ANTIBACTERIAL WOUND DRESSING FABRICATION

BY

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ABSTRACT

Antibacterial wound dressing is a medical device which is used for covering wounds to prevent bacterial infection. It also helps the wound heal by protecting it from the contaminated environment. The important properties of wound dressing are biodegradable, it must not be harmful and has an allergy reaction toward human skin. The objective of this project is to produce the antibacterial wound dressing, which can prevent the contaminated bacteria infected that can occur with a patient's skin. There are 4 kinds of antibacterial agents that used in this study including povidone, amoxicillin and ciprofloxacin (as a control), honey, curcumin (turmeric extract) and anthocyanins (strawberry extract). We initially check the antibacterial agent's ability to inhibit the growth of ***S. aureus*** and ***E. coli*** by spectrophotometer and cell culture of spreading methods. Firstly, we produce the polymer of lactic acid or polylactic acid (PLA), which is the safe material for human skin. The polymerization process is performed by microwaving at 620W about 6-9 minutes. More microwaving time increased the molecular weight of synthesized polylactic acid. The results were analyzed by Fourier Transfrom Infrared Spectroscopy (FTIR). The polymerization by microwave technique serves to construct the polymer before the crystallization or annealing process. It is annealed for 4.5 hours to solidify the polymer's sample. Secondly, we intend to study the appropriate antibacterial agent that is mixed with polylactic acid by soaking method. Lastly, we investigate the appropriate and optimal concentration of antibacterial agent for our polylactic acid by mixing directly. The antibacterial property of samples were tested on nutrient agar plates and measure the

diameter of the lacking bacteria grown area around the samples by the ImageJ program. The results have shown that curcumin did not affect to inhibit the growth of ***E. coli*** but it inhibits the growth of ***S. aureus*** at the minimum concentration of 1% (w/v). Meanwhile, anthocyanin inhibits the growth of ***E. coli*** at the minimum concentration of 1%. It did not show any significant effect to inhibit ***S. aureus*** compared to PLA without antibacterial agent. Therefore, it concluded that anthocyanin has the ability to inhibit both bacteria compared to curcumin can inhibit only ***S. aureus***.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbols/Abbreviations	Terms
LA	Lactic acid
PLA	Polylactic acid
ROP	Ring Opening Polymerization
H_2O_2	Hydrogen Peroxide
$NaOH$	Sodium hydoxide
Na_2CO_3	Sodium carbonate
w/v	weight per volume
v/v	volume per volume
NA	Not available / Nutrient agar
D.W.	Distilled water
CUR	Curcumin
ANTHO	Anthocyanin
POV	Povidone
SIIE	School of International Interdisciplinary Engineering Programs

CHAPTER 1
INTRODUCTION

This chapter begins with the background and significance of the research, which introduces the theory of antibacterial wound dressing. The polylactic acid (PLA) is the main material. According to theory, it states that the antibacterial agents including curcumin, anthocyanin and honey can inhibit the growth of bacteria under the suitable concentration [1,2,3]. We tested our sample against the growth of ***E. coli*** and ***S. aureus*** by comparing the results of the inhibition area on the nutrient agar plate. We use the diameter of inhibited area and statistics to analyze their inhibition efficacy. Subsequently, the research objectives are described, followed by the hypothesis and the scope of this project.

1.1 Background and significance of the study

Wound dressings are a type of bandages that cover wounds from contaminated environments and prevent bacteria growth. It should maintain a suitable moistness and be non-adherent to the wound [4]. The material being used to make wound dressings needs to be biodegradable, non-toxic, and skin-allergen free. There are two types of wound dressing including traditional wound dressings and modern wound dressings. The traditional wound dressing is employed for initial wound healing. It is appropriate for dry, clean wounds with little discharge to prevent tissue maceration [5]. For the modern type, this dressing is designed to prevent dehydration and support wound healing [6]. This study uses a synthetic polymer of polylactic acid (PLA) [7], which includes the property of antibacterial agents given, to conduct modern wound dressings.

A polymer of lactic acid called polylactic acid (PLA) is created when lactic acid condenses. It can be used in many ways for the medical field. PLA is biodegradable, biocompatible, and bioresorbable, moreover it is a safe material for human. PLA could make sutures, staples, dressings for wounds, surgical implants, and orthopedic fixation devices. In order to make the polymer of lactic acid (PLA), heat in a microwave would be used to condense the lactic acid molecule [8,9], which would then be polymerized and solidified by the crystallization and annealing processes [10].

The best wound dressings should offer a temporary physical barrier of protection as well as the moisture required to promote re-epithelialization [11] and have the ability to prevent the bacterial growth and infection. However, it could be better to reduce the bioburden of wounds by minimizing the bacteria colonization [12]. Moreover, the antibacterial wound dressing should help speed up the healing and lower the risk of bacterial infection. The purpose of this project is to fabricate an antibacterial wound dressing from PLA included several antibacterial agents. Since povidone (iodine), amoxicillin and ciprofloxacin are a widely available commercial antibiotic, its role is the experiment's positive control [13,14,15]. The appropriate pH and concentration of antibacterial agent have been investigated.

1.2 Objectives

1.2.1 Fabricate antibacterial wound dressing from lactic acid by microwaving with soaking antibacterial agents and mixing antibacterial agents directly in PLA.

1.2.2 To investigate the appropriate antibacterial agents and optimal concentration mixed with polylactic acid.

1.2.3 To determine the factors affecting the inhibition area.

1.3 Scope of the study

1.3.1 The polymerization of PLA by microwave was used in this study.

1.3.2 Precursor of 85% lactic acid was used to make PLA.

1.3.3 ***E. coli*** and ***S. aureus*** grow on nutrient agar by spreading technique.

1.3.4 Curcumin, anthocyanin, honey, amoxicillin and ciprofloxacin were used as the antibacterial agents.

1.3.5 The imageJ program was used to measure the inhibition area.

1.4 Report outline

The rest of this report is organized as follows:

Chapter 2 reviews the theory related

Chapter 3 describes the design and implementation of this project

Chapter 4 shows the results and discussion

Chapter 5 reviews all the results and the discussion and conclude the results related to the objective

CHAPTER 2
REVIEW OF THEORY RELATED

This chapter discusses the background, analysis and design of this project. Firstly, I wished to identify the wound dressing (section 2.1). Secondly, I wished to establish the polymerization of lactic acid (section 2.2). Thirdly, I wished to clarify the properties of curcumin, anthocyanin and honey (section 2.3). Finally, section 2.4 summarizes the chapter.

2.1 Wound dressing

The definition of a wound is a rupture in the continuity of the epithelium lining of the skin or mucosa brought on by thermal or physical injury. The wound is classified as either acute or chronic depending on the length and type of healing process. Skin damage that arises suddenly as a result of an accident or surgical procedure is known as an acute wound. Depending on the size, depth, and amount of the injury to the skin's epidermis and dermis layer, it heals within an anticipated time frame of typically 8 to 12 weeks. On the other hand, chronic wounds are unable to heal according to the usual stages and cannot be fixed in a timely and ordered method. Leg ulcers, decubitus ulcers, and burns are the most common causes of chronic wounds. The wound type, any associated pathological disorders, the type of dressing material, and other factors all play a significant role in how these phases progress. Technology has advanced to the point that all types of wounds can now be dressed using a variety of materials. However, choosing the right material for a specific wound is crucial for a quicker recovery [16].

2.1.1 Characteristics of an ideal wound dressing

A suitable dressing material must be applied based on the type of wound. The ability of the dressing should: a) create or maintain a moist environment; b) facilitate epidermal migration; c) encourage angiogenesis and the synthesis of connective tissue; d) permit gas exchange between injured tissue and the environment; e) maintain appropriate tissue temperature to improve blood flow to the wound bed and

facilitate epidermal migration; f) provide protection against bacterial infection; and g) should not stick to the wound and be simple to remove once it has healed h) must act as a dehumidifier to encourage leukocyte migration and boost the buildup of enzyme i) the item must be sterile, non-toxic, and allergy-free [16].

2.1.2 Types of wound dressings

- Traditional wound dressing

Gauze, lint, plasters, bandages (natural or synthetic), and cotton wool are common dry items used as primary or secondary dressings for wounds to prevent contamination. Traditional dressings are typically recommended for clean, dry wounds with low amounts of exudate or used as backup dressings. Traditional dressings have been replaced by modern dressings with more sophisticated compositions because they cannot maintain a moist environment around the wound [5].

- Modern wound dressing

Modern wound dressings have been created to help the wound heal. These bandages are intended to prevent dehydration of the wound and encourage recovery. There are several products on the market that can be chosen depending on the cause and kind of the wound. Modern wound dressings are categorized as passive, interactive, and bioactive products and are typically made of synthetic polymers. Gauze and tulle dressings are examples of passive products that are non-occlusive and used to cover wounds in order to restore their natural functions. Interactive dressings come in the shapes of films, foam, hydrogel, and hydrocolloids, and they are either semi-occlusive or occlusive. These dressings serve as a barrier to prevent bacteria from entering the wound environment [6].

- Semi-permeable film dressings

These dressings are made of adherent, transparent polyurethane that allows oxygen and carbon dioxide from the wound to pass through while simultaneously

allowing for autolytic debridement of eschar and being bacterially impermeable. Films were initially produced using nylon derivatives with adhesive polyethylene frames acting as the support, making them occlusive. Due to their poor absorption capabilities and tendency to produce maceration of both the wound and the healthy tissues surrounding it, nylon-derived film dressings were first avoided for severely exuding wounds. These dressings, however, can conform to any shape and are incredibly elastic and flexible, so they don't need any further tapping. Because of transparent films, it is also possible to inspect the wound closure without removing the wound dressing. Therefore, epithelialising wounds, superficial wounds, and shallow wounds with modest exudates are indicated for these dressings [6].

- Semi-permeable foam dressings

Foam dressings contain both hydrophobic and hydrophilic materials. Its hydrophobic features of the outer layer prevent liquid but permit gaseous exchange and vaporized water. Rubber foam with a silicone base conforms to the shape of the wound. Depending on the thickness of the wound, foam has the capacity to absorb a range of wound drainage. There are both sticky and non-adhesive foam treatments. Lower leg ulcers and mild to severely exuding wounds can also benefit from foam dressings, which are also recommended for granulating wounds. Due to their high absorption capacity and moisture vapor permeability, they are typically employed as primary dressings for absorption, necessitating no additional dressings [6].

- Hydrogels dressing

Insoluble hydrophilic materials known as hydrogels are created using synthetic polymers like poly(methacrylates) and polyvinyl pyrrolidone. Granulation tissues and epithelium benefit from hydrogels' high water content (70–90%) in a wet environment. Hydrogels' soft elastic properties allow for simple application and damage-free removal once a wound has healed. Hydrogels, which have a soothing and cooling effect, lower the temperature of skin wounds. For dry chronic wounds, necrotic wounds, pressure ulcers, and burn wounds, hydrogels are employed.

Dressings made of hydrogel are not abrasive, don't react with living tissue, and let metabolites pass through [6].

- Hydrocolloid dressing

The most popular interactive dressings are hydrocolloid dressings, which have two layers: an inner colloidal layer and an outside, water-impermeable layer. These dressings are a mixture of elastomers, adhesives, and the three gel-forming substances carboxymethylcellulose, gelatin, and pectin. Hydrocolloids have the ability to debride wounds, absorb wound exudates, and are permeable to water vapor but impenetrable to germs. They are applied lightly to moderately exuding wounds such traumatic wounds, small burns, and pressure sores. These dressings are especially suggested for managing wound care in children since they may be removed without discomfort. These hydrocolloids create gels when they come into touch with the wound exudate, which creates a moist environment that aids in protecting the granulation tissue by absorbing and holding onto exudates [6].

- Alginate dressing

The sodium and calcium salts of mannuronic and guluronic acid units are combined to create alginate dressings. Seaweed is the source of biodegradable and absorbent alginates. Strong hydrophilic gel formation, which reduces bacterial contamination and wound exudates, allows for absorption. Alginate dressings are appropriate for wounds with moderate to high drainage but are not advised for dry wounds, wounds with third-degree burns, or severe wounds with exposed bone. Additionally, these dressings need additional dressings since they may dry the wound, delaying healing [6].

- Bioactive wound dressings

Bioactive dressings, the final kind of modern wound care products, are made from biomaterials that are crucial to the healing process. These dressings, which are often created from synthetic materials including collagen, hyaluronic acid, chitosan, alginate, and elastin, are recognized for their biocompatibility, biodegradability, and

non-toxic properties. Depending on the kind of wound, one or more of these materials' polymers may be employed. To speed up the healing of wounds, growth factors and antimicrobials are occasionally added to biological dressings [6].

