DEBRE BERHAN UNIVERSITY

COLLEGE OF ENGINEERING

DEVELOPING AND CHARACTERIZATION OF CASSAVA STARCH-BASED ANTIMICROBIAL PACKAGING FILM BY INCORPORATING NETTLE LEAF EXTRACT

MSc. Thesis

By

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DEVELOPING AND CHARACTERIZATION OF CASSAVA STARCH-BASED ANTIMICROBIAL PACKAGING FILM BY INCORPORATING NETTLE LEAF EXTRACT

A Thesis Submitted to the Department of Chemical Engineering, College of Engineering, Debre Berhan University

Submitted In partial Fulfilment of the Requirements for the Degree of Master of Science in Chemical Engineering (Process Engineering)

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DEBRE BERHAN UNIVERSITY

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THESIS SUBMISSION FOR DEFENSE

APPROVAL SHEET – I

This is to certify that the thesis entitled: 'Developing and Characterization of Cassava Starch-Based Antimicrobial Packaging Film by Incorporating Nettle Leaf Extract' 'submitted in partial fulfillment of the requirements for the degree of Masters of Science with specialization in Process Engineering of the Graduate Program of the Chemical Engineering, College of Engineering, Debre Berhan University and is a record of original research carried out by Natnael Shibabaw Chekol (PGR/235/11), under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

The assistance and help received during this investigation have been duly acknowledged. Therefore, I recommend that it be accepted as fulfilling the thesis requirements.

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We, the undersigned members of the boarded of the examiners of the final open defense by Natnael Shibabaw Chekol have read and evaluated his thesis entitled 'Developing and Characterization of Cassava Starch-Based Antimicrobial Packaging Film by Incorporating Nettle Leaf Extract'' and examined the candidate. This is therefore to certify that the thesis/dissertation has been accepted in partial fulfillment of the requirements for the degree of Master of Science in Process Engineering.

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DECLARATION

I declare that this thesis 'Developing and Characterization of Cassava Starch-Based Antimicrobial Packaging Film by Incorporating Nettle Leaf Extract'' is my genuine work and that all sources of material used for this thesis have been profoundly acknowledged in the text and a list of reference given. This thesis has been submitted in partial fulfillment of the requirement for a master of science MSc. At Debre Berhan University and it is deposited at the university library to be made available for users under the rule of the library. I declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

Name: Natnael Shibabaw Chekol

Place: College of Engineering, Debre Berhan University

Date of Submission: June, 2021

ACRONYMS

2 FI	Two Factorial
NL	Nettle Leaf
AM	Anti-Microbial
S. Aureus	Staphylococcus Aureus
S. Typhi	Salmonella Typhi
NAM	Nutrient Agar Media
NBM	Nutrient Broth Media
Eva	Ethyl Vinyl Acetate
SD	Steam Distillation
EO	Essential Oil
ID	Inhibition Diameter
CFU	Colony Forming Units
MHA	Muller Hinton Agar
FAO	Food Agriculture organization

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ABSTRACT

Nowadays, to extend the shelf life of foods and to travel the foods safely, packaging that can protect food from chemical, biological and physical influences has become an integral part of food processing technology and its expenditure has reached more than 3,030 billion USA dollars per annum by the year 2020. However, plastics which are non-biodegradable and nonrenewable materials are the main packaging material used in the food industry. Therefore, this study is designed to overcome the limitations and add more important features to the existing packaging materials. In this study, antimicrobial packaging film was produced using a starch extracted from cassava roots, an antimicrobial agent extracted from nettle leaf, and glycerol as a plasticizer. The antimicrobial agent is extracted using ethanol. The antimicrobial activity of the extract was tested on gram-negative and gram-positive bacteria namely Staphylococcus aureus, Salmonella typhi, and E. coli and antifungal (Candida Albicans). Thereafter, the minimum concentration of nettle leaf required for inhibition is determined using the broth dilution method. Then, packaging film from cassava starch, glycerol, and extracted nettle leaf is prepared and its physical, antimicrobial, and mechanical properties are determined. The result shows that the extract exhibits strong antimicrobial activities as all microbes are completely inhibited to grow at an extract concentration of 10 mg/mL. Compared to the other microbes, candida Albicans are less resistant to the extract and ceases to grow at 6 mg/mL extract concentration. The ANOVA analysis, which is based on three factors [starch/glycerol ratio (A), antimicrobial concentration (B), and film thickness (C)] with mixed levels, indicated that all the factors have significant effects on all microbes and the AC and BC interaction effects are significant on all microbes. Regarding the properties of the film, the moisture content decrease from 35% to 22%, solubility increase from 50% to 70% and swelling capacity decrease from 30% to 20% as antimicrobial concentration increases while the mechanical properties which is tensile strength increase from 20Mpa to 27Mpa and elongation break also increase from 60% to 82% with an increase in the antimicrobial concentration. The overall analysis of the result suggests nettle leaf extract and cassava starch are the potential alternative to plastic packaging material for the production of antimicrobial, renewable, and biodegradable food packaging. Therefore, the researcher recommends the players in the packaging industry seriously consider nettle leaf extract and cassava starch as packaging materials of the future.

Keywords: *Antimicrobial activity; Cassava Starch; Packaging Film; Nettle leaf and moisture content, solubility, and swelling.*

1. INTRODUCTION

1.1 Background and justification

The fast-changing culture, lifestyle, and socio-economic dynamism in the globe are demanding a dynamic response to the ever-changing food preference and food packaging demands. To retain the desired quality of the food throughout its life, to keep food hygienically, and to provide a good look for the food, food packaging is vital. Despite its importance, food packaging has the following limitations: waste of resources and environmental harm, and health impact (some packaging materials release toxins when heated or radiated). As a result, food packaging materials have become the concern of food packaging industries as well as the scientists in the area. The ability to protect/preserve the food without losing.

In food packaging material selection and design, the food's taste and quality throughout its shelf life is a major concern. Currently, plastics are the major packaging materials used in the food industry. Although plastics are cheap and long-lived materials that are resistant to chemical attacks, environmentally unfriendly, and not biodegradable. Besides, they are mainly made from a non-renewable source, petroleum. In addition to retaining the quality of the food, avoiding food contamination from migrated chemicals from the packaging materials is an important factor in food packaging material selection or design. (Kuorwel, 2011). The study made proved that food contamination from packaging materials causes poisoning and health risk to the consumer.

To overcome the above limitations and to enhance the packaging materials' properties such as antimicrobial and controlled-atmosphere packaging, (R.Ahvenainen, 2012) suggested novel food packaging techniques. These techniques have great capability to improve the quality and safety of food with small or no subsidiaries and preservatives, thus reducing food poisoning and allergic reactions. Due to its biodegradability and availability, starch has become a potential packaging material alternative to plastic materials. Starch can be extracted from cereal seeds (corn, wheat, and rice), roots, and tubers (potato, taro, cassava). Starch-based films exhibit physical characteristics similar to synthetic polymers: transparent, odorless, tasteless, semipermeable to CO_2, and resistant to O_2 passage. To improve the physical and functional

properties of starch films, blending with other biopolymers, hydrophobic substances, and/ or antimicrobial compounds has been proposed. (Eraricar Salleh, 2009).

In Ethiopia, the packaging material is mainly the conventional plastic-based which is based on imported. Besides, there is no published literature on novel biodegradable and antimicrobial packaging materials, especially from locally available starch materials. Therefore, this study intends to develop a packaging film from starch extracted from Cassava, a widely available and rarely industrially used root crop, which is mainly growing in the Southern part of the country. Further, to introduce an antimicrobial effect on the starch-based packaging material, the study intends to extract essential oil from Urtica Pilulifera, commonly known as stinging nettle (SN). The plant is a year-wide-growing plant from Urticaceae botanical family and is rarely used for other purposes except in a few places as a food source.

1.2 Problem Statement

Nowadays, getting food packaging materials that are environmentally friendly and healthy to the packed food is a concern of food industries and scientists in the area. The widely used plastic materials are not biodegradable. Besides, some of the packaging materials when exposed to light, heat, and radiation, they introduce toxic chemicals to the packed food. Therefore, researching for packaging materials that are biodegradable, renewable and the ones that have an antimicrobial effect is a timely issue for the food industries as well as for the researchers in the area. Thus, in this study, a cassava starch-based packaging material is developed by impregnating nettle leaf extract for antimicrobial effect.

1.3 Objectives

1.3.1 General Objective

- The general objective of this study is to developed antimicrobial packaging film from cassava starch and nettle leaf extract.

1.3.2 Specific Objective

- To produce and analyze the phytochemicals of the nettle leaf extract.
- To investigate the antimicrobial activities of the extract of the nettle leaf on major food pathogenic microorganisms

- To produce packaging film in a different starch-nettle mass ratio.
- To characterize physio-chemical properties of produced packaging film.

1.4. Significance of the Study

Since, different methods used to preserve food have different disadvantages such as degrading the nutritional value (thermal preservation) and incurring toxicity and allergic to humans (chemical preservation), nettle extract impregnated starch-based packaging helps to minimize the aforementioned problems. Therefore, developing biodegradable, renewable, and antimicrobial packaging materials at the laboratory level effectively lays a foundation for large level production that helps to make the food industry more sustainable and greener ecologically. Further, it leads one step toward a healthy food packaging technology.

2. LITERATURE REVIEW

2.1 Packaging History

In the early time, nature-based materials (NBM) such as leaf's, hollowed tree limbs, and animal skins were commonly used packaging materials (containers). With civilization, more complex containers were developed to meet a specific purpose. The large ceramic vessel called amphora was used from 1500 BC to 500 AD to ship wine and other commercial products across the Mediterranean ocean. Greek and Roman empires were pioneers to use large-scale packaging materials. During the early ages, the design and choice of the packaging material were mainly based on compatibility for transportation, its cost, and ease of handling (Raheem, 2012). Following NBM and ceramics, plastic emerged with several comparative advantages in the packaging sector. Starting with styrene, packaging plastics have evolved through several generations by incorporating improvements. Insulation and cushioning materials, foam boxes, cups, and meat trays for the food industry, and vinyl chloride were the major development before the 19th century. In the 20th century, more plastic developments have come. In addition to petroleum-based plastics, wood pulp was introduced in 1900.

Figure 2.1 Some Examples of Packaging Material

2.2 The Role of Food Packaging

The principal roles of food packaging are (i) to preserve or protect food products from outside influences (light, oxygen, moisture, microbes, mechanical stresses, and dust) and damage, (ii)

to contain the food, (iii) to provide consumers with ingredient and nutritional information and (iv) to make distribution easier (Kenneth marsh, 2007). In addition to the aforementioned uses, packaging has several requirements such as (I) must provide adequate information on its labeling, (ii) must be convenient to the consumer, e.g., easy opening, re-closable lids and a suitable dosing mechanism, and (iii) must have good marketing properties, reasonable price, technical feasibility (e.g., suitability for automatic packaging machines, seal ability), suitability for food contact, low environmental stress and suitability for recycling or refilling. A package has to satisfy all these various requirements effectively and economically. Some requirements and demands are contradictory to each other, at least to some extent. For these reasons, a modern food package should be optimized and integrated effectively with the food supply chain (R.Ahvenainen, 2012).

