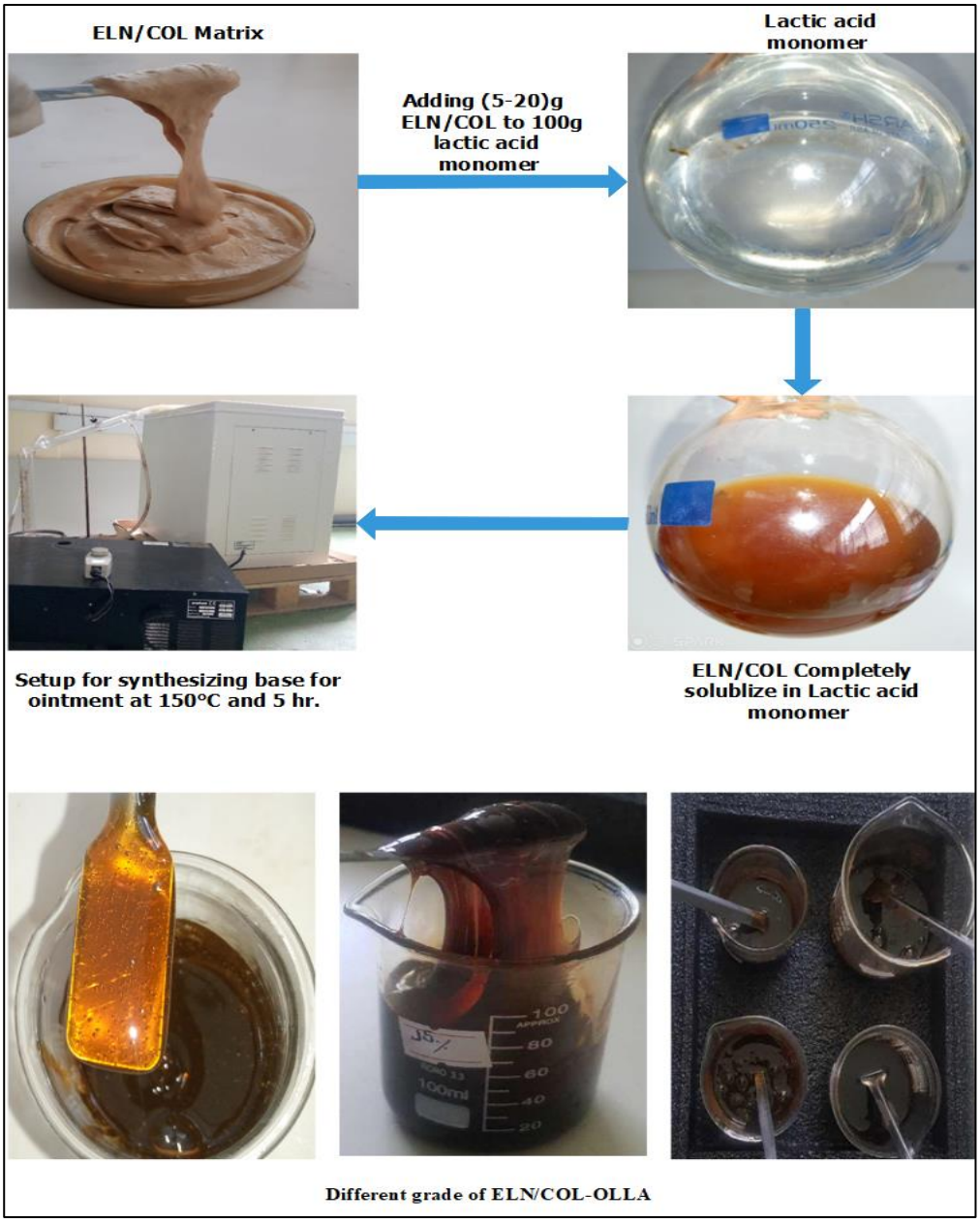

Figure 3-3: Different grade Ointment base Development.

3.3.3 Extraction *Ocimum lamiifolium* (ደማከሴ)

Extraction using Soxhlet: Fresh plant material was dried, powdered, and filled into porous cellulose thimble and placed in the 500 ml Soxhlet apparatus. Rotavapor was used to separate the crude extract from the solvent.

Design experiments for crude extraction of *Ocimum lamiifolium*: This work was focus on studying the effect of three factors (reaction temperature, mixing ratio, and reaction time) on the quality of the product. The responses studied were extraction yield. Levels of each

factor are tabulated in the following Table 3.2 and levels of factors were determined by doing a preliminary experiment.

Table 3-2: Level of each factor.

S. No	Factor	Unit	Level	
			Low	High
1	Temperature	℃	55	75
2	Time	hr	3	6
3	Mixing ratio	$Wt/Wt\%$	2	3.6

Design Expert® version 11.1.0.1 software was used for the randomized design of the experiment and analysis of variance (ANOVA) was also used for the statistical significance of the experimental data.

Box-Behnken method design was used to design the experiment. The experimental design involved three factors with two levels of temperature, reaction time, and mixing ratio were chosen as independent variables giving 17 experimental runs, and extracted yield was the output response.

3.3.4 Formulation of Herbal Ointment

In this method, 1%, 3%, 5%, and 10% crude extract of *Ocimum lamiifolium* were dispersed in synthesized ointment base having a high viscosity (20%ELN/COL-OLLA) and mix, stirred gently and continuously until a homogenous dispersion was obtained by fusion method at 70 ℃. There are different types of ointments with different viscosity and spredability which was reported by different researchers'; therefore, all the synthesized ointment base could be used for the formulation of herbal ointment. For carrying active ingredient for plant extract having antimicrobial and antifungal characteristics that have low spredability with high viscosity was selected for treating the infected body surface with small sheer force, for this investigation 20%ELN/COL-OLLA was chosen for further analysis for producing herbal ointments. Using hot pate for specified temperature four different types of herbal ointment with increasing concentration of ethanoic extracted oil were prepared [9]–[14].

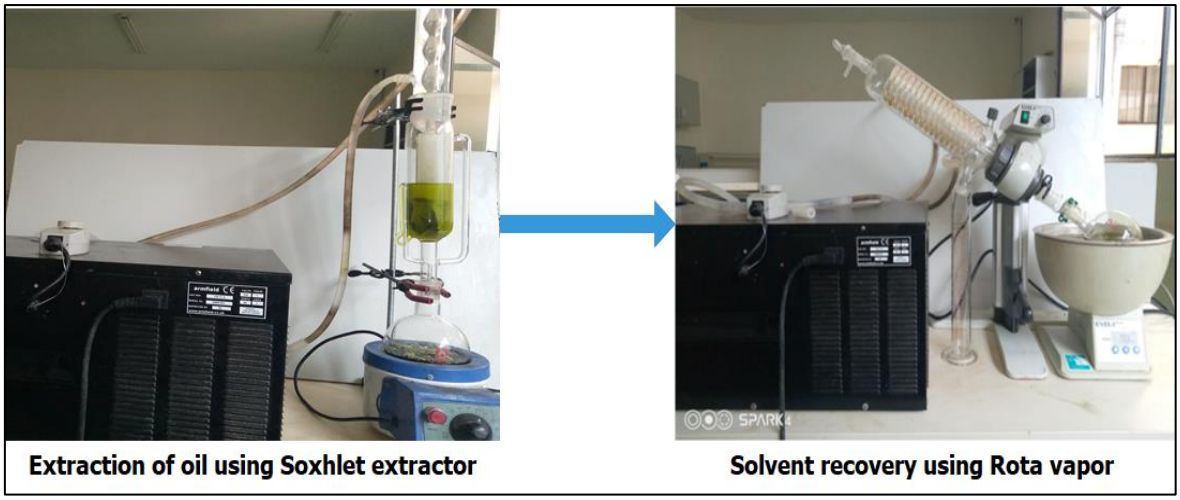

Figure 3-4: Extraction and Recovery of Ocimum lamiifolium

3.3.5 Antibacterial Activity of Formulated Herbal Ointments

Disc Diffusion Method

In vitro antibacterial susceptibility test was determined by the disc diffusion method. The medium was prepared by dissolving 38 g of Mueller Hinton agar medium in 1000 mL of distilled water and then autoclaved at 121 °C for 15 min. The autoclaved medium was poured into sterile plates (20-25 mL/plate) and the plates were allowed to solidify under the sterile condition at room temperature. After solidification, the plates were seeded with overnight grown culture approximately 1.5 X 10^8 CFU/mL by swabbing evenly onto the medium's surface with a sterile cotton swab.

1mg/mL amount of different concentrations of formulated herbal ointment, OL, and 20%ELN/COL-OLLA were prepared by dissolving in DMSO. Whatman filter paper no. 1 was used to prepare discs approximately 6 mm in diameter. The sterile discs were infused with the samples and position on the surface of the medium with sterile forceps and gently pressed down to ensure contact with the MHA. Ampicillin was used as a standard antibiotic and then plates were inverted and then incubated at 37 °C for 24 hrs. After incubation, the inhibition zone produced by the samples for the Disk Susceptibility Tests was evaluated by measuring the diameter (mm) of the clear zone around the disc against the test organisms using a ruler [100].

3.4 Experimental Protocol for Characterization

Experimental protocols for the extraction of ELN/COL matrix; Ointment base development; Extraction of *Ocimium lamiifolim,* and formulation of antimicrobial ointment were discussed in the following subheadings.

3.4.1 Proximate Analysis of Extracted ELN/COL matrix

Moisture content: The empty dish was dried in the oven at 105℃ for 3h and it was weighed after it was transferred to the desiccator to cool. 3g of sample (raw skin) was weighed and spread uniformly to the dish and the dish with the sample was placed in the oven at 105℃ for 3 hrs., drying the dish was transferred to the desiccator to cool until a constant weight was gained [101].

Calculation

$$\boldsymbol{Moisture}\ (\%) = \frac{\boldsymbol{W1-W2}}{\boldsymbol{W1}} * \mathbf{100} \text{..... Equation (1)}$$

Where: W1 is the weight (g) of the sample before drying and W2 is the weight (g) of the sample after drying

Ash content: Method for ash content calculation was taken**,** first the 3 crucibles were placed in the furnace until a constant weight was gained. 5g of the sample (broiler skin) was placed into the crucible and placed in a furnace at 550ºC overnight. The ash content in the sample was determined by the ratio of the weight of ash to the weight of the sample multiplied by 100% [101].

$$\boldsymbol{Ash\ content}\ (\%) = \frac{\boldsymbol{W2}}{\boldsymbol{W1}} * \mathbf{100} \text{..... Equation (2)}$$

Where: W1 is the weight (g) of sample and W2 is the weight (g) of ash

Fat content: Method for calculating fat content was taken **(AOAC,2000)** with some modification**,** first the bottle was placed in the oven at 105℃ overnight to ensure that the weight of the bottle is stable, then 5g of sample (broiler skin) to into extraction thimble and transfer into Soxhlet. Petroleum ether was used to wash the sample on the heating mantle. After 14 hours extraction the solvent was evaporated using Rotavapor and finally, the bottle

was placed in the oven at 80 ℃ ± 5 ℃ until the solvent is completely evaporated and the bottle is completely dry. After drying, the bottle was transferred to the desiccator to cool and reweight the bottle and its dried content [101].

$$\boldsymbol{Fat\ content}\ (\%) = \frac{\boldsymbol{W2}}{\boldsymbol{W1}} * \mathbf{100} \ldots\ldots\ldots\ldots\ldots\ldots \textbf{Equation (3)}$$

Where: W1 is the weight (g) of the sample and W2 is the weight (g) of fat

A Biuret Test for protein: A Biuret test is a chemical test that is used to assess the presence of a substance's peptide bond. It is based on the biuret reaction in which when treated with alkaline copper sulfate, a peptide structure containing at least two peptide connections produces a violet color. If the sample has two or more peptide bonds, the peptide bonds in the Biuret reagents give a positive result for the test (violet or purple color). The general qualitative test is known to be for compounds (proteins and peptides) with two or more peptide (CO-NH) bonds[102]. The Biuret reagent has been synthesized with a solution composed of sodium hydroxide (10% NaOH) or potassium hydroxide (1% KOH).