- Tissue engineered skin substitutes

Two forms of tissue-engineered replacements for human skin, or dermal equivalent (HSE), are available; one imitates the layer of skin made up of keratinocytes and fibroblasts on a collagen matrix (Cell containing matrix). Only the dermal components with fibroblasts on the collagen matrix are present in the second (Acellular matrix). HSE's main mechanism is to release and promote wound growth factor, which results in epithelialization. Bioengineered organisms have the ability to adjust to their surroundings and release the growth factors and cytokines used in dressings [6].

- Medicated dressings

By removing necrotic tissues, medicated dressings with built-in medications contribute significantly to the healing process. This has been accomplished by disinfecting or debriding agents for necrotic tissue, antimicrobials that stimulate tissue regeneration and prevent infection [6].

- Composite dressing

Each layer of a composite or combination dressing is biologically unique and contains numerous layers. The majority of composite dressings include three layers. Composite dressings may also include a transparent film or non-woven fabric tape border that adheres to the skin. They may be used in conjunction with topical drugs and can serve as a main or secondary dressing for a variety of wounds. The topmost layer serves to shield the wound from infection, the intermediate layer is often made up of absorbent material to help keep the environment wet and aid with autolytic debridement, and the bottom layer is made up of non-adherent material to avoid attaching to newly granulation tissues. Composite dressings are more expensive and less flexible [4,7,16].

2.2 Polymerization of lactic acid

In tissue engineering, polylactic acid (PLA) is the main biodegradable and bioresorbable polymer employed. It is made from lactic acid, an organic acid that occurs naturally and can be created through fermentation. Lactic acid's affordable pricing and widespread commercial availability are critical factors in the development of PLA. Because of their outstanding biocompatibility and biodegradability, PLA and its copolymers are used in the biomedical field as implants or devices. Stereoisomers of PLA include poly(L-lactide), poly(D-lactide), and poly(DL-lactide) (PDLLA). While PDLLA is atactic and optically inactive, PLLA and PDLA are isotactic and optically active. However, the high crystallinity of PLLA's fragments and its prolonged breakdown durations can result in inflammatory responses in the body. This can be avoided by using PLLA, a polymer made of a combination of D, L-lactic acid and L-lactic acid monomers, the latter of which degrades quickly without producing crystalline pieces [17].

PLA can be gained by a variety of methods. In general, three methods can be used to create high molecular mass PLA. Direct condensation polymerization, ring-opening polymerization and using heating in the microwave are three examples of polymerization (as shown in Figure 1) [17].

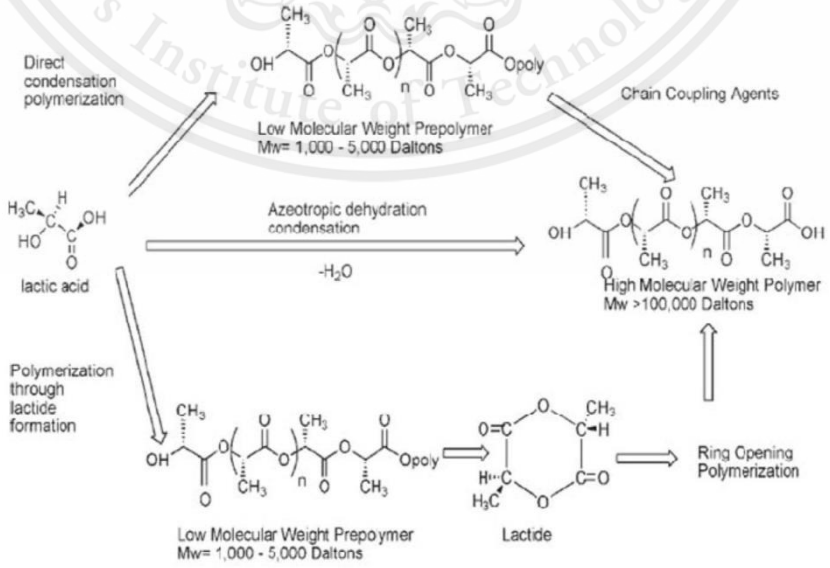


Figure 1. Polylactic acid (PLA) synthesis methods [17].

2.2.1 Direct condensation polymerization

The direct condensation theory proposes an alternate approach of synthesizing PLA in which lactic acid monomers directly polycondensate rather of going through an intermediary stage of lactic acid generation. This approach tries to streamline the entire production process, potentially lowering costs and lactic acid fermentation's negative effects on the environment.

In order to synthesize the higher molecular weight poly(lactic acids) by direct condensation polymerization of lactic acid, there were 6 steps to obtain. We initially selected the appropriate lactic acid monomers. In the second part, using a suitable catalyst such as dipentaerythritol was used as a chain branching agent and an antimony trioxide catalyst. Thirdly, the direct condensation reaction conditions should be established. The temperature, pressure, and response time are all factors to consider. Depending on the catalyst and monomer system being utilized, the exact requirements could change. Furtheremore, the purification is needed due to any contaminants that can interfere with the polymerization process. Techniques like distillation or recrystallization can be used for this purify process. Then, setting up the reaction vessel and adding the catalyst and purified lactic acid monomers to begin the reaction. The reaction container needs the proper stirring and temperature control equipment. Lastly, starting the direct condensation process by raising the temperature of the reaction mixture and holding it there for the allotted amount of time. The catalyst makes it easier for the lactic acid monomers to polycondensate, which produces PLA chains. This step was the main factor that polymerize lactic acid to PLA [17].

2.2.2 Ring-opening polymerization (ROP)

A commonly used method for creating PLA, which has uses in a variety of sectors including packaging, textiles, and biomedical applications, is polylactic acid ring-opening polymerization.

The lactide's ring-opening polymerization (ROP), which requires a catalyst but produces PLA with precise molecular weight. Depending on the monomer employed

and the circumstances governing the reaction. It is possible to control the ratio and sequence of D- and L-lactic acid units in the final polymer.

Lactic acid polymerization is started by dehydrating the monomer, which results in prepolymer chains made of oligomers and PLA with a low molecular weight. Starting with lactic acid, this procedure includes three separate steps: lactide production, polycondensation, and ring-opening polymerization.

The initiation process must be applied before ROP. This process was using common initiators include metal-based catalysts such as stannous octoate. It was utilized as a catalyst in the polymerization process, which used lactic acid (LA reagent grade) with an 85% purity level. Using the reaction setup depicted in Figure 2, lactic acid was first dehydrated to generate oligomers. The lactide's carbonyl group and the initiator work together to coordinate, which helps the ring-opening process.

The ester bond inside the lactide ring is severed after the lactide monomers have begun to undergo ring opening. A nucleophilic assault by an active species, such as the initiator or the developing polymer chain end, might cause this cleavage. An intermediate formed as a result of the nucleophilic assault is an alkoxide or carboxylate.

Through nucleophilic attack, the alkoxide or carboxylate intermediate combines with a different lactide monomer to create a new ester bond and lengthen the polymer chain. The expansion of the PLA polymer chain is caused by the repetition of this process of monomer addition and ester bond formation.

Until all of the lactide monomers are used up or until a termination event has place, the lactide ROP can go on. Different processes, such as coupling reactions, disproportionation, or chain transfer events, might result in termination. The termination step, as well as the choice of reaction conditions and the presence of any chain transfer agents, might affect the PLA's molecular weight and polydispersity [17].

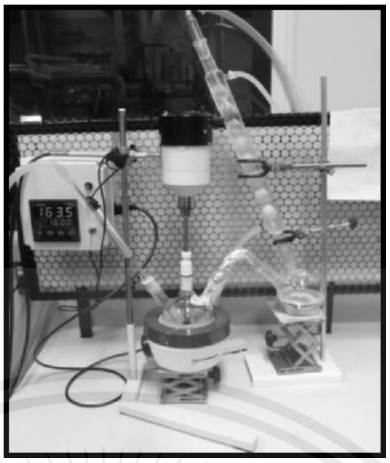


Figure 2. Polymerization experiment system [17].

2.2.3 Using heating in microwave

One of the fields that is expanding the most quickly in organic synthesis is microwave activation. First off, the length of chemical reactions has been drastically shortened (by tens, hundreds, and thousands of times). The objective of this research is to create novel, efficient ways to generate low molecular weight PLA, with a particular emphasis on its usage in medicine. (as shown in Figure 3).

Microwaves are electromagnetic waves that interact with polar molecules or ions to produce heat. When microwave radiation is applied to a material having polar groups, the molecules attempt to align with the quickly fluctuating electric field, which causes the molecules to rotate and subsequently produce heat.

Microwave-assisted polymerization can enhance reaction control and selectivity. Rapid and targeted heating can decrease undesirable byproducts, improve stereochemistry control, and lessen adverse effects. It is possible to customize the polymerization process and create polymers with the required characteristics by carefully adjusting the microwave power, irradiation period, and reaction conditions.

The polymerization can occasionally be done with decreased or no solvent systems. Microwaves may effectively heat reactions so that they can continue without requiring huge amounts of solvent, which can simplify purification procedures and have a less negative environmental impact.

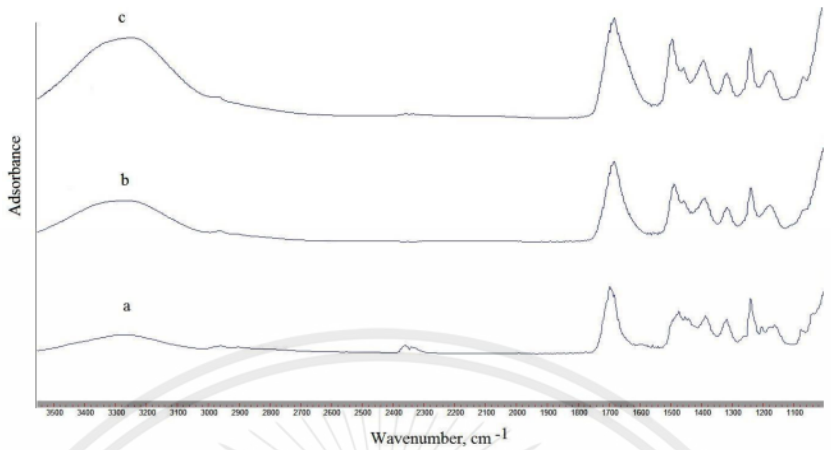


Figure 3. IR spectra of tetranol 9a0 and synthesized tetraol derivatives: compound I (b) and compound II (c) [18].

Microwave heating has been discovered to remove water from materials and speed up polymerization processes by a factor of ten to one hundred compared to traditional heating. In other words, microwave heating is recognized to be rapid volumetric, and selective compared to traditional heating. As a result, this method seems to be particularly intriguing for the processing of polymers and chemistry. At the lab or industrial sizes, several initiatives have been implemented with success. Microwave heating has already demonstrated its advantages in terms of time and energy savings, raising the pace and yield of chemical processes, reducing side reactions, and, in certain situations, producing goods with high purity and superior qualities [18].

2.3 Antibacterial agents

A category of substances known as antibacterial agents work to prevent pathogenic bacteria. Thus, the harmful effect of bacteria in biological settings will be reduced by killing them or decreasing their metabolic activity.

2.3.1 Curcumin (turmeric)

Popular Indian spice curcumin has been used for generations to cure a number of illnesses, including rheumatism, diabetic ulcers, anorexia, cough, and sinusitis. The primary curcuminoid in turmeric, diferuloylmethane, gives it its characteristic yellow

hue. Significant anti-inflammatory, antioxidant, anti-mutagenic, anti-carcinogenic, anti-clotting, and anti-infective properties of curcumin have been demonstrated. Additionally, it has been demonstrated that curcumin possesses potent wound-healing abilities. It affects several phases of the body's natural wound-healing cycle to speed up healing. This review reviews and summarizes recent research publications on curcumin's impact on skin wound healing. The reviews' featured research show that curcumin can lessen the body's normal reactions to cutaneous wounds, including oxidation and inflammation. Curcumin's capacity to promote granulation tissue development, collagen deposition, tissue remodeling, and wound contraction is further supported by current literature on the compound's wound healing characteristics [19]. The phenolic compound called flavonoid polyphenol, weas shown to be the growth inhibitors in some related studies [20].

- Wound healing activities of curcumin

The biochemical properties of curcumin, such as its anti-inflammatory, anti-infectious, and antioxidant activities, are thought to be responsible for its ability to heal wounds. Through its role in tissue remodeling, granulation, tissue creation, and collagen deposition, curcumin has also been demonstrated to improve the healing of cutaneous wounds. Numerous studies have demonstrated that applying curcumin to a wound also promotes epithelial regeneration, boosts fibroblast proliferation, and improves vascular density [19].