Today, the ways food products are produced, distributed, stored, and retailed are dynamically changing. The dynamic food packaging is requiring continuing change for improved safety, quality, shelf-life for packaged foods, and freshness of the foods. This, in turn, is placing greater demands for the improvement of food packaging materials. It is to be noted that preserving food without or with the least additives and preservatives helps to avoid associated allergies and food poisonings (R.Ahvenainen, 2012).

2.3 Food Born Bacteria

For food and health scientists, foodborne bacteria are a serious concern. It is expected that antimicrobial packaging methods can reduce the risk by controlling the growth of microorganisms in foods (Ferede, 2014). Depending on the type of raw materials, production methods, handling hygiene, and processing, the types of bacteria in foods are different. In the food, spoilage bacteria might induce reactions that provoke changes in flavor, odor, color, or other sensory properties. In addition to spoilage bacteria, there are pathogenic microorganisms that can cause illness when contaminated foods are consumed, even without bringing changes in sensory properties. However, today, different methods help to delay these processes and hence prolong the shelf-life of the foods. These methods mainly involve alteration of the intrinsic factors such as pH and water activity, or extrinsic factors such as storage time and temperature. Every bacterium found in foods has its specific nutritional requirements and is

predictably affected by environmental conditions. (Jay Loessner & Golden, 2005) Two of the common pathogenic bacteria in foods are *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* (Jay Loessner & Golden, 2005). In experiments, these bacteria are often used as a standard for Gram-negative and Gram-positive bacteria, respectively. They were therefore selected as test organisms for the experiments. Both bacteria can cause serious illness, and food contaminated with *E. coli* and *S. aureus* represents a risk to human health.

2.3.1 *Staphylococcus Aureus*

S. aureus is a facultative anaerobe, gram-positive cocci that can cause a range of illnesses in humans (Antonio Bevilacqua, 2017). It is a foodborne pathogen, and risky to public health. It is a bacterium that can grow in many different environments, as it tolerates a wide range of temperature, salt concentration, and pH. Although it can grow at wide ranges of temperature (7-48°C) and pH (6.0-7.0), the optimum ranges are 7-48°C and 4.0-9.8, respectively. Regarding salt concentration, most strains of the bacterium tolerate concentrations up to 10%, and few species can grow up to 20% (Ferede, 2014).

In experiments, *S. aureus* is used as the standard for Gram-positive bacteria. It is also a good indicator for hygiene in food production where human handling is involved. As 20-30% of humans are constantly carrying the bacterium on their skin and in their mucosal membranes, there is a major risk for contamination with the bacterium from hands that handle food products if they do not wear gloves. Although the bacterium itself is heat intolerant, it produces heat-resistant enterotoxins in the food. Food poisoning by *staphylococci* is recognized by acute symptoms including pain in the stomach, diarrhea, and vomiting. Being a common pathogen in the world, in different regions such as the European Union, staphylococcal toxins are considered the major cause of food-related illness. Thus, developing methods to limit or suppress the growth of *S. aureus* and its toxin production in foods is one of the attention areas in studies associated with food hygiene (Antonio Bevilacqua, 2017).

2.3.2 *Escherichia Coli*

E. coli is a rod-shaped, facultative anaerobe and gram-negative bacterium that belongs to the Enterobacteriaceae family. It is part of the normal flora in the intestinal tract of warm-blooded animals and can be used as an indicator organism for fecal contamination of both foods and

water (Eker, 2016). The bacterium is common in the intestine. So, it can survive a pH 2 (it must pass through the acidic environment in the stomach to reach the intestine). However, its ideal pH range for growth is 5-9. For survival, however, it can survive also in most pH levels. It can grow from the 7-48°C temperature range with an optimum temperature of 37°C (Jay Loessner & Golden, 2005). Therefore, human body temperature is favorable for this bacterium's growth.

Although, *E. coli* is a part of the normal intestinal macrobiotic in humans and other mammals, some of its strains are potentially pathogenic in humans (Baker and Pariz Maluta , 2014). These strains might cause different illnesses among which serious foodborne diseases are common. Like *S. aureus*, it does not form spores, which means that it does not survive at high temperatures and its toxins are produced inside the consumer. Thus, it is enough to inactivate the vegetative cells in the food to avoid its poisoning.

2.3.3 *Salmonella Typhi* (*S. Typhi*)

Salmonella is one of the leading causes of bacterial foodborne disease in industrialized as well as developing countries even though the incidence seems to vary between countries. (Ferede, 2014). In Ethiopia, because of the limited studies, lack of coordinated epidemiological surveillance systems, under-reporting of cases, and the presence of other high-priority diseases, variations of Salmonellosis are poorly known. The epidemiology of salmonellosis is complex largely because there are more than 2,500 distinct serotypes (serovars) with different reservoirs and diverse geographic incidences. Changes in food consumption, production, and distribution have led to an increasing frequency of multistate outbreaks associated with fresh produced and processed foods (Rounds, et al., 2010).

2.3.4. C. Albicans

a con-shaped, facultative anaerobe and gram-negative bacterium that belongs to the Enterobacteriaceae family.

2.4 Microbial Food Spoilage and Preservation Techniques

Food spoilage results when microbiological, chemical or physical changes occur, rendering the food product unacceptable to the consumer. Microbiological food spoilage is caused by the growth of microorganisms that produce enzymes that lead to objectionable by-products in the

food. The range of spoilage microorganisms is wide. Bacteria are responsible for some of the most rapid and evident spoilage events of proteinaceous foods such as meat, poultry, fish, shellfish, milk, and some dairy products. The growth of yeasts and molds is generally slower than that of bacteria, but the wide variety of ecological niches they can exploit, the ability to utilize a variety of substrates, and tolerance of more extreme conditions than (vegetative) bacteria makes them formidable spoilage. (Antonio Bevilacqua, 2017).

2.4.1. Preservation Techniques

In many city centers, eating and drinking in public institutions, like Hotels, Restaurants, and Snack bars is a common method in many countries. This prepares, handles, and serves a large number of food and drink to large groups of persons in a short period implying a possible risk of infections if sanitary and hygienic norms are not strictly followed. The world health status review indicates that the health problem of developing nations is mainly linked to inadequate sanitation. Better education of food industry workers in basic food safety and restaurant inspection procedures may prevent cross-contamination. Food handling errors can lead to outbreaks. Improvements in farm animal hygiene, in slaughter plant practices, and in vegetable and fruit harvesting and packing operations may help prevent salmonellosis caused by contaminated foods (Ferede, 2014).

2.5 Antimicrobial Packaging Film

Starch, protein, lipid, and polylactate are some of the materials used to prepare packaging materials. Selecting packaging material requires considering several things such as the interaction of the material with an antimicrobial agent as well as the interaction with food material. For instance, starch-based materials are biodegradable without having any interaction effect with the food material (Antonio Bevilacqua, 2017). However, these materials alone lack some important features such as extended shelf life, especially to fit with the current need for longer food storage time and distribution distances. To overcome this and other limitations, antimicrobial properties on these packaging materials can be incorporated by adding different natural extracts or chemical preservatives. The incorporation of the antimicrobial agent into starch-based film extends the bacterial lag phase (which is the cause for food spoilage), slowing the growth rate of micro-organisms.

When selecting antimicrobial agents, several factors such as the interaction between the polymer matrix and the active agent, the presence of other functional additives in the formulation, the type and properties of the packaged food and their effectiveness against the target microorganisms and so on must be considered. However, antimicrobial (AM) agents are environmentally sensitive. So, they require proper conditions such as temperature and pressure (Eraricar Salleh, et al., 2009).

2.6 Biodegradable and Active Packaging

Nowadays, plastic which is made from non-renewable material is non-biodegradable and is being massively used for packaging and other uses (M. Jawaid, et al., 2015). Thus, it has an enormous environmental impact. To overcome this and other limitations of plastic-based packaging materials, renewable and biodegradable materials such as biomass-based materials (e.g., starch, protein, and lipids) have become potential alternatives. Besides, the technology to produce plastic-based packaging material is more costly than that of renewable one Since direct addition of antimicrobial substances into food formulations or onto food surfaces may not be sufficient to prevent the growth of microorganisms (pathogenic and spoilage) as antimicrobial substances applied could be partially inactivated or absorbed by the food (M. Jawaid, et al., 2015), the use of active bio-based films which is biodegradable and renewable is a promising alternative. Although most biodegradable films are made of edible crops, they are widely available, low cost, and can be grown in wide ranges of climate conditions.

2.7. Raw Materials for Production of Antimicrobial Films

For the production of packaging films, the main raw materials are starch, plasticizers, and AM agent. The details of these materials are given in the following section.

2.7.1. Starch

Starch a white, granular, and organic chemical that is produced by all green plants. Starch is a soft, white, tasteless powder that is insoluble in cold water, alcohol, or other solvents. The basic unit of the starch molecule is ($C_6H_{10}O_5$). Starch is a poly saccharide comprising glucose monomers joined in α 1,4 linkages. The simplest form of starch is the linear polymer amylose; amylopectin is the branched form (Baldwin, et al., 1995). Starch is a semi-crystalline, very hydrophilic material. The amorphous and crystalline phases affect the physical

and chemical properties of starch-based films such as the mechanical and gas barrier properties. Films manufactured from starch-based materials have better gas barrier properties (when dry) than synthetic polymer films but their mechanical properties are poor. Starch can be derived from tubers and cereals, such as cassava, potatoes, maize, yam, rice, etc. Starch is one of the naturally occurring biopolymers, which are economical biodegradable resource and has easy availability. Of late, starch has received extensive attention in packaging industries for producing commercial thermoplastic polymers (Kenneth marsh, 2007).

2.7.2 Cassava

Cassava (Manihot esculenta Crantz) is a perennial with starchy tuberous edible root and is one of the most important and highly exploited starchy foods in the world. It is cultivated throughout tropical world. It is the fourth most important energy source crop for farmers in tropics after rice, wheat and sugar, consumed by up to a billion people globally (FAOSTAT, 2010). It is a major source of calories to more than 250 million people in the sub-Saharan Africa and 600 million people globally (Obiero et al., 2007). In Ethiopia cassava used as both food security crop and source of income. Currently the use of cassava and its by-products is not mainly restricted to human and animal consumption, but also to serve various industrial needs, including bio fuel production, waxy starch, bio plastics, glue, textile and paper (Saelim et al., 2009). The average total coverage and production of cassava per annum in southern Ethiopia is 4942 hectares with the yield of 53036.2 tones which implies the average productivity of cassava in Ethiopia is not more than 10 tons per hectare (Snnpr, Boa, 2000). It can be transformed into different forms and stored for several years. It is the third most sources of calories to tropical area. Cassava multiplied mainly by stem cutting (vegetatively) that show slow process, affected by season compared to grain crops (Santan et al,.2009). This crop affected by diseases like cassava mosaic disease (Pheneas and James, 2007). Thus, infections transmit from one generation to other through cutting, which contributes to the spread of diseases that leading to poor yields in successive seasons (Roca and Mroginski, 1991). This is also a major limitation in germplasm maintenance and exchange of materials across borders. The multiplication rate of cuttings is also very low compared to grain crops, which are propagated by true seeds. Other challenges with the cuttings include high perishing ability since they dry up within a few days,

high handling and transport costs and inconvenient weight and bulk of the material (Escobar et al., 2006).