3.4.2 Quality control of the Synthesized Ointment bases and Formulated Herbal Ointment (20%ELN/COL-OLLA -%OL)

Physical evaluations: Preliminary evaluation of the synthesized ointment bases and the formulated herbal ointments at different concentrations was carried out as follows:

Organoleptic parameters: Organoleptic parameters like color, homogeneity, texture, phase separation, immediate skin feel, and odor of the synthesized ointment base and the formulated herbal ointments were carried out by visual examination.

pH: The pH of various formulations was determined by using a digital pH meter. 1 g of the synthesized ointment base and the formulated herbal ointments were weighed and dispersed in 100 mL of distilled water and the pH was noted [12].

Figure 3-5: pH measurement for the synthesized ointment

Viscosity: The measurement of viscosity of prepared ointment bases was carried out with VK-2000 Viscometer. The values of each formulation were done in triplicate and average values were depicted.

Spreadability: The Spreadability of the synthesized ointment basses and formulated herbal ointments were determined by measuring the spreading diameter of 1g sample of between two horizontal plates (9.5 cmx9.5 cm). The standard weight applied to the upper plate was 100 g. Spreadibility was determined using the formula (S=M*L/T). This method is adapted from Toppo et al., with some modifications [12].

$$\boldsymbol{S = (M * L)/T}$$ **……. Equation (4)**

Where S, M, L, T refers to spredability, weight applied, length of the glass slides, and time taken to separate the glass slides from each other respectively.

Figure 3-6: Spredability of the synthesized Ointment

3.4.3 Instrumental Characterization of the Synthesized Ointment base and the Formulated Herbal Ointments

For identifying the functional groups and reaction time without degradation (thermal stabilities) the extracted elastin/collagen; different grades of the synthesized ointment bases which are formed by in-situ oligomerization of lactic acid with elastin/collagen and the formulated herbal ointments were characterized by Fourier transferred infrared radiation (FTIR) and Thermal gravimetric analysis (TGA) respectively. Gas chromatography-mass spectrometry (GCMS) is used for identifying the phytocomponents of extracted oil (OL) which are responsible for the antimicrobial activity.

Functional group analysis: The extracted elastin/collagen; different grades of the synthesized ointment bases which are formed by in-situ oligomerization of lactic acid with elastin/collagen and the formulated herbal ointments was monitored using Fourier transferred infrared radiation (FTIR with Model name FTIR-6600 type A and Serial number A013861790) to know the functional group and predict the interaction occurs between lactic acid monomer and elastin/collagen matrix. And this FTIR attenuated with total reflectance (ATR) mode in the range of 4000 to 500 cm^{-1} with 4 cm^{-1} resolution and 64 scan rates.

Figure 3-7: FTIR-6600 for functional analysis.

Thermogravimetric analysis (TGA): Thermal gravimetric analysis (TGA) analysis was performed using flow rate of Nitrogen at 20.0 mL/min to determine the thermal stability of the extracted elastin/collagen; different grades of the synthesized ointment bases which are formed by in-situ oligomerization of lactic acid with elastin/collagen and the formulated herbal ointments extracted elastin sample. The temperature was adjusted between 25 ℃ to 800 ℃ with a heating rate of 20 ℃ and the decomposition of sample weight (%) against temperature (℃) was recorded.

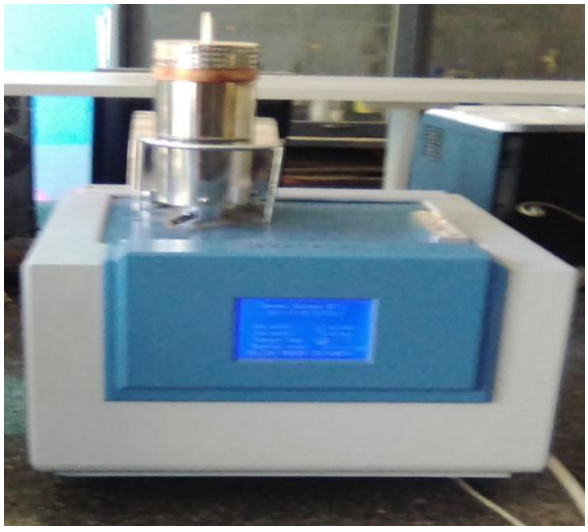

Figure 3-8: TGA for study thermal properties.

Determination of Major Component using GC-MS: GC-MS analysis of various crude organic extracts of *Ocimum lamiifolium* leaves was performed on Agilent Technologies 7890B GC System, fitted with Agilent capillary column (30 m * 0.25 mm inner diameter,

*0.25 μm film thickness; maximum temperature, 325 ℃), coupled to Agilent 240 Ion Trap mass spectrometer (MS). Ultra-high purity helium was used as carrier gas at a constant flow rate of 1.0 mL/min.

The injection, ion source, and transfer line temperatures were 290 °C. The ionizing energy was 70 eV. Electron multiplier voltage was obtained from autotune. The oven temperature was programmed from 60 °C (hold for 2 min) to 280 °C at a rate of 3 °C/min. The crude samples were diluted with an appropriate solvent (1/100, v/v) and filtered. The particle-free diluted crude extracts (1 μL) were taken in a syringe and injected into the injector with a split ratio of 30:1. All data were attained by collecting the full-scan mass spectra within the scan range 40-550 amu. The percentage composition of the crude extract constituents was expressed as a percentage by peak area. The identification and characterization of chemical compounds in various crude extracts were based on GC retention time. The mass spectra were computer matched with those of standards available in mass spectrum libraries [103].

Figure 3-9: Agilent Technologies 7890B GC System used for identifying Phytochemical components.

CHAPTER FOUR

4 RESULT AND DISCUSSIONS

4.1 Characterizations of Extracted Elastin/Collagen matrix

The hydrolyzed elastin/collagen matrix was successfully extracted with an alkaline solution from local broiler skin. Both elastin and collagen are fibrous proteins in the extracellular matrix (ECM) and the extracted ELN/COL matrix was characterized for its physical and chemical compositions.

4.1.1 Proximate Analysis of Broiler skin

The proximate analysis (fat content, moisture content, and ash content) of broiler skin is dispelled in Table 4.1. Ash content represents incombustible components that are remaining after the sample is completely burned. Table 4.1 shows the ash in ELN/COL matrix was significantly less (<1 %), indicating that the sample contains small inorganic residue. This indicates the raw material is derived from completely organic matter. The fat and moisture content after extraction was decreased due to defatting with acetone and drying, respectively. A Similar report were done on the fat content of extracted ELN/COL from broiler skin and the result obtained was 0.94 ± 0.06 % [104].

Table 4-1: Proximate analysis of Broiler skin

Proximate Analysis	Raw skin	ELN/COL Matrix
Moisture Content (%)	**4.25**	**1.8**
Ash Content (%)	**< 1**	**< 1**
Fat Content (%)	**3.8**	**1.02**
Biuret Test for protein	**NC***	**Positive (Violet color)**

* NC= Not applicable

In the presence of an alkaline solution, blue-colored copper II ion can form a complex with the peptide bonds since the peptide had unshared electron pairs in nitrogen and oxygen of water. The colored coordination complex is formed between Cu^{2+} ion and carbonyl oxygen C=O) and amide nitrogen (=NH) of the peptide bond. Once this complex has been formed, the solution turns from blue to purple. The deeper the purple color, the higher is the number of peptide-copper complexes. From Figure 4.1, the intensity of color after biuret test was high, therefore, qualitatively there could be more peptide in the extracted elastin/collagen matrix.

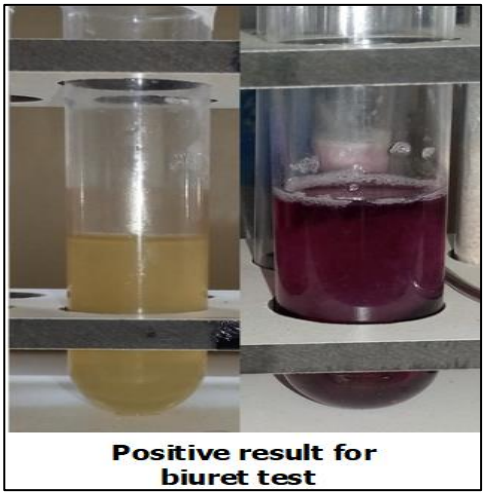

Figure 4-1: Positive result for Biuret Reagents.

4.1.2 FTIR Analysis of Elastin/Collagen

The chemical structure analyzed by FTIR spectroscopy indicates that the amide I, amide II, and amide III groups of elastin and collagen were observed at 1605-1770 cm^{-1},1488-1605

$cm^{-1,}$ and 1266-1360 cm^{-1} respectively. A similar result was also reported by Rabee Cheheltani et al., for elastin and collagen extracted from the aorta [105]. In Figure 4-2, the CH_3 wagging vibration of the proline side chain which is commonly happening for both collagen and elastin is indicated at **1338** cm^{-1}. Many researchers indicate the FTIR spectra similarity of elastin and collagen. The presence of hydroxyproline in collagen peptide bond makes it different from that of elastin along with the secondary structural variation. Collagen is commonly known for its α helical structure while elastin is having a β sheet structure. The peak at 974 cm^{-1} and 1647 cm^{-1} confirms α helical and β sheet secondary structures of collagen and elastin respectively. However, Rabee Cheheltani et al., reported that the α helical and β sheet secondary structures of pure collagen and elastin occur at 928 cm^{-1} and 1635 cm^{-1} respectively. The same report also showed the shifting of amide I and amide II peaks into higher wavenumbers with increasing concentration of elastin [105]. Therefore, the increment of the wavenumber for the extracted protein is due to the coexistence of elastin and collagen. Additionally, the amide II group of elastin and collagen shows C-H in-plane bending, C-N-C=O symmetric vibration, CH_3 out of plane bending vibration at FTIR spectra of 1409 cm^{-1},1493 cm^{-1}, and 1450 cm^{-1} respectively.