- Curcumin reduces oxidation: a major cause of inflammation

Reactive oxygen species (ROS), which are by-products of aerobic respiration that are unavoidable, play a crucial role in a number of cellular and biochemical functions, including intracellular communication, differentiation, cell growth, apoptosis, and immunology. ROS have a role in wound healing as well since the immune system needs them to defend against microorganisms. However, oxidative stress is produced when ROS are present for an extended period of time at high quantities and can seriously harm human cells. The process of healing a wound is

significantly influenced by oxidative stress, which normally prevents tissue remodeling [19].

Free radicals must be properly foraged since they also attack and harm tissue proteins. Superoxide dismutase, glutathione peroxidase, catalase, and other antioxidant enzymes guard human cells from harmful reactive oxygen species. Antioxidants having the ability to scavenge free radicals have been demonstrated to dramatically enhance wound healing when given topically. Superoxide radicals are known to be non-enzymatically reduced by curcumin, which lowers oxidative stress [19,20].

- Effects of curcumin on the proliferative phase of wound healing

The production of granulation tissue, collagen deposition (the creation of the extracellular protein matrix), fibroblast proliferation, epithelialization, and the death of cancerous cells are all part of the proliferative phase of wound healing. Numerous research have examined the impact of curcumin on these procedures while comparing the time it takes for wounds to heal in curcumin-treated animals to controls [19].

- Effects of curcumin on apoptosis

When a wound is healing, a variety of apoptotic processes take place to remove undesirable inflammatory cells from the wound site in order for the wound to move onto subsequent stages. As a result, the wound might develop and go on to the proliferative stage. Although the precise method of action is unknown and depends on the kind of cell, it has been proposed that curcumin's capacity to produce ROS is what allows it to be able to trigger apoptosis. As dead cells were found as early as four days after treatment with the COP bandage in a rat wound model, DNA fragmentation experiments have shown that curcumin is apoptotic in the early stages of wound healing [19,21].

Curcumin was able to speed up the healing cycle into the proliferative phase with little prolonging of the inflammatory phase as compared to control treatments, where low apoptosis rates were found in the early stages of wound healing. Eleven

days after wounding, researchers found that the control group had a higher degree of apoptosis, but wounds treated with curcumin had none at this time. This demonstrates that untreated wounds in the control group are still in the early stages of wound healing, whereas lesions that had been treated with curcumin have advanced into the proliferation phase [19,21].

- Effects of curcumin on re-epithelialization and remodeling

The epidermis, the top layer of skin, acts as an essential barrier between an organism and its surroundings, shielding the host from microbiological, chemical, and physical harm. Keratinocytes move and divide during the process of epithelialization from the lower skin layers. Re-epithelization, which is the last stage of wound healing (together with remodeling), has to be a strong procedure to restore the epidermis's proper barrier function [1, 22].

Curcumin has powerful modifying effects on how quickly wounds heal. According to studies, curcumin accomplishes this through influencing the inflammatory, proliferative, and remodeling stages of the healing process, which shortens the time needed for wound healing. Curcumin's poor solubility, quick metabolism, reduced bioavailability, and light sensitivity are unfortunate drawbacks [1,21,22].

2.3.2 Anthocyanins

By dissolving the cell wall of germs that cause food poisoning, anthocyanins can function as an antibacterial. Flavonoid polyphenolic pigments known as anthocyanins are frequently found in the stems and leaves of flowers, fruits, seeds, and other plant life [23]. Plants naturally contain anthocyanins in the form of glycosides that also contain glucose, galactose, arabinose, rhamnose, and xylose. Multiple phenolic hydroxyl groups included in the structure of anthocyanins shield plants against oxidizable substances [24,25]. Due to its distinctive functionality, it is utilized to fight free radicals, prevent diabetes, reduce weight, safeguard vision, and more. It also has anti-tumor, anti-cancer, and anti-inflammatory properties. Anthocyanins can operate as an antioxidant to get rid of reactive oxygen species

(ROS) since they have the capacity to absorb oxygen free radicals in vitro and sequester oxygen and hydroxyl radicals.

Anthocyanins can cause the activation of the Nrf2 signaling pathway by stimulating antioxidant enzymes like NAD(P)H oxidase. This property raises the possibility that anthocyanins may protect cardiovascular and neurological disorders. Additionally, research has demonstrated that anthocyanins have anti-inflammatory properties. They encourage the activation of the MAPK pathway while inhibiting the NF-B pathway. By preventing cells from progressing through the S and G2 stages of the cell cycle, anthocyanins also have a therapeutic impact on cancer. In this manner, they also prevent the growth of tumor cell [25].

According to reports [26], wheat and wheat-grass have antibacterial action against a variety of common human diseases. Using well diffusion experiments, researchers discovered that ethyl acetate extracts from several wheat cultivars were efficient against the pathogens ***Escherichia coli (E. coli), Salmonella typhimurium (S. typhimurium)***, and ***Staphylococcus aureus (S. aureus)***. The potential of wheat germ as a natural antibacterial and food preserving agent was discovered when researchers examined its antimicrobial action against ***S. aureus***, ***E. coli, S. typhimurium***, and ***Bacillus cereus.*** According to other reports, wheat-grass extracts have antibacterial action against a variety of bacteria, including several foodborne pathogens like ***Yersinia enterocolitica*** and ***Listeria monocytogenes***. Additionally, the scientists observed that wheat-grass juice had antibacterial properties against the diseases ***Candida albicans***, ***E. coli***, ***Pseudomonas aeruginosa***, and ***S. aureus***.

Commonly grown wheat (***T. aestivum***) is amber in color and contains much less anthocyanins, however colorful wheat cultivars of ***T. aestivum*** (blue, black, purple, and red) are abundant in anthocyanins and other phytochemicals and are currently gaining popularity worldwide. Anthocyanins, bioactive substances found in colored wheat, have several health advantages, including the prevention and treatment of a wide range of chronic illnesses, including cancer, cardiovascular disease, diabetes, inflammation, obesity, aging, liver dysfunction, and hypertension. Additionally, it has been shown that anthocyanins have potent antibacterial action

against a variety of Gram-positive and Gram-negative human infections. Anthocyanin-rich American cranberries were shown by researchers to inhibit the development of ***E. coli*** after receiving anthocyanin extract therapy. pomace from sour cherries, red cabbage, and ***Lonicera caerulea L. Haskap*** berries are an excellent source of anthocyanins, and their extracts are employed as organic antibacterial agents to stop foodborne outbreaks connected to ***E. coli***, ***S. aureus***, and ***L. monocytes***, ***S. typhimurium*** and ***B. cereus***.

- Antimicrobial activity of colored wheat anthocyanins against microbial strains using Agar-Overlay method

The findings showed that, in comparison to white wheat, anthocyanin extracts from colored wheat flour and wheat-grass juice had higher antibacterial action against human infections. The extracts' antibacterial activity was dose-dependent.

From the paper of Evaluation of Anthocyanin Content, Antioxidant Potential and Antimicrobial Activity of Black, Purple and Blue Colored Wheat Flour and Wheat-Grass Juice Against Common Human Pathogens (Natasha Sharma, Vandita Tiwari, Shreya Vats, Anita Kumari, Venkatesh Chunduri, Satveer Kaur, Payal Kapoor and Monika Garg), anthocyanin extracts from black wheat flour (50–200 mg/mL) shown antibacterial efficacy against all pathogens. Black wheat was more antimicrobial than purple wheat, blue wheat, or white wheat among extracts of anthocyanins from wheat flour. When used against ***S. aureus***, ***E. coli***, ***P. aeruginosa***, and ***C. albicans***, all extracts created obvious halo zones (as shown in Figure 4) [26].

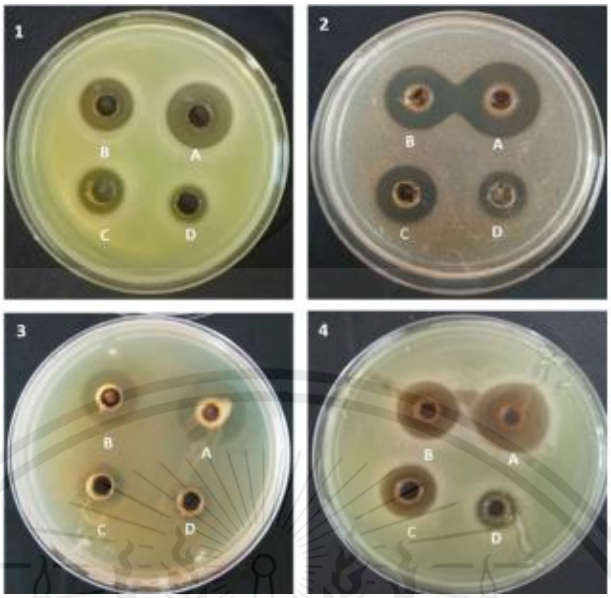


Figure 4. Antagonistic activity of anthocyanin extracts of black wheat flour against 4 bacteria [26].

Compared to all other flour extracts, black wheat flour extracts (200 mg/mL) demonstrated much greater zones of inhibition against ***Candida albicans*** (2.57 cm) and ***Staphylococcus aureus*** (2.50 cm). In comparison to other flour extracts, black and purple wheat flour extracts (200 mg/mL) demonstrated the largest but statistically equal zones of inhibition against ***P. aeruginosa*** (2.73 and 2.60 cm) and ***E. coli*** (2.50 and 2.37 cm).

Blue wheat flour did not exhibit any antimicrobial action against ***S. aureus*** and ***C. albicans*** at this dose. Even at low extract concentrations (50 mg/mL), black and purple wheat flour extracts were active against all pathogens, but white flour extracts were inactive against ***Candida albicans***.

In comparison to the other extracts, black wheat-grass juice extracts (200 mg/mL) considerably increased the zones of inhibition against ***P. aeruginosa*** (2.60 cm) and ***C. albicans*** (2.37 cm). ***S. aureus*** (2.23 and 2.20 cm) and ***E. coli*** demonstrated noticeably greater zones of inhibition when exposed to black and purple wheat-grass juice extracts (200 mg/mL) (1.93 and 2.0 cm). Even at a dosage of 50 mg/mL, black and purple wheat-grass juice extracts showed promising efficacy against all four pathogens, but blue wheat flour exhibited no activity at all against ***S.***

aureus and ***C. albicans***. Additionally, white wheat-grass juice extracts failed to exhibit any anti-***C. albicans*** action at this dosage.

- Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) of colored wheat anthocyanin extracts against human pathogens

In comparison to white wheat extracts, the results showed colored wheat anthocyanin extracts to have a strong and promising antibacterial activity against human infections. The antibacterial activity of all the extracts was dosage dependent, peaking at 200 mg/mL extract concentration. Black wheat flour extracts demonstrated the most antibacterial activity, followed by purple flour extracts (as shown in Table 1) [26].

Table 1. Minimum inhibitory concentration of colored wheat anthocyanin [26].

Wheat Sample	Inhibition	*S. aureus*		*P. aeruginosa*		*E. coli*		*C. albicans*	
		WF [1]	WG [2]	WF [1]	WG [2]	WF [1]	WG [2]	WF [1]	WG [2]
Black	MIC *	50	100	50	150	100	100	100	150
	mmC **	100	200	150	200	200	-	200	-
Purple	MIC *	50	150	50	150	100	150	150	150
	mmC **	150	200	150	-	-	-	-	-
Blue	MIC *	150	200	100	150	150	200	-	-
	mmC **	200	-	200	-	-	-	-	-
White	MIC *	100	150	100	150	-	200	200	-
	mmC **	200	-	150		-	-	-	-

* MIC—minimum inhibitory concentration; ** mmC—minimum microbicidal concentration; 1—WF (wheat flour); 2—WG (wheat-grass juice).

Purple wheat flour demonstrated MIC against all pathogens (50-150 mg/mL) and demonstrated mmC of 150 mg/mL against ***P. aeruginosa*** and ***S. aureus***. However, blue wheat flour extracts had the least antibacterial efficacy among the colored wheat flour extracts. It displayed 200 mg/mL of mmC against ***P. aeruginosa***, ***S. aureus***, and ***E. coli***, with MICs of 100 mg/mL for ***P. aeruginosa*** and 150 mg/mL for ***S. aureus*** and ***E. coli***. It did not exhibit any MIC toward ***C. albicans***. contrasted with colorful wheat flours Extracts from white wheat flour did not exhibit any mmC against ***E. coli*** and ***C. albicans***. The MIC against ***S. aureus***, ***P. aeruginosa***, and ***E. coli*** was 100 mg/mL. and 200 mg/mL against ***E. coli***. ***C. albicans*** and shown no antibacterial action at concentrations lower than 100 mg/mL of extract.