2.7.2.1 Proximate composition

The cassava storage root is essentially a carbohydrate source. Its composition shows 60-65% moisture, 20-31% carbohydrate, 0.2 to 0.6% fat%,1-2 percent crude protein and a comparatively low content of vitamins and minerals and contain no trypsin inhibitor, but create a problem due to presence of cyanide which is removed by post-harvest treatments and cooking (Fasuyi, 2005). Though the protein content is said to be low, the quality of cassava root protein is however, fairly good as far as the proportion of essential amino acid as a percentage of total nitrogen is concerned. The main amino-acids present in cassava proteins are arginine histidine, isoleucine, leucine and lysine. Sulphur containing amino-acids are deficient particularly lysine, methionine, tryptophan, cysteine and cystine. These are limiting amino- acids in the root. The mineral content of the dry bark is higher than that of the cortex. Calcium values in the whole root range from 15-129 mg/100 g, while phosphorous values are approximately 100 mg/100 g (Chijindu and Boateng, 2008). Vitamin C content of raw roots range from 38.5-64.6 mg. drying reduces the vitamin C content apparently, with values going down to 2-13 mg/100 g. Even though the roots of cassava contain significant amounts of vitamin C in its raw form i.e., about 38.5-64.6 per 100 g fresh weight (Onwueme, 1978; Ngudi et al., 2003), the thiamine, riboflavin, and niacin contents are not as high. Large proportions of these nutrients have been reported to be lost during processing. All of these should be taken into account in cassava-processing in order to retain as much as possible of these nutrients.

2.7.2.2. Cassava Starch

Starches are now made in many countries from many different starchy raw materials, such as wheat, barley, maize, rice, sweet potatoes, cassava, sago palm and waxy maize. Although they have similar chemical reactions and are usually interchangeable, starches from different sources have different granular structures which affect their physical properties. Unlike another tuber starches, extraction of starch from cassava is simple and the isolated starch is pure white in color and relatively free from other chemical impurities (Tetchi et al., 2007). Starch granules in storage tissues can vary in shape, size and composition. The shape and size of the granules depend on the source, which allows one to identify the botanical source of the starch by

microscopic examination (Narayana et al., 2002). Cassava starch granules are spherical in shape, exhibiting normal particle size distribution (12.71 μm to 14.34 μm) and A type crystallinity. The proximate composition of the starches on dry weight basis were found to be 0.001 - 0.01% protein, 0.08 - 0.11% fat, 0.03 - 0.1% ash, and 85.7% - 87.5% starch (Hoover 2001; Paulos et al., 2009).

2.7.2.3. Production Process of Cassava Starch

The cassava starch production process is illustrated in Figure 2.3 and the following production processes are carried out.

- **Cleaning and screening**: - All foreign materials are removed like Stones tops. The skin is peeled-off and the cassava is cut into small pieces. Screening separates starch from pulp. The pulp can be reground and a second extraction performed to obtain a total starch yield of 12-19%, based on raw cassava.
- **Grinding** The suspensions are ground in a mass collider to form slurries. After grinding dilution with water (10 –200 %) in the presence of 0.02% sodium desulphated to prevent enzymatic browning.
- **Removal water from starch** the starch is extracted by filtering the starch solution from the fibrous part and washed several times to remove the impurities. Filtration on rotating vacuum drum filters out puts water content 38 – 40 %.
- **Drying** The starch is dried in air 140 –160 °C (temperature < 175 °C chemical changes of starch), output temperature 46 –49 °C and ground to 150 μm in size.

  Finishing The final product is stored bulk silos and packing in bags or big bags.

Figure 2.1 Cassava starch production process

Cassava starch can be used as a raw material for biodegradable film production. The natural biodegradable starch films are based on gelatinization that occurs above 70°C in the presence of excess water. The gelatinized starch, when cooled, forms a thin film similar to cellulose film that is resistant and transparent, which are desirable characteristics for fruits and vegetables packaging. However, improved functional properties are required in order to make these

biodegradable films commercially available. The use of modified starches could be an alternative method for improving the functional properties of these films. Oxidized starches are used in the cellulose and

paper industry to increase paper resistance. Oxidized starch also promotes viscosity reduction, generates higher stability, increased clarity, improved adhesion properties, and has a better ability to form clear, continuous, and homogeneous films which have less tendency to shrink and crack. Oxidized starch produces a hard and strong film with promising features for use in food preservation, which have been reported by Zanudio-Flores et al. and Thunwall et al. When considering the film forming ability, besides the advantages of oxidized starches, due to their strong hydrophilic nature related with the presence of carboxyl groups, the films made with them are more soluble in water when compared with those made with the unmodified parent starches. The water vapor permeability should be defined as the water vapor transmission rate based on the unit area and the thickness of the material as well as the partial pressure difference between the two surfaces under specific conditions of temperature and relative humidity.

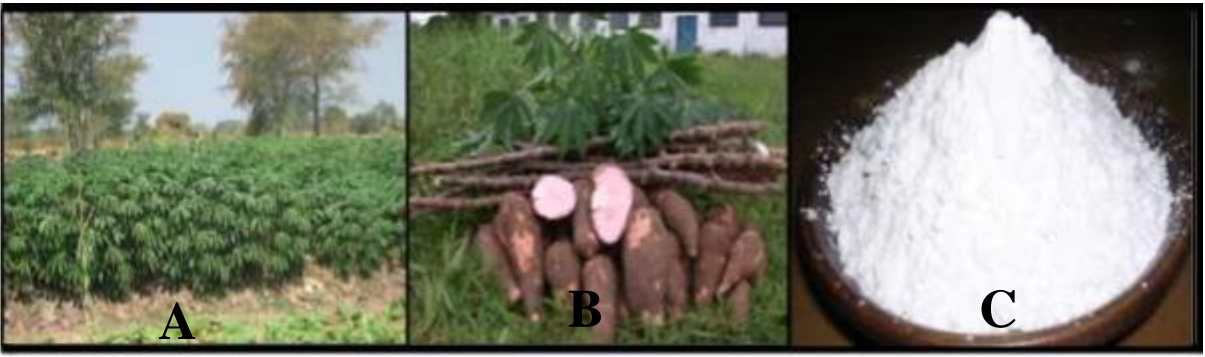


Figure 2.2 Cassava Tree (A), Cassava root (B), Cassava Starch (C)

2.7.3. Plasticizers

Plasticizers are relatively low molecular weight compounds that can be co-polymerized with the polymer or added to it to reduce the intermolecular force and increase the mobility of the polymeric chains (Garcia, et al., 2000). Plasticizers are usually mixed with biopolymers such as starch-based materials to aid processing, improve film flexibility and lower the glass transition temperature of the polymer (Brody, 2005). The type and amount of plasticizer, its number of functional hydroxyl groups, and its compatibility with the polymer may affect the properties of the resultant films. Films made from pure polymers tend to be brittle and

frequently crack when dried, the addition of food-grade plasticizers to the film-forming solution mitigates these issues (Da Roz, et al. , 2006). Examples of plasticizers that are commonly used with biopolymers include polyols such as glycerol, sorbitol, and mannitol, monosaccharides such as fructose, glucose, and mannose, and polyethylene glycol (Brody, 2005). Water is another important plasticizer for bio-based films although moisture may affect the film properties (Van Soest, et al., 1997)). Water can be added to a starch-based film to break its native granular structure and hydrogen bonding. When a high concentration of plasticizer is used, the mechanical strength, barrier properties, and rigidity are decreased. According to (McHugh, et al., 1994), plasticizers that are added to film formulations decrease the film density and increase the free volume of the film matrix which, in turn, increases the permeability of the films to gases and vapors. For example, glycerol molecules interfere with starch packing, thus decreasing intermolecular attraction and increasing polymer mobility (Kuorwel, 2011).

2.7.4. Antimicrobial Agent (Nettle leaf's)

Nettle (Urtica) has a long history as herbal medicine and nourishing food (soups, salads, and curry). Then, it has been used as industrial crops (textile, pharmaceutical etc.) (Guil-Guerrero et al., 2003). Up to four years, the nettle crop can productive, however, if grown longer, weed infestations tend to increase and yields reduce. Extensively nettle can be grown for 10 – 15 years, or even without a time limit (Vogl C.R., 2003).

Although more than 1000 plant species of the nettle family (Urticaceae) are known across the world, the most common is Urtica dioica Land and Urtica urens L. The former has different names such as stinging nettle or fibber nettle or common nettle. It is characterized by a height ranging from 30 – 150 cm and has a light greenish color. Its Leaf's are ovate, rarely lanceolate acuminate. The male and female flower heads are similar in form, branched. The female flowers have a purplish stigma.

- **Urtica urens L**: - known as annual nettle, dwarf nettle, small nettle, and dog nettle or burning nettle. It is an annual herb, monoecious, 10 – 60 cm in height, clear green in color. Leaf is ovate and deeply serrate. The male and female flowers are numerous, centrally globous, or sparsely hispid on the back. It reminds the common nettle in habit but has smaller leaf and short flowers (G, 2003).

- **Urtica dioica L: -** often called common nettle or stinging nettle, fibber nettle. It is a perennial herb, 30 – 150 cm in height, lightly green in color, usually dioecious. Leaf are ovate, rarely lanceolate acuminate. The male and female flower heads are similar in form, branched. The female flowers have a purplish stigma.

Figure 2.3 Nettle leaf

Nettle can be used for consumption and medical purposes. Its nutritional value is very high, much higher than other vegetables and herbs usually grown in the gardens.

Table 2. 1 Different composition of nettle leaf.

NO	Source of Vitamins and minerals	Amounts
1	Vitamins C	20-60 mg/100 gram of nettle
2	Vitamins K	0.16-0.64 mg/100 gram of nettle
3	Calcium	0.1 mg
4	Iron	0.09 mg
5	Magnesium	0.085 mg
6	Phosphorus	0.0075 mg
7	Potassium	0.065 mg
8	Sodium	0.05 mg

Source: - Suppakul et al., 2003; Gibis and Rieblinger, 2011; Lopez et al., 2012.

Nettle leaves contain nine carotenoids. Lutein and lutein isomers, β-carotene are the basic carotenoids in nettle leaf's (Guil-Guerrero et al., 2003). Also, important substances are amino acids, glucokinase, and chlorophylls (Graff A.,et al., 2011).

2.7.5. Antimicrobial Activity of Nettle

Nettles possess noticeable antimicrobial activity against Gram-positive and Gram-negative bacteria compared to the standard and strong antimicrobial compounds such as miconazole nitrate, amoxicillin-clavulanic acid, ofloxacin, and netilmicin (Gulçin, I et al., 2004). Different fractions of various Urtica species (Urtica ssp.) have been studied to determine their antimicrobial activity and the result is given in Tables 2.2and 2.3.

Table 2.2 The activity of *Utrica ssp*. Against microorganism.