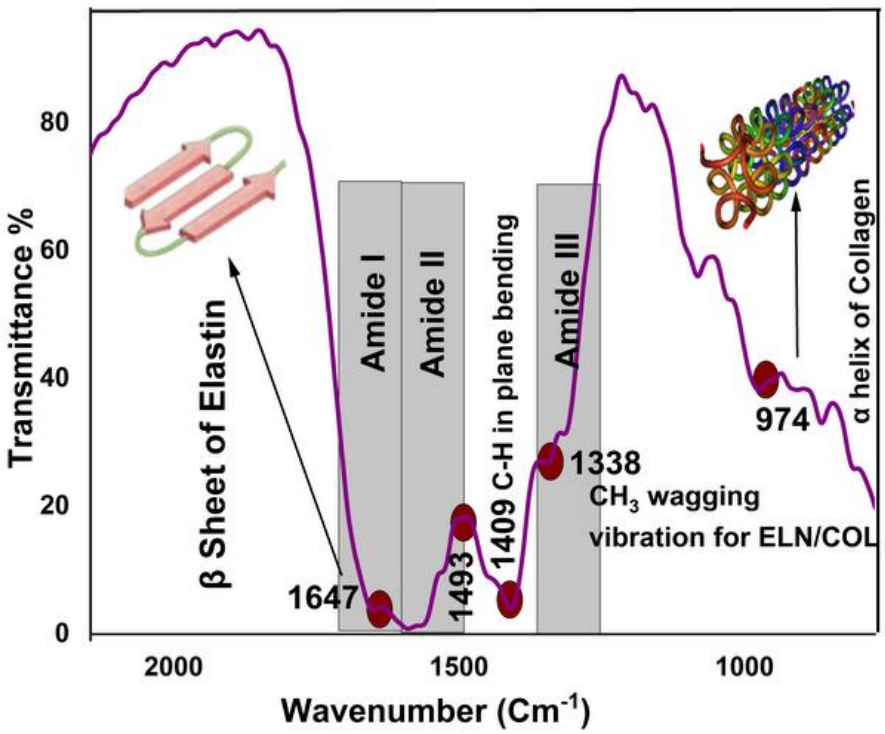

Figure 4-2: FTIR spectra of Elastin /Collagen matrix

4.1.3 Thermal Stability Analysis of Elastin/Collagen

The thermal gravimetric analysis thermogram is shown in Figure 4-3 with decomposition peaks as a function of temperature. Based on the TGA thermogram result, the extracted ELN/COL matrix shows major degradation peaks in six mass loss steps. These decomposition peaks are seen clearly in the DTG curve.

The first decomposition peak shows 34% weight loss in the temperature range of about 30-120 ℃. The second, third, fourth, fifth, and sixth have weight loss 21%, 30%, 6%, 1%, and 6% which is in the range of 120 ℃-217 ℃, 217 ℃-446 ℃, 446 ℃-557 ℃, 687 ℃-713 ℃, and 713 ℃-775 ℃ respectively. Moreover, the decomposition temperature when weight loss is at T_{10}, T_{50}, Tmax, and T_{90} are 74 ℃, 171 ℃, 344 ℃, and 516 ℃ respectively.

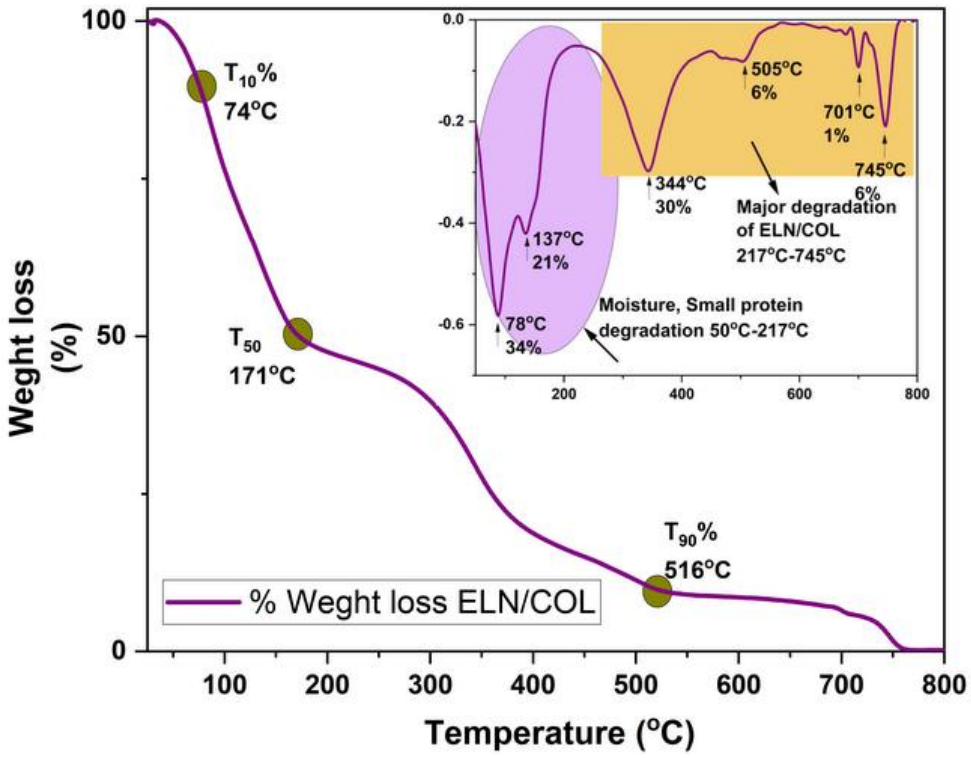

Figure 4-3: TGA and DTG curve of Elastin/Collagen matrix.

Below 217 ℃ hydration (water removal) stage and small protein in extracted ELN/COL could be removed. The macromolecule of ELN/COL shows degradation above 217 ℃. Therefore, below 171℃ (T_{50}) for the reaction mechanism was an appropriate temperature that may be used for saving 50% of material degradation from the sample. Reaction

temperature at 150 ℃ ± 5 was a convenient temperature for the reaction of lactic acid and ELN/COL.

This analysis was used to define a suitable temperature for using the extracted protein for other purposes like an ingredient for skin therapy. From TGA results, it can be observed that the maximum ELN/COL decomposition or weight loss was approximately 30% which occurs in the temperature range from 217 ℃ to 446 ℃. Therefore, for ELN/COL chemical reaction takes place it should be below the maximum degradation. However, Zerihun Yoseph et al., report elastin extraction from tannery waste and maximum degradation (30%) weight loss when the temperature ranges between 298-367 ℃ [43], which slightly deviates from the extracted ELN/COL matrix, this difference occurs may be the presence of collagen in the ELN matrix.

4.2 Synthesis and Characterization of ELN/COL-OLLA Bioconjugate for Ointment base matrix

4.2.1 The solubility of Elastin/Collagen in Lactic acid monomer

The solubility of proteins is considered as the proportion of nitrogen in a protein product that is in the soluble state under specific conditions. Solubility is the amount of protein in a sample that dissolves into a solution. Knowledge of protein solubility can give useful information on the potential utilization of proteins and their functionality, especially in foams, emulsions, and gels [106].

Figure 4-4 shows that the extracted protein from broiler skin (ELN/COL) has been completely dissolved in a lactic acid monomer, which is considered as weak organic at room temperature. Solubilization of the extracted protein within the lactic acid monomer might facilitate the delamination of α helix and β sheet stacks of collagen and elastin, respectively through a weakening of the secondary structure formed by the NH and C=O bond of the consecutive chains of the peptide bonds. This vital phenomenon supports the initiation of oligomerization of lactic acid within the protein-peptide chains and could be the reason for the formation of a new secondary structure between elastin/collagen and lactic acid oligomer.

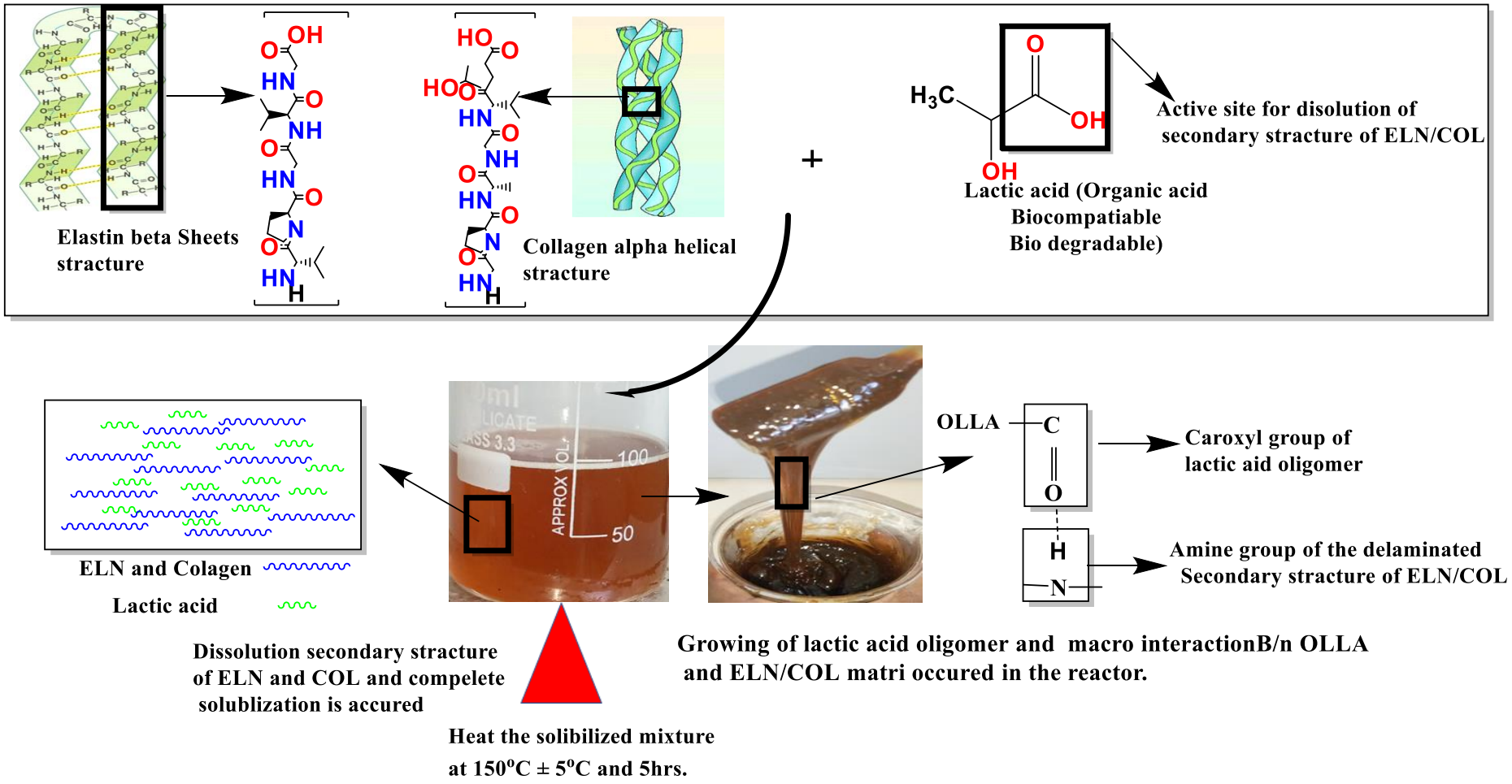

Figure 4-4: Solubility and Reaction mechanism of ELN/COL-OLLA Bioconjugates

4.2.2 Reaction Mechanism of Elastin/Collagen- Lactic acid

After complete solubilizing ELN/COL matrix in lactic acid monomer, polycondensation reactions were done at 150 ℃ ± 5 ℃ for 5hours. The question is that what would happen in the reactor for the selected time and temperature. Oligomerization lactic acid takes place in the RBF reactor after the delamination of secondary structure of the extracted protein and increases its chain by giving water as a byproduct.