Blue wheat-grass juice extracts had the least antibacterial activity of all the colored wheat extracts. The blue wheat-grass juice extracts had MICs of 150 mg/mL for ***P. aeruginosa*** and 200 mg/mL for ***S. aureus*** and ***E. coli*** but no mmC against any of the pathogens. It did not exhibit any MIC against ***C. albicans*** and ***E. coli***. Similar to white wheat-grass juice extracts, which exhibited MICs of 150 mg/mL against ***S. aureus*** and ***P. aeruginosa*** and 200 mg/mL against ***E. coli***, white wheat-grass juice extracts did not demonstrate mmC against any of the pathogens. All pathogens' development was fully suppressed by antibiotics at a dosage of (10 g/mL). In comparison to white wheat, the study finds that anthocyanin-rich colored wheat flour and wheat-grass juice have great antioxidant capacities against free radicals and excellent antibacterial potential against a variety of human infections. In conclusion, the black wheat have shown to be one of the effective antibacterial agents [26].

2.3.3 Honey

The only concentrated sweetener that can be found in nature is honey, which is an example of a naturally occurring substance. Even before it was known what causes infections, it has been used for many years as a form of therapy in several nations. It has a reputation for being extremely successful in practically all infection situations and for accelerating healing, particularly in burn injuries and wounds. As a result, several research have examined the chemical and physical characteristics of honey to see how they may contribute to its potential efficacy against certain pathogens.

It is obvious that there are many different types of honey around the world, and as different geographical areas will have different flora, this can influence the production and activity of various types of honey. Honey may also be divided into two primary categories: floral honey (also known as blossom honey), which is created from the nectar of blossoms, and honeydew honey, which is made from the secretions of live plants or the excretions of insects that feed on plants [3].

- Honey composition

The sugar solution in honey is highly saturated. It comprises at least 181 distinct chemicals, and its makeup is complicated and varied. The major

components, such as glucose and fructose, and the minor compounds, which include amino acids, enzymes, vitamins, minerals, and polyphenols, may be broadly separated into two classes. While regional variations (floral sources) account for some variations in honey composition, seasonal variations can also play a significant role. The nectar, volatile essential oils, pollen, propolis, and other elements that bees gather to make honey will all have an impact on the makeup of the final product [3].

The antibacterial activity of honey can be increased by the primary components, fructose and glucose, which are always the principal sugars present, are often unaffected by these fluctuations in the components of honey. The color of honey is a reflection of the numerous ingredients that are present, including polyphenols, minerals, and pollen, with black honey containing more pigments like flavonoids. Honey comes in a variety of hues, from light yellow to amber, dark reddish amber, and almost black. Estevinho et al.'s findings demonstrate that black honey contains a high concentration of phenolic chemicals, which has been well correlated with its stronger antibacterial action [3].

- Methods of Measurements of Antibacterial Activity

  Other techniques have been employed to evaluate the antibacterialeffectiveness of honey. Numerous techniques, including the broth (micro) dilution test, the well/disk diffusion assay, the agar dilution methods, and the time-kill experiment, can be used to quantitatively evaluate the sensitivity of bacteria to honey. In following the recommendations of the CLSI (Clinical & Laboratory Standards Institute), these procedures are often employed in microbiological labs. The agar diffusion test approach, for instance, involves cutting a well into a nutrient agar plate that has already been infected with a microbial culture, then applying a tiny amount of honey or a solution of honey to the middle of the well (approximately 6 mm in diameter). The honey diffuses out into the agar from the place of application while the plate is incubation [3].

Table 2. Honey inhine number and its relationship with honey concentration [3].

Inhibine number	Bacterial growth	Honey concentration	
		(% w/w)	(% v/v)
5	No growth	6.10	5
4	No growth	11.9	10
3	No growth	17.4	15
2	No growth	22.7	20
1	No growth	27.8	25

The zone of inhibition (ZOI), a clear zone surrounding the honey application site, is a gauge of the honey's effectiveness. The effective antibacterial concentration in this experiment, however, may be lower than the concentration given to the agar since honey is diluted during diffusion. In other techniques, the nutrient agar or the nutrient broth in which the bacterial culture is cultivated include honey. The broth micro- or macro-dilution experiment is the most used bacterial susceptibility test [3].

In order to perform the approach, two-fold dilutions of honey are prepared in broth and then dispensed onto tubes (for the macro-dilution version) or 96-well microtiter plates (for the micro-dilution version). The standardized test microorganisms are introduced into each tube or well before being incubated. A spectrophotometric evaluation of the bacterial growth (change in turbidity) is performed. It is feasible to establish the minimum inhibitory concentration (MIC) for each variety of honey under study by utilizing a series of various concentrations of honey in the broth or agar.

The lowest concentration of an antibacterial agent that would prevent the apparent development of microorganisms following an overnight incubation is known as the MIC and is used to assess an antibacterial substance's in vitro activity. Fluorimetry and spectrophotometric methods for measuring absorbance have higher sensitivity, particularly when employed with low honey quantities. The broth micro-dilution test, which measures bacterial growth inhibition spectrophotometrically, is the most suitable technique due to its sensitivity. In addition to the traditional plate count, this approach is typically employed to determine the MIC and MBC values.

Additional approaches, such as direct microscopic counts or the evaluation of a particular metabolite, such as lactic acid, can measure a growth indicator. In general, it is essential to recognize that the findings will be greatly influenced by the methodology and scientific judgment; this needs to be taken into account when comparing results obtained using various methodologies [3].

- Features of Honey Relevant to Its Antimicrobial Activity

Numerous elements have been proven to support honey's antibacterial action, including its high viscosity, which is primarily caused by a high sugar concentration and low water content and helps to create a barrier of defense against infection. Additionally, the hydrogen peroxide concentration and low acidity have apparent antibacterial properties [3].

- Low Water Activity

The amount of unbound water in food is measured by water activity; the less unbound water there is, the more difficult it is for bacteria to thrive in food. Honey has a very low water availability to support the development of any microbes, lower than the range where bacterial growth is totally prevented, according to its water activity, which varies from 0.562 to 0.62.

In this regard, the osmosis process is crucial to honey's antibacterial action, and the degree of inhibition will vary depending on the quantity of honey and the type of bacteria being researched. Because of the high sugar content, osmosis happens. The high concentration of sugar in honey puts osmotic pressure on bacterial cells, which causes water to be transported out of bacterial cells by osmosis. It follows that undiluted honey has the power to entirely inhibit the development of bacteria.

It also has been demonstrated that a concentration of 15% (w/v) carbohydrate (fructose, glucose, and glucose and fructose combinations) was sufficient to have a similar inhibitory effect as honey on all 28 tested isolates of

Helicobacter pylori, despite the fact that some bacterial strains are more sensitive to the osmotic effects of carbohydrate monomers and dimers than others [3].

- Acidity

Since most bacteria prefer a pH between 6.5 and 7.5, honey's acidity, which ranges from 3.2 to 4.5, is another significant element in its antibacterial effectiveness. The presence of organic acids, in particular the 0.5% (w/v) presence of gluconic acid, is what causes this acidity. According to certain studies, endogenous glucose oxidase converts glucose into gluconic acid, which is a potent antibacterial agent. Although this low pH can be a potent antibacterial agent in pure honey, when diluted in food or bodily fluids, it will not be sufficient to prevent the development of many bacterial species.

- Hydrogen Peroxide (H_2O_2)

An essential oxidizing and sanitizing agent is hydrogen peroxide. It is created by an enzyme in honey and can have a significant role in the antibacterial action. Although glucose oxidase is a naturally occurring enzyme, the low pH levels in undiluted honey prevent it from becoming active. However, when honey is diluted, glucose oxidase is activated, allowing it to react with endogenous glucose to create hydrogen peroxide. In fact, a honey dilution of 30–50% can yield the highest quantity of hydrogen peroxide.

Bang et al. claim that depending on the method of dilution, the hydrogen peroxide generation in some honey samples might grow continuously over time to a certain degree. In fact, honey's H2O2 levels can rise to 2.5 mmol in 30 minutes, and they can double with further incubation time. Researchers have measured the amount of hydrogen peroxide in several samples of honey, as shown in Table 3 [3].

Table 3. Levels of hydrogen peroxide in diluted honey [3].

Number of samples	Honey concentration (v/v %)	H_2O_2 content (mM/l)	Incubation duration (minutes)	Reference
31	NA	0–0.95	NA	Bogdanov [42]
90	14	0–2.12	1 hour	Roth et al. [43]
8	30–40	~2.5	30 min	Bang [41]
8	25	0.24–2.68	NA	Brudzynski et al. [44]
133	6.25, 12.5 and 25	0.4–2.6	0	Brudzynski et al. [45]
5	10–100	0.34–1.11	NA	Al-Waili et al. [46]

It is significant to remember that catalase's existence and activity also affect how much hydrogen peroxide is present in honey. In fact, a similar study found a significant correlation between this enzyme's and glucose oxidase's levels and the antibacterial efficiency that resulted. Additionally, it is believed that a high quantity of hydrogen peroxide would be related to a high level of glucose oxidase. A low catalase level would also indicate a high hydrogen peroxide level.

It was once thought that the antibacterial action of diluted honey was only caused by hydrogen peroxide, and that adding catalase would fully neutralize the antibacterial effect of honey. However, the presence of phytochemical substances in honey can affect how sensitive bacteria are to hydrogen peroxide generated in honey.

Some investigations have revealed that adding catalase to honey is inadequate to completely eliminate the antibacterial activity, indicating that the antibacterial action of honey is likely related to factors other than the activity of glucose oxidase. This emphasizes the significance of additional elements that may influence how hydrogen peroxide and honey's acidity work together to exert their antibacterial effects [3,27].

2.4 Chapter Summary

In Chapter 1 we proposed the background and significance of the research, which introduces the theory of antibacterial wound dressing. Polylactic acid (PLA) is the major material used. Testing if the antibacterial agents curcumin, anthocyanin and honey can inhibit bacterial growth at the proper concentration. Moreover, we also used povidone, amoxicillin and ciprofloxacin as the control antibiotic commercial to

see the difference between their inhibitory potencial toward bacteria. Analyzing the diameter and statistics to evaluate the effectiveness of their inhibition area.

In this chapter, the theory related was categorized into wound dressing (section 2.1) and the polymerization of polylactic acid or PLA (section 2.2). Observations were made on the systems and antibacterial agents reviewed (section 2.3), and the relevance of the objective of research was summarized (section 2.4).

The next chapter, we present the design or methodology of this research, which is a system intended to produce polylactic acid (PLA) by microwaving at different times and using power around 620W. Then, we specifically rechecked food-grade antibacterial agents including curcumin, anthocyanin and honey to see what least concentrations can inhibit the growth of bacteria. Furthermore, we used three commercial antibiotics including povidone, amoxicillin and ciprofloxacin for comparing their potencial and efficacy with the previous food-grade. After that we will compare the method of putting antibacterial agents inside PLA between soaking method and mixing directly. We compared them by testing with the bacteria culture. Using the dilution and incubation for preparing. Lastly, there was the use of the imageJ program that was being used to analyze the product's efficacy toward bacterial growth.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter, we describe the fabrication of antibacterial wound dressings. A process focuses on synthesized polylactic acid with appropriate times and power of microwaving technique. In addition, we intended to initially check the antibacterial activity of food-grade agents by spectrophotometer and cell culture by spreading methods. We identified the lowest concentration that can inhibit the growth of ***S. aureus*** and ***E. coli***. The antibacterial agents included curcumin, anthocyanin, honey, povidone, amoxicillin and ciprofloxacin. Furthermore, we investigated the method of soaking and mixing directly of antibacterial agent to PLA on the bacteria culture to determine which method is more suitable.

3.2 The ability of antibacterial agents to inhibit the growth of bacteria.

The antibacterial agents that we used were food grade, which might have some additional substances mixed lead to deteriorate the efficacy of agents. These lead us to recheck its antibacterial acitivity by following these steps.

i. Incubating ***S. aureus*** and ***E.coli***

Preparing 2 liquid nutrient 50 ml contained in erlenmayers flasks. Then, we put each bacteria colony in both flasks and incubated for 24 hours at a temperature of 37°C and 150 rpm for shaking.

ii. Diluting bacteria

Diluting the bacteria by $10^{-1}$to 10^{-3}. In experiment, we used the cell culture diluted at 10^{-3} to test the inhibition area. The liquid culture media of 5 ml ; were contained in test tube with lid. Then sterilized by autoclaving at 121°C for 15 minutes.

iii. Preparation of various antibacterial agents concentration

- Curcumin

  There were seven tubes in total. The first four tubes would have 300 ul of both bacteria along with different concentrations of curcumin as follows; 0%, 1%, 5%, and 10% (w/v). The last three tubes would have no bacteria, but they still had the same concentration as the previous test tubes. These three were for positive control. Then, they were put in the incubator shaker for 24 hours.