Urtica.Spp. Extract	Microorganisms	Location
U.dioica L. Ethyl acetate Extract	Aeromonal hydrophila	Iran
	S.typhi	
	S.aureus	
	B. cereus	
	E. coli	
U.dioica L. water Extract	salmonella spp	Iran
	proteus spp	
	S.aureus	
	p. aeruginosa	
	E. coli	
U.dioica L. root aqueous Extract	Aeromonal hydrophila	Turkey
	S.typhi	
	S.aureus	
	B. cereus	
	E. coli	
U.dioica L. Ethanol Extract	Aeromonal hydrophila	Iran
	S.typhi	
	S.aureus	
	B. cereus	
	E. coli	

Source: - Suppakul et al., 2003; Gibis and Rieblinger, 2011; Lopez et al., 2012.

Table 2.3 Activity of *Urtica spp*. Against microorganisms: minimal inhibitory concentrations

Urtica spp. Extract	Micro organisms	Minimal Inhibitory Concentration (MIC)	Location
U. dioica L. ethanol extract	Bacillus subtilis	36.21 mg/mL	Serbian
	Escherichia coli (food-origin)	36.21 mg/mL	
	Escherichia coli (urne-origin)	72.43 mg/mL	
	Pseudomonas aeruginosa	72.43 mg/mL	
	Lactobacillus plantarum	72.43 mg/mL	
U.dioica L. hexane extract	Staphylococcus aureus MRSA	66.66 mg/mL	Iran
	Bacillus cereus	16.66 mg/mL	
	Bacillus spizizenii ATCC 663	16.66 mg/mL	
	Vibrio parahaemolyticus	66.66 mg/mL	
U.dioica L. chloroform extract	Bacillus cereus	33.33 mg/mL	
	Vibrio parahaemolyticus	4.16 mg/mL	
U.dioica L.	Acinetobacter calcoaceticus	33.33 mg/mL	
U.dioica L. ethanol extract I	Bacillus cereus	8.33 mg/mL	
	Vibrio parahaemolyticus	16.66 mg/mL	
	Saccharomyces cerecisiae	2.08 mg/mL	
U. dioica L ethyl acetate extract II	Vibrio parahaemolyticus	0.13 mg/mL	
U.dioica L. methanol extract	Acinetobacter calcoaceticus	16.66 mg/mL	
U.dioica L. butanol extract	Escherichia coli	16.66 mg/mL	
	Bacillus subtilis	8.33 mg/mL	
	Staphylococcus aureus MRSA	16.66 mg/mL	

Source: - Suppakul et al., 2003; Gibis and Rieblinger, 2011; Lopez et al., 2012.

The results presented in Table 2.2 show that the antimicrobial activities of various nettle extract obtained using different solvents on different microorganisms. As can be seen, some nettle extracts show activity at a concentration of 72 mg/mL and others at 1 g/ml. These differences appear excessive, and the results should therefore be viewed with caution. Such variations may be associated with the location of the plant habitat and climactic conditions, as well as being due to the use of different extraction techniques and evaluation methods. Despite their

significant differences, however, the results of these studies show that nettle plants exhibit antimicrobial activity against a wide spectrum of microbial strains, often isolated from foods of low microbiological quality. A study by (Kukric, 2012) revealed that nettle extracts had inhibitory effects on various Gram-positive and Gram-negative bacteria including *Bacillus subtilis, Lactobacillus Plantarum, Pseudomonas aeruginosa*, and *Escherichia coli.*

2.8. Extraction Technique and Recent Technology.

Extraction is an important step for the separation, identification, and use of valuable compounds from different plants The choice of an acceptable technique to obtain maximum yield and highest purity varies according to the nature of the target compound. Three main methods have been identified for the extraction of the oil/fats/lipids: (i) Mechanical Extraction, (ii) Chemical Extraction (Solvent Extraction), and (iii) Biological Extraction. The most commonly used commercialized methods of oil extraction are solvent extraction and mechanical extraction (Gisila, 2018).

2.8.1. Mechanical Extraction

Mechanical extraction of the oil is accomplished by exerting sufficient force on the confined seed. Under this condition, pressure is high enough to rupture the cells and force oil from the seed to “escape.” Extraction is accomplished by compressing the material in a container that has small perforations, either round or slotted, that allow the liquid component to leaf. This operation may be done in either a batch process or a continuous process. The advantages and disadvantages of mechanical extraction process is given in Table 2.4.

Table 2.4 Advantage and dis advantage of mechanical extraction

Mechanical Extraction	
Advantage	**Dis-advantage**
Natural oil is more sought-after extraction	High solid residue
No potential for solvent contamination	Time and labor-intensive
Low operation cost	Requires experienced Operators
	Relatively low oil yield
	High kernel moisture content

A batch process involves extracting the oil from one batch of seeds completely, before cleaning out the container and starting over. In this operation, the seeds are placed into a container with a moveable surface, most often the top. Then hydraulic pressure is applied to this moveable surface, which compresses the material, forcing the liquid component of the material to escape, usually through a perforated section in the bottom. In some cases, screw pressure was used instead of hydraulic pressure. The continuous mechanical extraction of oil regardless of the size of the operation. Expeller presses range in capacity from less than 1 ton to over 50 tons per day (Bhargavi, 2017).

2.8.2. Solvent Extraction

For the extraction of polyphenols or other bioactive compounds from plant materials, water and organic solvents (ethanol, methanol, acetone, and diethyl ether) are used. Additionally, during the extraction process, the percent recovery depends mainly on the type of solvent and the extraction methods being adapted. Solvents with low viscosity have low density and high diffusivity that allow them to easily diffuse into the pores of the plant materials to leach out the bioactive constituents (Sukri, 2012). For optimal recovery of total phenolic content from fresh sample matrix to the diverse chemical structures of polyphenolics ranging from simple and free to conjugated and polymerized forms (lipophilic) might consequently affect their solubility behaviour and there is no specific or appropriate extraction solvent is recommended. The selection of solvent systems was made based on the efficiency in extracting polyphenols and other antioxidant compounds from the fresh sample matrix (Gisila, 2018). The advantages and disadvantages of solvent extraction process is given in Table 2.5.

Table 2.5 Advantage and dis advantage of solvent extraction

Solvent Extraction	
Advantage	Dis-advantage
Low energy consumption	Less efficient for polar lipids extraction
Large production capacity	Agitation is not possible
The non-soluble portion of the extracted solid remains in the thimble	Take long extraction time.

2.8.3. Steam Distillation

Steam distillation (SD) is one of the ancestral methods applied for the recovery of EOs. It is also amongst the best-suited methods to obtain high-quality of EOs. For the past decades, large quantities of the EOs of herbs and spices for commercial use have been usually extracted by SD (Burt, 2004).SD is suitable to extract heat-sensitive components (*e.g.*, volatiles), and purify them by the application of steam. Distilled water is used as the steam source for this technique. The extraction process works in a way that the plant material containing the compounds of interest is distilled at a temperature below their boiling point and the application of steam would volatile them at a temperature lower than 100 °C, at atmospheric pressure (Rojas, J., & Buitrago, A., 2015). The volatile compounds are driven by the steam, which passes through the aromatic plant from the bottom of the alembic to the top. The steam destroys the structure of plant cells, releases the contained molecules, and takes away most of the volatile components. The oil is then carried by the steam out of the column and into a cooling system (condenser) where the steam is condensed back to a mixture of water-oil liquid. The advantages and disadvantages of Steam extraction process is given in Table 2.6.

Table 2.6 Advantage and dis advantage of steam extraction

Steam Extraction	
Advantage	**Dis-advantage**
Maximum yield is obtained	High Consumption of Energy
Thermal decomposition can be avoided	Long extraction time
No solvent required	
Low production cost	

Source: (Halne R. R., 1966).

2.8.4 Ultrasonic Extraction

Ultrasound, the term used to describe sounds ranging from 20 kHz to 1 GHz, is usually generated by a transducer that converts mechanical or electrical energy into high-frequency vibrations. (Pecorino-Issartier et al., 2013) reported that the enhancement of extraction efficiency of organic compounds using ultrasound is attributed to a phenomenon called

cavitation produced in the solvent by the passage of an ultrasonic wave. They found that cavitation bubbles are produced and compressed during the application of ultrasound, allowing higher penetration of the solvent into the raw plant materials and intracellular products released by disrupting the cell walls. Ultrasound has been shown to aid extraction in several plant materials by significantly reducing extraction time and increasing maximum extraction yield, respectively. Recent studies have shown that ultrasound-assisted extraction can enhance extraction efficiency through acoustic cavitation and some mechanical effects. Acoustic cavitation can disrupt cell walls facilitating solvent to penetrate the plant material and allowing the intracellular product release. Another mechanical effect caused by ultrasound may also be the agitation of the solvent used for extraction, thus increasing the contact surface area between the solvent and targeted compounds by permitting greater penetration of solvent into the sample matrix (Zhang, et al., 2006). The advantages and disadvantages of Ultra-Sonic extraction process is given in Table 2.7.

Table 2. 7 Advantage and dis advantage of ultra-sonic extraction.

Ultra-Sonic Extraction	
Advantage	**Dis-advantage**
Lower extraction time	Energy-intensive
Higher contact surface area between the solvent and targeted compounds	Filtration required
Maximum extraction yield	Solvent intensive

Source: (Haln R. R., 1966).

2.8.3 Microwave Extraction

Microwaves are high-frequency electromagnetic radiation with a typical wavelength of 1mm to 1 m. Many microwaves, both industrial and domestic, operate at a wavelength of around 12.2 cm (or a frequency of 2.45 GHz) to prevent interference with radio transmissions. Microwaves are split into two parts, the electric field, and the magnetic field component. These are perpendicular to each other and the direction of propagation (travel) and vary sinusoidally. Microwaves are comparable to light in their characteristics. They are said to have particulate character as well as acting like waves. The ‘particles' of microwave energy are known as photons. These photons are absorbed by the molecule in the lower energy state (Eo) and the

energy raises and electrons to a higher energy level (Ei). Since electrons occupy definite energy levels, changes in these levels are discrete and therefore do not occur continuously.

The energy is said to be quantized. Only charged particles are affected by the electric field component of the microwave. If the charged particles or polar molecules are free to move, this causes a current in the material. However, if they are bound strongly within the compound and, consequently, are not mobile within the material, a different effect occurs. The particles re-orientate themselves so that they are in phase with the electric field. This is known as dielectric polarization. Microwave heating has been recently used for the isolation and analysis of essential oils (Zhang, et al., 2006). Solvent-free microwave extraction (SFME) is a new technique that combines microwave heating with dry distillation at atmospheric pressure for the isolation and concentration of the essential oils in fresh plant materials. In the SFME method, there is no need to add any solvent or water if fresh plant material is used. If dry plant material is used, the sample is rehydrated by soaking in water for some time and then draining off the excess water. The advantages and disadvantages of the micro-wave extraction process are given in Table 2.8.