When the lactic acid chain increases, the amount of water out from the reactor increases, and interaction between lactic acid oligomer (OLLA) and the secondary structure of elastin/collagen matrix was beginning. Because of this macro interaction, secondary weak bond was formed between the carboxyl group (C=O) and the NH group of delaminated sheets of elastin/collagen matrix.

As the elastin/collagen concentration increases a formation of week bond (Vander walls force) might be high that can drag its function to a lower wavelength shown in FTIR spectra. From Figure 4-4, it is clearly shown that lactic acid has begun to grow and the secondary structure of ELN/COL was delaminated and create an interaction between the carboxyl group of oligomer and NH group of ELN/COL. Because of the interaction of oligomer of L-lactic acid and ELN/COL matrix's secondary structure, the synthesis base has different viscosity. When the ELN/COL matrix increases at a constant lactic acid amount the viscosity of synthesized ointment increases and this shows a strong secondary macro interaction between the lactic acid oligomer and delaminated secondary structure of the ELN/COL matrix.

4.2.3 Physical Evaluation of Synthesized Ointment bases

Organoleptic Characteristics

The organoleptic properties, including physical appearance, color, texture, phase separation, and homogeneity of the selected base conjugate, were displayed in Table 4.3. Results showed that the ointment base had a cosmetically appealing appearance and smooth texture, and they were all homogenous with no signs of phase separation. The synthesized ointment base has a brown and dark brown color.

Spreadability

Spreadability of semisolid formulations, that is, the ability of an ointment to evenly spread on the skin, plays an important role in the administration of a standard dose of a medicated formulation to the skin and the efficacy of topical therapy. Figure 4-5 shows the spreading values, that was, diameters observed for the synthesized ointment bases. The values refer to the extent to which the formulations readily spread on the application surface by applying a small amount of shear.

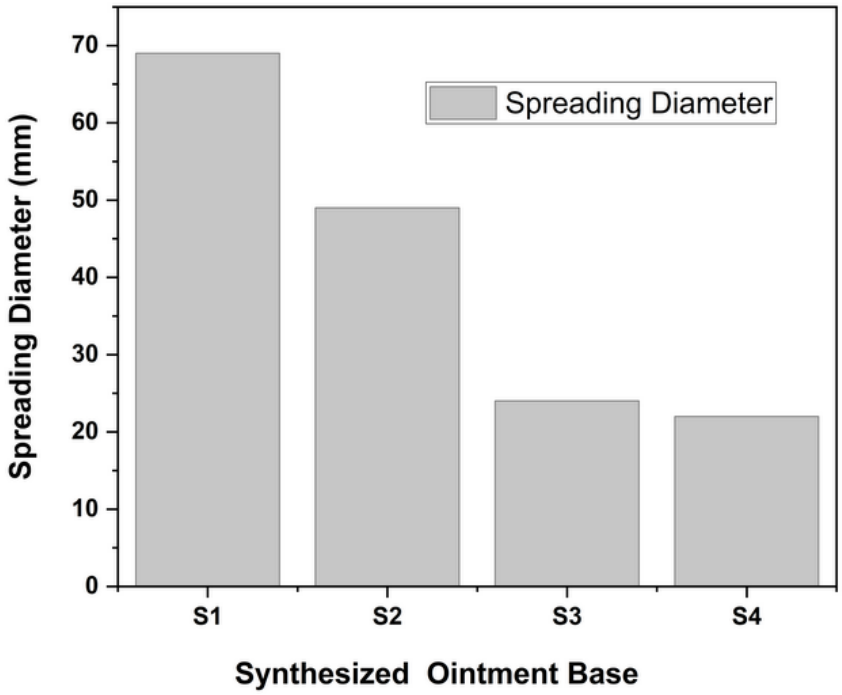

Figure 4-5: Spreadability values for the synthesized Ointment Base.

From Figure 4-5, it is shown that the spreadability of the synthesized ointment base decreases with increasing viscosity (which is directly proportional to the concentration of ELN/COL) and its values that it is shown in Table 4.2, ranges between 20 ± 2 (S4) - 62 ± 7 (S1) (g.cm/s) after the time used for separating the two plates and lesser the time taken for the separation of two slides, better the spreadability.

The synthesized ointment base matrix is spreadable with lubricant characteristics. The results indicated that the synthesized ointment base could be applied easily without being runoff. This assures that the formulation maintains a good wet contact time when applied to

the site of application. Similar reports on the spreadability of simple ointment base and topical herbal formulations were done by Bhopal et al., - the spreadability of formulated topical herbal ointment range between 42-69 (g.cm/s), which indicates good spreadability of simple ointment base and topical herbal formulations. The results indicated that the formulation could be applied easily without being runoff [12].

pH Values

The pH of all the synthesized ointment bases which are shown in Table 4.2. was found to be in the range of 5.20 ± 0.02 - 5.76 ± 0.02, which lies in the normal pH range of the skin [10] and would not produce any skin irritation. There was no significant change in pH values as a function of time. The results obtained showed that the pH of the synthesized ointment base increased with increasing ELN/COL concentration.

Viscosity Measurement

From Figure 4-6, it is shown a preliminary experiment on synthesizing bioconjugate, and it has different colors, orange (at a lower temperature (135 ℃)), brown (165,150 ℃), and black color (180 ℃). The viscosity of the ointment bases was determined by using VK viscometer and viscosity from the preliminary study ranges from 3036-221 cp. The amount of viscosity depends on the amount of water removal when the oligomerization of L-lactic acid proceeds. Oligomerization begins from 120-160 ℃, - as the time increases the oligomer produced from lactic acid monomer increases and the amount of water removal increases.

Figure 4-6: Preliminary study of ointment base synthesis.

Based on thermal analysis of ELN/COL; 150 ℃ ± 5 ℃ was chosen for ointment base synthesis since major degradation of compounds was begin above this temperature (171 ℃). The final synthesized ointment base(S1-S4) has more than 5000 Cp which is not measured in the viscometer (VK 200rpm).

Bhopal et al. also study the viscosity of formulated herbal gel and creams, the viscosity ranges from 5120 to 6480 cp. It concludes that viscosity is the most crucial parameter in the evaluation as it governs the many properties of the formulation such as spreadability, pourability of the product from the container, etc. The viscosity of the formulated ointment generally reflects its consistency [12].

Table 4-2: Evaluation parameter for the synthesis base matrix ELN/COL-OLLA

Base Synthesis	Composition	Moisture Content (%)	Spreadability	pH	Viscosity (cp)
S_1	5 g ELN/COL 100g L-Lactic acid	< 1%	62 ± 7	5.20 ± 0.02	>5000
S_2	10 g ELN/COL 100g L-Lactic acid	< 1%	46 ± 3	5.43 ± 0.03	>5000
S_3	15 g ELN/COL 100g L-Lactic acid	< 1%	22 ± 2	5.56 ± 0.02	>5000
S_4	20 g ELN/COL 100g L-Lactic acid	< 1%	20 ± 2	5.76 ± 0.02	>5000

S_1-S_4: Stands for the synthesis of Ointment base.

Table 4-3: Sensory analysis for the synthesized Ointment base

Base Synthesis	Appearance	Homogeneity	Texture	Phase Separation
S_1	Light Brown	Completely homogenized	Smooth (Lubricant)	no
S2	Brown	Completely homogenized	Smooth (Lubricant)	no
S3	Dark Brown	Completely homogenized	Smooth (Lubricant)	no
S4	Dark Brown	Completely homogenized	Smooth (Lubricant)	no

S_1-S_4: Stands for the synthesis of Ointment base.

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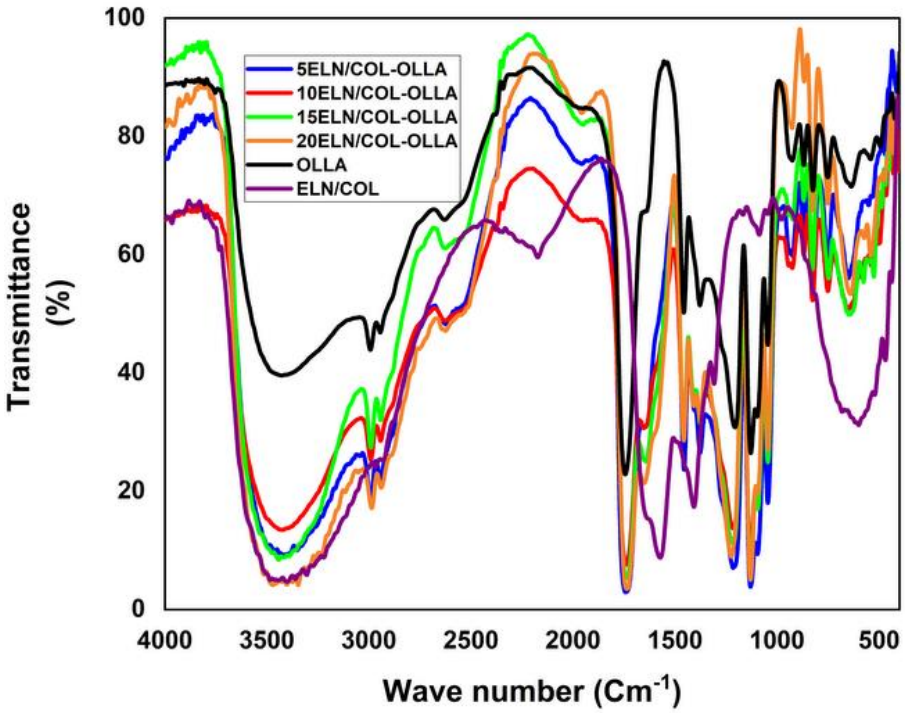
4.2.4 FTIR Spectral Features of the Synthesized Ointment Base

From the reaction mechanism of the synthesized base, it is seen that an increases lactic acid chain, and there was also a formation of secondary weak interaction (like hydrogen bond) between lactic acid oligomer C=O group and the delaminated NH group of the secondary structure of elastin and collagen. This secondary interaction that was formed between C=O of lactic acid oligomer and NH group of ELN/COL matrix was supported by the formation of a broad shoulder like peak at 1745 cm^{-1} with the addition of elastin/collagen composition within the lactic acid oligomer as it is seen in Figure 4.7. Similarly shifting of the major peak from 1745 cm^{-1} to 1737 cm^{-1} is observed due to the interaction. More interestingly, with increasing ELN/COL concentration within the oligomer matrix a new FTIR peak is observed at 1581 cm^{-1}. Moreover at 1634 cm^{-1} the shoulder like peak clearly observed for lactic acid oligomer and it is shifted to 1639 cm^{-1} ,1641 cm^{-1},1643 cm^{-1} ,1649 cm^{-1} for 5,10,15 and 20%ELN/COL-OLLA samples respectively. This can be correlated with the interaction of the amide I group of elastin/collagen with C=O of a lactic acid oligomer.



