Development and Characterization of Sustainable Coatings on Surgical Sutures for Wound Healing Application

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A thesis submitted to the National University of Sciences and Technology, Islamabad,

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Supervisor: Dr. Shoaib Butt

School of Chemical and Materials Engineering

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(2024)

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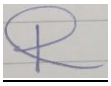
Student Signature: 

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DEDICATION

"I dedicate this dissertation to my beloved parents, siblings, and friends for their continuous help and support."

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Hafiz Hamza Ghafoor

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

SSI	Surgical Site Infections
SEM	Scanning Electron Microscope
XRD	X-ray Powder Diffraction
FTIR	Fourier-Transform Infrared Spectroscopy
PBS	Phosphate-buffered saline
HDFa	Human primary dermal fibroblast
MTT	3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide

ABSTRACT

Surgical site infections (SSIs) cause considerable morbidity and cost in surgery all across the world. Anti-microbial resistance is becoming an increasingly serious concern for the future of humanity. Common antibiotics are failing to treat microbial infections at an accelerating rate. As the importance of antibacterial natural compounds is increasingly recognized, the number of studies on their extraction, purification, and characterization is progressing rapidly. Therefore, the present research aimed to develop and evaluate antibacterial Clove/gelatin-coated sutures that could help reduce the incidences of SSI. Syzygium aromaticum was assessed as a potential antimicrobial agent for suture coating, and gelatin as a biocompatible matrix for silk-braided suture materials. Syzygium aromaticum -coated sutures were prepared by dip-coating surgical sutures of different concentrations (5%, 10%, 15%, and 20%) to form biofilm. Surface morphology, chemical composition, and mechanical strength were characterized using a scanning electron microscope, Fourier Transform Infrared Spectroscopy, and tensile strength. Zone of inhibition assays showed antimicrobial effects of the sutures against (Staph aureus, Escherichia coli, Klebsiella pneumoniae, and Enterococcus faecalis), where 20% Clove/gelatin formation showed potent antibacterial properties up to 17.5± 0.875 mm and low cytotoxicity in MTT assay human primary dermal fibroblast. Hemolytic assays showed that all composite concentrations showed <1% hemolytic activity. Approximately 93% of drug release was observed in 20% composite up to 72 hours which shows promising results compared to untreated sutures. Sutures coated with Syzygium aromaticum can potentially be useful for antimicrobial and wound healing applications.

Keywords: Syzygium aromaticum, surgical site infections, antimicrobial activity, hemolytic activity, wound healing, MTT assay

CHAPTER 1: INTRODUCTION

1.1 Biomedical science

Biomedical science is used to diagnose and treat illnesses and diseases by conducting scientific tests on human fluids, cells, and tissue samples within a laboratory. Biomedical scientists can work in different specialisms, including infections, blood, cells, and genetics. Biomedical science studies how the human body functions at a molecular level. It involves researching and developing new medical treatments for various infections, including drug-delivery methods [1]. Clinical epidemiology is also a part of biomedical science, closely related to healthcare and public health. The main goal of biomedical science is to support the healthcare of the human body. It is the broadest area of science that aids modern medicine. Biomedical science involves a deep analysis of the human body in both normal and diseased states, focusing on diseases and their symptoms. Technological advancements in biomedical science have led to the development of various anti-bacterial and anti-inflammatory medicines, particularly for wound healing applications. These modern medication methods are highly beneficial in treating a wide range of diseases. Microbiology, which is a part of biomedical science, involves the study of living organisms such as bacteria, fungi, and other organisms that can cause infections. By investigating infections, we can effectively treat them using antibiotics or anti-bacterial drugs at the site of infection.

1.1.1 Types of Biomedical Science

Biomedical science is mainly divided into four main disciplines.

1. Infection science

2. Blood science

3. Cell science

4. Genetic and microbiology

1.1.2 Biomedical Applications

The field of biomedical science has several uses. Biomedical research can assist us in creating a medicine delivery system. For example, we can help wounds heal by using surgical sutures and anti-microbial or anti-inflammatory medications [2]. Fighting off various germs that are harmful to the human body requires the anti-microbial effect. Bioimaging, the practice of viewing biological activity and functional pictures in living objects, is another field in which biomedical applications find extensive use. Furthermore, biomedical science plays a major role in developing biosensors, instruments that generate quantifiable signals corresponding to the target's concentration. Pathogens in food are found using biosensors. For instance, by examining changes in pH brought on by ammonia, biosensors can be used to identify the presence of Escherichia coli in vegetables.

1.2 Wounds

Wounds are injuries in which our body cells and tissues are damaged. Wounds can be categorized in various ways based on the healing time. Generally, wounds are divided into two main types: open wounds and closed wounds. Sudden acts like cuts, falls, or knocks cause them. Examples of wounds include cuts, grazes, and lacerations. Cuts are typically caused by sharp objects like knives, glass, or even a very sharp and narrow sheet of paper [3].

1.2.1 Types of Wounds

There are two types of wounds.

I. Open wound

II. Closed wounds

Open wound: Open injuries Wounds classified as open are those that are exposed to the outside air or surroundings. Wounds that reveal the underlying tissues and organs are known as open wounds. Blunt force trauma and penetrating wounds are the best examples of open wounds. Many types of wounds, including thermal chemical burns,

electrical burns, puncture wounds, and surgical wounds, fall within the category of penetrating wounds. Skin tears, cuts, and abrasions are all possible outcomes of blunt-force trauma.

Closed wounds: Closed Wounds are those that are closed and do not contact the surrounding environment or tissues. They also do not come into contact with the outer atmosphere. The three main categories of closed wounds are crush injuries, blisters, and contusions.

1.2.2 Stages of Wound Healings

Wound healing is a complex biological process in the human body, involving four precisely and highly programmed phases [4].

1. Homeostasis

2. Inflammation

3. Proliferation

4. Remodeling

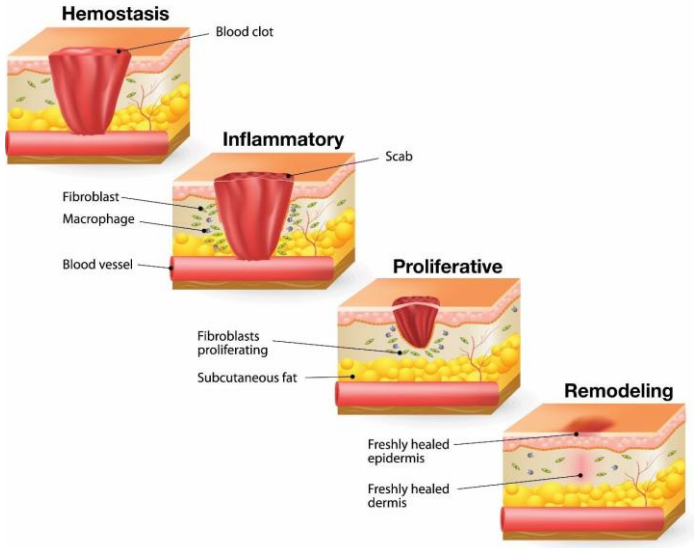


Figure 1.1: Different Stages of wound healing

1.2.3 Treatment for the healing of wounds

Here are the revised instructions for caring for a new injury:

a) Gently and frequently clean the injury to remove dirt and debris, preferably in the shower.

b) In some cases of severe injury, getting a tetanus shot may be recommended.

c) To conduct a thorough examination of a deep injury, a local anesthetic will be administered before the examination.

d) If necessary, carefully remove dead skin with the help of a local anesthetic.

e) Large wounds should be closed with stitches or staples.

f) Dress the wound based on your doctor's recommendation, which depends on the type and severity of the injury. For most chronic wounds, a moist dressing is recommended.

g) Manage pain with appropriate medications.

h) Take prescribed antibiotics and use antimicrobial dressings as directed.

i) Be aware that certain medications, such as anti-inflammatory drugs and steroids, can interfere with the body's healing process.

1.3 Aim and scope

The goal of this study is to provide a dual-drug delivery silk braided suture with high and advanced anti-microbial properties. This research aims to investigate the entrapment of clove extract in gelatin and then evaluate its biocompatibility, physicochemical properties, and drug release characteristics. By doing this, we can enhance the patients' smart sutures' wound healing process. The study will provide insights into the potential of these composite of clove/gelatin for targeted drug delivery in biomedical applications.

1.4 Objectives

The objectives of this research work are as follows:

- Preparation of an aqueous clove extract involves mixing clove powder with a combination of ethanol and deionized water.
- Preparation of aqueous Gelatin solution using pure gelatin powder.
- Preparation of composite of clove/gelatin.
- Coating a silk braided suture with a clove/gel solution.
- Material characterization using Antibacterial, FTIR, SEM, Hemolysis, Drug release test, and mechanical testing.
- Study the effect of coated silk braided sutures for biomedical applications, such as wound healing.

CHAPTER 2: LITERATURE REVIEW

2.1 Surgical sutures

The most common term for sutures is "stitching." We use sterile surgical sutures for wound healing. The choice of sutures depends on the type of wound, with some scars requiring alternative methods such as metal staples instead of sutures. Surgical sutures are susceptible to biofilm development and bacterial infections [5].

Pain and unwanted, excessive inflammation at the suture site are also harmful to wound healing [6]. By creating a surgical suture coated with Clove, it is now possible to administer anti-inflammatory and antimicrobial medications locally.

Cephalosporins are the most often prescribed treatment class for prevention in patients who develop SSI (73.2%), with third-generation cephalosporins being given to patients in 60.7% of cases [7]. Researchers are exploring using natural and synthetic materials, such as plant extracts and polymers, to coat medical devices like surgical implants or sutures. This coating aims to prevent surgical site infections, which can be difficult to treat and lead to complications in severe cases [8].

The addition of antibiotics to these coating biomaterials enables the localized delivery of antibiotics directly at the implantation or suture site, thereby reducing the risk of infection. [9].

The primary goal of this study is to develop new polymeric composite films with natural anti-inflammatory properties and to create antimicrobial coatings for silk braided sutures.

The plan is to combine a biodegradable polymer with a natural herb to create a drug-eluting agent that can provide antibacterial and anti-inflammatory effects, as illustrated in Fig 1.2. By combining the properties of these two materials, we aim to enhance wound healing and prevent surgical site infections [10].