Table 2.8 Advantage and dis advantage of micro-wave extraction

Microwave Extraction	
Advantage	**Dis-advantage**
Moderate Investment	The operation is more complicated than others.
Loss of volatile compound is avoidable	The vessel should be cooled to avoid loss of volatile component
No provision of hazardous fumes	The solution has low Temperature

Source: (Pierce, 1987)

2.8.4. Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is one of the emerging, environmental-friendly, and ‘green’ technologies. SFE technology is recognized as an effective technique that has been used for laboratory-scale level, to preparative scale, to pilot scale, and up to larger-scale industrial commercial production, with high efficiency comparable with that of existing chemical analysis procedures (Gisila, 2018). It is possible to obtain more information on the extraction processes

and mechanisms using SFE, which is useful in the quantitative evaluation of the extraction efficiency and appropriate optimization of the process (Wai, 2001). The extracting solvent in this process is a fluid (dense gas) at a temperature and pressure above the critical values, Tc and Pc. SFE is applicable for the qualitative and quantitative identification of constituents of natural products, including heat-labile compounds. In SFE, several factors like temperature, pressure, sample volume, cosolvent addition, and flow and pressure control play a vital role during extraction by SFE. In conditions that are fairly higher than the critical temperature and pressure, supercritical fluids exhibit properties intermediate to those of the liquid and gaseous phases. This fluid possesses properties bounded by the extremes of the gaseous and liquid states, and these properties could be adjusted with alteration of the applied pressure and temperature (Raynie, 2010). The advantages and disadvantages of the supercritical fluid extraction process are given in Table 2.9.

Table 2.9 Advantage and dis advantage of supercritical fluid extraction.

Supercritical Fluid Extraction	
Advantage	**Dis-advantage**
No filtration is required	Many parameters to optimized
It is an automated system	Technically complex
No hazardous wastes	Suspectable for loss of volatile substances
Fast extraction	Risk of system clogging

Source: (Haln R. R., 1966).

3. MATERIAL AND METHOD

3.1 Raw Material and Chemicals

The research materials for this study were stinging nettle leaf, cassava root, and glycerol. The fresh leaf of nettle leaf (*Urtica pilulifera*) and *cassava seed* were collected from Debre Birhan (130 km from Addis Ababa, Amhara regional state, and North Shoa Zone or district) and Arbaminch city, respectively. Then, species of the nettle (*Urtica pilulifera*) were identified at the department of botany at Addis Ababa University (Arat Kilo campus). Similarly, Cassava roots were bought from Arbaminch city local market and transport to Debre Berhan University, Chemical Engineering laboratories. NL (nettle leaf) powder Muller Hinton agar and broth, Sabroud dextrose agar and broth, food graded ethanol, starch and glycerol are some of the chemicals used in this study.

3.2. Equipment's

The main types of equipment used in this study are Soxhlet extractor, water bath, Electric heater, thimble, Digital weighting balance, Water bath, Beakers, Round bottom flask, peri dish, vacuum distillation, Centrifuge, air dryer, hood, filter cloth, sieve, glass bottles, thermometer, condenser, crusher, separating funnel, vacuum pump, oven, different size conical flasks, beakers, measuring cylinders, burette, micropipettes, Incubator, UV Spectro photo meter.

3.3 Methodology

3.3.1. Nettle leaves preparation & Extraction

Once the nettle leaves are collected, they have to be cleaned, dried and milled to get a powder. This involves cleaning by rubbing to remove the burning part, picking the leaves from the stem, rinsing with water and soaking in boiling water for 10 min to soften the texture and then rinsing. Thereafter, it has been dried by putting it in an air dryer at 45°C for 24 hrs. After complete drying, it was powdered by using ball mill. Finally, the sample was stored in a plastic bag at room temperature before use.

The solvent extraction method was used to extract oil from nettle leaves, using food graded ethanol as the extracting solvent. This experiment was done in the Chemical Engineering Department's laboratory, Debre Berhan University. For extraction, a Soxhlet apparatus fitted

with a 250 mL round-bottom flask and a condenser was used. The extraction was executed in a water bath 150 mL of food graded ethanol was poured into a round bottom flask and 40 grams of powdered nettle leaves was placed in a filter cloth (thimble) and inserted in the center of the extractor. When the solvent had boiled, the vapor rose through the vertical tube into the condenser which is located at the top. Thereafter, the liquid condensate dripped into the thimble that contained the sample (nettle leaves) in the center. Then, the extract trickled through the pores of the thimble and filled the siphon tube, where it flew back down into the round bottom flask. This was repeatedly continued for three different extraction times allowed (4, 6, 8 hr). Following this, the ethanol was recovered by evaporation using a water bath at 80°C and the extracted oil was collected. The extracts were filtered through Whitman filter paper (№ 1) and then concentrated in a vacuum at 40°C using a rotary evaporator. Finally, the extract was transferred to glass vials and kept at 4°C before use.

3.3.2. Preliminary Phytochemical Screening of Nettle leaf Extract.

The crude leaves extract constituents of different chemicals such as alkaloids, flavonoids, saponins, phenolic components, nettle, and glycosides as describe in Zoran et al. (2012). In this study, the major components (saponins, phenols, and tannins) of the nettle leaves extract are identified. To detect the presence of saponins, the extract was shaken with distilled water and then heated to a boil. The appearance of bubbles showed the presence of saponins. Similarly, after forming an aqueous nettle solution using distilled water, 10% aqueous iron chloride ($FeCl_3$) was added to the solution. Then, the formation of dark green color indicated the presence of tannin and phenol.

3.3.3. Preparation of Media for Antimicrobial Tests

Muller Hinton broth and Muller Hinton agar (MHA) were used for antibacterial activating testing since Muller Hinton agar is drug-sensitive. On other hand, Sabouraud dextrose agar (SDA) and broth were used for antifungal activity. To proceed with the tests, first, the media were prepared according to the instruction labeled on medium. Thus, the Muller Hinton agar medium was prepared by dissolving 38 g in 1000 mL of distilled water. The 1000 mL medium SDA was prepared by dissolving 62 g SDA in distilled water. Also, the Muller Hinton broth was prepared by dissolving 21 g in 1000 mL of distilled water. Therefore, the measurements

and calculations for medium preparation were based on these standards. Following the media preparation, to get complete dissolution, the SDA-distilled water mix was heated and shaken. After that, the amount of medium required for the experiment was calculated and measured. Then, the measured agar was covered by an aluminum foil and autoclaved at 121°C for 15 minutes. Lastly, it cooled down to 45°C, and then it was used for bacteria growth (media).

3.3.5. Preparation and Standardization of Inoculum

First microorganisms (fungi and bacteria) were refreshed to reactivate them. This was made by incubating the bacteria and fungi for 24 hours in 5 mL MHA and 5mL SDA, respectively. After refreshment, cultures of the test organisms were prepared using Chicezie (2017) procedure. Then, five morphologically identical colonies were inoculated in 5 mL of MH Broth and SD Broth into different test tubes for each microorganism. The turbidity of broth cultures was then equilibrated with similar broth to match that of half of McFarland standard (1 to 2 x 108 CFU/ml). Then, the turbidity of microorganisms was read using a UV spectrophotometer. MH and SD broths were controlled at 625 nm and the absorbance was adjusted to keep it between 0.08-0.13 by adding broth or microorganisms. It is to be noted that the absorbance 0.08 to 0.13 at 625 nm wavelength is equivalent to half of McFarland standard (1 to 2 x 108 CFU/ml). After standardizing the suspension (broth plus microbes), 200 µL bacterial suspension was diluted with 2 mL MH broth to obtain the minimum required microorganism number which is 107 CFU/ml. Thereafter, the absorbance of each microorganism read to be 0.085, 0.09, 0.13, and 0.123 for *E. coli*, *salmonella*, and *staphylococcus aureu*s, and *Candida albicans* respectively.

3.3.6. Antimicrobial Test for Nettle Extract

Anti-bacterial and antifungal activity tests were the two main antimicrobial tests used in this study. The tests were carried out for the extract by using the hole plate diffusion method (Paiva et al., 2003). The standard organisms for the test included in this study were *Staphylococcus aureus* (ATCC25923), *E. coli* (ATCC25922), and *Salmonella* (ATCC13311), and *Candida albicans* (ATCC25942). Thereafter, 20 mL (for each) MH and SD agar from the prepared agar media were poured into two plates at 45°C in a sterile condition. Then, each standard microorganism (200 mL) was added to the plate containing the agar. The bacteria (*S. aureus, E. coli,* and *Salmonella*) were added to MH agar whereas, the fungus (*candida Albicans*) was

added to SD agar. After adding the microorganisms onto agar-containing plates, the mixture was spread with the help of a sterile glass spreader and allowed to solidify at room temperature. After solidifying the prepared mix (SPM), it was holed using a sterilized inoculation loop (8 mm). A 1000 mg of the extract was measured and dissolved in 5 mL dimethyl sulfoxide (5% concentration). Afterward, this solution was diluted to get four different solutions with 200, 100, 50, and 25 mg/mL concentrations. Then, 200 µL from each solution was added into each 8 mm holed SPM using a micropipette. Next, the filled SPM was left at room temperature for 1h for diffusion. On the other hand, for comparison/benchmark, the above procedure but without nettle were repeated. It means 5% dimethyl sulfoxide without nettle extract as a negative control and standard antimicrobial (with nettle extract) as a positive control were used. The antimicrobial (with nettle extract) and the benchmark (without nettle extract) samples were incubated at 37 °C for 24 hours (for bacteria) and 28 °C for 72 hours (fungus).

3.3.7. Minimum Inhibition Concentration for Nettle Leaf Extract

The minimum inhibitory concentration (MIC) for the extract was determined by the agar dilution method (Daniel Ikhane, Kolawole Banwo, and Ogunremi Omotade, 2014). Two mL of the dilutions (200, 100, 50, and 25 mg/mL) of the extract were mixed with 18 mL of Mueller Hinton agar and poured into sterile Petri dishes to set. The surface of the agar was allowed to dry before streaking with the test organisms. Extract-free media was used as a negative control, while that containing 1 mg/mL nettle extract was used as a positive control. The plates were incubated at 30°C for 18 hours. The lowest concentration of plant extract that prevented visible growth of the organisms was taken as the MIC of the extract.

Finally, the presence or absence of growth at each concentration of nettle was examined. The MIC that completely inhibited the growth of bacteria for the test compound was recorded. All the tests were carried out in duplicate and the results were reported as the average of these replications.

3.3.8. Preparation of Antimicrobial Starch-Based Packaging Films

Once the cassava root was cleaned, peeled, chopped, and ground (using a juicer), the cassava juice was filtered with a muslin cloth. The starch inside the juice was settled for 24 hours and then the supernatant was removed. Thereafter, the settled starch was centrifuged with 3000

RPM to remove fibrous materials. Lastly, the centrifuged starch was dried in an air dryer. This cassava starch was used to prepare the film for packaging material. The films were prepared by the conventional solution-casting technique (Eraricar Salleh, et al., 2009). Three different ratios of starch/ glycerol (70:30, 80:20, and 90:10 (wt. /wt.) and antimicrobial agents (8, 9, and 10 g/mL) were used. The films were prepared with two different thicknesses: 0.5 and 1mm.