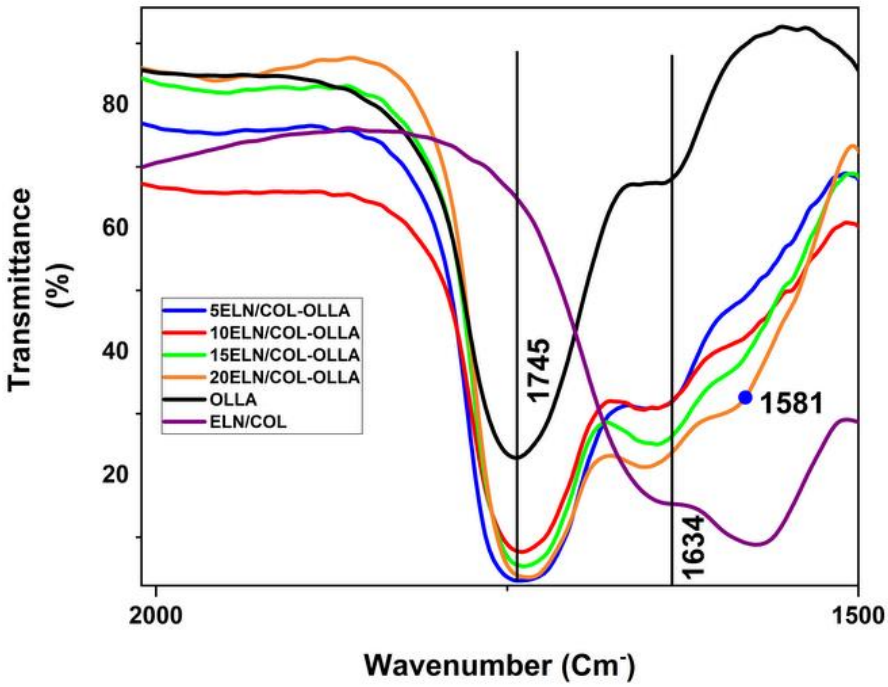


Figure 4-7: (A) Full range of IR spectra for synthesized ointment base. (B) C=O stretching of OLLA, ELN/COL, and ELN/COL-OLLA.

4.2.5 Thermal Stability Analysis of Synthesized Ointment base

Thermal analysis of ointment base was compared with the raw ELN/COL matrix. From DTG curve, it is seen that there was the formation of huge hump having thermal stability compared to the ELN/COL matrix. ELN/COL matrix has major six degradations and the maximum thermal degradation temperature of the extracted protein which decomposes in different steps shifts to a higher temperature due to the reaction of lactic acid oligomer and ELN/COL. The thermal property of bioconjugate shifts and changes the multi-component degradation to single component degradation. The thermal degradation of the synthesized ointment base shows similar degradation with lactic acid oligomer degradation and the six-mass loss in the ELN/COL matrix was not seen in the synthesized ointment base. The reason for showing similar degradation characteristics might be due to secondary week interaction that happened between the carboxyl group of lactic acid oligomer and amide group of ELN/COL matrix.

The major six peaks from ELN/COL thermal analysis were changed to a major peak temperature reign 148-320 ℃. Below 171 ℃ dehydration (water removal) stage and small

protein in extracted ELN/COL may be removed. The macromolecule of ELN/COL shows degradation above 171 ℃. Therefore, using below 171 ℃ was the appropriate temperature for the reaction mechanism and it might be used for saving 50% of material degradation from the sample. Reaction temperature at 150 ℃ ± 5 ℃ was a convenient temperature for the reaction of lactic acid and ELN/COL. Viscosity was monitored by oligomerization of L lactic acid, above 150 ℃ ± 5℃ there were higher viscosity and more chain formation but, ELN/COL practically degrade.

Figure 4-8 shows that 50% weight loss (T_{50}) in ELN/COL matrix that occurred at 171 ℃ was not seen on the synthesized ointment base (ELN/COL-OLLA). This may be due to the macro interaction or secondary week bond oligomerization of lactic acid and extracted protein.

Table 4-4: TGA analysis for selected weight loss and maximum degradation

	T_{10} (℃)	T_{50} (℃)	T_{90} (℃)
ELN/COL	74	171	516
OLLA	164	249	319
5%ELN/COL-OLLA	178	249	295
10%ELN/COL-OLLA	184	260	319
15%ELN/COL-OLLA	179	256	329
20%ELN/COL-OLLA	179	261	329

The reaction between ELN/COL and oligomer of L lactic acid (OLLA) enhances the maximum degradation of extracted protein occurs at 171 ℃ to 390 ℃. From Table 4.4, it is clearly shown that as the concentration of ELN/COL increases its thermal stability also increases.

Generally, due to secondary force between the lactic acid oligomer and ELN/COL matrix the multicomponent degradation which appears in ELN/COL changed to one major peak this shows that the synthesized ointment has higher thermal property than the extracted protein and lactic acid oligomer.

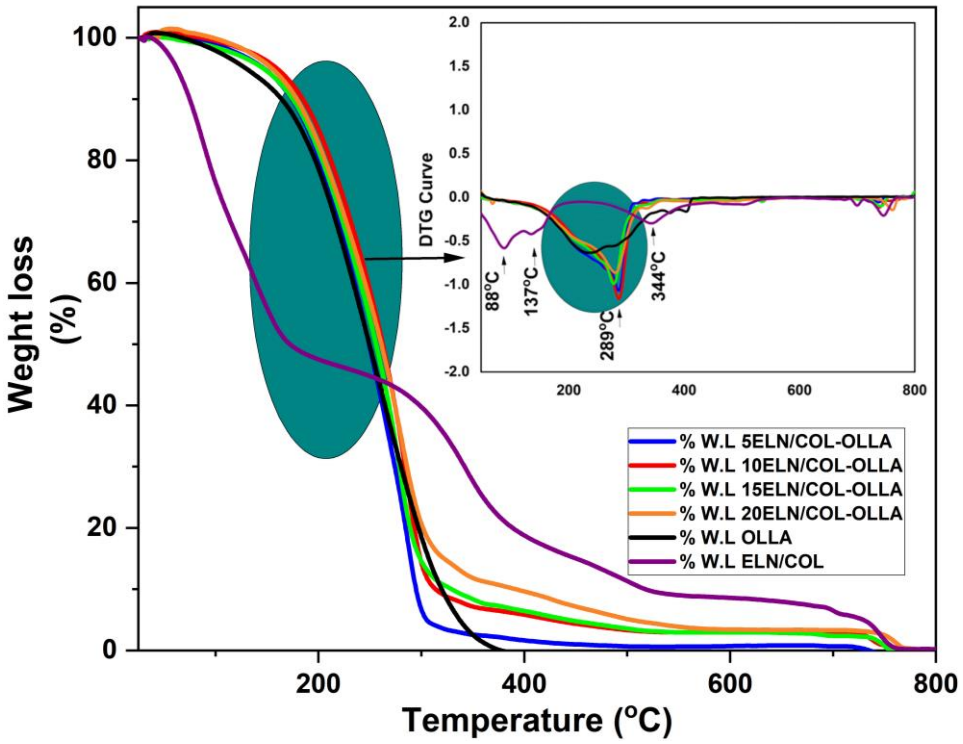


Figure 4-8: Thermal analysis of Synthesized Ointment base.

4.3 Quantitative Analysis of the Extracted Oil from *Ocimum lamiifolium.*

4.3.1 Statistical Analysis

The Design Expert program was used with Box Behnken response surface method having 17 runs in the regression analysis and analysis of variance (ANOVA). The Statistical software program was used to generate surface plots, using the fitted equation obtained from the regression analysis.

Table 4-5: Build Information for Response Surface Methodology

Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	17
Design Model	Quadratic	**Blocks**	No Blocks

As observed from the experiment run, the highest average yield (11.8%) was obtained at 65 °C, 4.5 hours, and 2.8g of mixing ratio. Whereas the lowest value of the essential oil yield was 8% and this value was obtained at the temperature level of 65 °C, and 6 hours extraction time. From this result, it can be noted that the optimal extraction time and mixing ratio for

all temperatures of this study was about 4.5 hours and 2.8g of plant powder with a specified solvent amount. The extractable oil decreases as the temperature are above and below 65 °C. When the temperature increases above 65 °C the extracted yield decreases and for a prolonged time (6hours), the extracted oil was decreased. The quantitative study can give an insight into the effect of the temperature, extraction time, and mixing ratio on the extraction process. As indicated in Figure 4-9, the extracted and solvent recovered *Ocimum lamiifolium* has a lubricant characteristic and close to yellowish color.

Figure 4-9: Extracted and solvent recovered of Ocimum lamiifolium.

4.3.2 Qualitative Analysis of the Extracted *Ocimum lamiifolium*

Determination of Major Component using GC-MS

A total of sixty-four components with different retention times were identified from the GC column from those compounds there are thirty-two major compounds as indicated by the chromatogram in Figure 4-10 and the major component were displayed in appendix A and From the table, the maximum probability is 97.7 % (Benzene, 1,2,3-trimethoxy-5-(2-propenyl)), and the minimum probability of 53.03% (Squalene). There are many areas that this leaf extract will be researched so there should be further analyzed for its antioxidant, antimalaria, and anticancer nature of the plant.

The major compound includes: Benzene, 1,2,3-trimethoxy-5-(2-propenyl) (16.93%), 9,12,15 Octadecatrienoicacid,methylester (22.02%), Hexadecanoicacid, methylester(12.60%), Squalene (7.65%), Phenol, 2-methoxy-4-(2-propenyl)(2.58%),

Octadecanoic acid, methylester (1.47%), n-Hexadecanoic acid (32.08%) and Eicosanoic acid, methyl ester (0.9%).

From those photochemical compounds which have antimicrobial, anticancer, and antioxidant nature from plant extract were identified by R. Preethi et al., [107], *Ocimum lamiifolium* leaf extract has seven compounds: n-Hexadecanoic acid, Hexadecanoic acid, methyl ester, 9,12,15, - Octadecatrienoic acid, Octadecanoic acid, Eicosanoic acid, and Squalene which was screened by GCMS. Having this component in this leaf extract shows *Ocimum lamiifolium* may be used in many pharmaceutical industries such as antimicrobial ointment, antioxidant ointment, and creams. Similarly, Teklit Gebregiorgis Amabye et al., reported that *Ocimum lamiifolium* has a phytochemical responsible for antioxidant and antimicrobial activities [95].

Due to this OH functional group which was seen in Table 4.6, the extract had an antimicrobial effect. Mallappa Kumara Swamy et al., report methanolic extract of *Lantana camara* has antimicrobial, and antioxidant behavior because of Hexadecanoic acid constituent in its GCMS analysis [108].