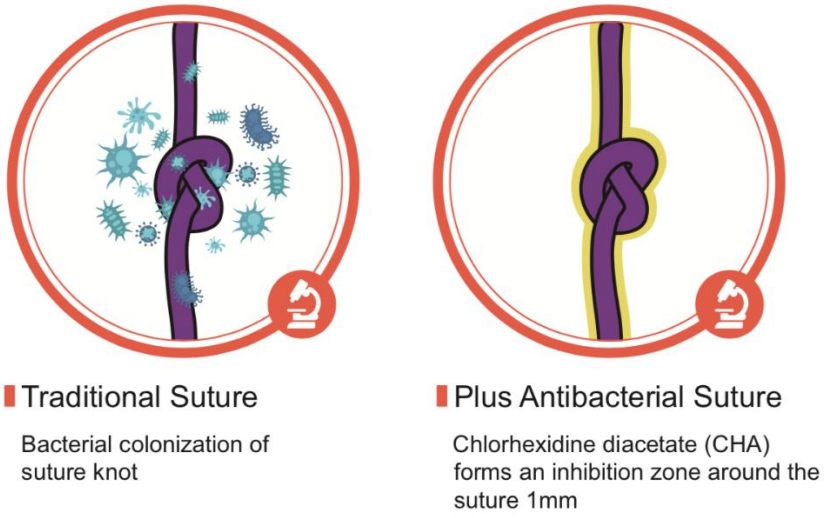


Figure 2.1: The traditional and antibacterial coated suture

2.1.1 Properties of Sutures

After an accident or surgery, surgical sutures are crucial to the body's tissue healing process. The sizes and materials of surgical sutures vary widely. The suture site determines whether surgical sutures are used. Absorbable and non-absorbable sutures are the two primary categories of surgical sutures. Unlike non-absorbable surgical sutures, absorbable surgical sutures break down over time and do not require a standard removal procedure. In four to eight weeks, absorbable sutures break down on their own. For the accessible and internal portions of the suture sites, absorbable surgical sutures work incredibly well [11].

Surgical sutures with the potential to cure wounds and be used in biological applications are now possible. Antimicrobial and bioactive sutures, such as coated and drug-eluting sutures, are included in surgical sutures. In addition to conventional surgical sutures, there is currently a market for coated sutures that are antimicrobial and anti-inflammatory [12]. These sutures have a great deal of potential to prevent bacterial growth or unintended inflammatory reactions [13]. In 2002, a polyglactin suture coated with triclosan was reported to be the first FDA-approved antibacterial surgical suture. It was successful in lowering the surgery site infection rate.

The creation of anti-microbial and anti-inflammatory surgical sutures is made possible by this FDA-approved antibacterial suture. Further, expanded stitch choices are

desirable due to the growing protection against triclosan and a cutoff to the compelling and supported usage of Vicryl Plus. When microbial effects are taken into account, the environments around sutures are less likely to support disease, and the stitches themselves are less likely to form biofilms, which is a challenging process of long-distance bacterial colonization. In general, treating contamination and biofilm anticipation will result in less expensive clinical care and easier patient healing.

Infection control is a key problem for every sutured lesion due to the restricted selection of antimicrobial sutures available. During stitching, patients experience agony at the location of the wound. Medication like anti-inflammatory medications is frequently used to prevent this pain. Manufacturing surgical sutures with antimicrobial and anti-inflammatory properties for biomedical applications that promote improved wound healing and localized pain alleviation. We now have a whole new type of surgical suture called a coated suture. Coated antibacterial and antimicrobial surgical sutures are used because they target the tissues with localized drug release at the same time. The healing process at the suture sites is negatively impacted by the addition of direct medication administration from surgical sutures. The most often used antibiotic is rifampicin, which is an excellent anti-microbial medication because of its high degree of effectiveness and low minimum inhibitory concentration (MIC) against the strains of common bacteria, including S. aureus and E. coli. Red wine contains a significant amount of the powerful anti-inflammatory compound resveratrol. Its biomedical application for wound healing is highly effective due to its free radicals.

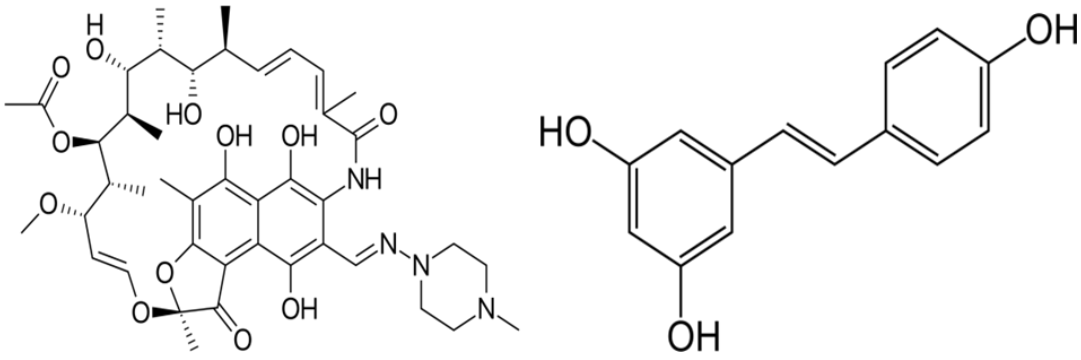


Figure 2.2: Anti-microbial molecule rifampicin (left) and anti-inflammatory molecule resveratrol (right)

Most research findings indicate that most medications are released for a brief duration, often lasting between one and two weeks. An infection may take a long time to recover. The process of wound healing often requires four long weeks of tissue remolding. For the best recuperation, we employ therapeutic medications at every stage of the procedure [14].

The body takes around two months to break down and remove absorbable sutures. Four weeks is an ideal time frame for drug delivery and therapeutic dosage, depending on the circumstances.

A number of their manufacturing processes impact the mechanical characteristics of drug-eluting sutures. A non-absorbable silk suture can withstand a 20 N tensile force. The most crucial characteristic of surgical sutures is their tensile strength, even though polyester and polypropylene sutures have a tensile force of 11 N. At a minimum, the strength of drug-eluting sutures is comparable to that of polyester and polypropylene sutures.

2.1.2 Types of sutures

There are two types of the sutures

1. Absorbable sutures
2. Non absorbable sutures

Absorbable sutures: Absorbable sutures are known as biodegradable sutures. Most doctors believe the body's absorbable sutures don't need to be removed. Our body's many enzymes will naturally break them down.

Wounds inside the body are usually treated with absorbable sutures. It is advised to use absorbable sutures for closed wound healing.

Non absorbable sutures: Biodegradable sutures are not those that are non-absorbable. Depending on the process, doctors extract them from the human body within a few days or weeks. Non-absorbable sutures work best when used for open-wound healing.

Figure 2.3: Diff. b/w absorbable and non-absorbable

1.1.3 Sutures materials

Sutures materials are made up of different types of materials. we classify the two main categories of suture materials

1. Natural
2. Synthetic

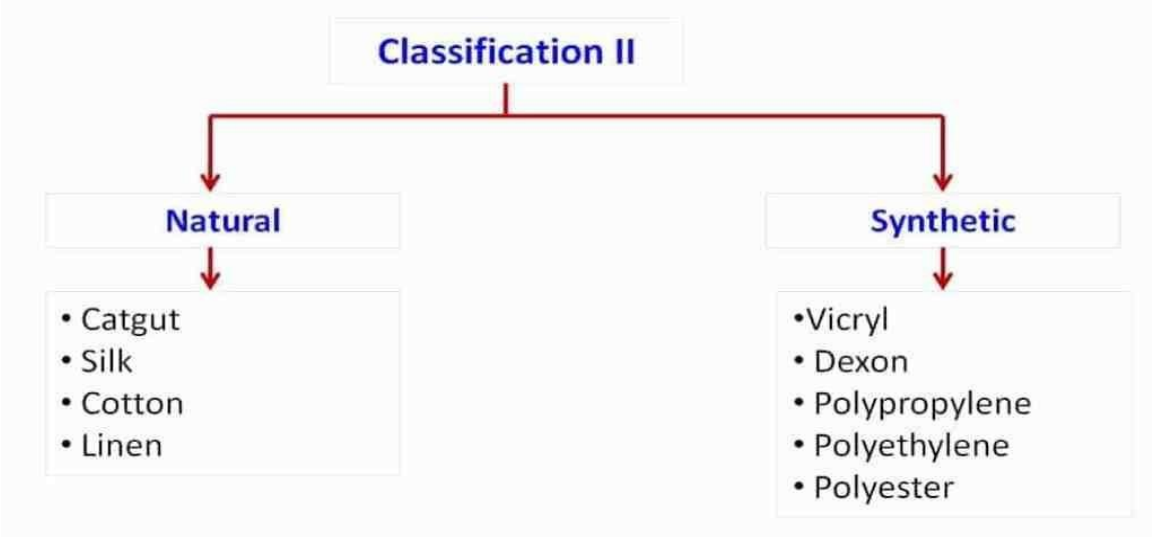


Figure 2.4: Types of sutures material

1.1.4 Uses of Sutures

To close the wound, we stitched it up. We are wounded in two ways. both closed and open wounds. For closed wounds, absorbable sutures are used. Open wounds typically require the use of non-absorbable sutures. Most of the time, we employ unconventional techniques like metal staples to treat wounds [15].

Table 2.1: Different types of sutures and Different structures of the surgical sutures

Suture type	**Absorbable**	**Non absorbable**	**Monofilament**	**multifilament**
Silk	NO	Yes	NO	Yes
Vicryl	Yes	NO	NO	Yes
PDS	Yes	NO	yes	NO
Monocryl	Yes	NO	yes	NO
Nylon	NO	Yes	yes	NO
Prolene	NO	Yes	yes	NO

1.1.5 Structure of Surgical Sutures

There are two types of structures for surgical sutures.

1. Monofilaments
2. Multifilament

Monofilaments: The word "mono" refers to single, and a single-stranded filament suture exists in monofilament. The monofilaments nylon and proline are two excellent examples. Although monofilament has a very minimal risk of infection, it has two disadvantages: it is easy to handle and has poor knot security.

Multifilament: A multifilament suture is made up of several twisted filaments. The most widely used type of multifilament suture is the silk-branded suture. While these sutures are easily handled and in acceptable condition for knot security, there is a chance of infection with multifilament sutures.

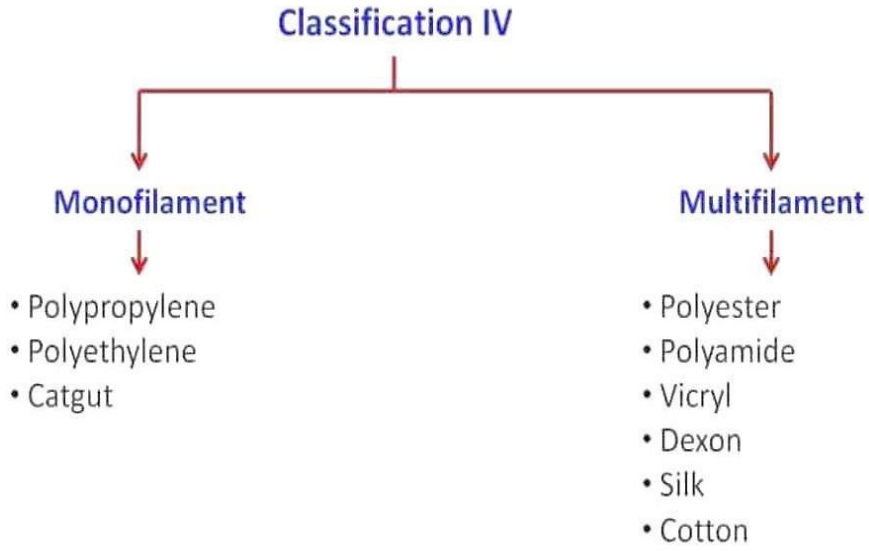


Figure 2.5: Structure of sutures material

1.1.6 Problems Related to Surgical Sutures

Surgical sutures also have drawbacks because of various issues, such as cross-hatching. Making little modifications to the infection site is quite challenging. When surgical sutures are extracted from the human body, there is a chance that they will die. The tissue being sutured and the type of suture material utilized determine the complications associated with suture hypersensitivity. The degree of congestion, inflammation, and foreign body reactions can make it more difficult for any tissue to heal a wound. Irritation from the clipped ends of the suture material is the primary drawback of this suture material tissue**.**

1.1.7 Solutions to Problems Related to Surgical Sutures

- Sutures frequently lead to dehiscence, infection, suture responses, and ligature loop failure. It's crucial to use the right suture materials and techniques to lessen these

problems. One way to lower the risk of suture reactions and infections is to use synthetic sutures that are less reactive [16].