In the process of film production, first, 8% (wt./wt) aqueous cassava starch solution using produced cassava starch and distilled water was prepared. Then, by heating, the solution at 80°C for 15 min under constant stirring in a hot water bath, homogeneously dispersed gelatinized cassava starch was prepared. Thereafter, the different ratios of plasticizer (glycerol) were added into the gelatinized solution: 30%, 20%, and 10% (w/w). The heating process continued for an additional 15 min at 80 °C. Then, the solution was cooled down to 50°C. Then, nettle extract with different concentrations (8,9 and 10 g/mL) at around 50°C was added to the cooled solution. Film-forming solution (plasticized and gelatinized starch solution and nettle extract), while it was hot, was transferred into casting in glass Petri-dishes having 200 mm diameter by 15 mm depth. The glass Petri-dishes served as casting surfaces, enabling the film to have a smooth and flat surface. Since the antimicrobial agent is temperature-sensitive, the dishes were placed for four days in an oven set at 45°C for drying. In the meantime, the film without nettle extract was prepared and used as a negative control. After 4 days of drying, the films were detached from the casting surfaces and stored in a desiccator as given in Eraricar et al (2009). After preparing the films (with and without nettle leaf extract), the phytochemical activities (flavonoids, alkaloids, and saponins) and biological activity (the presence or absence of microorganisms) of the films have been evaluated.

3.3.9. Antibacterial activity Test on Films

Testing of the antimicrobial activities of the films was carried out using the agar diffusion method according to Mau-Chang Chen (1996). The edible films were cut into squares (l cm x l cm) and were placed on Mueller Hinton agar plates and potato dextrose agar plates. These plates had been previously seeded with 0.1 ml of inoculum containing 1.5 X 108 CFU/ml and 104 - 105 CFU/ml of test bacteria and fungus; respectively. The plates were incubated at 37ºC for 48 hours and at 27ºC for 72 hours for bacterial and fungal cultures; respectively. The plates were

visually examined for zones of inhibition around the film discs, and the size of the clear zone diameter was measured at two cross-sectional points and the average was taken as the inhibition zone (Mau-Change Chen, et al., 1996).

3.4. Antimicrobial Packaging Films Characterization

In this section, physical properties (moisture, solubility, and swelling power) of AM packaging film were characterized.

3.4.1. Moisture Content of AM Packaging Films

The moisture content was determined using the method developed by Zinash Assefa and Shimelis Admassu (2013). The sample was heated for 25 min at 110°C. The experiment of moisture content was performed in 3 replicates and the average was then recorded. The moisture content (MC) of the starch-based film was determined gravimetrically using equation 3.1.

$$MC(\%) = \frac{Mf - Mi}{Mi} * 100 \quad 3.1$$

Where: MC-Moisture content of the starch-based film, Mf *is* the final moisture content of the starch-based film and Mi is the initial moisture content of the starch-based film.

3.4.2. Solubility and Swelling Power of AM Packaging Films

Similarly, the solubility and swelling power of the starch were determined using the method adapted by Zinash Assefa and Shimelis Admassu (2013). Thus, one gram of starch was transferred into a dried test tube and weighed (W_1). Then, within 50 mL of distilled water. The starch was heated at 40, 50, 60, and 70°C for 1 h while gently stirring and by rapid cooling to room temperature then centrifuged at 3,500 rpm for 30 min. Then five ml of the supernatant was dried to a constant weight at 120°C. The residue obtained after drying the supernatant represented the amount of starch solubilized in water. The solubility of starch was calculated in percent. The residue obtained from the experiment after centrifugation with water was transferred to a clean, dried test tube and reweighed (W_2) and the percentage swelling was calculated as

$$\text{Swelling power of starch}(\%) = \frac{W2 - W1}{W1} * 100 \quad 3.2$$

Where: W_1-Starch in a dried test tube, W_2 - Residue after centrifugation.

3.4.3. Mechanical Property Test of Antimicrobial Packaging Films

Mechanical properties including tensile strength, and elongation were measured using a texture analyzer (Model CD-500, BBOYD instrument, 2003, China). Uniform film specimens of 100 mm by 5 mm size were prepared from the starch-based film samples. The film strips were placed in the pneumatic grips of the texture analyzer, which were set at an initial separation of 50 mm. The crosshead speed was set at 50 mm $min^{-1.}$ Values were reported.

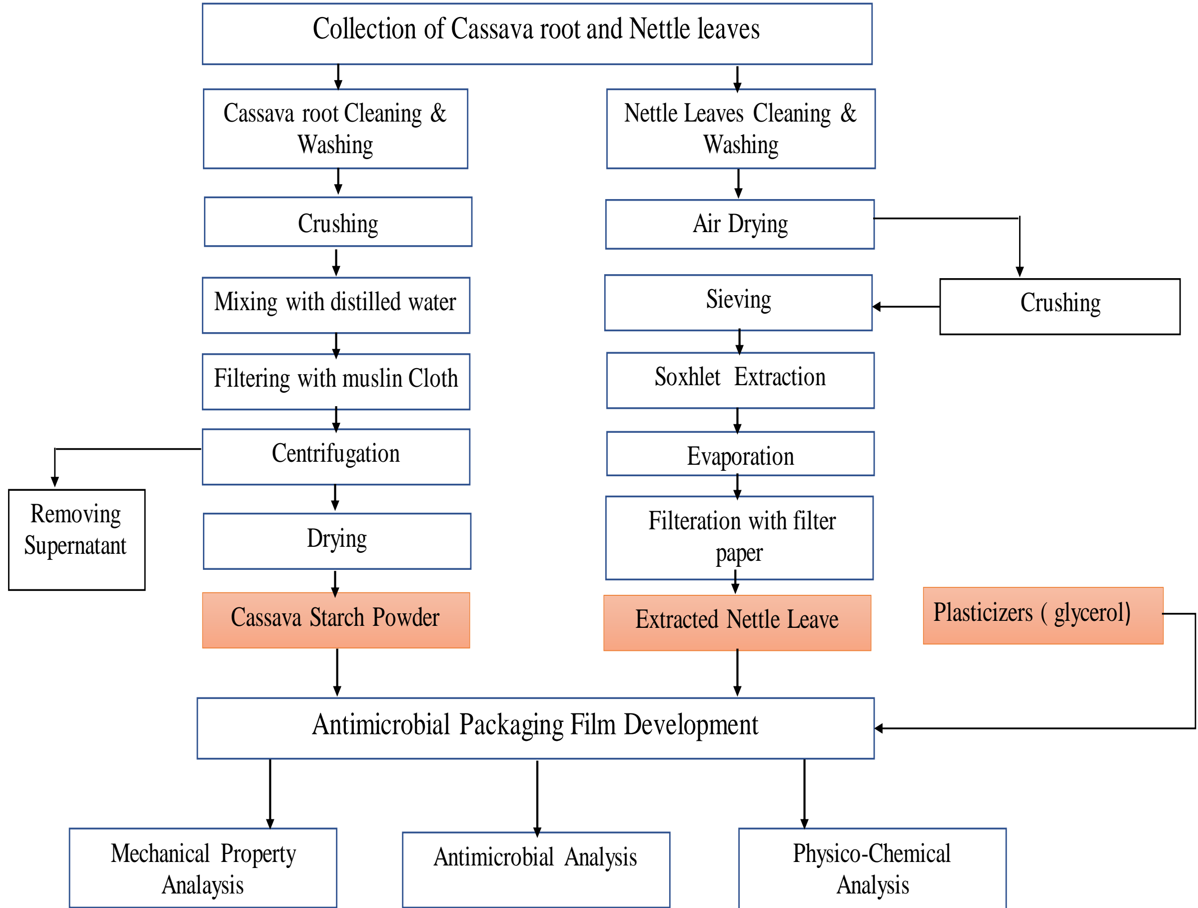


Figure 3.1 General Experimental Framework.

4. RESULT AND DISCUSSION

As discussed in the methodology, (i) the phytochemical constituents of the nettle leaf, antimicrobial activities of the nettle leaf extracts and (iii) mechanical strength analysis of packaging films are determined. The detail results of the analysis are given in the following sections.

4.1. Phytochemical Constituents of nettle leaf

Saponins, phenols, and tannins were found in crude extracted from nettle leaf using ethanol crude and the method adopted by Astuti (2014). Tannin and phenols were determined by Braymer's test method. The test gave a dark green color which indicates the presence of phenols and tannin. For saponins, the method adopted by Eldhose (2013) was used and a miss creamy froth color appeared that indicates the presence of saponins. Both dark greenish and creamy colour of the constituents is given in appendix C**.** The presence of these phytochemicals in the nettle leaf indicates as the nettle extract is the potential for inhibiting the growth of microorganisms (Astuti, 2014).

4.2. Antimicrobial Activities of the Extracts

The antimicrobial activities of films were evaluated using the agar diffusion method. The growth inhibition of test microorganisms *E. coli*, *Staphylococcus aureus, S. typhi, and Candida albicans* by nettle leaf extract at four different concentrations (200 mg/mL, 100 mg/mL 50 mg/mL, and 25 mg/mL) are shown in Table 4.1.

Table 4.1 Inhibition Diameter of Microorganisms at Different Concentrations.

Concentration of NL	Inhibition diameter(mm) for extracts			
Extract(mg/mL)	***S. typhi***	***E. coli***	***S. Aureus***	***Candida albicans***
200	35	25	30	27
100	30	21	26	20
50	25	18	20	16
25	22	17	18	12

The bacteria show different degrees of susceptibility to the staining nettle leaf; it is specified by their respective clear inhibition zones. As shown in Table 4.1, for all bacteria, as the

concentration of the nettle leaf increases the inhibition diameter increases. With increasing nettle leaf concentration from 25 mg/mL to 200 mg/mL. The inhibitory activity was measured based on the average diameter of the clear inhibition zone. If there is no clear zone surrounding the bioactive, it is assumed that there is no zone of inhibitory effect. The inhibitory effect of the nettle leaf against *Escherichia coli and Candida albicans* increases with increasing their concentration. Among the four tested microorganisms, *Escherichia coli* is the most resistant bacteria and *Candida albicans* also resisted fungi. When compared the resistance of the four tested target microorganisms against the nettle the first resistant was *Candida albicans*, the second was *Escherichia coli,* the third one was *Staphylococcus aureus*, and *Staphylococcus typhi*. This comparison was done based on the diameter of the inhibition zone. This means with the same concentration of the nettle leaf; the inhibition zone is maximum against *Staphylococcus typhi* and it is minimum for *Candida albicans.*

The ethanol extract of the nettle leaf on *Enterococcus spps*, Klebsiella pneumonia, Lactobacillus acidophilus *Staphylococcus typhi, Staphylococcus aureus*, and *Candida albicans* was stated by (Shaza Sitrallah and Joumaa Merza, 2018), in this paperwork, approximately the same results were found on *Staphylococcus typhi and Staphylococcus aureus.*

4.3. Minimum Inhibition Concentration of Nettle Leaf Extract

Further, to get the minimum concentration (MIC) of the nettle extract required to bring an inhibition, an experiment was run at 6, 10, and 12 mg/mL. The MIC that completely inhibited the growth of bacteria for the test compound was recorded and the results are presented in Table 4.2.