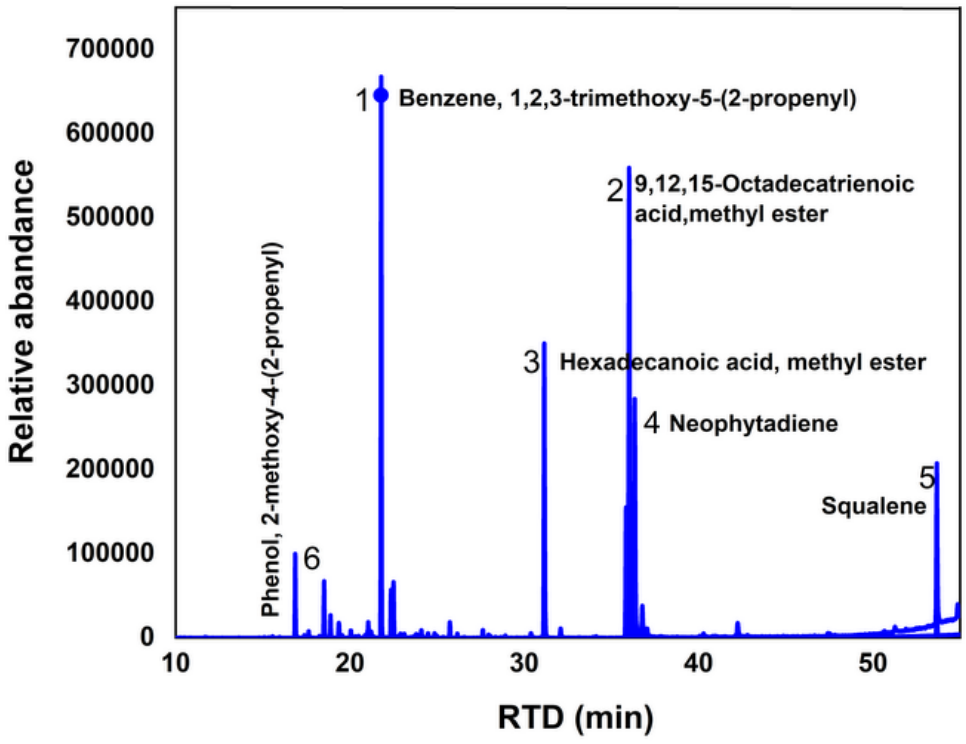
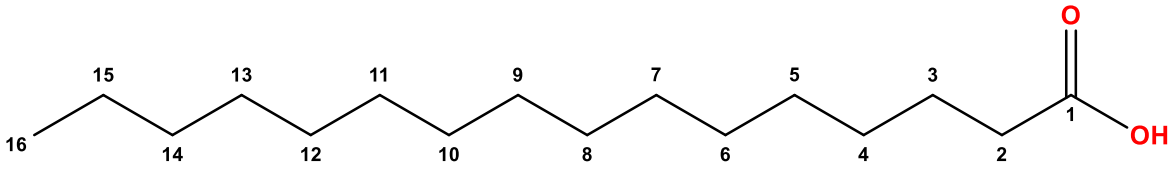
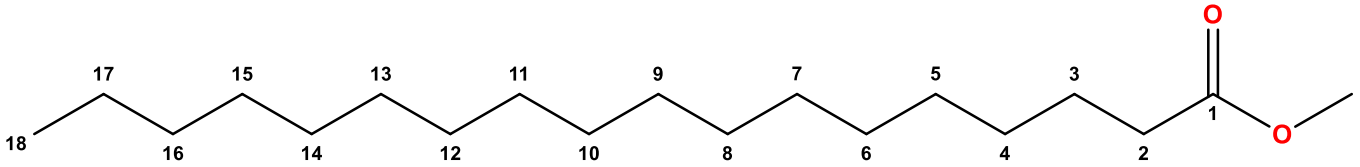
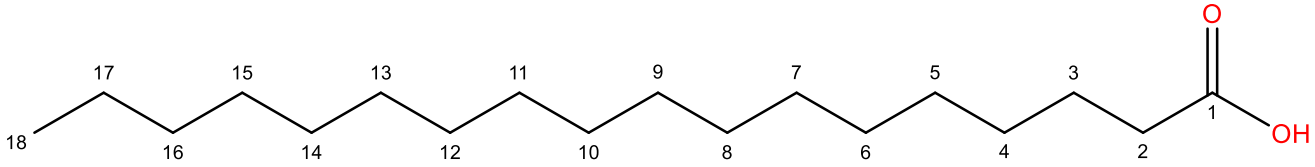
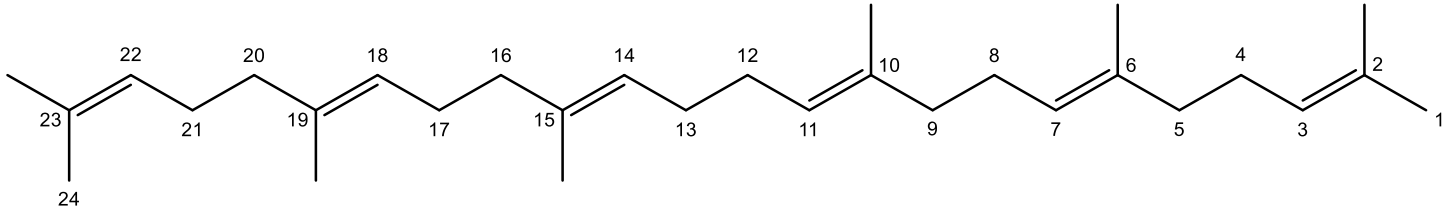
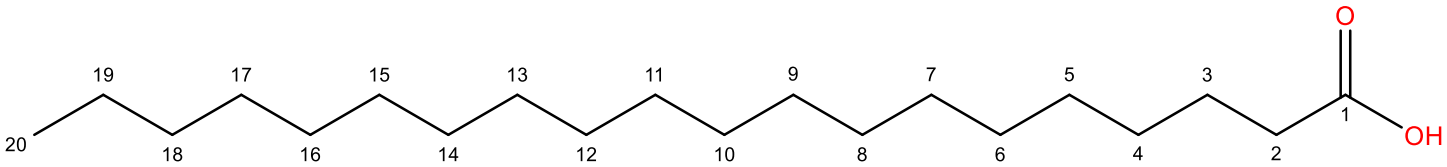
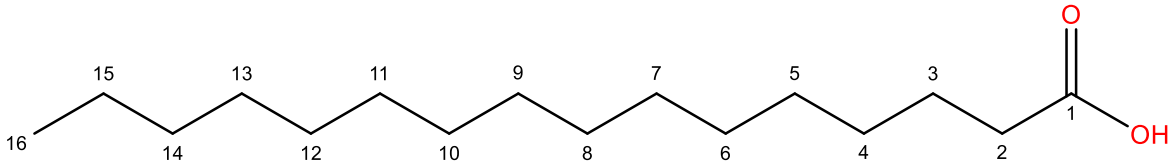
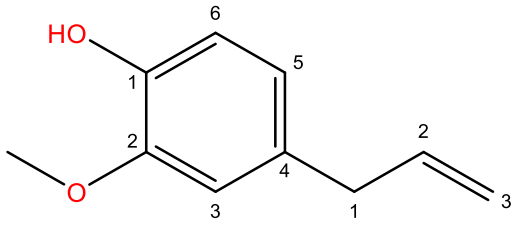
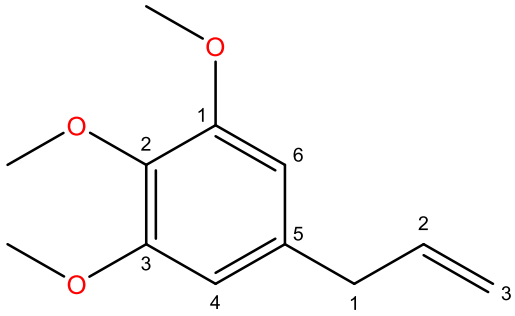


Figure 4-10: GC-MS chromatography of Ethanolic leaf extract of Ocimum lamiifolium.

Table 4-6: Structure of selected Phyto-chemical compounds.

Phyto chemical species	Structure
Hexadecenoic acid	
9,12,15, - Octadecatrienoic acid	
Octadecanoic acid	
Squalene	

Eicosanoic acid	
n-Hexadecanoic acid	
Phenol, 2-methoxy-4-(2-propenyl)	
Benzene, 1,2,3-trimethoxy-5-(2-propenyl)	

4.4 Formulation of Herbal Ointment

The oil extract of *Ocimum lamiifolium* was formulated as an ointment with different concentrations of 1%, 3%, 5%, and 10%. High viscous (20%ELN/COL-OLLA), pH, and having lubricant characteristics comparing with the rest synthesized ointment base was chosen for carrying different concentrations of plant extract to produce an antimicrobial ointment.

4.4.1 Physical Evaluation of the Formulated Ointment

The organoleptic properties, including physical appearance, color, texture, phase separation, and homogeneity for the formulated ointment were displayed in Table 4.7. Results showed that the formulated ointment had a smooth texture, dark brown color and all homogenous with no phase separation signs. The formulated herbal ointment was easily spreadable by a small amount of shear. The formulated antimicrobial ointment shows emollient characteristics due to the immersion of OL extract.

The time used for spreading the formulated ointment was 20-35 sec and the value of spreadability decreases after the dispersion of extracted oil to the synthesized ointment base. This is due to the increasing viscosity as the concentration of oil increases in the formulation. From Table 4.8, it is clearly shown that the pH of the formulated antimicrobial ointment has shown a significant change from the ointment base. F4 showed higher pH (6.02) that is in the human skin range with increasing viscosity and decreasing spreadability.

The report on elastin and collagen shows there was antioxidant and antihypertensive activity and collagen that used for wound healing ability and provides the building block of elastin. Therefore, using this biomaterial for ointment base might give extra advantage and creates insight for others researchers to work on this area. Lactic acid provides additional moisture to the outer layer so the synthesized ointment and formulated antimicrobial ointment have a moisturizing effect and with easily washable with water.

Figure 4-11: Formulated antimicrobial ointment using 20%ELN/COL-OLLA Ointment base.

4.4.2 Thermal analysis of Formulated Ointment

The thermal analysis of extracted oil from *Ocimum lamiifolium* shows higher thermal stability than the synthesized base (20%ELN/COL-OLLA). Having this higher thermal property means it has a higher resistance to decomposition and it is safely used for below degradation temperature. From Figure 4-12 it is seen that the maximum thermal degradation of extracted oil occurred at 447 ℃ and compared to the synthesized base having maximum degradation at 280 ℃, it is a thermally stable material. This stability at higher temperature shows that the phytochemical compounds that are responsible for antimicrobial activity maybe not be affected or degrade during formulation below 447 ℃.

For producing this antimicrobial ointment on an industrial scale using the fusion method needs two vessels for synthesizing base and for the formulation of antimicrobial ointment. Because of the high thermal stability of extracted oil, it can be produced as soon as the synthesized base is done at 150 ℃ ± 5 ℃ simply by physical mixing. Doing this formulation may decrease the cost for the second vessel with a mixer.