- Preventive antibiotics should be taken into consideration to reduce the risk of infection, particularly in high-risk individuals. Planning for infection prevention should consider variables like the duration of the surgical process, the existence of inflamed operative sites, and patient characteristics (e.g., age, immunocompromised status) [16].
- It's critical to select the appropriate suture type (absorbable versus non-absorbable, monofilament, or multifilament) based on the patient's demands and the features of the wound. Infection rates may be lowered by the fact that monofilament sutures often exhibit less bacterial adhesion than multifilament sutures [16].
- Employing proper suturing techniques can prevent complications like suture spitting and tracking. Techniques such as the simple interrupted suture or the vertical mattress suture should be used appropriately based on the wound type and location [17].
- When sutures are removed within the ideal window of time, which is typically 7 to 10 days, issues including foreign body reactions and scarring that result from the prolonged presence of sutures can be avoided[17].
- New developments in suture materials, like drug-eluting or antibacterial-coated sutures, can speed up wound healing and lower infection rates. These cutting-edge sutures feature surface changes to stop bacterial colonization or can distribute drugs locally [17].

1.1.8 Cost Effectiveness

Different kinds of materials are used to make surgical sutures. Polydioxanone is a more costly surgical suture compared to synthetic monofilament absorbable sutures like polyglycolic acid and polytrimethylene carbonate. The cost per box of our tru braided non-absorbable suture is USD 2.81. There are 12 distinct surgical sutures in each package. Each piece of our silk-braided suture costs USD 0.23.

1.1.9 Features of Trusilk

1. Natural, braided fibers of silk
2. Excellent handling
3. High knot security
4. Treated with wax/silicone for added strength
5. Commonly used on mucosal and intertriginous areas as it is soft and pliable

A non-absorbable, inert suture renowned for its smoothness and knot security is called trusilk. Tight braiding guarantees improved strength, functionality, and precise knot placement.

Silk sutures are not advised for use in biliary and urinary system surgery due to their moderate tissue reactivity. It should be used in situations where the least amount of suture reaction is preferred.

The technical details are as follows:

1. Made from 100% protein fiber spun
2. Coating of wax/silicone
3. Available in black or ivory colors
4. Sterilized by ethylene oxide
5. Shelf life of 5 years

The range is as follows:

1. USP sizes available: 6-0 to 4
2. Length available with a needle: 35 cm to 90 cm

3. Needle length: 12mm to 50mm across all needlepoint profiles

1.1.10 Applications

1. General surgery
2. Ophthalmic surgery
3. Cardiovascular surgery
4. Gastrointestinal surgery
5. Orthopedic surgery

1.2 Clove Extract

The evergreen Clove (Syzygium aromaticum) tree is Indigenous to Indonesia, but it is also grown in Madagascar, Tanzania, and Sri Lanka [18]. Clove trees have tiny white blooms and dark green leaves, and they can reach a height of 12 m [18].

The first step in the production of cloves is planting seedlings of clove trees, which are typically raised in nurseries for up to a year before being placed in the field [19]. Well-draining soil and a warm, humid climate are essential for clove tree growth. To get enough sunshine and air circulation, trees are often spaced 8 to 10 meters apart [20].

Apart from its conventional uses, clove valorization—the process of turning cloves into higher-value products—has attracted increasing attention in recent years.

Analgesic and anti-inflammatory qualities have been found in eugenol, among other health advantages [21]. Additionally, it has been found that acetyl eugenol possesses anticancer characteristics, suggesting that it could be used as a cancer therapeutic agent [22].

Cloves and other plants, such as cinnamon and black pepper, contain the bioactive substance caryophyllene. It works well to alleviate pain and inflammation because of its analgesic and anti-inflammatory qualities [23].

Table 2.2: Composition of active compounds from clove extract [24]

Compound Concentration	Range (mg/g)
Eugenol	480-630
Acetyl Eugenol	22-37
β-Caryophyllene	14-17
α-Humulene	2.2-2.9
α-Caryophyllene oxide	2.5-3.0
α-Murolene	1.6-2.0

2.2.1 Components of Clove

Eugenol: Clove extract's primary bioactive ingredient, eugenol, is seen in Figure 2.6 and is responsible for many of its advantageous qualities [25]. Up to 85% of clove essential oil is made up of eugenol, the primary bioactive component [26]. It has been discovered to have many health advantages, including analgesic, antioxidant, and anti-inflammatory properties. Eugenol's proven antibacterial and antifungal properties make it effective against a wide range of infections [27].

Eugenol is one of the most well-known substances because of its analgesic effect, which effectively reduces pain. It has been shown that eugenol inhibits the activity of certain enzymes involved in pain perception, including lipoxygenase (LOX) and cyclooxygenase-2 (COX-2) [28].

Its potential as a medicine delivery agent has been studied. Eugenol has been shown to improve the solubility and bioavailability of some medications because of its lipophilic nature [29].

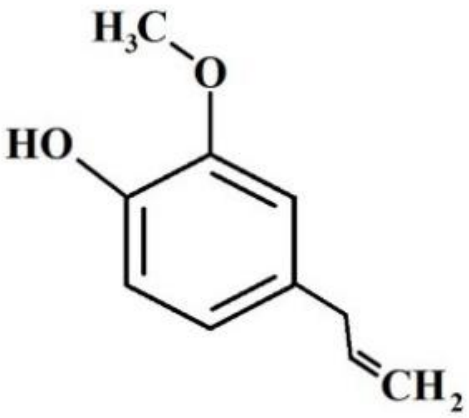


Figure 2.6: Chemical Structure of eugenol [30]

Acetyl Eugenol: Another significant bioactive ingredient in cloves is acetyleugenol, which makes up 15% of the essential oil **[31]**.

Additionally, it has been found that acetyl eugenol possesses antibacterial qualities that enable it to effectively combat infections. Acetyl eugenol is effective against a variety of bacteria and fungi, suggesting that it could be used as a substitute for artificial antibacterial treatments [32].

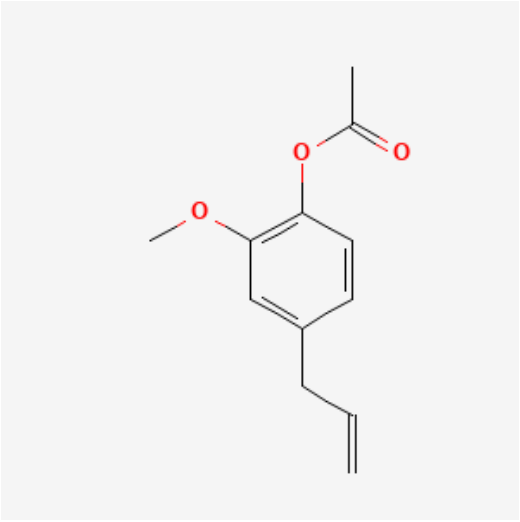


Figure 2.7: Chemical Structure of acetyl eugenol

Caryophyllene: One naturally occurring substance found in clove extract essential oil is caryophyllene. It is a member of the class of substances called sesquiterpenes, which are found naturally in plants and have a variety of biological functions **[33]**.

Caryophyllene's capacity to function as a selective agonist of cannabinoid receptor type 2 (CB2) is one of its most amazing qualities. Immunological cells are the main cells that express CB2 receptors, which are involved in controlling inflammation and the immunological response. Research has indicated that through the activation of CB2 receptors, which regulate the immune response, caryophyllene can lessen pain and inflammation. This suggests that caryophyllene may be used as a medicinal drug to treat a variety of inflammatory conditions, including inflammatory bowel disease, asthma, and arthritis **[34]**.

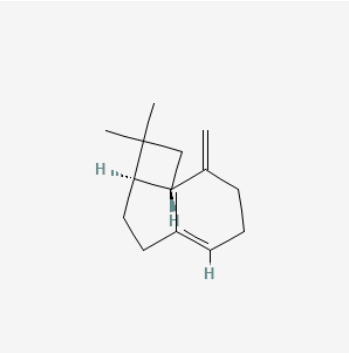


Figure 2.8: Chemical Structure of Caryophyllene

2.2.2 Applications of Clove

The following are major applications of clove extract

Food and beverage industry: Clove extract is a natural food flavoring and preservative because of its strong flavor and antibacterial qualities. It is a common ingredient in drinks, chewing gum, and baked products[35].

Oral care products: Toothache, gum disease, and poor teeth are some oral health conditions that clove extract can help naturally. It is a common ingredient in mouthwash, dental floss, and toothpaste [36].

Skincare products: Because of its antibacterial and antioxidant qualities, clove extract is a useful treatment for wrinkles, acne, and other skin issues. Typically, cleansers, lotions, and creams contain this component [37]. Bioactive substances with potential health benefits can be found in abundance in clove extract. It is often taken as a dietary supplement to improve immune system function, lower inflammation, and improve digestion [38].

Pharmaceuticals: The numerous medicinal qualities of clove extract and its bioactive constituents make them useful in the management of a wide range of illnesses, such as diabetes, cancer, and arthritis. It is frequently found in nutritional supplements and natural treatments [39].

2.3 Gelatin

Gelatin, a natural polymer derived from the hydrolysis of collagen protein, offers numerous medical benefits due to its unique amino acid structure [40]. It can be easily dissolved in water before intake and is frequently available as pills, granules, or powders [41]. The use of gelatin as a scaffold for tissue engineering and as a matrix for three-dimensional cell culture is a topic of active research [42]. Furthermore, gelatin is a high-protein food that can be used in place of fats and carbohydrates in some nutritionally balanced dishes [43]. The most prevalent protein in both humans and animals is collagen,

which is mostly found in the skin, bones, tendons, and ligaments [44]. Collagen must be boiled or hydrolyzed, sometimes with the help of enzymes, to release gelatin, which is then utilized as a flavorless and colorless gelling ingredient in food preparation [45].

Gelatin possesses potent emulsifying properties that could prevent milk, soy milk, and other proteins from clumping together into gastric acid once they reach the stomach, hence aiding in the process of food digestion.