- Anthocyanin

  There were seven tubes in total. The first four tubes would have 300 ul of both bacteria along with different concentrations of anthocyanin as follows; 0%, 1%, 5% and 10% (w/v). The last three tubes would have no bacteria, but they still have the same concentration as the previous test tubes. These three were for positive control. Then, they were put in the incubator shaker for 24 hours.

- Honey

  There were seven tubes in total. The first four tubes would have 300 ul of both bacteria along with different concentration of honey as follows; 0%, 1%, 5% and 10% (v/v). The last three tubes would have no bacteria, but they still have the same concentration as the previous test tubes. These three were for positive control. Then, they were put in the incubator shaker for 24 hours.

- Ciprofloxacin (control)

  There were two tubes in total. In the first tube, we intended to put 3 ul of ciprofloxacin along with bacteria. In the second tube, there were no bacteria, but it still had the same concentration. Then, they were put in the incubator shaker for 24 hours.

- Amoxicillin (control)

  There were two tubes in total. In the first tube, we intended to put 3 ul of amoxicillin along with bacteria. In the second tube, there were no bacteria, but

it still had the same concentration. Then, they were put in the incubator shaker for 24 hours.

iv. Using spectrophotometer

The spectrophotometer was set at wavelength of 660 nm to measure the absorbance value of sample. The values were compared with the control test tube to see some significant changes. However, the values were over the range of measurable. It led us to dilute the test tubes by ten times. Then, we prepared the spreading method for the sample that couldn't be validated.

v. Spreading

Nutrient agar plates (NA) were prepared for this experiment. We spreaded 300 ul of the sample that has antibacterial agents incubated with bacteria, which used 1% and 5% (w/v) of both curcumin and anthocyanin. The 10% tube had an intense pigment and sendimen that couldn't be spreaded. In honey case, the three concentration can be spreaded due to its being liquid. Following this, we'll place them in the incubator for 24 hours at 37°C.

3.3 Polylactic acid (PLA) wound dressing fabrication

3.3.1 Polylactic acid (PLA) synthesis

20 ml of 85% (w/w) lactic acid (KemAus) was microwaved for 9 minutes at 620 W, removing the water by condensation [7,8], to create polylactic acid. The PLA's molecular weight was efficiently raised by this procedure. According to Figure 5, which depicts the changes in the chemical structure inside the samples through the comparison between the absorbance or transmittance spectra and different times, the acquired PLA was examined using Fourier transform infrared spectroscopy (FTIR, FT/IR-4600, JASCO Corporation).

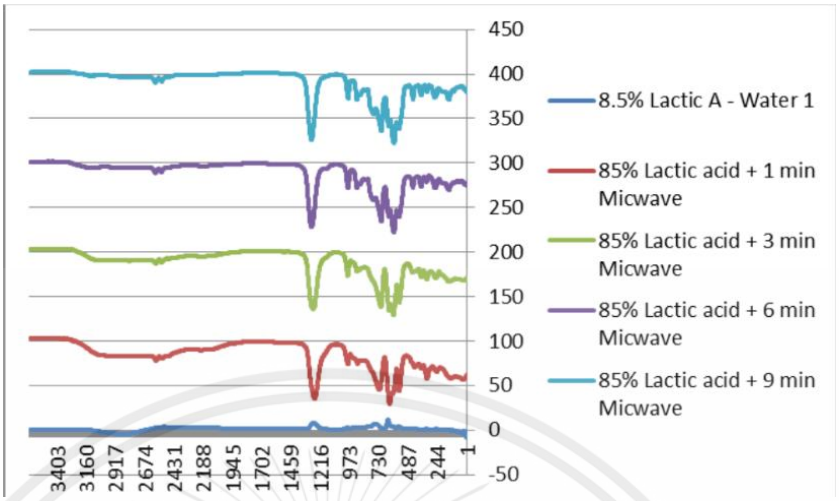


Figure 5. FTIR results for PLA at different times.

3.3.2 Annealing and Crystallization

According to the research, it is proven that the polylactic acid can be solidified by annealing at 90°C for 4.5 hours [10]. We intend to use this annealing method with appropriate concentrations of each antibacterial agents mixing with pure PLA for futher tests.

3.3.3 antibacterial agents into PLA

After the confirmation results in 3.2, we used two different method for mixing with polylactic acid as follows;

i. Soaking method

Each antibacterial agent was prepared by combining with distilled water (D.W.) at the proper concentrations; for curcumin, 10%, 15%, 20%, 25%, 30%, and 35% (w/v). We did analyze some studies on anthocyanin cases and it is claimed that concentrations of at least 20% (w/v) begin to stop the bacterial growth. Their concentrations at 20%, 25%, 30%, and 35% (w/v) were indicated. We utilized 10% povidone, which doesn't require mixing with distilled water because it is already liquid. Then, we soaked each synthesized PLA sample with the prepared antibacterial agents and tested with bacteria.

ii.Mixing directly method

We combined each antibacterial agent with synthesized PLA after lactic acid had been microwaved. We intended to combine their textures by rotating them. Then, we solidified the sample with the annealing and crystallization methods.

3.3.4 Making sample

The samples were assembled into a circle's sandwich layers. Foil paper, filter paper, hardened PLA, and a paper punch are the four materials. Foil paper served as the foundation layer, while filtered paper with PLA served as the surface or top layer. After that, they will be cut and maintained thickness easier before annealing. By using a paper punch to create a 5 mm circle, the sample size was managed (as shown in Figure 6).

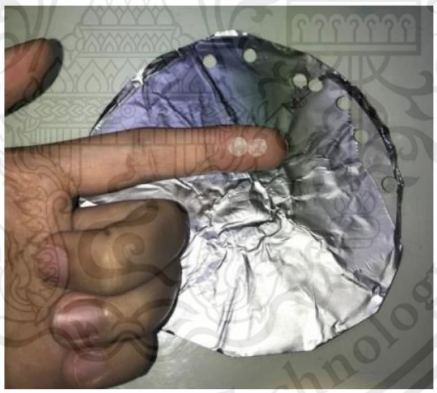


Figure 6. 5mm of PLA sample.

3.3.5 Bacteria Culture

Staphylococcus aureus (***S. aureus***) and ***Escherichia coli*** (***E. coli***) were selected to test the antibacterial activity of agents. We pre-cultured these bacteria in nutrient media for 24 hr at 37°C, 150 rpm. A series of diluted cell cultures were carried out, ranging from 10^{-1} to 10^{-3}. Triplicate were used to test the growth of bacteria with antibacterial containing samples placed on. If two of them create an inhibition area, we can assume that they are capable of inhibition toward bacteria.

3.3.6 Measurement of the diameter

The imageJ program and ruler were used for measuring diameter of inhibition area in this process. Firstly, the image was added and set the scale for measurement. By click analyze > set scale > draw a line in cross section at the indicated distances > type the known distance and unit of the length > OK. The diameter of inhibition area will show in the table as a length column. Then, collected all of the data to analyze in the Microsoft excel and downloaded the add-ins and resource files(xrealstat) into this program for plotting graphs to clarify the average and SD value of each samples more clearly. The statistics that was being used here called one way anova because our results have than one variable.

CHAPTER 4

EXPERIMENTAL RESULT AND DISCUSSION

4.1 Introduction

In this chapter, we present a testing method and its results that show polylactic acid (PLA) produced from the condensation of lactic acid, antibacterial agents mixed with polylactic lactic acid and we tested them for the bacteria inhibition.The chapter is organized as follows: Section 1 we introduce the creation of polylactic acid and crystallization method. We specifically describe what volume we use to produce it and how it is done through the microwave and annealing method. Next, we present the confirmation results of food-grade antibacterial agents including curcumin, anthocyanin, honey, ciprofloxacin and amoxicillin. We describe the results of spectrophotometer method and spreading bacteria cultivated to find out the minimum concentration that can inhibit the growth of bacteria.

4.2 Result and Discussion

4.2.1 Antibacterial agents toward bacteria inhibitory ability

After performing spectrophotometer and cell culture by spreading method (as shown in Table 4), the results have shown that curcumin can inhibit only ***S. aureus*** at the minimum concentration of 1% (as shown in Figure 7). Meanwhile, anthocyanin can inhibit ***E. coli*** and ***S. aureus*** at the minimum concentration of 1% (as shown in Figure 8-9). As for the honey case, it didn't inhibit the growth of both bacteria (as shown in Figure 10). These results also prove that the inhibitory substance in honey which is hydrogen peroxide might not have the appropriate volume to inhibit the growth. We conducted graphical results (as shown in Figure 11-12) and it didn't show much significant change. For the antibiotics control, we have used ciprofloxacin and amoxicillin at their minimum inhibitory concentrations and they clearly inhibited both bacterial growth. These results indicated that honey hasn't had the ability to inhibit the growth of bacteria.

Table 4. The absorbance value for each antibacteria agents.

Name of antibacterial agents	Absorbance at 660 nm
Ciprofloxacin (control)	0.1 x 1
Amoxicillin (control)	0.210 x 1
Ciprofloxacin (***S. aureus***)	0.005 x 1
Amoxicillin (***S. aureus***)	0.320 x 1
Ciprofloxacin (***E.coli***)	0.296 x 1
Amoxicillin (***E.coli***)	0.307 x1
Honey 10% (control)	0.594 x 1
Honey 20% (control)	0.212 x 1
Honey 30% (control)	0.378 x 1
Honey 10% (***S. aureus***)	0.192 x 10 (dilute 10 times)
Honey 20% (***S. aureus***)	0.150 x 10 (dilute 10 times)
Honey 30% (***S. aureus***)	0.145 x 10 (dilute 10 times)
Honey 10% (***E.coli***)	0.096 x 10 (dilute 10 times)
Honey 20% (***E.coli***)	0.094 x 10 (dilute 10 times)
Honey 30% (***E.coli***)	0.102 x 10 (dilute 10 times)
Curcumin 1% (control)	0.747 x 10 (dilute 10 times)
Curcumin 5% (control)	0.962 x 10 (dilute 10 times)
Curcumin 1% (***S. aureus***)	0.116 x 10 (dilute 10 times)
Curcumin 5% (***S. aureus***)	0.992 x 10 (dilute 10 times)
Curcumin 1% (***E.coli***)	0.134 x 10 (dilute 10 times)
Curcumin 5% (***E.coli***)	0.955 x 10 (dilute 10 times)
Anthocyanin 1% (control)	0.242 x 10 (dilute 10 times)
Anthocyanin 1% (***S. aureus***)	0.191 x 10 (dilute 10 times)

Anthocyanin 1% (***E.coli***)	0.294x 10 (dilute 10 times)
No agents (***S. aureus***)	0.059 x 10 (dilute 10 times)
No agents (***E.coli***)	0.026 x 10 (dilute 10 times)