Table 4.2 Minimum Inhibition Concentration for each Organism

	Visible growth of microorganisms			
Concentration of NL Extract(mg/ml)	*S. typhi*	*E. coli*	*S. Aureus*	*Candida albicans*
6	Yes	Yes	Yes	No
10	No	No	No	No
12	No	No	No	No
Control without Antimicrobial Agent	Yes	Yes	Yes	Yes

From the above Table 4.2, 6mg/mL concentration of the extract completely inhibited the growth of the three microorganisms other than *Candida albicans.* The growth of *staphylococcus*

aureus, *salmonella typhi,* and *E. coli* was completely inhibited by 10mg/ml of liquid medium. *Thus, the more MIC requirement indicates as the microbes is resistant to the extract. Thus, Candida albicans* is less resistant to the nettle extract. Compared to the study made by Lezama et al. (2020), our study gives a better inhibition at the same concentration (10, 10, 10, 6 mg/ml) of antimicrobial agents on *salmonella typhi, staphylococcus aureus, E. coli,* and *Candida albicans,* respectively. (The comparison table of Lezama et al. (2020) is given in appendix D). Based on this MIC, three concentration levels (8, 9, and 10 mg/mL of the experiment are taken.

4.4 The Process Variables of Antimicrobial Packaging Film

The starch/glycerol ratio, concentration of the antimicrobial agent, and film thickness be taken as the process variables to show the effect of variables on the antimicrobial activity of the film. Furthermore, physical and mechanical properties of packaging film. It was possible to observe that the films with high antimicrobial concentration and low glycerol content have a good crystallite. The film prepared from a high starch/glycerol ratio, high concentration of staining nettle leaf extract, and thick film thickness were displayed good antimicrobial and antifungal activity which is established later on in section 4.5. But the films were prepared from a low starch/glycerol ratio with thick film thickness were found good physical and mechanical properties of packaging films. Solubility and swelling properties of the film were increased as the ratio of starch/glycerol ratio increased. Also, one could see that was prepared from a low ratio of starch/glycerol is flexible, easy to peel, and have a high release rate of active components incorporated within the film. This concept was supported with the analysis of variance of the raw data were collected during the experiment which is demonstrated later on in section 4.5.

4.5 Data Analysis of Response

The three factors are Starch to glycerol ratio (A), the concentration of the antimicrobial agent (B), and the thickness of films (C). The general factorial method with three factors and mixed levels (three levels for A and B and two levels for C) were selected. The response was the inhibition zone of each microbe The Design Expert 7.0.0 software was used in the analysis of variance (ANOVA). The actual experimental data at the different formulations of packaging films were recorded. The summary of the experiment design and factors is given in Tables 4.3

and 4.4. Besides, the physical and mechanical properties of the packaging films are also graphically described in Figures 4.15-4.20 and Figure 4.21-4.23, respectively.

Table 4.3 Design Summary

Study Type	Factorial	Runs	36
Initial Design	Full Factorial	Blocks	2
Design Model	2FI		

Each response of the packaging films was used to develop a mathematical model that correlates the antimicrobial activity as well as physical and mechanical properties of the packaging films with the three factors. The model fit summary for each response and statistical analysis of the ANOVA are given in below. The multiple regression coefficients were obtained by employing a least square technique to predict a two factorial (2FI) model for the responses.

Table 4.4 Factor Summary

Factor	Name	Units	Type	Low Actual	High Actual		**levels**
A	Starch/Glycerol	%	Categoric	70:30	80:20	90:10	3
B	AM Concen.	g/ml	Categoric	8	9	10	3
C	Film Thickness	mm	Categoric	0.5	-	1	2

The model fit summary for each response and statistical analysis of the ANOVA are given in Tables 4.5 and 4.6. The multiple regression coefficients were obtained by employing a least square technique to predict a two factorial (2FI) model for the responses.

Table 4.5 Response Summary

Response	Name	Units	Obs	Analysis	Minimum	Maximum
Y1	Inhibition Diameter E. Coli	mm	36	Factorial	2	14
Y2	Inhibition Diameter S. typhi	mm	36	Factorial	26	35
Y3	Inhibition Diameter S. aureus	mm	36	Factorial	16	25
Y4	Inhibition Diameter Candida albicans	mm	36	Factorial	1	14

Table 4.6 Design and the experimental response of dependent variable

Run	Block	Factor 1 A: Starch/Glycerol	Factor 2 B: Antimicrobial Concentration	Factor 3 C: Film Thickness	Response 1 ID E. coli	Response 2 ID S. Typhi	Response 3 ID S. Aureus	Response ID C. albica
1	Block 1	80:20	10	1	11	34	24	11
2	Block 1	80:20	10	0.5	8	33	23	8
3	Block 1	80:20	9	1	7	30	20	7
4	Block 1	90:10	8	1	2	27	17	1
5	Block 1	90:10	10	1	9	35	25	8
6	Block 1	80:20	9	0.5	6	32	22	6
7	Block 1	80:20	8	0.5	3	28	18	3
8	Block 1	70:30	8	0.5	4	31	21	3
9	Block 1	70:30	8	1	6	30	20	6
10	Block 1	70:30	9	1	8	34	24	8
11	Block 1	70:30	9	0.5	7	33	23	7
12	Block 1	90:10	9	0.5	5	29	19	5
13	Block 1	90:10	10	0.5	9	27	17	9
14	Block 1	90:10	8	0.5	2	27	17	2
15	Block 1	80:20	8	1	4	28	18	4
16	Block 1	90:10	9	1	7	31	21	6
17	Block 1	70:30	10	1	14	35	25	13
18	Block 1	70:30	10	0.5	9	35	25	8
19	Block 2	70:30	10	1	14	35	25	14
20	Block 2	70:30	8	1	7	31	21	7
21	Block 2	90:10	10	1	10	35	25	10
22	Block 2	70:30	8	0.5	4	32	20	4
23	Block 2	70:30	9	1	8	35	25	8
24	Block 2	90:10	8	0.5	2	27	17	2
25	Block 2	80:20	8	0.5	3	27	17	3
26	Block 2	70:30	9	0.5	7	32	22	7
27	Block 2	80:20	10	0.5	9	33	23	9
28	Block 2	90:10	8	1	3	27	17	3
29	Block 2	80:20	9	1	8	31	21	8
30	Block 2	90:10	9	1	6	31	21	6
31	Block 2	90:10	9	0.5	5	29	19	5
32	Block 2	80:20	8	1	5	26	16	5
33	Block 2	90:10	10	0.5	9	28	18	9
34	Block 2	70:30	10	0.5	10	35	25	10
35	Block 2	80:20	10	1	13	34	24	13
36	Block 2	80:20	9	0.5	6	32	22	6

The analysis of variance (ANOVA)

The ANOVA was carried out to determine the significance of the model's equation and the model's terms (Tables 4.7- 4.10). The effects of the three variables and their mutual interactions and the effect of these variables on inhibition diameter of the bacteria were examined by Analysis of Variance (ANOVA) using Design-Expert software. In this research, 95 % confidence level or 5% significant level as the critical P-value was considered. To examine the effect of the parameters (variables) on the inhibition diameter, the critical and obtained P-values were compared. For three-level variables, the levels are high (+1), center (0), and low (1), whereas for two-level variables the levels are high (+1) and low (1). The response for *E. coli* (F value, P-value, and the sum of square (SS) is given in Table 4.7. It is to be noted that the larger the F value, the more significant the corresponding term is. However, a small p-value indicates the rejection of the null hypothesis (Prabha, 2017). As the value of SS increased the significances of that variable also increases.

Table 4.7 Analysis of Variance (ANOVA) Response 1 for Inhibition diameter for *E. coli*.

Source	SS	df	Mean Square	F Value	p-value Prob > F	
Block	1.78	1	1.78			
Model	346.44	13	26.65	57.89	< 0.0001	significant
A-Starch/Glycerol	35.06	2	17.53	38.08	< 0.0001	significant
B-Antimicrobial Con	268.06	2	134.03	291.16	< 0.0001	significant
C-Film Thickness	32.11	1	32.11	69.76	< 0.0001	significant
AB	1.78	4	0.44	0.97	0.4470	non-significant
AC	5.39	2	2.69	5.85	0.0095	significant
BC	4.06	2	2.03	4.41	0.0253	significant
Residual	9.67	21	0.46			
Cor Total	357.89	35				

Table 4.7. Shows the statistical results of the Inhibition diameter E. coli obtained using ANOVA. The model's F-value is 57.89 that indicates high significance for the proposed model. There was only a 0.01% chance that a "Model F-value" this large could occur due to noise. Also, the value of ''prob > F" for the model was less than 0.0001. The P-values below 0.05

showed model terms were significant. In this case, the first and second-order effects of Starch/Glycerol (A), Antimicrobial Concentration (B), Film Thickness (C), AC, and BC were significant model terms, the model has not aliased P-value < 0.0001. If two effects are aliased together, we can estimate their combined effect, but cannot separate the size of each effect. For values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit Tests" table compares residual error with "Pure Error" from replicated design points. The two-factorial model, identified earlier as the likely model for all responses, does not show a significant lack of fit (Table 4.7.).

Table 4.8 Analysis of Variance (ANOVA) Response 2 for Inhibition diameter for *S. typhi.*

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	0.028	1	0.028			
Model	286.14	13	22.01	17.39	< 0.0001	significant
A-Starch/Glycerol	87.50	2	43.75	34.56	< 0.0001	significant
B-Antimicrobial Con	144.67	2	72.33	57.14	< 0.0001	significant
C-Film Thickness	10.03	1	10.03	7.92	0.0104	significant
AB	6.33	4	1.58	1.25	0.3203	Non-significant
AC	20.72	2	10.36	8.18	0.0024	significant
BC	16.89	2	8.44	6.67	0.0057	significant
Residual	26.58	21	1.27			
Cor Total	312.75	35				

Table 4.8. presents the statistical results of the Inhibition diameter *E. coli* obtained using ANOVA. The model's F-value is 17.39 that indicates high significance for the proposed model. There was only a 0.01% chance that a "Model F-value" this large could occur due to noise. Also, the value of ''prob > F" for the model was less than 0.0001. The P-values below 0.05 showed model terms were significant. In this case, the first and second-order effects of Starch/Glycerol (A), Antimicrobial Concentration (B), AC, and BC were significant model terms, the model has not aliased P-value < 0.0001. If two effects are aliased together, we can estimate their combined effect, but cannot separate the size of each effect.

For values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit Tests" table compares residual error with "Pure Error" from replicated design points. The two-factorial model, identified earlier as the likely model for all responses, does not show a significant lack of fit (Table 4.8.).

Table 4.9 Analysis of Variance (ANOVA) Response 3 for Inhibition diameter for *S. Aureus*.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	0.028	1	0.028			
Model	285.47	13	21.96	16.79	< 0.0001	significant
A-Starch/Glycerol	79.39	2	39.69	30.34	< 0.0001	significant
B-Antimicrobial Con	155.56	2	77.78	59.45	< 0.0001	significant
C-Film Thickness	12.25	1	12.25	9.36	0.0059	significant
AB	4.78	4	1.19	0.91	0.4746	Non-significant
AC	19.50	2	9.75	7.45	0.0036	significant
BC	14.00	2	7.00	5.35	0.0132	significant
Residual	27.47	21	1.31			
Cor Total	312.97	35				

Table 4.9. This shows that the model F value was 16.79 with a corresponding P-value of <0.00011 and high SS (285.47) implied that this model was significant and can appropriately explain the relationship between response and independent variable. There is only a 0.01% chance that a model F-value this large could occur because of noise. Values of "Prob>F" less than identified model terms are significant. Values greater than 0.1000 identify the model terms aren't significant. In this case, A, B, C, AC, and BC factors were significant model terms whereas BC was insignificant to the response.