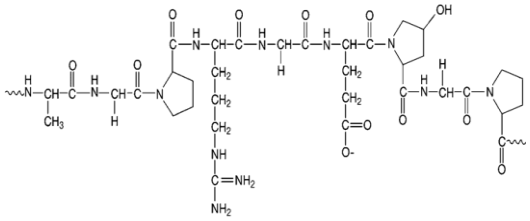


Fig 2.9: Basic chemical structure of Gelatin

2.3.1 Types of Gelatin

In general, two different types of Gelatin may be produced based on the pre-treatment of collagen

1. Type A gelatin

2. Type B gelatin

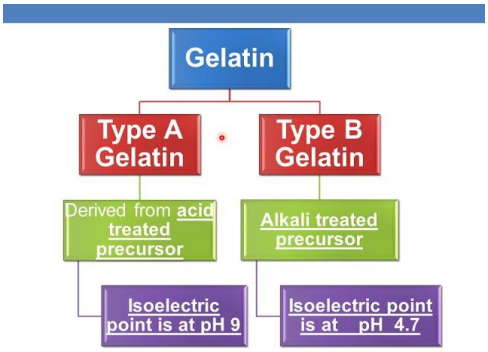


Fig 2.10: Types of Gelatin

2.3.2 Applications of Gelatin

Gelatin is a crucial component in modern cuisine and the food industry, thanks to its gelling properties. For instance, it stabilizes food structure and is utilized in the creation of gourmet desserts for texture, foaming, and clarity [46]. Gelatin can be utilized as an edible film and coating material because of its high nutritional content and exceptional film-forming capabilities [47].

Gelatin is utilized in the medical field to make hydrogels, cell transplant carriers, nanofibers, nano microsphere containers, and medicinal additives [40, 48]. In the world of medicine, gelatin is crucial for processes like hemostasis (stopping bleeding). At the bleeding site, gelatin and thrombin can form a durable clot. Gelatin particles can swell and offer a location for a fibrin clot to stop blood flow and create a mechanically stable matrix surrounding the bleeding site [46, 49].

In addition, Zeng has created injectable gelatin microcrystals (GMs) to enhance the efficacy of cell therapy for treating deep wounds by introducing cells into the tissue's deep layer. This technique reduces site-targeted pain and minimizes invasive side effects by injecting a deep wound layer using a microsyringe needle[46].

CHAPTER 3: MATERIALS AND METHODS

This research focuses on synthesizing a clove/gel composite using natural clove extracts and gelatin. The clove extract is then entrapped into gelatin films for characterization, drug release efficiency, and cytotoxicity testing. This research was conducted in the Materials Laboratory of the School of Chemical and Material Engineering (SCME), National University of Science and Technology (NUST), Islamabad.

Non-absorbable surgical Silk braided sutures were acquired from D Watson Blue Area in Islamabad. Clove extract was purchased from G-11 Islamabad. Dr. Muhammad Shoaib Butt from SCME, NUST, donated the Gelatin powder. The Institute of National University of Science and Technology Islamabad's biomedical lab had all the additional chemicals, solvents, and reagents needed. Dr. Romeza Hnif from the Atta Ur Rehman School of Applied Bioscience, ASAB NUST, provided all the required strains.

1.1 METHODS

3.1.1 Dip Coating

Dip coating is a process where the substrate is submerged in a tank containing coating material. Once removed from the tank, the substrate is allowed to drain. The coated substrate can then be dried using forced drying or a baking process. Dip coating is a well-known technique for achieving uniform substrate coating [50].

3.1.2 Stages of Dip Coating

There are three-stage of the dip-coating methods

Immersion

Immersion is the stage in dip coating where we fully submerge the substrate in a tank containing the coating material at a constant speed. The substrate remains immersed in the tank while the coating material starts to coat the substrate.

Withdrawal

After immersion, withdrawal is the stage in the dip coating process. During withdrawal, the substrate is pulled out at a constant speed to avoid any jerking movements. The withdrawal speed is a critical factor that affects the thickness of the coating on the substrate. Increasing the speed at which the substrate is withdrawn after immersion results in a thicker layer of coating material being formed on the substrate's surface.

Evaporation

After immersing the substrate in the tank, we proceed to the evaporation stage in the dip coating process. During this stage, we dry the substrate by baking it, resulting in the deposition of a thin and uniform layer of the coating material on the substrate's surface.

3.1.3 Advantages and disadvantages of the dip coatings

Dip coating is a very simple coating process. The thickness of the coating material on the substrate is controlled by the viscosity of the coating material and the rate of the withdrawal of the substrate from the immersion tank. Dip coating is a coating process in which we can coat the different shapes of objects. We can also coat the different sizes of the object in the immersion tank.

There are some drawbacks to the dip coating. mostly the thickness of the layer that is coated on the substrate varies from top to bottom due to the wedge effect, so due to this effect most of the time we have unequal coating deposits on the surface of the substrate.

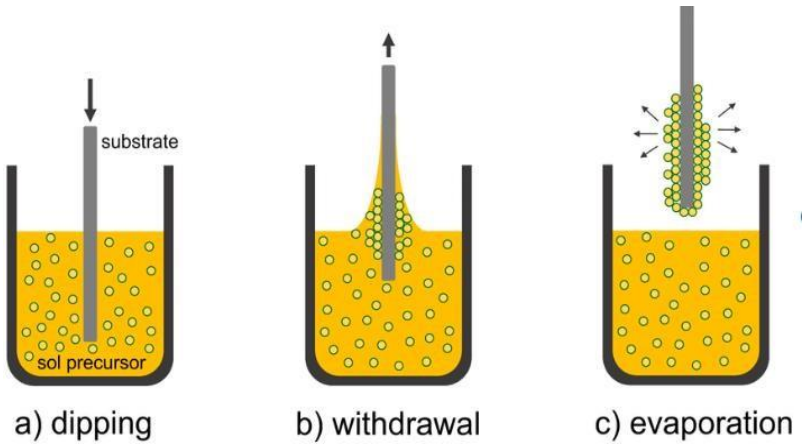


Figure 3.1: Schematic of dip-coating method

3.1.4 Comparison between EPD, DIP coating, and Electrospinning coating

Table 3.1: Comparison between EPD, DIP coating, and Electrospinning coating

Types of coating	**Thickness**	**Advantages**	**Disadvantages**
Electrophoretic deposition (EPD)	0.1 to 2.0mm	Uniform coating thickness, rapid deposition, coat complex substance	Required high sintering temperature, high cost of precursors
Dip coating	20 nm up to 50 μm	Thickness of the layer by controlling the viscosity of the coating material speed of withdrawal of substrate	Unequal coating layer due to the wedge effect from top to bottom of the substrate
Electrospinning	93.18 μm to 619.22 μm	Very thin fibers to the order of a few nanometers with large surface areas	Scaffold fibers are closely packed, which results in poor cell infiltration and migration.

3.1.5 Methodology for wound healing

Table 3.2: Methodology for wound healing

Types of absorbable Suture material	**Materials to make suture conductive**	**Types of coating to coat suture**	**Materials that can be coated for healing**	**Their importance in wound healing**
Natural Silk	Gold coating for suture	Dip coating	Moringa Olifera Leaf	Protect your skin from skin infection, and inflammation, and even treats burn scars
Synthetic Polyamide (nylon)	Sliver coating to make suture conductor	Electrophoretic deposition	Turmeric, anti-inflammatory, anti-viral, anti-bacterial, anti-fungal, anti-carcinogenic, and anti-mutagenic	For copper, it promotes angiogenesis and skin ECM formation and stabilization
Synthetic polyester (Dacron)	Copper coating to make suture conductors	Possibly CVD Chemical vapor deposition, PVD, Physical vapor deposition	Mango, ginger Antipyretic Amba Haldi is an Anti-Bacterial, Anti-Fungal, Anti-Oxidant	Itching in the skin wounds. It helps in treating skin problems
Synthetic polypropylene (proline)	Cross-linking polymers	Electrospinning	Gandhak, Rasayan Detoxified Sulfur	Antibacterial, antiviral, Anti-microbial medicine

3.2 Synthesis of Clove/Gelatin Composite

3.2.1 Synthesis of Clove Extract

First of all, a 40-ml homogeneous solution of ethanol (80%) and DI water (20%) was used to create an aqueous solution of clove extract by adding 0.4g of clove powder. The solution was shaken in a water-shaking bath set at sixty degrees Celsius for thirty minutes to form a homogeneous solution.

Subsequently, the mixture was subjected to a 10-minute 4000 rpm centrifugation to filter out the residuals, and the result was filtered using Whatman paper No. 1 for a complete filtration process.

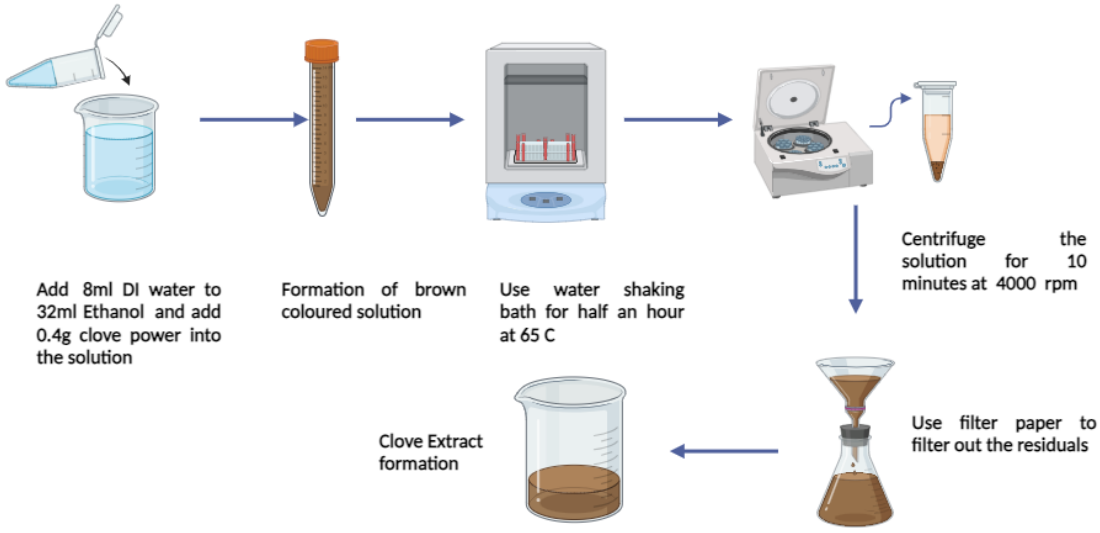


Figure 3.2: Synthesis of clove extract

3.2.2 Synthesis of Gelatin Solution

The gelatin solution was prepared by dissolving gelatin powder (2.5 g) in 60 ml of DI water. The mixture was magnetically stirred for almost half an hour until a homogenized solution was formed.