Figure 7. Curcumin 1% and 5% (w/v) in ***S. aureus.***

Figure 8. Anthocyanin 1% (w/v) in ***S. aureus.***

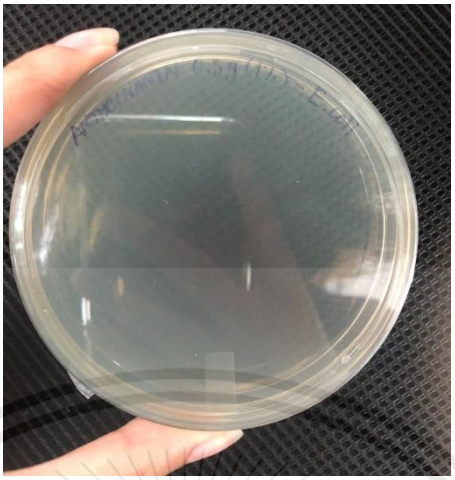


Figure 9. Anthocyanin 1% (w/v) in ***E. coli.***

Figure 10. Honey 10% (w/v) in ***S. aureus.***

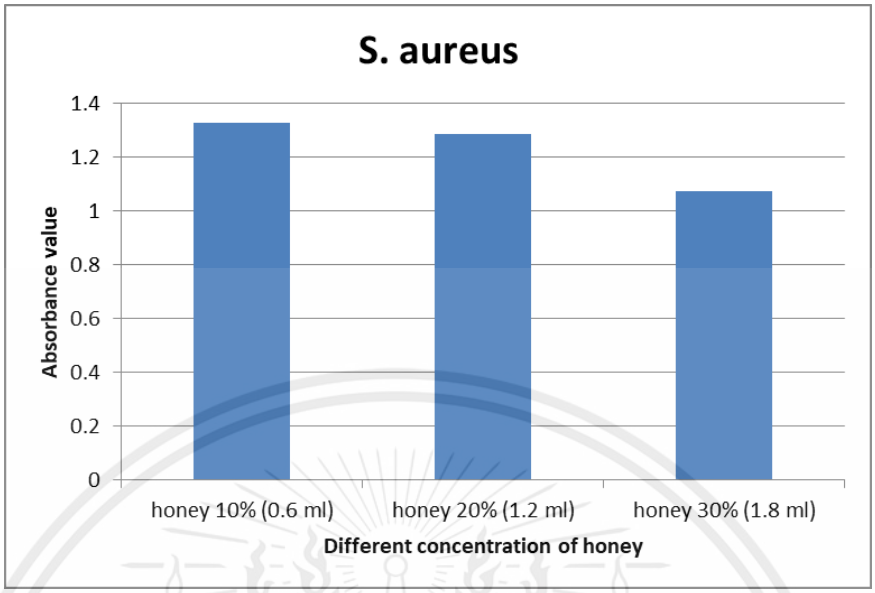


Figure 11. The graph of honey absorbance value in ***S. aureus.***

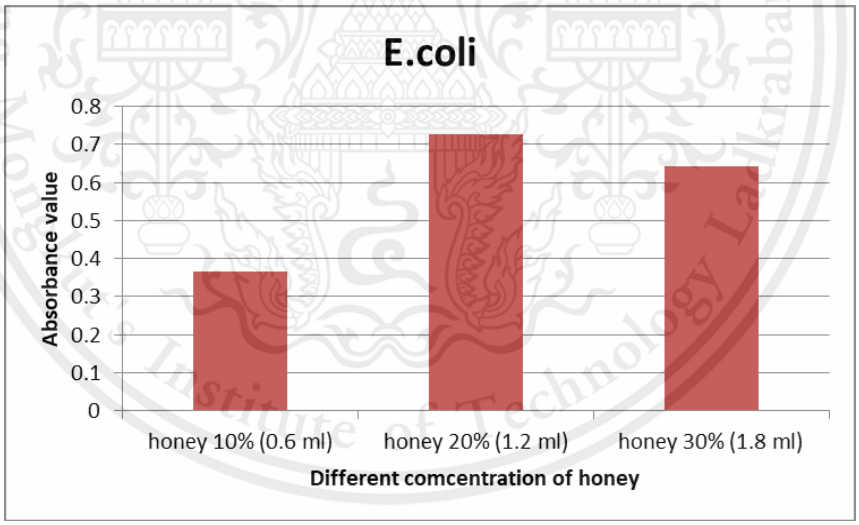


Figure 12. The graph of honey absorbance value in ***E. coli.***

4.2.2 Fabrication of PLA wound dressing

- Condensation of lactic acid

We have used the condensation method to produce polylactic acid (PLA) by microwaving lactic acid at different times starting from 1 min, 3 min, 6 min and 9 min with the power around 620W. The 9 min was able to turn lactic acid

into PLA and this result was checked by FTIR. Furthermore, the volume of lactic acid that we used are 5 ml, 10 ml and 20 ml. We discovered that 20 ml produces at least 3-4 grams of polylactic acid is considered an appropriate volume for testing. It started to become polylactic acid after 6 min forward. However, the results have shown that the 9 min of lactic acid are perfectly changed into polylactic acid by the the FTIR analysis.

- Annealing and crystallization method

  After the lactic acid were condensed, its texture was too viscous to test. According to the research, the annealing method can solidify the polylactic acid into a more stable state. After, we annealed it about 4 hours and 30 minutes with a temperature around 90 Celsius. The results have shown that its texture becomes more solidified, less viscous and thinner than before. However, it also came up with some limitation that being at room temperature for longer than 10 or 15 minutes could increase the viscosity of this material. To solve this problem, we have packed it up in the refrigerator to keep it modelized.

- Soaking with antibacterial agents

  These tests have indicated that soaking methods create an uncontrollable environment towards the inhibition area's results. The more liquid substance soaked, the liquid spread more around the sample. Some of the area became larger and crossed over each other. Moreover, the reason why we used this method is because the state of povidone is liquid which can't be mixed directly with polylactic acid. We also tried to use curcumin, anthocyanin with this method and the results showed liquid substance also spread more. It is hard to control the dampness of the soaking filter paper which leads to lots of human errors in this experiment.

- Mixing with antibacterial agents

The test illustrated that it is easier for antibacterial agents in solid or powder state mixing in polylactic acid. Though its color pigment, more concentration we added, the more thick and intense the color it became. However, the agents still leaked its pigment and liquidity out on the spreading in incubating methods. To conclude this method, we could say that soaking or mixing directly didn't make much significant change of inhibition area.

4.2.3 pH of PLA affects the growth of bacteria

After several testing, we discovered that polylactic acid creates an unstable inhibition area. The PLA produces a larger inhibition area than the PLA mixed with antibacterial agents. We have checked the pH of the PLA and it turns out to be 4, whichis still considered as acid. Adding curcumin did not significantly inhibit the growth of bacteria than PLA without antibacterial agents. We have used 1M of sodium hydroxide (NaOH) , the strong base to neutralize the pH. The samples's pH turned to 6 by 60-65 drops of NaOH (as shown in Figure 13-14).

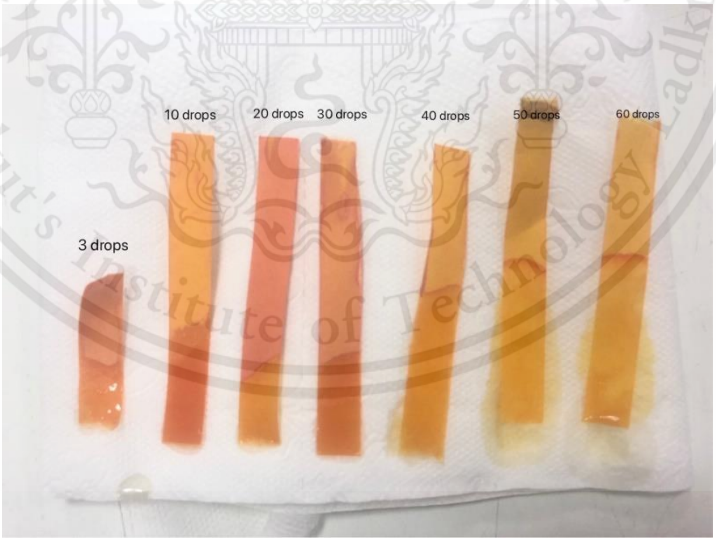


Figure 13. 10-60 drops of NaOH in PLA.

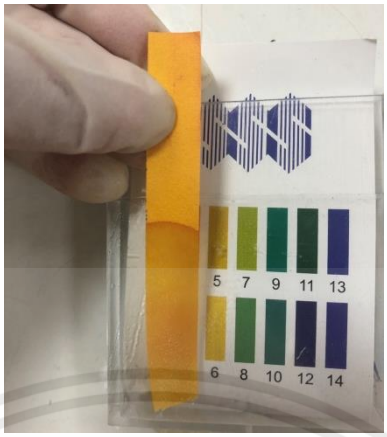
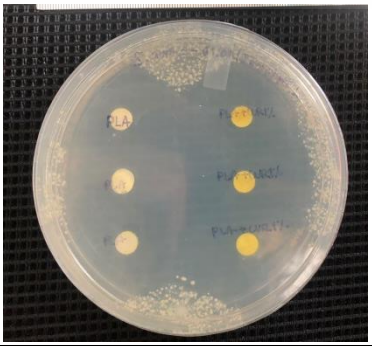
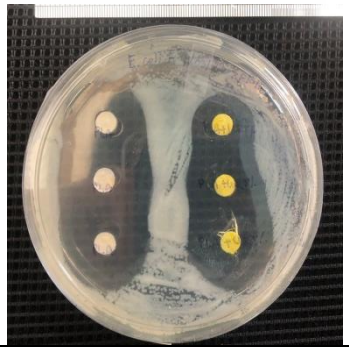


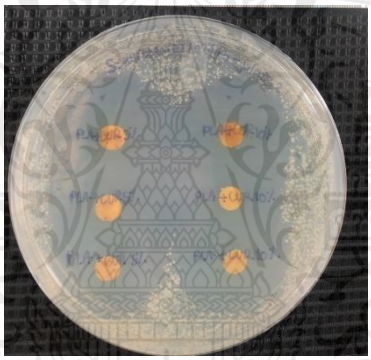
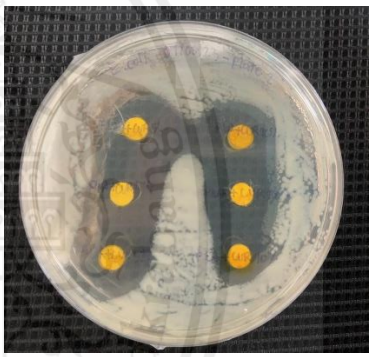
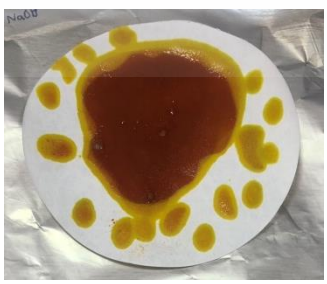
Figure 14. The pH is 6 at 60 drops.

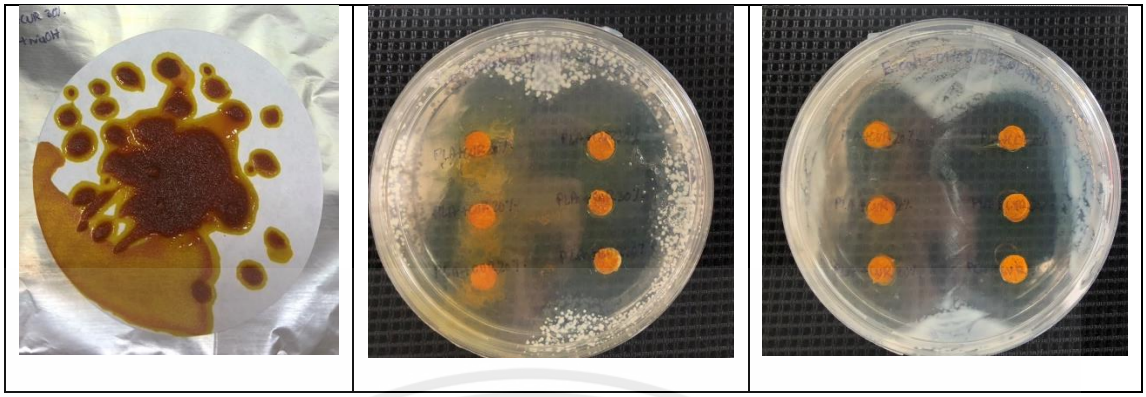
- Sodium hydroxide ($NaOH$) affecting inhibition area

The tests evaluated that all of the samples could inhibit the bacteria growth which made no sense because the adjusted pH of PLA to be neutral. It shouldn't inhibit the growth of bacteria. The inhibition area of curcumin should be significantly greater than the PLA adjusted pH. Besides, the inhibition areas were crossing each other, which made it was difficult to measure the diameter (as shown in Table 5).

Table 5. Different concentrations of curcumin in PLA and inhibition results.

Name	Inhibition area in ***S. aureus.***	Inhibition area in ***E. coli.***
PLA+NaOH 		
PLA+CUR 1% + NaOH		

		
PLA+CUR 5% + NaOH 		
PLA+CUR 10% + NaOH 		
PLA+CUR 20% + NaOH 		
PLA+CUR 30% + NaOH		



- Using distilled water to subside sodium hydroxide ($NaOH$)

According to the adjusted pH on PLA to be neutral, it still inhibited the growth of bacteria. Mixing antibacterial agent curcurmin didn't significantly inhibit bacteria compared to only PLA. Therefore, we concluded that NaOH may affect the growth of bacteria. We decided to use distilled water to remove the excess NaOH. Each adjusted PLA with curcumin have soaked in the distilled water and dry on the plate for a few minutes. Then, we put them to test the inhibition area which were smaller than previous (as shown in Figure 15-16). The use of distilled water in this experiment has shown that the inhibition area became more certainly than the previous results. It showed a definite area of each sample. Even though each area was still crossing each other. This also proved that sodium hydroxide (NaOH) has caused inaccurate results of the inhibition of bacteria growth.