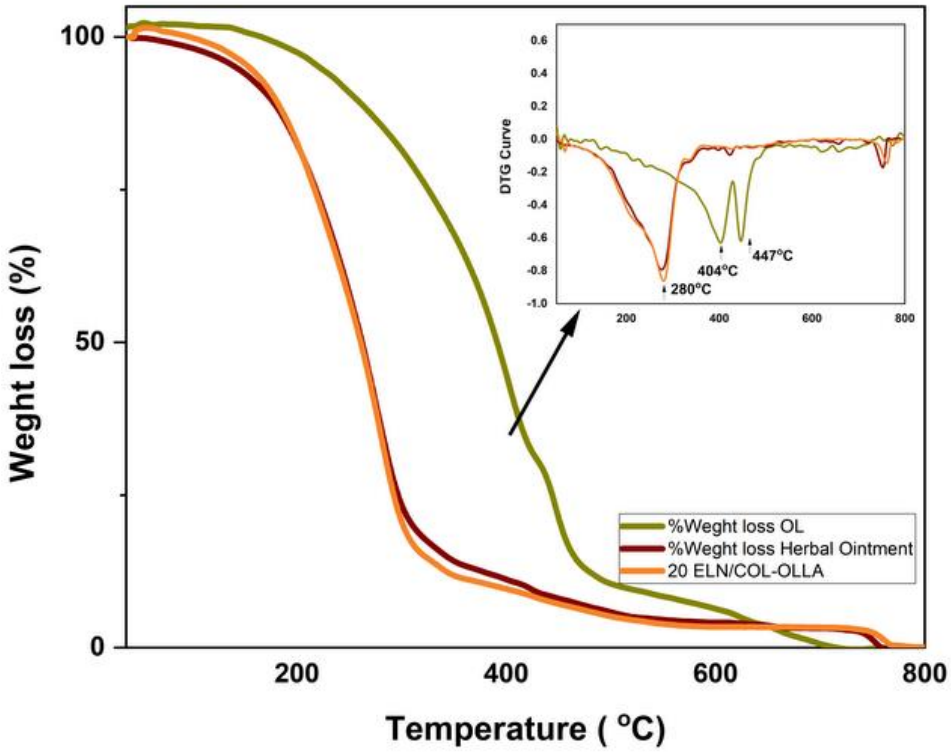


Figure 4-12: Thermal analysis of Formulated Antimicrobial ointment

4.4.3 FTIR Analysis of Antimicrobial Ointment

FTIR spectra of the formulated antimicrobial ointment (20%ELN/COL-OLLA-OL) show similar spectra as the synthesized base (20%ELN/COL-OLL). The peak at 3300 Cm^{-1} which was responsible for NH and OH stretching appeared for all samples, which are OL, ointment base (20%ELN/COL-OLLA), and formulated antimicrobial ointment (20%ELN/COL-OLLA-OL). Moreover, due to adding ointment base to the extracted oil, CH_3 stretching for extracted oil (OL) appeared at 2915 Cm^{-1} the spectra show higher intensity to a higher wavenumber, which is 2993 Cm^{-1}.

The peak at 2622 Cm^{-1} and 2618 Cm^{-1} that was not shown for extracted oil (OL) appeared for antimicrobial ointment and synthesized base. This peak has been shown for the formulated ointment because of the OH symmetric vibration of the synthesized base which appeared at 2618 Cm^{-1}. From figure 4-13, it is clearly seen that, no a formation of new peak in the formulated ointment that is differ from the extracted oil and synthesized ointments. Therefore, conclusion can be made that there was no any chemical interaction occurs between the synthesized ointment base and extracted oil.

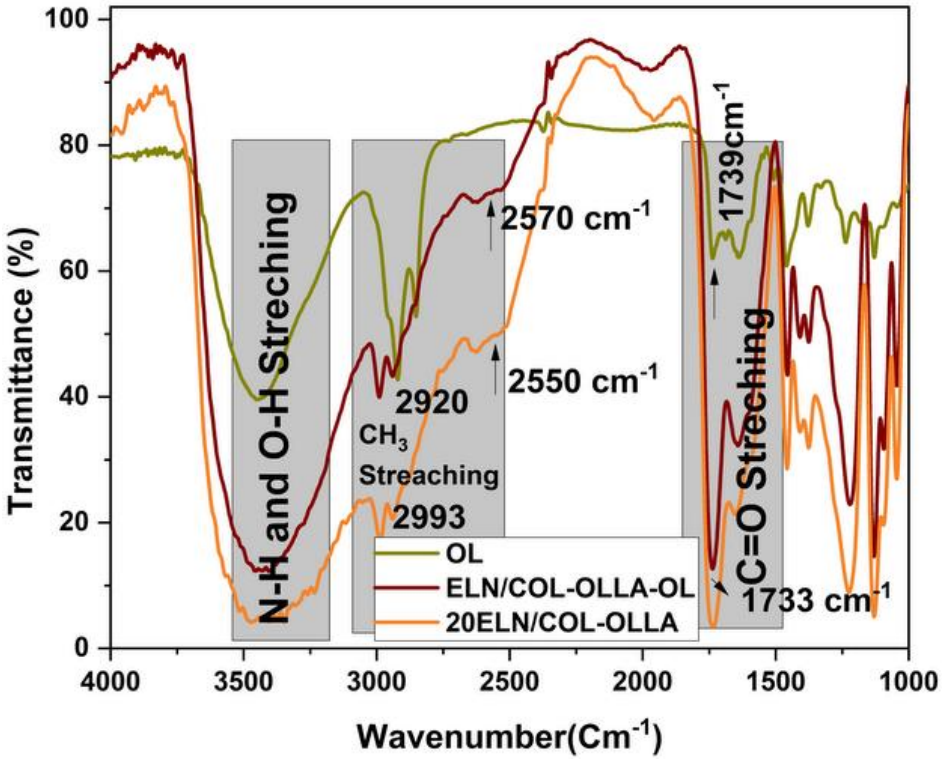


Figure 4-13: FTIR spectra for the formulated ointment.

Table 4-7: Evaluation of Sensory analysis for the formulated ointment

Formulation	Appearance	Homogeneity	Texture	Phase Separation
Base	Dark Brown	Completely homogenized	Smooth (Lubricant)	no
F1	Dark Brown	Completely homogenized	Smooth (Lubricant)	no
F2	Dark Brown	Completely homogenized	Smooth (Lubricant)	no
F3	Dark Brown	Completely homogenized	Smooth (Lubricant)	no
F4	Dark	Completely homogenized	Smooth	no

Table 4-8: Evaluation parameter for the formulated Antimicrobial ointment

Formulation of ointment	Composition	Spreadability (g.cm/s)	T (Sec)	pH
Ointment base	20 g ELN/COL-100g L-Lactic acid	20 ± 2	17	5.76
F1	5 g Ointment base 1% extracted leaf of *O. lamiifolium*	17.27	20	5.92
F2	5 g Ointment base,3% *O. lamiifolium*	13.81	25	5.94
F3	5 g Ointment base,5% of *O. lamiifolium*	10.46	33	5.98
F4	5 g Ointment base,10%*O. lamiifolium*	9.87	35	6.02

F_1-F_4: Stands for the synthesis of formulated ointment, T=Time required to move the slides.

4.4.4 Antimicrobial property of Herbal Ointment

The antimicrobial study showed that all developed ointment formulations have an inhibitory effect on S. aureus and E. coli. The in vitro antimicrobial activities were calculated in terms of the zone of inhibition diameter (mm), the result recorded in Table 4.8. F4 showed a higher zone of inhibition against S. aureus and E. coli.

Table 4-8: Zone of inhibition in mm for synthesized Antimicrobial ointment.

Formulated ointment (1mg/ml)	Zone of inhibition in mm at different concentration of OL	
	Escherichia coli (E. coli)	Staphylococcus aureus (S.aureus)
Base(20%ELN/COL-OLLA)	12	14
Extracted Oil (OL)	9	11
F1	20	20
F2	21	23
F3	22	24
F4	23	25
Ampicillin (Amp) Positive Control	7	6

From Figure 4-14 and 4-15, it is clearly shown that F4 has a higher zone of inhibition for both pathogens. The developed biocompatible ointment base also shows resistance especially gram-positive bacteria. This is a new sight for the scientific world. Compared to the formulated base, the positive control (Ampicillin) an antibiotic giving for both bacteria, shows no resistance. Especially for E. coli bacteria, this positive control was given for preventing form constricting cell wall by killing the bacteria.

As the ratio of extracted oil dispersion in ointment base increases, from 1% to 10% in 5g ointment base, the inhibition zone increases. This result indicating that using culturally known plant extract for ointment for a different purpose. The prepared ointments may be used for wound healing activity by studying its drug release.

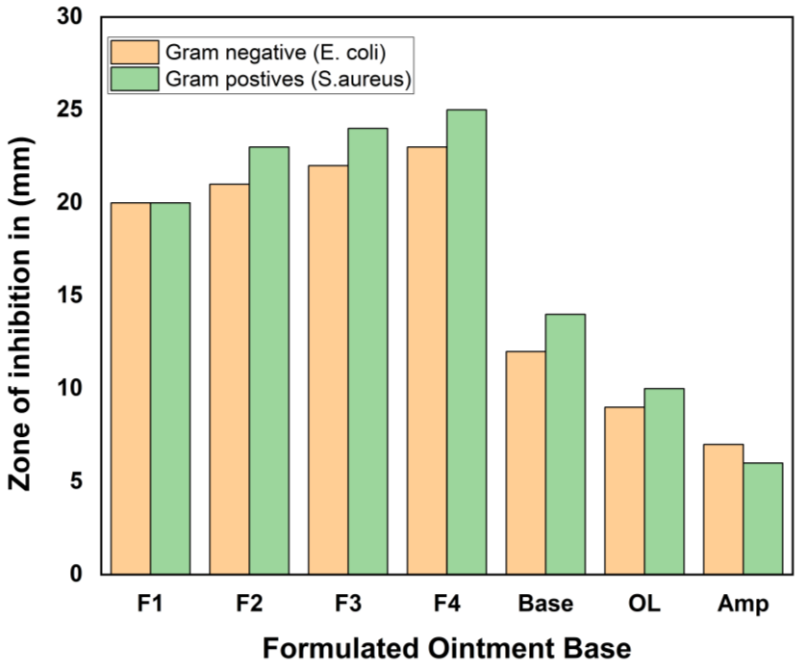


Figure 4-14: Zones of inhibition for topical ointment formulation.

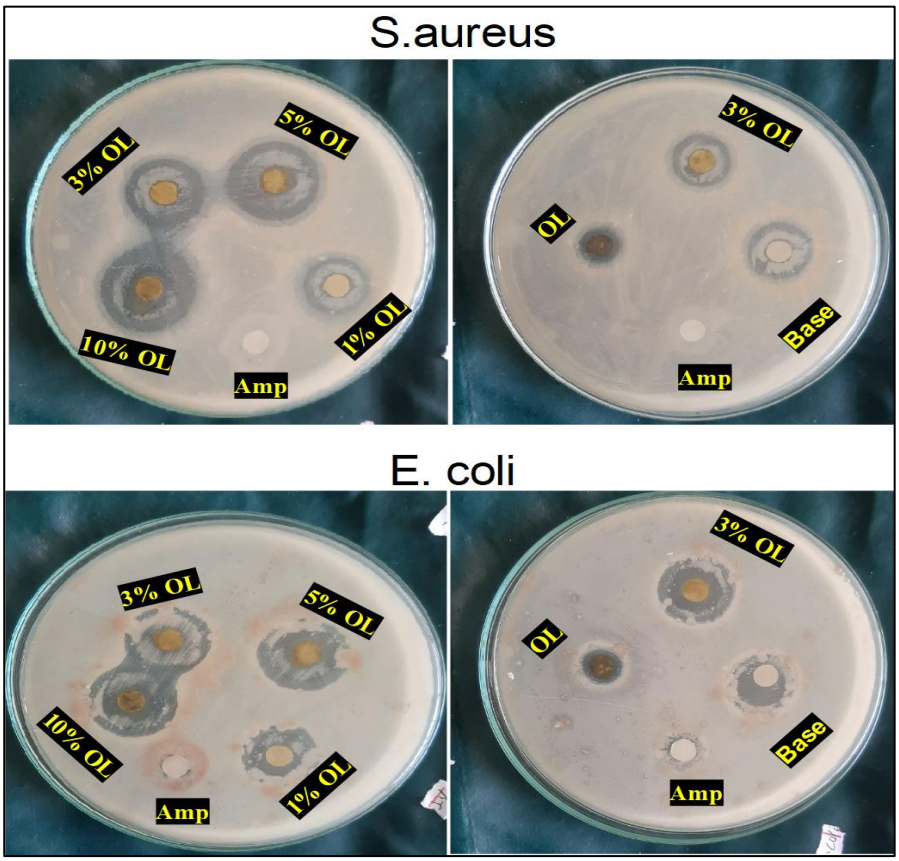


Figure 4-15: Zone of inhibition of the formulated herbal ointment.