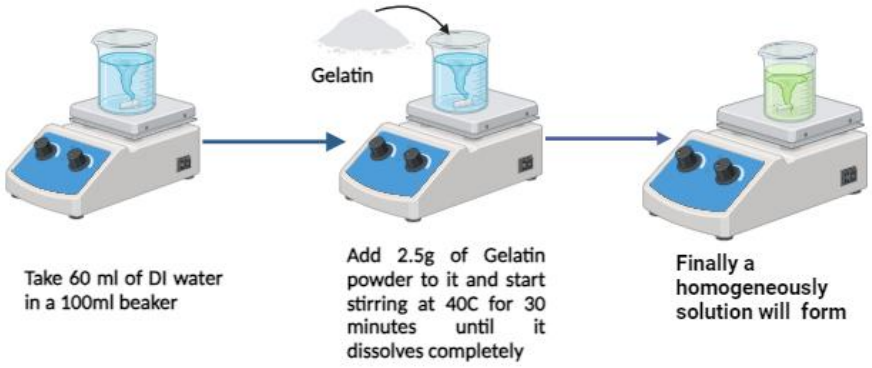


Figure 3.3: Synthesis of gelatin solution

3.2.3 Synthesis of Clove/Gelatin Composite

The clove/gel composite was formed by combining an aqueous solution of freshly prepared gelatin solution and clove extract solution (prepared in ethanol) in fixed amounts of 5%, 10%, 15%, and 20%.

For 5%, 14.25 ml of gelatin solution and 750 microliters using a microliter pipette of clove extract were added to a beaker and mixed well to form a homogeneous solution. Similarly, for 10%, 13.5 ml gel and 1500 microliter clove, for 15%, 12.5 ml gel and 2250 microliter clove, and 20%, 12 ml gel and 3000 microliter aqueous clove extract were used, respectively.

The dip coating technique was used to coat the silk-braided sutures with the prepared mixture. The mixture of the coating composite was poured into the beaker with varying concentrations of clove extract (5%, 10%, 15%, 20%), and the sutures were cut to a size of 1 cm, dipped in the solution for overnight, and placed overnight to air dry. Once dried, the sutures were ready for use in subsequent experiments.

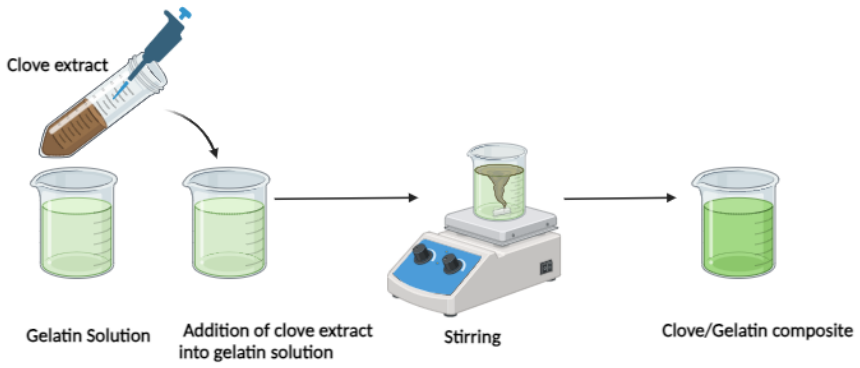


Figure 3.4: Synthesis of Clove/gelatin composite

3.3 Characterizations

3.3.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is utilized for the characterization of surface morphology of materials. In our project, we use SEM (JSM-6490A-JEOL Japan) to describe the silk-braided nonabsorbable suture with or without a coating of Clove extract. Firstly, we prepare the sample silk-braided nonabsorbable suture to characterize the scanning electron microscopy. To prepare the sample for SEM, our silk-braided nonabsorbable suture is coated with gold of 5 nm thickness by using gold sputter in a vacuum. JEOL (SEM) took images at an excitation voltage of 20 kV.

When the electron beam interacts with the material surface, it results in various signals including transmitted electrons, backscattered electrons, secondary electrons, cathodoluminescence, and characteristic X-rays.[51].

3.3.2 Fourier transforms infrared (FTIR)

Fourier transforms infrared radiation (FTIR) spectrum to identify the different functional groups. From FTIR, we have a fingerprint region through which we can calculate the functional groups attached to the materials [52].

KBr pellet was used as the carrier as KBr does not have any absorption spectrum in the Infra-Red region. KBr was pressed into a pellet using a hydraulic press and a sample

drop was added to the pellet. After getting the FTIR spectra of samples, the graph was plotted using GraphPad Prism (Version 8.0.1).

3.3.3 Mechanical properties of silk sutures

We can find the mechanical properties of the silk-braided nonabsorbable suture with the help of a pull test by the UTM (universal testing machine SHIMAD2U 20KN). We take a sample of approximately 2.5" silk sutures for the pull test. Our silk-braided nonabsorbable sutures, with or without coating, are kept in an optimum environment. We perform a pull test at room temperature. UTM (Universal Testing Machine) is used to measure the tensile strength of the silk-braided nonabsorbable sutures.

The gauge length for the pull test is 1”. In UTM, silk sutures were pulled at 10 mm/min for the tensile test until the complete fracture point occurred. In UTM, we use the 100 N load cells for the measurement of force and displacement graph. The force and displacement curves are obtained from the measurements of the pull test at UTM. The tensile test is used to measure the maximum load, stiffness, and work-to-failure.

3.3.4 Antibacterial Testing

The antimicrobial activity of the silk nonabsorbable suture coated with clove extract is measured using antibacterial tests. An inhibitory zone forms during an antibacterial test to verify the antimicrobial activity of our nonabsorbable silk suture coated with clove, indicating the effectiveness of the antimicrobial activity. Different strains are used to assess the clove-coated silk suture. First, we cultivate the strains of bacteria in a drying oven for the entire night. After the plates have been autoclaved, we spread the culture bacteria throughout the media. We dispersed the strains of bacteria and then placed the silk nonabsorbable suture, either coated or uncoated, on the freshly autoclaved bacteria plates.

The autoclaved plates are incubated overnight at 37 °C in the drying oven. We evaluate the antibacterial drug's effects on a nonabsorbable silk suture after 24 hours. The formation of an inhibitory zone against the bacterium demonstrates the silk suture covered with clove's antimicrobial properties.

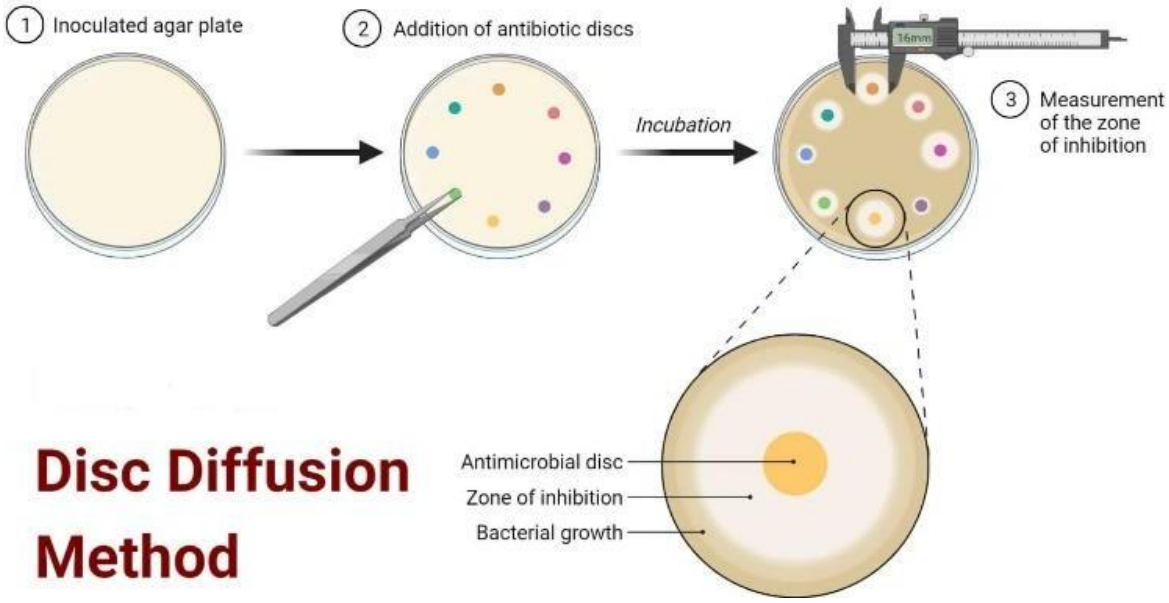


Figure 3.5: Schematic of Anti-bacterial testing

3.3.5 Clove release test

To examine the behavior of our medication release into human blood, we employed the drug release assay. First, fill a 50-ml white plastic tube with 50 ml of DI water containing a PBS tablet to prepare the phosphate-buffered saline (PBS) solution. PBS is a solution that is maintained at a regular temperature and pH level, and it closely resembles human blood plasma. We initially prepared a coating of clove extract and gelatin on a silk suture to assess the effectiveness of the clove extract and gelatin release in the body. Now, in a shaking incubator set to 37.5 °C and a continuous stirring at 100 rpm for 2, 4, 6, 24, 48, and 72 hours, immerse the coated suture sample in a PBS solution in falcon tubes or Eppendorf tubes.

We will take half of the sample solution after every required hour. To maintain the original volume of 50 ml, an equivalent amount of fresh PBS was added after each extraction. The experiment was repeated in triplicates. We may examine how the gelatin and clove have been deposited on the surgical sutures. by measuring the weight both before and after the surgical sutures are coated. We can measure the amount of coating on the surgical sutures in this manner.

3.3.7 Clove release Kinetics

Of the different ways of setup used in evaluating the drug release, information on the release of molecules from the matrix plays a very crucial role. Several kinetic models are used on the in vitro drug release assay to ascertain the mechanism of release.

3.3.8 Hixson and Crowell model

The Hixson-Crowell model identifies the releases from the systems wherein the change in surface area and changes in the diameter of the particles [53].

Hixson-Crowell obtained the equation that defines the rate of dissolution in terms of the cube root of the weight of particles and the radius of the particle is not assumed to be constant which can be expressed by the following equation,

$$Mo^{\frac{1}{3}} - Mt^{\frac{1}{3}} = Kt$$

where M0 is the initial amount of drug in the pharmaceutical dosage form, Mt is the remaining amount of drug in the pharmaceutical dosage form at a time 't' and κ is the proportionality constant.

3.3.9 Hemolytic Assay

A hemolysis assay assessed the hemolytic activity of gelatin and clove extract and their composite. Initially, the blood was centrifuged at 10,000 rpm for 10 minutes to extract the supernatant. Fresh human blood was drawn and put in an EDTA tube for the experiment.

The blood was centrifuged at 10,000 rpm for 10 minutes to extract the supernatant afterward. This specific process was carried out five times using PBS at a pH of 7.4, resulting in a pure pellet.

The subsequent step involved the addition of clove extract, gelatin, and their five different concentrations of composite (5%, 10%, 15%, and 20%) to the blood sample while simultaneously adjusting the final volume to 1 mL using PBS.