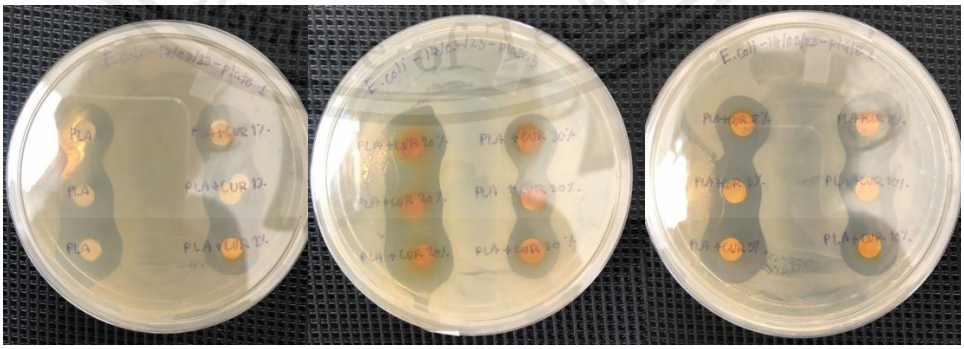


Figure 15. 3 plates of ***E. coli*** testing with adjusted PLA mixed with curcumin.

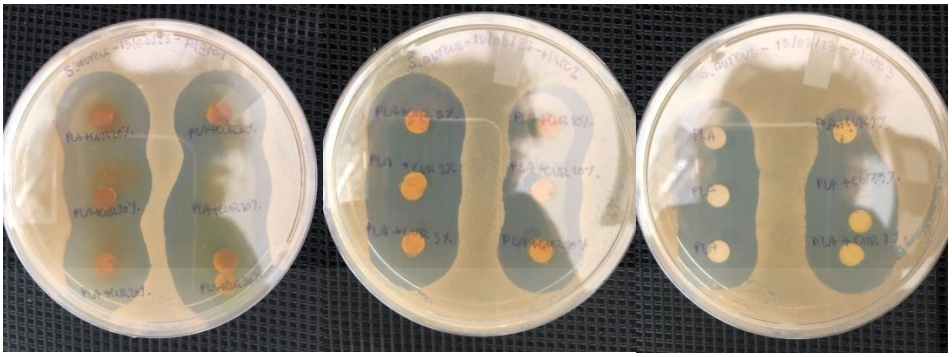
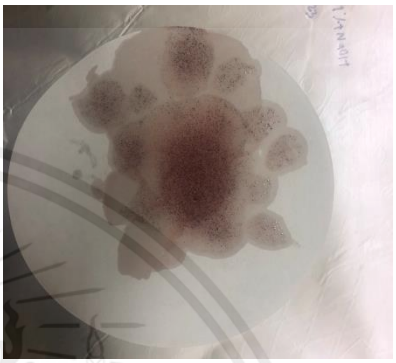
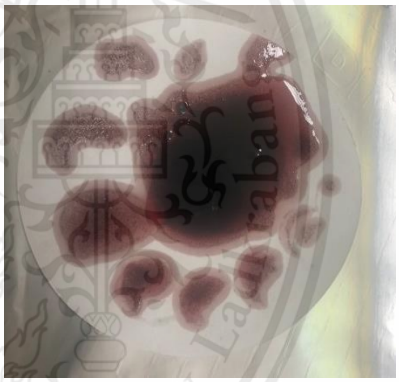


Figure 16. 3 plates of ***S. aureus*** testing with adjusted PLA mixed with curcumin.

- Anthocyanin with the mix of PLA and D.W.

The results have shown that the higher concentration of anthocyanin, the more intense the color and thickness of sample because it did not dissolve homogenously(as shown in Table 6). Moreover, the use of distilled water also reduce the effect of $NaOH$ by making more certainly inhibition area. In addition, the higher concentration of anthocyanin have shown more significant different but the 5%-10% and 20%-30% almost have similar areas on ***S. aureus*** (as shown in Figure 17). For ***E. coli*** cases, it formed a smaller area than ***S. aureus*** but one sample of 30% (w/v) showed a smaller area than the other concentrations (as shown in Figure 18). These results can be concluded that distilled water did decrease the effect of sodium hydroxide, but this result probably due to the removed NaOH could make the pH of PLA returned to be acidic, which could inhibit inherently.

Table 6. Different concentrations of anthocyanin in adjusted PLA.

Name	Results (color and texture)
PLA + ANTHO 1% + NaOH	
PLA + ANTHO 5% + NaOH	
PLA + ANTHO 10% + NaOH	
PLA + ANTHO 20% + NaOH	

	
PLA + ANTHO 30% + NaOH	

Figure 17. Different concentrations of anthocyanin on ***S. aureus.***

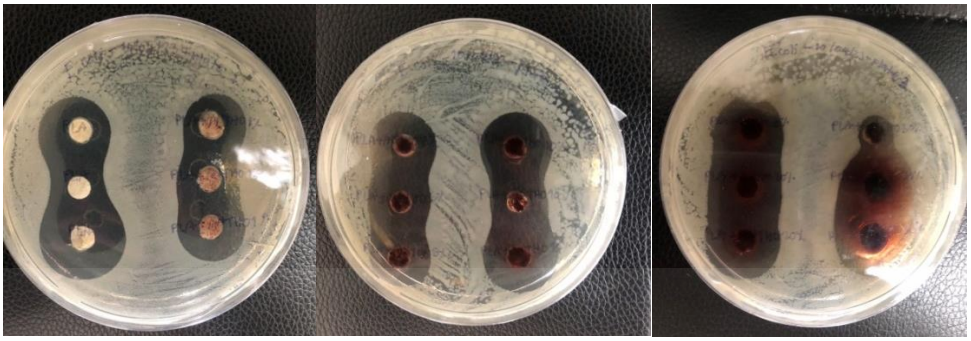


Figure 18. Different concentrations of anthocyanin on ***E. coli.***

- Amoxicillin and ciprofloxacin as positive control

These tests were executed because we intend to compare the antibacterial agents's potency with the inhibitory ability of both well-known antibiotics. The results have shown that both antibiotics created similar inhibition area in both ***S. aureus*** and ***E. coli*** (as shown in Figure 19-20). Their inhibition area still crossed each other. These prove that each of the drugs have a great inhibition property toward bacteria.

Figure 19. Amoxicillin and Ciprofloxacin on ***S. aureus***.

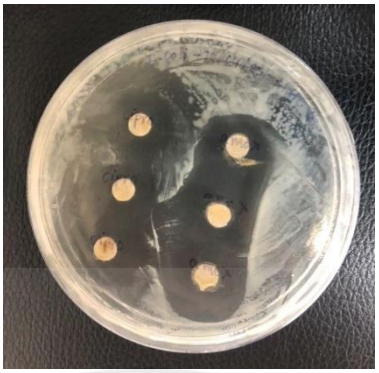


Figure 20. Amoxicillin and Ciprofloxacin on ***E. coli***.

- Measurement of diameter

The results have demonstrated that curcumin did not inhibit the growth of ***E. coli*** but it inhibited the growth of ***S. aureus*** at the minimum concentration of 5% (as shown in Figure 21-22). Anthocyanin inhibited the growth of ***E. coli*** at minimum concentration of 10% (as shown in Figure 23). Moreover, it did not show any significant effect to inhibit ***S. aureus*** compared to PLA only (as shown in Figure 24). PLA still had the ability to inhibit the growth of both bacteria.

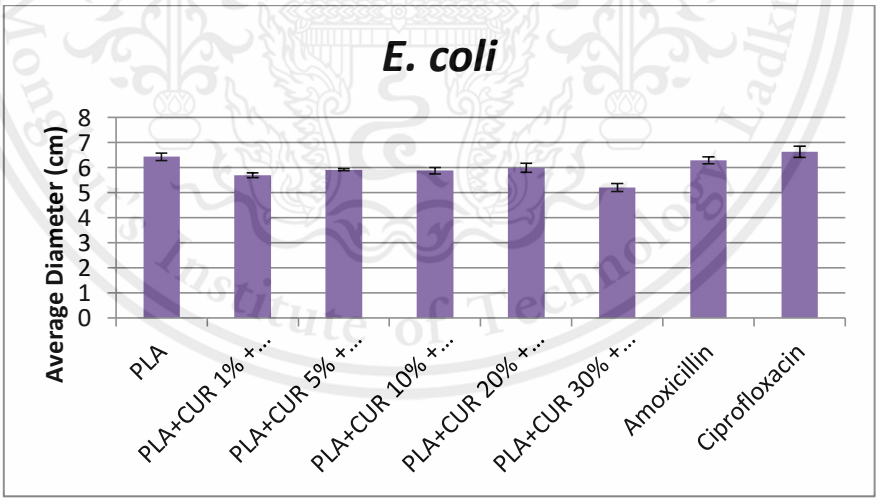


Figure 21. Curcumin's graph on ***E. coli.***

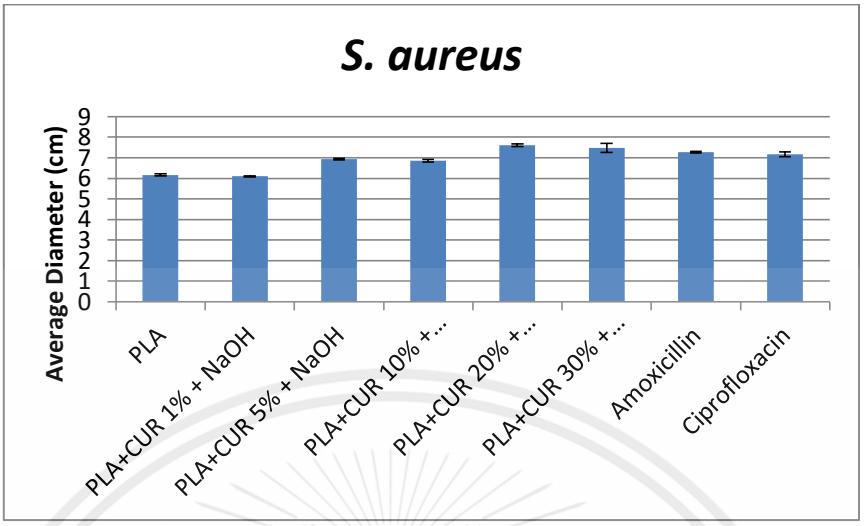


Figure 22. Curcumin's graph on ***S. aureus*.**

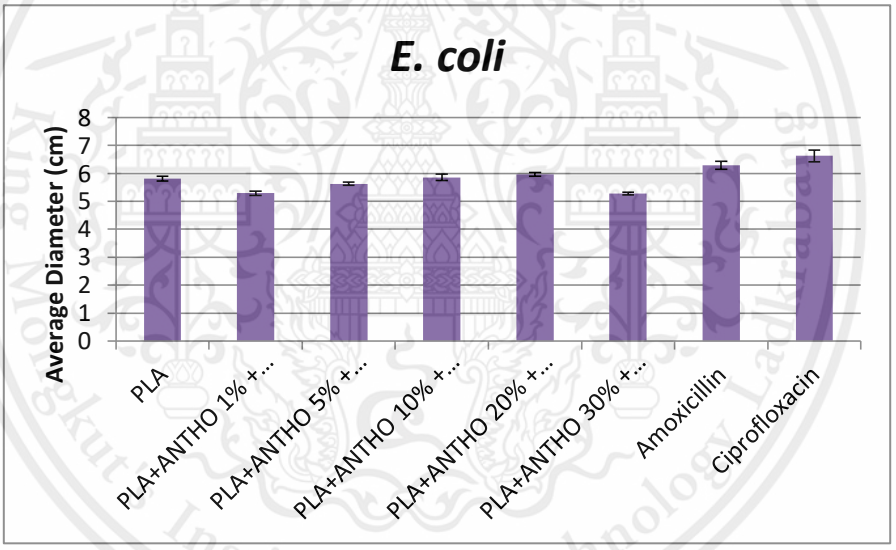


Figure 23. Anthocyanin's graph on ***E. coli*.**

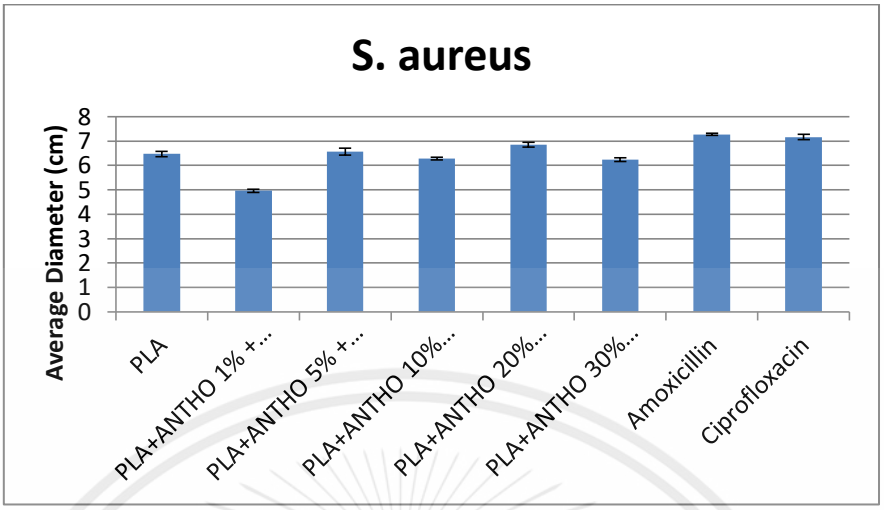


Figure 24. Anthocyanin's graph on ***S. aureus.***

4.3 Concentrations of antibacterial agents

To find an appropriate concentrations, we increased concentrations of curcumin as follow: 0.05%, 0.075% and 0.1% (w/v). We also use povidone, commercial antibacterial agent to be the positive control. According to the theory, the more concentration they have, the larger inhibition area may obtained. However, the 0.025% (w/v) of curcumin have more area than the 0.05% - 0.1% (w/v) which made no sense (as shown in Figure 25-26 and Table 7-9). These results indicated the unstable inhibition area results and made we assumed that the concentration of curcumin might be too low to inhibit and it was the food grade which contained others components. Moreover, we also supposed that low pH of polylactic acid affects to inhibit the growth of bacteria instead of curcumin.