Table 4-9: Zone of inhibitions for selected ointment and antibacterial drugs

Author name	Zone of inhibition in mm at different concentration of plant extract	
	Gram-Negative Escherichia coli (E. coli)	Gram-Positive Staphylococcus aureus (S.aureus)
Apas Kumar Pal et al., (1mg/mL) [109]	13.67	13.00
Pandit Deepika et al., (20mg/mL) [110]	14	17
Mahendran Sekar et al., (20mg/mL) [111]	14.33	14.60
Gentamycin [110]	22.4	20
Streptomycin	25	20
Ofloxacin (1mg/ml) [109]	22.33	18.33
Vancomycin (1mg/ml) [111]. Penicillin	17.90 Resistant	19.57 Resistant
Ampicillin (1mg/mL)	7	6
20%ELN/COL-OLLA (1mg/mL) (current investigation)	**12**	**14**
20%ELN/COL-OLLA-10%OL(F4) (1mg/mL) (current investigation)	**23**	**25**

Table 4.9 shows different researches on antimicrobial ointment, the diameters of the inhibition zone of the synthesized ointment base also show higher inhibition zone compared to the formulated topical ointment from petroleum derivative base and Biophytum sensitivum extract [109], and the formulated ointments were comparable with the standard antibiotic drugs which are listed in the above Table 4-9. The ointment formulation showed a broad spectrum of activity at 10% concentration extracted oil, with the zone of inhibition of 25mm against S. aureus and 23 mm against E. coli.

Compared to recent articles published on antimicrobial ointment having petroleum derivative bases, this synthesized base shows an inhibition zone for the principal pathogens causing skin infections which are Staphylococcus aureus and Escherichia coli. The

inhibition zone increases after the dispersion plant extract in the ointment base matrix. Both ointment base and extracted oil show significant inhibition but after the formulation using fusion methods, the inhibition zone was increased as the concentration of oil increases. Because of its biocompatibility with living things, the synthesized ointment base may be used for carrying different kinds of plant extract for producing antimicrobial ointments. To produce new therapeutic agents, plants are important sources of potentially useful components because most of them are safe with very few side effects.

From my knowledge, there was no any report on synthesizing ointment base from ELN/COL-OLLA bioconjugate. Therefore, with all this result I can concludes that the synthesized ointment base may be used in a different production of ointment formulation, and it is a promise biocompatible material to use as a drug carrier for antimicrobial activity.

CHAPTER FIVE

5 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

An ointment is a homogeneous, viscous, semi-solid preparation intended for external use on the skin or mucous membranes. Commonly they are greasy with a high viscoelastic property. Numerous researches were done on the formulation of herbal ointment from petroleum derivatives ointment bases to carry active components of plant extract for producing antimicrobial, antifungal, and anti-inflammatory ointment. However, these petroleum derivatives ointments have a negative impact due to their chemical compositions. Petroleum derivatives base products destroy the skin in the long run.

This research focuses on synthesizing and characterizing ointment base from elastin/collagen and lactic acid which are biobased, green, and biocompatible materials with living systems. Poultry byproducts were used for extracting hydrolyzed elastin and collage from local broiler distributer in Adama city. The chemical structure of the extracted ELN/COL matrix analyzed by FTIR spectroscopy indicates the amide I, amide II, and amide III groups of elastin and collagen were observed at 1605-1770 cm^{-1},1488-1605 $cm^{-1,}$ and 1266-1360 cm^{-1} respectively and CH_2 wagging vibration for both collagen and elastin were indicated at **1338** cm^{-1}.

ELN/COL matrix was completely soluble in the lactic acid monomer. This may facilitate the delamination of α helix and β sheet stacks of collagen and elastin respectively through a weakening of the secondary structure formed by the NH and C=O bond of the consecutive chains of the peptide bonds. The synthesis of oligomer L-lactic acid (OLLA) and lactic acid modified elastin/collagen (OLLA-*m*-ELN/COL) copolymer was conducted by in-situ polycondensation reaction. The synthesized lactic acid modified elastin/collagen matrix (OLLA-m-ELN/COL) copolymer was characterized through various physicochemical techniques.

The six-mass loss in the ELN/COL matrix was not seen in the synthesized ointment base. The reason for showing similar degradation characteristics might be due to secondary week

interaction that happened between the carboxyl group of lactic acid oligomer and the amide group of ELN/COL matrix. This phenomenon is supported by the formation of a broad shoulder like a peak at 1745 cm^{-1} with the addition of elastin/collagen composition within the lactic acid oligomer. More interestingly, with increasing ELN/COL concentration within the oligomer matrix a new FTIR peak is observed at 1581 cm^{-1}.

Formulation of antimicrobial ointment was done by dispersion 1%, 3%, 5%, and 10% *ocimium lamifoliium* extracts in biocompatible, high viscous, and lubricant nature ointment base (20%ELN/COL-OLLA). The synthesized ointment base and formulated ointment had a smooth texture, dark brown color and all homogenous with no phase separation signs.

The time used for spreading the formulated ointment was 20-35 sec and the value of spreadability decreases after the dispersion of extracted oil to the synthesized ointment base. As the concentration of ELN/COL and OL extract increases, the viscosity and pH of the synthesized base and formulated antimicrobial ointment increases while the spreadbility of the synthesized base decreases.

The formulated ointment effectively inhibited the growth of E. coli and S. aureus with minimum concentration (1mg/mL) and antibacterial activity for the formulated ointment was increased with an increasing OL extract concentration from 1% to 10%. The diameters of the inhibition zone of the synthesized base (20%ELN/COL-OLLA) also show inhibition than the formulated topical ointment from a petroleum derivative base[109].

The ointment formulation showed a broad spectrum of activity at 10% concentration extracted oil, with the zone of inhibition of 25mm against S. aureus and 23 mm against E. coli. Compared to recent articles published on antimicrobial ointment having petroleum derivative bases this synthesized base shows an inhibition zone for the principal pathogens causing skin infections are S. aureus and E. coli. The inhibition zone increases after the dispersion plant extract in the ointment base matrix.

As far as my knowledge, there was not any report on synthesizing ointment base using biomaterials (ELN/COL-OLLA) which shows an antimicrobial effect, and biocompatible due to its composition. Therefore, with all this result I can conclude that the synthesized ointment base which is biocompatible, may be used for drug carrier and production of different ointment types with lubricant characteristics.

5.2 Recommendation

The synthesized ointment base has been studied and compared with other studies. The synthesized base has a promising property with the required ointment characterization such as spredability, pH, homogeneity, texture, viscosity, and washability. Base on the present study, some recommendations can be made for future work.

- Test for the rate of absorption, penetration, drug release, stability, rheological properties, and shelf-life characteristics of ointment produced should be done.
- Antioxidant and antihypertensive nature of ELN and COL should be studied for producing antioxidant and antihypertensive ointment base.
- By using the synthesized ointment base antiaging ointment can be produced by studying cell growth ability.
- The preliminary work for the synthesized ointment base shows an excellent plasticizing property that may be used for enhancing the brittleness nature of Polylactic acid. Further study should be done on mechanical properties of the material to define the maximum elongation that the material will be fractured.

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APPENDIXES

Appendix A: Major peaks of Extracted *Ocimum lamiifolium* using GCMS analysis:

The major Phyto-compounds detected in the ethanolic leaf extract OL by GC-MS analysis.

S. No	Name of the Compound	Retention time (min)	AREA %	Score %
1	Cyclohexanone	4.681	0.388334	72.13
2	5-Methyl-6-oxa-1-aza-bicyclo [3.1.0] hexane	8.349	0.336994	88.11
3	Phenol, 2-methoxy-4-(2-propenyl)	16.856	2.589549	95.76
4	Isocaryophyllene	18.513	1.778872	93.79
5	1,3,6,10-Dodecatetraene,3,7,11-trimethyl-, (Z, E)	18.883	0.669994	94.83
6	2,7:3,6-Dimethanonaphthalene, decahydro	19.362	0.541227	72.39
7	1H-Cyclopenta [1,3] cyclopropa[1,2]benzene, 3a,3b,4,5,6,7-hexahydro-3,7-dimethyl-4-(1-methylethyl)-,[3aS (3a.alpha.,3b.beta.,4.beta.,7.alpha.,7aS*)]	20.044	0.280852	83.38
8	beta. -Cadinene	21.043	0.741001	82.96
9	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)	21.788	16.93713	97.73
10	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-,[1ar-(1a.alpha.,4a.alpha.,7.beta.,7a.beta.,7b.alpha.)]-	22.354	1.565299	91.33
11	5-Oxatricyclo [8.2.0.04,6] dodecane,4,12,12-trimethyl-9-methylene-, (1R,4R,6R,10S)	22.493	1.953297	86.6
12	4-Isopropyl-1,6-dimethyl-1,2,3,4,4a,7,8,8a-octahydro-1-naphthalenol	24.092	0.315798	71.12
13	Myristic acid, methyl ester	25.727	0.53885	87.49
14	alpha. -Phellandrene, dimer	27.627	0.307976	85.28
15	Hexadecanoic acid, methyl ester	31.144	12.60894	91.44
16	n-Hexadecanoic acid	32.08	0.48033	76.33
17	Methyl 9-cis,11-trans-octadecadienoate	35.834	6.333032	93.21
18	9,12,15-Octadecatrienoic acid, methyl ester	36.013	22.27064	89.84

19	Neophytadiene	36.33	11.36659	89.45
20	Octadecanoic acid, methyl ester	36.764	1.472474	86.66
21	Chloroacetic acid, dodec-9-ynyl ester	37.035	0.368893	75.83
22	Eicosanoic acid, methyl ester	42.233	0.901785	75.1
23	Phenyl 4-[bis(ethoxycarbonyl)but-3-ynyl]-2,3,4-trideoxy-. alpha.,L-glcero-pent-2-enopyranoside	47.413	0.268877	53.03
24	(S)-(E) -(-)-4-Acetoxy-1-phenyl-2-dodecen-1-one	51.266	0.244525	55.86
25	Squalene	53.662	7.648766	76.24
26	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl	54.852	0.781412	56.67
27	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl	55.401	0.252386	61.95
28	Hexasiloxane,1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl	57.214	0.227461	67.95
29	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl	58.052	3.454573	61.75
30	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	58.658	0.673811	67.43
31	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethy	59.969	0.610222	64.94
32	GC stationary phase (UCC-W-982) P1529	61.084	1.090098	64.46