The resulting samples were subsequently placed in a shaking incubator for 4 hours, with the incubator set to a speed of 80 rpm. Following this incubation period, the samples were subjected to centrifugation for 10 minutes at a speed of 5000 rpm, and the absorbance of the supernatant collected was measured at a wavelength of 540 nm in triplicate.

To establish positive and negative controls, Triton-X-100 and blood samples in PBS were employed, respectively. The percentage of hemolytic activity was calculated using the equation mentioned below.

$$hemolysis\ (\%) = \frac{Absorption\ Sample - \ Absorption\ Negative\ Control}{Absorption\ Positive\ Control - Negative\ Control} \times 100$$

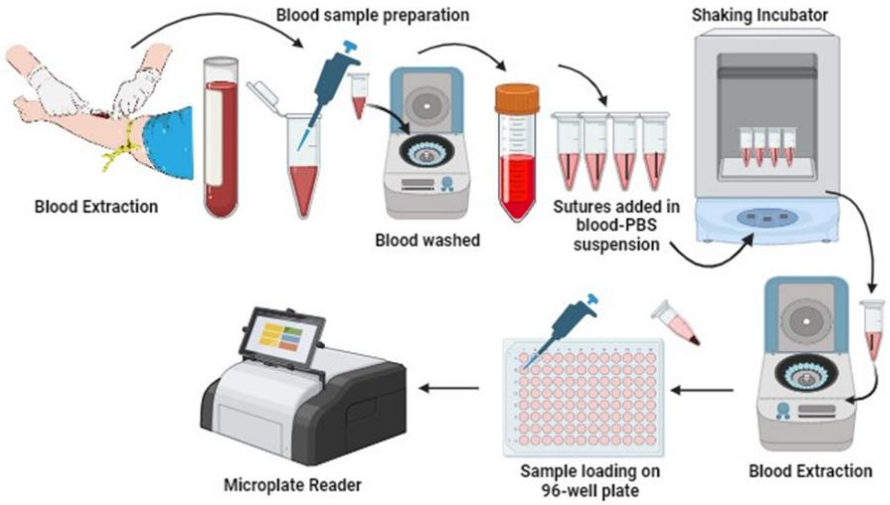


Fig 3.6: Hemolytic assay to evaluate the biocompatibility

3.3.9 Cytocompatibility of coated sutures

The cytotoxicity of the Clove/gelatin composite was determined using the human primary dermal fibroblast (HDFa) cell culture. The HDFa were cultured in liquid media containing DMEM (89 %), Fetal Bovine Serum (10 %), and Penicillin/Streptomycin (1 %) all were procured from Gibco. The composite was cultured with 5×10^4 HDFa (counted through a hemocytometer) along with a control well with HDFa only and cultivation was

performed at an ambient temperature of 37 °C in a CO_2 incubator for 48 hours. The cells were then allowed to grow on both control and composite solution for 48 h in a CO_2 incubator at the above-stated conditions. Subsequently, the culture medium was removed from each well and washed with PBS (3 times) followed by the addition of 1% WST-8 (Elabsciences) in PBS. The WST-8 added wells were then incubated for 1 h. The absorbance was taken at 450 nm using the microplate reader (Accuris, smart reader 96 – MR 9600).

$$\text{Cell viability (\%)} = \frac{Absorbance\ test}{Absorbance\ control} \times 100$$

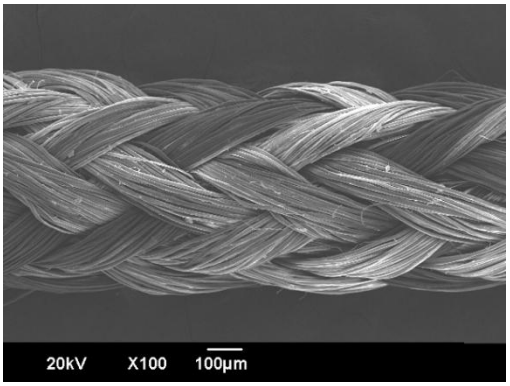
CHAPTER 4: CHARACTERIZATION

Clove/gel composite undergoes various characterizations to confirm their performance relative to individual solutions. The characterizations are provided below.

4.1 Scanning electron microscopy (SEM) analysis

Scanning electron microscopy images Fig 4.1(a) show that the uncoated silk braided nonabsorbable sutures and Fig 4.1(b) show that the coated silk braided nonabsorbable sutures are completely coated with the clove extract. The coated images of the silk braided nonabsorbable suture show that coated sutures are uniform.

For the scanning electron imaging, we prepare a sample in a vacuum that converts our clove/gelatin-coated suture into a dry sample. Therefore these little micro-cracks are present in the SEM images due to stresses experienced during drying. Our coating is uniform before drying.



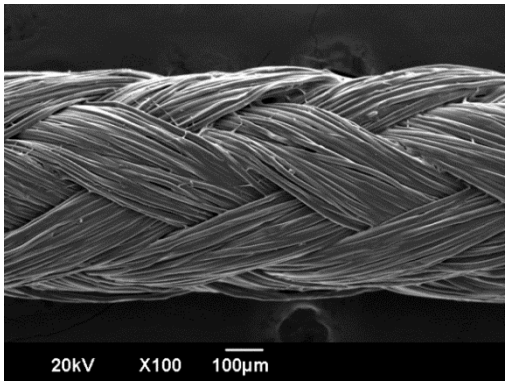


Figure 4.1(a): uncoated silk braided suture **(b):** coated silk braided suture

4.2 Fourier transforms infrared

4.2.1 FTIR of Clove extract

The Fourier transforms infrared spectrum shows the absorption range from 4000 cm^{-1} to 400 cm^{-1} in Fig 4.2. In the spectrum, the peak at 3427 cm^{-1} indicates the presence of the OH group, suggesting the presence of phenol, which confirms the existence of the

eugenol component. The peak at 2932 cm^{-1} shows the presence of the Alkyl CH stretch (sp3). The peak at 1723 cm^{-1} shows the presence of the easter group (C-O). The peak at 1617 cm^{-1} shows the presence of the aliphatic alkenes. A very sharp peak is shown at 1512 cm^{-1} showing the presence of the aromatic group. The peak at 1451 cm^{-1} shows the presence of the methylene (CH2). Similarly, peaks at the 1358 cm^{-1}, 1043 cm^{-1}, 916 cm^{-1}, and 760 cm^{-1} show the presence of methyl group (CH3), C-O methylene, and C=C respectively [54].

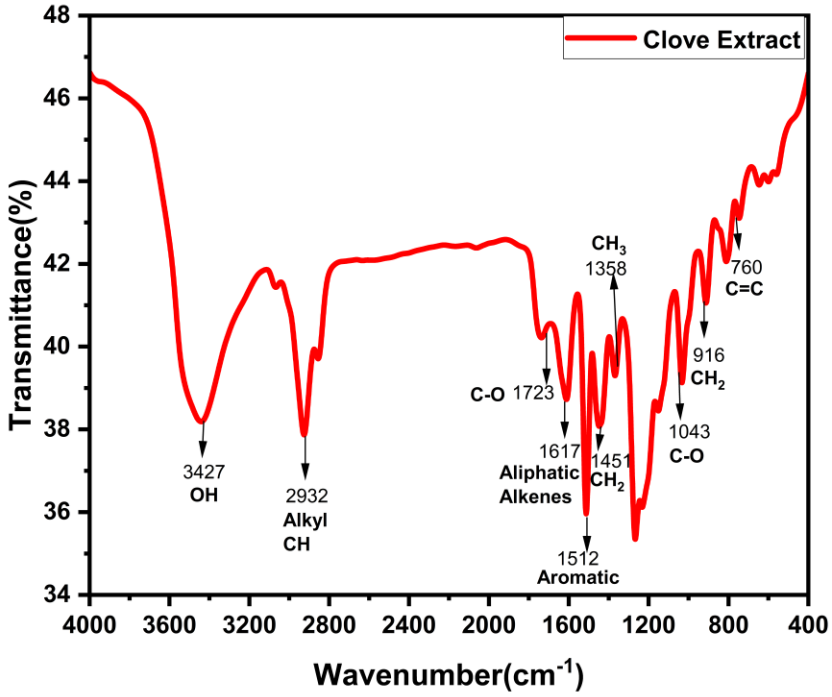


Figure 4.2: Shows the FTIR spectrum analysis of clove extract

4.2.2 FTIR of Gelatin

In Fig 4.3, the peak at 3433 cm^{-1} indicates the presence of the Hydrogen bond and Amide A group. A sharp peak at 1630 cm^{-1} shows the presence of the Amide I group. A peak is shown at 1565 cm^{-1} showing the presence of the Amide II group. The peaks at 1460 cm^{-1} and 1380 show the presence of the methyl group (CH3). The peak at 1240 cm^{-1} shows the presence of the Amide III group[55] [56, 57].

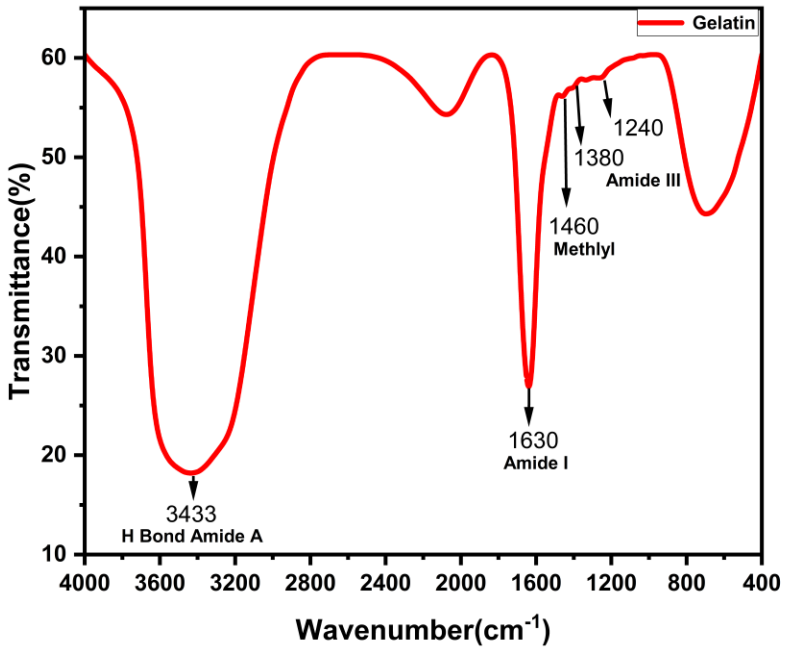


Fig 4.3: Shows the FTIR spectrum analysis of pure gelatin

4.2.3 FTIR of Clove/Gelatin composite

Due to hydrogen bonding between the polypeptide chain's N-H group, a small shift to lower wavenumbers was seen in the amide-A region when clove extract was added to the gelatin [58].

The amide-I peak primarily corresponds to the C=O stretching vibration and was identified in the range of 1630 cm^{-1} to 1650 cm^{-1}.