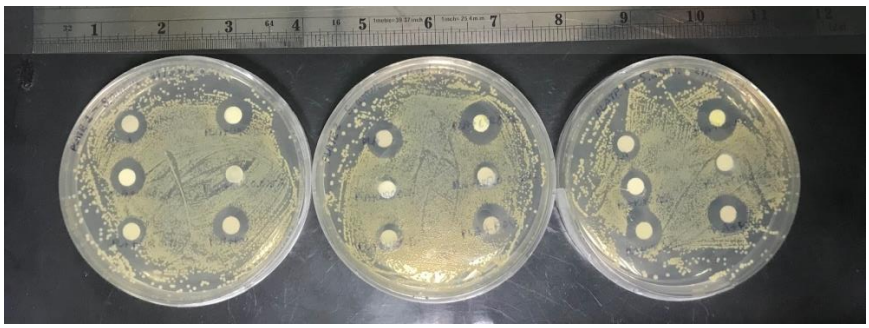


Figure 25. The inhibition area with difference concentrations of curcumin on ***S. aureus.***

Table 7. Area and diameter sample for ***S. aureus*** plate 1.

Sample1-***S. aureus***	Inhibition area plate 1.1	Inhibition area plate 1.2	Inhibition area plate 1.3	Diameter of inhibited area plate no.1.1	Diameter of inhibited area plate no.1.2	Diameter of inhibited area plate no.1.3
PLA	1.138	1.209	1.138	1.204	1.241	1.204
PLA+CUR 0.025%	1.391	1.257	1.458	1.331	1.265	1.362
PLA+CUR 0.05%	1.531	1.531	1.536	1.396	1.396	1.398
PLA+CUR 0.075%	0.618	0.644	0.695	0.887	0.906	0.941
PLA+CUR 0.1%	0.849	0.984	0.901	1.039	1.12	1.071
PLA+POV	1.655	1.745	1.688	1.452	1.491	1.466

Table 8. Area and diameter sample for ***S. aureus*** plate 2.

Sample2-***S. aureus***	Inhibition area plate 2.1	Inhibition area plate 2.2	Inhibition area plate 2.3	Diameter of inhibited area plate no.2.1	Diameter of inhibited area plate no.2.2	Diameter of inhibited area plate no.2.3
PLA	1.767	1.874	1.869	1.499	1.545	1.543
PLA+CUR 0.025%	2.614	2.653	2.525	1.824	1.838	1.793
PLA+CUR 0.05%	0.78	0.672	0.726	0.997	0.925	0.961
PLA+CUR 0.075%	1.006	1.06	1.007	1.132	1.162	1.132
PLA+CUR 0.1%	1.181	1.39	1.198	1.226	1.33	1.235

PLA+POV	2.633	2.786	2.404	1.831	1.883	1.749

Table 9. Area and diameter sample for ***S. aureus*** plate 3.

Sample3-***S. aureus***	Inhibition area plate 3.1	Inhibition area plate 3.2	Inhibition area plate 3.3	Diameter of inhibited area plate no.3.1	Diameter of inhibited area plate no.3.2	Diameter of inhibited area plate no.3.3
PLA	0.946	0.965	1.007	1.097	1.108	1.132
PLA+CUR 0.025%	2.321	2.289	2.034	1.719	1.707	1.609
PLA+CUR 0.05%	1.556	1.443	1.59	1.408	1.355	1.423
PLA+CUR 0.075%	0.511	0.449	0.525	0.807	0.756	0.818
PLA+CUR 0.1%	2.034	2.164	2.043	1.609	1.659	1.613
PLA+POV	2.355	2.207	2.224	1.731	1.676	1.683

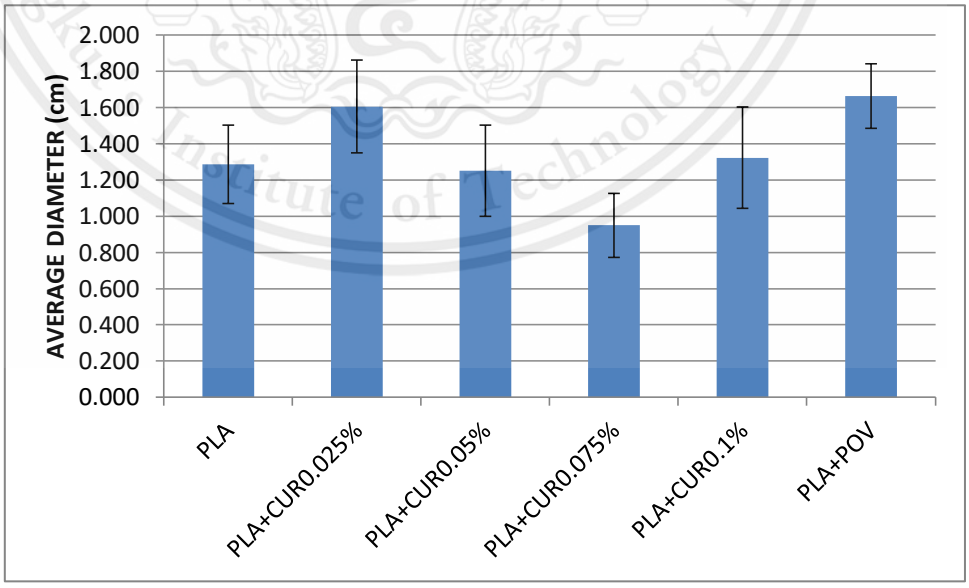


Figure 26. Average diameter of different concentrations of curcumin and povidone as a posotive control.

- pH of polylactic acid

  After we supposed the pH affects the growth of bacteria, we did measure the pH of every concentrations of curcumin that was mixed with polylactic acid by pH indicators. The results have turned out to be 4 (as shown in Figure 27) which was still considered as weak acid. This has proven that low pH of polylactic did affect the inhibition area and also the concentration of the curcumin didn't show significantly changes since its volume was too little.

Figure 27. pH results of different concentrations of curcumin.

- The pigments of antibacterial agents

  During the soaking method, we discovered that the pigment of each agents was a very intense color to see the inhibition area clearly(as shown in Figure 28). It couldn't measure by using the imageJ. Meanwhile, the mixing directly method can provide us more precise results because the pigments didn't spread out or leak too much but the inhibition area were crossing each other to the point where there was no more clear circle form.

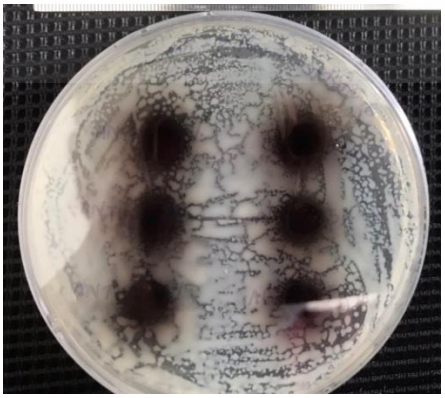


Figure 28. The intense pigments of anthocyanin on ***E. coli*.**

- The leaking of liquidity during bacteria culture with samples experiment

  The leaking of curcumin and anthocyanin liquid (as shown in Figure 29) led us to obtain unconsistant results because we could not control the volume of curcumin on the PLA by soaking. It did unexpectedly increase the human error in soaking method. Each sample spread liquidity with different volume.

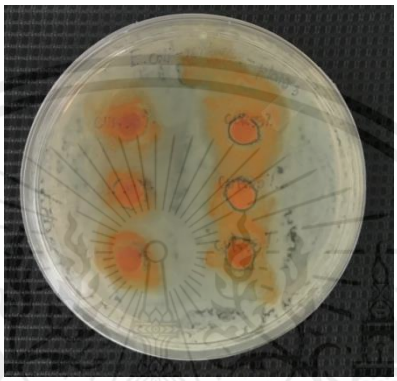


Figure 29. The leaking of curcumin after soaking method.

4.4 Summary

This chapter demonstrated the results of natural antibacterial agents mixed with polylactic acid (PLA) through the bacteria culture. Then, ImageJ was used to measure diameter at the inhibition area. During fabrication process by microwaving method, 9 min was considered as the appropriate time that can produce PLA from 85% of lactic acid. In case of antibacterial agents, the spreading and spectrophotometer methods were used to check their ability to inhibit the growth of bacteria. Their results showed that 1% concentrations of curcumin can inhibit only ***S. aureus*** but 1% concentration of anthocyanin can inhibit ***S. aureus*** and ***E. coli***. The testing with bacteria used two different method which are soaking and mixing directly. However, the low pH of the PLA had affected it by causing pure PLA to have more larger area than the added antibacterial agents during the soaking method. There were some leaking and pigment over the area which made it hard to measure. After that, an experiment of increasing pH were conducted with the use of sodium hydroxide (NaOH). It turned the pH from 4 (acid) to 6 by 60 drops but NaOH also caused the inhibition area to be larger than the PLA without it. These experiment were used mix directly method to avoid the leaking. Then, a series of using distilled water

to soak out NaOH from the adjusted PLA have been using. This solution created more definite area for each sample even though the area still crossed each other. At the very least, PLA still inhibit the growth of both bacteria. Moreover, soaking method and mixing directly didn't show much significant change.

CHAPTER 5
CONCLUSION

5.1 Conclusion

The aim of this project was to fabricate antibacterial wound dressing from lactic acid by microwaving with soaking and mixing antibacterial agents directly in PLA. We investigated the appropriate antibacterial agents and optimal concentration mixed with polylactic acid and determine the factors affecting the inhibition area. We chose to focus on their ability to inhibit the growth of ***S. aureus*** and ***E. coli***. In the first part, we have produced the PLA from 85% by microwave technique. It was proved that 9 minutes in microwave could fabricate PLA, which was analyzed by the FTIR. For the second part, the natural antibacterial agents including anthocyanin and curcumin were tested to find the appropriate concentration that can inhibit the bacteria by spectrophotometer and cell culture on spreading methods. It was shown that anthocyanin has the ability to inhibit both bacteria at the minimum concentration of 1% (w/v). Meanwhile, curcumin can only inhibit ***S. aureus*** at the minimum concentration of 1% (w/v). In third part, there were 2 different methods including PLA's soaking and mixing with antibacterial agents to test with bacteria. The results indicated that these two methods did not show significantly effects to inhibit the growth of ***S. aureus*** and ***E. coli*** compared to pure PLA. These results lead us to conclude that there were lots of factors that affected the inhibition property of antibacterial polylactic acid including pH of PLA, concentration and the type of antibacterial agents. We investigated the pH of PLA and tried to turn it from acidic to neutral by 60 drops of sodium hydroxide (NaOH). However, the NaOH also affected greatly at the inhibition area. Following this problem, we soaked the adjusted PLA out with distilled water and the results showed more definite area. Even though the pure PLA still showed the ability to inhibit greater than PLA with curcumin and anthocyanin. Therefore, we can conclude that the adjusted PLA may have some NaOH left and these antibacterial agents used in this project may not be pure due to it is a food product.

5.2 Suggestion

There are several potential avenues for further development and utilization of this project. One possibility is to improve the increasing pH of polylactic acid by some other technique. Another way is to find other suitable base material instead of PLA. That material must prioritize the safety toward human skin and can be mix with antibacterial agents. In addition, it is also possible to use other natural antibacterial agents.

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