The presence of clove extract affected the conformation and placement of polypeptide chains in different film samples, which was the primary cause of the amide-I region spectrum variations [8].

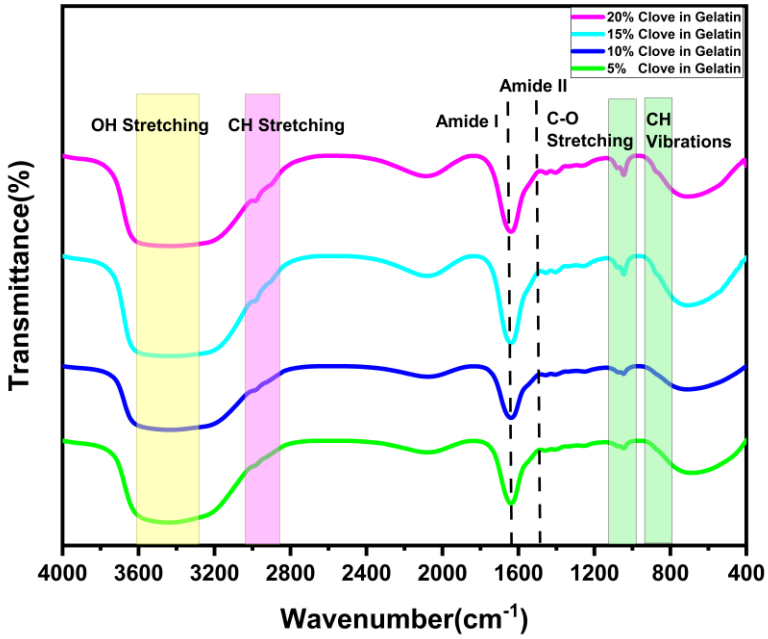


Fig 4.4: Shows the FTIR spectrum analysis of the Clove/gel composite

4.3 Mechanical properties of silk sutures

The statistical analysis of a sample found that the tensile strength of silk braided suture was slightly lower than that of coated suture, sustaining 47N. It shows that the analytical mechanical properties of clove particle-coated sutures exhibit higher average tensile strength than the uncoated silk braided sutures. This can be probably explained by the fact that fibers in the silk braided suture have enhanced binding with the aid of gelatin which implies that the combination presents a higher resistance to the axial load as illustrated in Figure 4.5.

Sutures that have a high coefficient of friction encounter the problem of tissue drag when being passed through the biological tissue and thus experience even a high value of tissue injury. Thus, one has to be acquainted with the frictional properties that are inherent to a suture material. [59, 60]. Therefore, the results of his study reveal that the coefficient of friction of the treated silk braided suture is lesser compared to untreated silk braided

suture, the treated silk braided suture's knot is not weakened as easily as compared to the untreated silk braided knot.

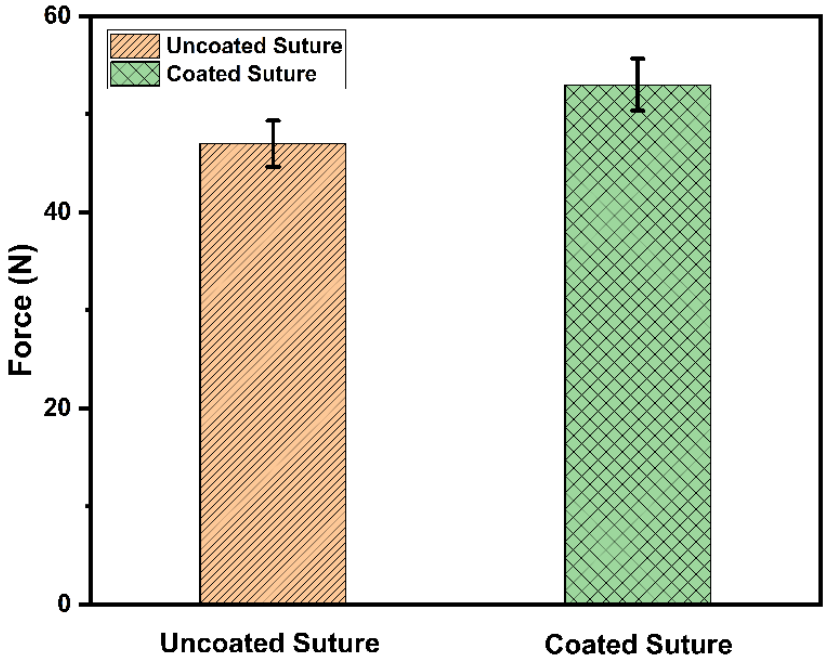


Figure 4.5: Tensile strength of Clove/gelatin composite

4.4 Antibacterial Activity

To check the antimicrobial activity, we placed our clove extract-coated silk braided nonabsorbable suture against all strains. Fig 4.5 shows an inhibition zone formed against the Klebsiella pneumoniae, Enterococcus faecalis, S. aureus, and against E. coli for at least 24 hours.

Clove/gelatin composite samples show increasing antibacterial activity with higher concentrations, and as the concentration of clove increases in the composite, a higher inhabitation zone is formed, which shows that clove has more antibacterial activity than gelatin and their composite has more enhanced results as compared to individual ones.

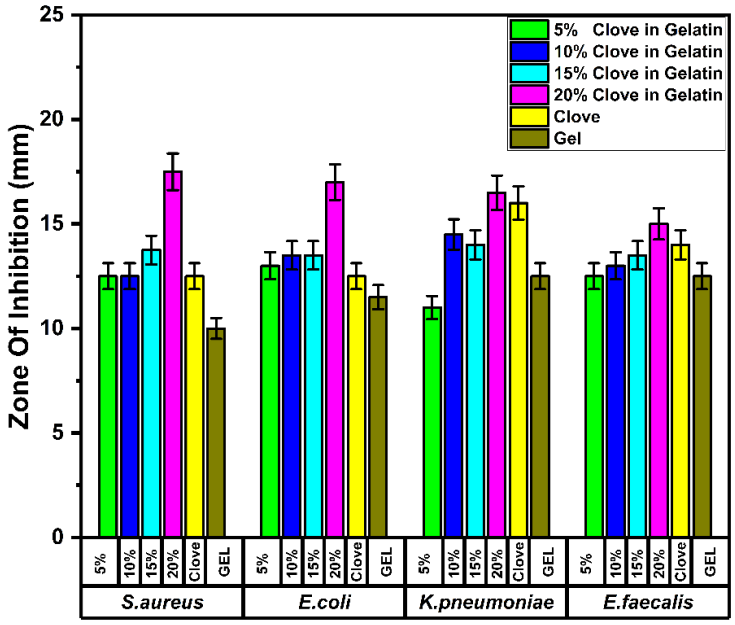


Figure 4.6: Shows Antimicrobial activity of Clove/gelatin suture

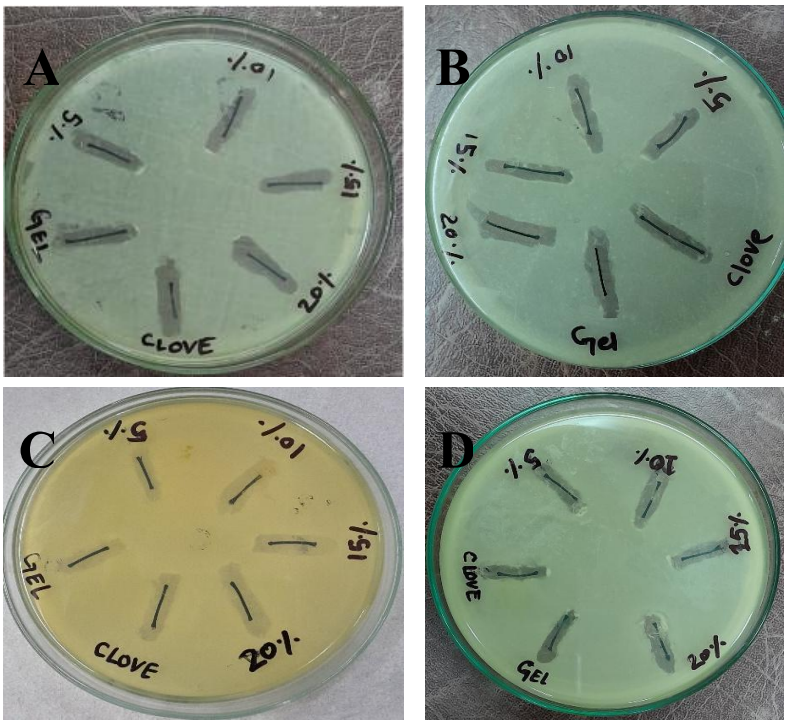


Figure 4.7: Shows Antimicrobial activity of Clove/gelatin suture in (A) S. aureus (B) E. coli (C) K. pneumoniae (D) E. faecalis

4.5 Clove Release Profile

The drug release assay of clove/gelatin composite was conducted and the results are depicted in Figure 4.6. As observed, all release profiles exhibit first-order release kinetics, which is indicative of a diffusion process [61].

Initially, there was an exponential rise in the drug release, which continued for the first 10 hours for all four concentrations (5%,10%,15%,20%). The highest drug release was observed in a 5% clove/gelatin composite with the greatest concentration of gelatin (95%). These profiles vary in that as the gelatin content rises, so does the total amount of drug released.

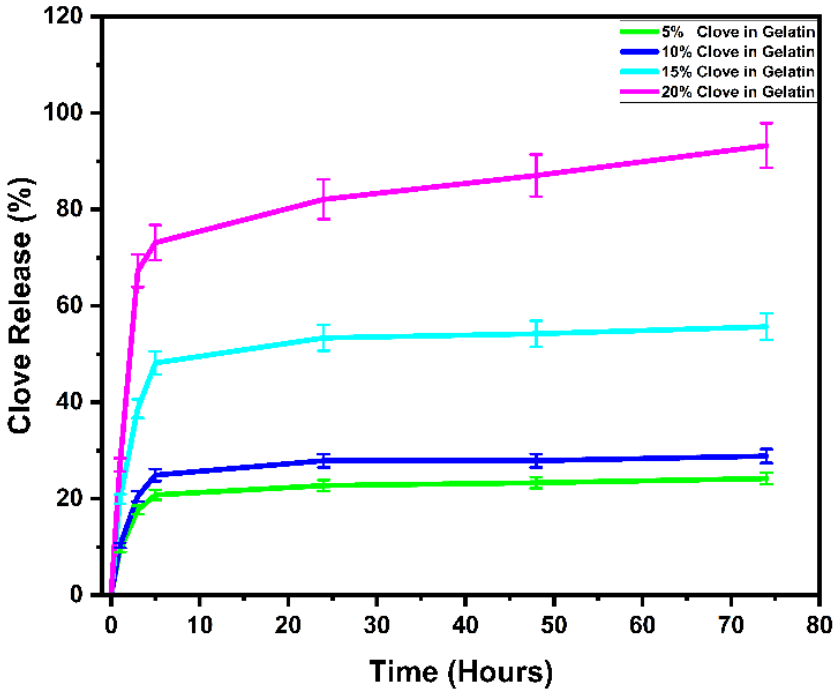


Figure 4.8: Drug release profile of different concentrations

4.5.1 Clove Release Kinetics

The Hixson-Crowell time equation model was considered best compared to all kinetic models because its R^2 value is very close to 1 as shown in Table I.

Table 4.1: The Hixson-Crowell kinetic model on drug release

Composite	Hixson-Crowell	
Sr. No (%)	R^2	K_1
Clove/gelatin composite 5 %	0.9877	-0.0219
Clove/gelatin composite 10 %	0.9877	-0.0219
Clove/gelatin composite 15 %	0.9877	-0.0219
Clove/gelatin composite 20 %	0.9877	-0.0219

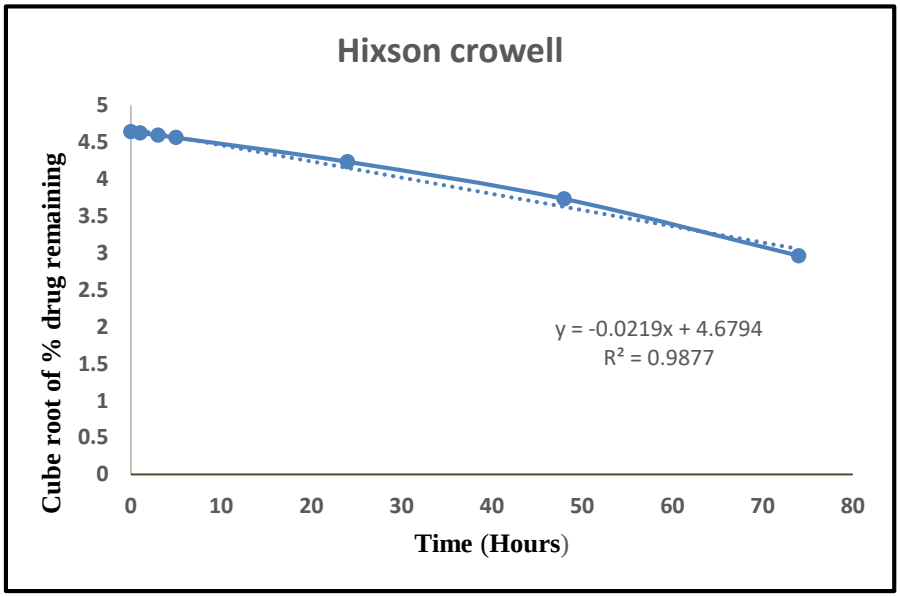


Figure 4.9: The Hixson-Crowell kinetic model on drug release

4.6 Hemolysis Activity

The hemolytic assay assessed the ability of clove extract, gelatin, and their combination to break down red blood cells (RBCs). This process causes the RBC membranes to rupture and release their contents, leading to the lysis of RBCs. The assay

used four different concentrations (5%, 10%, 15%, and 20%) of the clove/gelatin composite, as shown in Figure 4.5.

The results indicated that the hemolytic activity of the composite increased as the concentration of clove extract increased. The hemolytic activity of the clove/gelatin composite at the highest clove concentration (20%) was approximately 0.54%, compared to 0.075% hemolysis exhibited by the composite with the lowest clove extract concentration (5%). According to the ISO/TR 7406 standard, less than 5% hemolytic rates are generally classified as nonhemolytic, indicating that the clove/gelatin composite is safe for drug carriers.

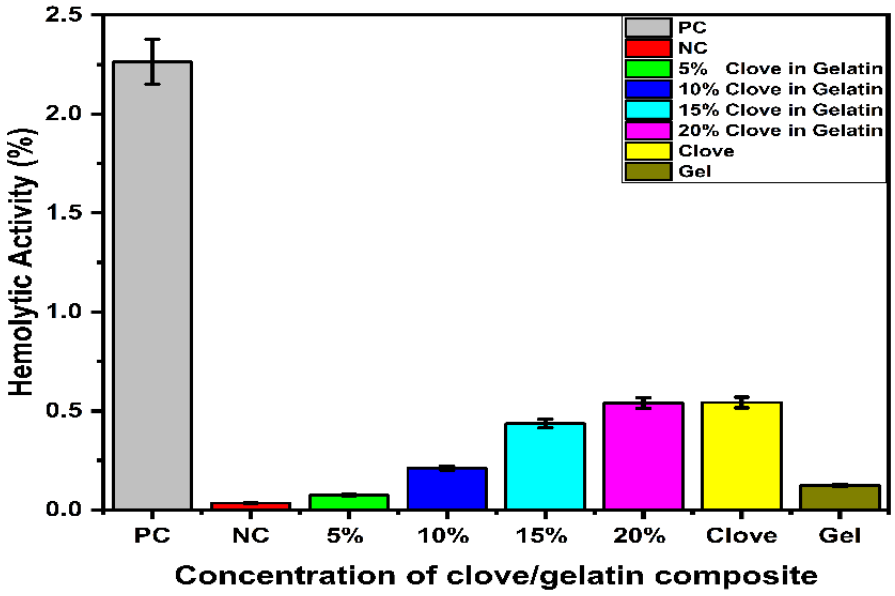


Figure 4.10: Hemolytic activity of clove/gelatin composite at different concentrations

4.7 Cytocompatibility

The percentage cellular viability of the bare sample and the composite sample was evaluated by employing the WST-8 assay. Clove/gelatin composite had a slight or negligible cytotoxicity response in the human primary dermal fibroblast (HDFa). The viability of the HDFa cells, after a post-incubation period of 48h, was 111.2, 103.7, 95.2,

and 87.7 for 5%,10%,15%, and 20% respectively as shown in Figure 10. The control group produced 100% cell viability. The 5% and 10% composite concentrations showed more than 100% cell viability because of the presence of eugenol eugenol, the main bioactive compound in clove extract has been reported to enhance wound healing through stimulation of angiogenesis, eugenol has been shown to up-regulate the expression of growth factors that are involved in the wound healing includes the vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β). The results of this investigation establish the non-toxic nature of the tested materials .

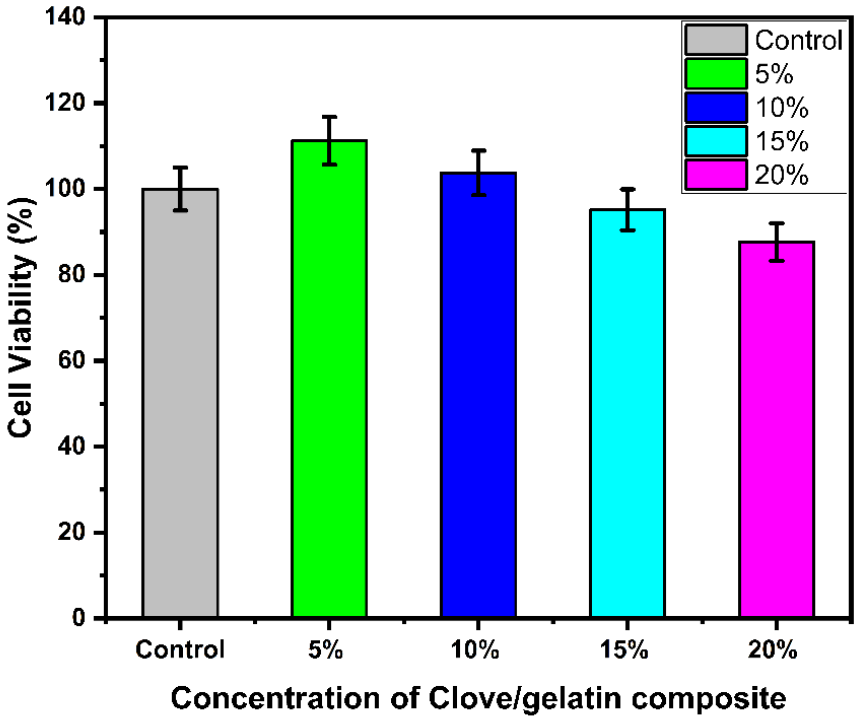


Figure 4.11: MTT assay showing cell viability of Clove/gelatin coated at various concentrations.

CHAPTER 5: CONCLUSIONS AND FUTURE RECOMMENDATION

5.1 Conclusion

Syzygium aromaticum as a traditional source has been explored for its antibacterial nature. Sutures have been successfully coated with the *syzygium aromaticum* powder via a dip coating. The outcomes of the present investigations have shown that *syzygium aromaticum* strongly adhered to the sutures and exhibited large antimicrobial activity against E. coli, S. aureus, K. pneumoniae, and E. faecalis strains with the mean zone of inhibition value 17 mm, 17.5 mm, 16.5 mm, and 15mm respectively. Furthermore, despite the release of Moringa from the coated sutures during the degradation process of the suture, the amount of *Syzygium aromaticum* measured by hemolytic activity (less than 1%), and low toxicity through MTT assays as compared to the other reported results. Cytotoxicity of human primary dermal fibroblast showed that coated sutures do not affect cell viability. In particular, sutures coated with *Syzygium aromaticum* may offer antimicrobial and antibiofilm performance that can be beneficial for successful surgeries and postoperative wound healing.

5.2 Future Recommendation

We are focusing on the non-absorbable silk-braided suture for our thesis project because it does not break down independently. We apply a gelatin-based clove coating to maximize the coating's effects and give the silk suture a special surface texture. In my view, there is still room for improvement in the coating procedure and the variety of non-absorbable sutures that can be employed in surgical procedures.

5.2.1 Surface modification of the silk suture

We can modify the surface of the silk suture by introducing the plasma (N2) treatment. We can do EDX or XPS to check the increase in nitrogen on the silk sutu re. We can also add some cross-linking polymers to activate the amides on the silk suture, which helps to increase the reactivity of the suture.

5.2.2 Surface Modifications Affect Mechanical Properties

It is possible to determine whether or not surface alterations have an impact on the mechanical characteristics of a silk suture. We perform a pull test again for that reason. The bending angle at which failure or cracks begin to spread on the silk suture's surface can also be determined by performing a T-bend test. The lap shear test is another test that we can perform to determine the strength of the connection between the clove coating and the silk suture surface.

There will be a change in the mechanical characteristics of the silk sutures when they are dehydrated. Most of the testing for our thesis work is conducted on the suture in a dry state. As a result, some cracks form on the surface of the coated suture during the EPD coating process since the suture is passed through an aqueous suspension. It is necessary to test the coated suture in various conditions to assess the change in mechanics and verify the mechanical properties on the surface of the suture.

5.2.3 Alternative coatings

Sutures used in surgery come in two varieties. An absorbable suture breaks down on its own. For internal surgery, absorbable silk sutures are typically used by surgeons. The surgeon favors absorbable sutures since we cannot perform numerous internal procedures to remove the sutures. A dual medication delivery method is also an option for absorbable silk sutures. We coat the absorbable suture; however, there's a concern about the degradation profile.

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