The "Lack of Fit Tests" table compares residual error with "Pure Error" from replicated design points. The two-factorial model, identified earlier as the likely model for all responses, does not show a significant lack of fit (Table 4.9).

Table 4.10 Analysis of Variance (ANOVA) Response 4 for Inhibition diameter for *C. Albicans.*

Source	Sum of square	df	Mean	F-value	p-value	
Block	5.44	1	5.44			
Model	339.44	13	26.11	48.38	< 0.0001	significant
A-Starch/Glycerol	35.39	2	17.69	32.79	< 0.0001	
B-Antimicrobial Con	260.72	2	130.36	241.55	< 0.0001	
C-Film Thickness	28.44	1	28.44	52.71	< 0.0001	
AB	1.11	4	0.28	0.51	0.7257	
AC	10.06	2	5.03	9.32	0.0013	
BC	3.72	2	1.86	3.45	0.0507	
Residual	11.33	21	0.54			
Cor Total	356.22	35				

Table 4.10. Shows the statistical results of the Inhibition diameter E. coli obtained using ANOVA. The model's F-value is 48.38 that indicates high significance for the proposed models. There was only a 0.01% chance that a "Model F-value" this large could occur due to noise. Also, the value of ‘‘prob > F" for the model was less than 0.0001. The P-values below 0.05 showed model terms were significant. In this case, the first and second-order effects of Starch/Glycerol (A), Antimicrobial Concentration (B), and AC were significant model terms, the model has not aliased P-value < 0.0001. If two effects are aliased together, we can estimate their combined effect, but cannot separate the size of each effect. For values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit Tests" table compares residual error with "Pure Error" from replicated design points. The two-factorial model, identified earlier as the likely model for all responses, does not show a significant lack of fit (Table 4.10).

4.6. Model Adequacy Check

The model adequacy checking is essential to confirm whether the fitted model provides an adequate approximation of the actual values or not. It was tested by analysis of variance and some diagnostic plots which have been indicated in Table 4.10 and Figure 4.1-4.3.

Table 4.11 model adequacy checking parameters for each bacterium.

	R-squared	Adj R-Squared	Pred R-Squared	Adeq Precision
E. coli	0.9729	0.9561	0.9202	27.337
S. typhi	0.9150	0.8624	0.7502	13.655
S. aureus	0.9122	0.8579	0.7420	13.773
C. albicans	0.9677	0.9477	0.9051	25.130

As shown in the Table 4.11, the quality of the model developed was evaluated based on the coefficient of corelation (R^2 value), adj- R^2 and pred-$R^2$and adeq precision. For R^2 value, the model developed is effective since the R^2 value is closer to unity. This depicts as the predicted value is closer to the actual value for the response. The R-square reported in this study 0.9729, 0.9150, 0.9122, and 0.9677 for *E.* coli, S. typhi, S. *aureus,* and *C. albicans* respectively indicates, the model accounts for over 0.9729, 0.9150, 0.9122, and 0.9677 of the variability in the response. This implies that about 99% of the variability in the data was explained by the model. The adjusted R-squared (R^2-adj) was also very high which confirms that the models were highly significant. Also, The R squared (R^2) and the adjusted R-squared (R^2-adj) are close to each other, which shows that the model does not include insignificant parameters.

The "Pred R-Squared" of 0.9202, 0.7502, 0.7420 and 0.9051 was in reasonable agreement with the "Adj R-Squared" of 0.9561, 0.8624, 0.8579 and 0.9477. for E. coli, S. *typhi*, *S. aureus,* and *C. albicans* respectively. A difference greater than 0.20 between the "Pred R-Squared" and the "Adj R Squared" indicates a possible problem with the model and/or data. So, in this study, the difference value between "Pred R-Squared" and the "Adj R Squared" was 0.0045 this indicates that there is no problem on a model as well as the data.

The signal-to-noise ratio is measured by adeq precision ratio. A ratio greater than 4 is desirable. In this experimental work, the adeq precision ratio for each organism are 27.337, 13.655, 13.733, and 25.130 indicate an adequate signal. Therefore, this model can be used to navigate the design space. Table 4.10 given in above indicates that the "Pred R-Squared" of 0.9202,

0.7502, 0.7420, and 0.9051 are in reasonable agreement with Adj R-squared 0.9561, 0.8624, 0.8579, and 0.9477for each organism, respectively.

The regression coefficients and the corresponding 95% Confidence Interval of the model are presented in appendixes B1 up to B4. If zero was in the range High and Low 95% Confidence Interval, the factors do not affect. From the 95% CI High and Low values of each model term, it could be concluded that the regression coefficients of Starch/glycerol Ratio, Antimicrobial concentration, Film thickness and the interaction terms of starch/glycerol Ratio with Film thickness and antimicrobial concentration with Film thickness has a highly significant effect on antimicrobial packaging film.

Final Equation in terms of coded factors

$$\text{Inhibition diameter E. coli} = 6.94 - 1.21A(1) + 0.014 * A(2) + 3.33 * B(1) + 0.14 * B(2) + 0.94 * C + 0.12 - 0.46 * A(1)C - 0.069 * A(2)C + 0.33 * B(1)C + 0.14 * B(2)C \quad \text{Eq: 4.1}$$

$$\text{Inhibition diameter S. typhi} = 31.08 - 1.87 * A(1) + 0.21 * A(2) + 2.42 * B(1) - 0.25 * B(2) + 0.53 * C + 0.67 * A(1)C + 0.35 * A(2)C + 0.83 * B(1)C + 0.056 * B(2)C \quad \text{Eq: 4.2}$$

$$\text{Inhibition diameter S. Aureus} = 21.03 - 1.79 * A(1) + 0.18 * A(2) + 2.50 * B(1) - 0.28 * B(2) + 0.58 * C + 0.62 * A(1)C + 0.38 * A(2)C + 0.75 * B(1)C + 0.083 * B(2)C \quad \text{Eq: 4.3}$$

$$\text{Inhibition diameter C. Albicans} = 6.78 - 1.21 * A(1) - 0.069 * A(2) + 3.29 * B(1) - 0.097 * B(2) + 0.89 * C - 0.62 * A(1)C - 0.097 * A(2)C \quad \text{Eq: 4.4}$$

The adequacy of the model was further checked through graphical analysis of the normal plot of residual versus internal studentized residual, residual versus predicted, and predicted versus actual, as shown in Figures 4.1- 4.3), respectively.

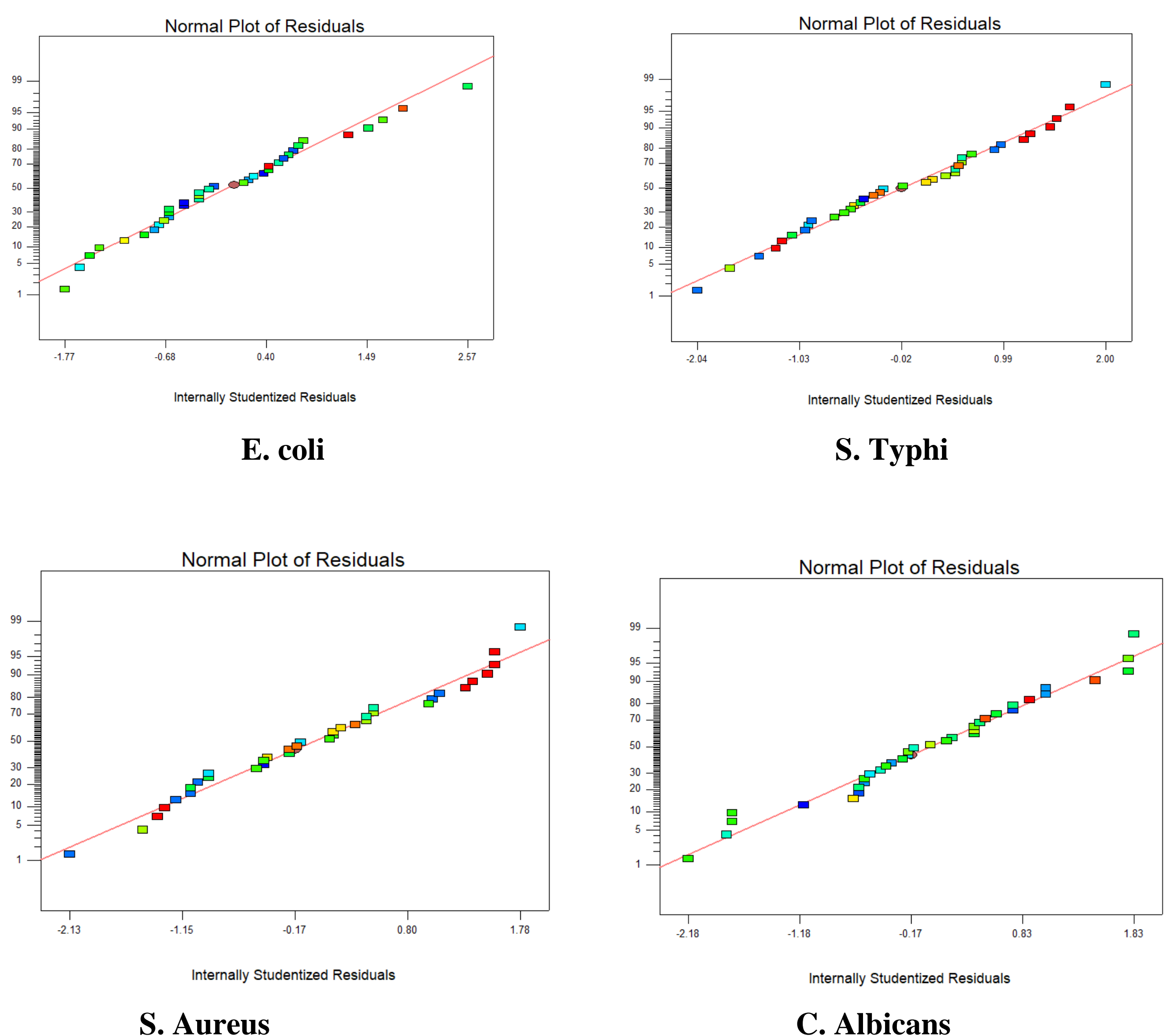


Figure 4. 1 Normal plot of residuals

Figure 4.1. gives the plots of the normal probability vs. the studentized residuals for inhibition diameter for *E. coli*, *S. typhi*, *S. aureus,* and *C. Albicans***,** respectively. The plot of the normal probability indicates if the residuals are normal and independent distribution. It means the points must follow a straight line (Rahmanian,m.pakizeh., 2011). It is observed that error values related to the response variable of each response coefficient were almost along with the straight normal line and it shows the error distribution is normal.

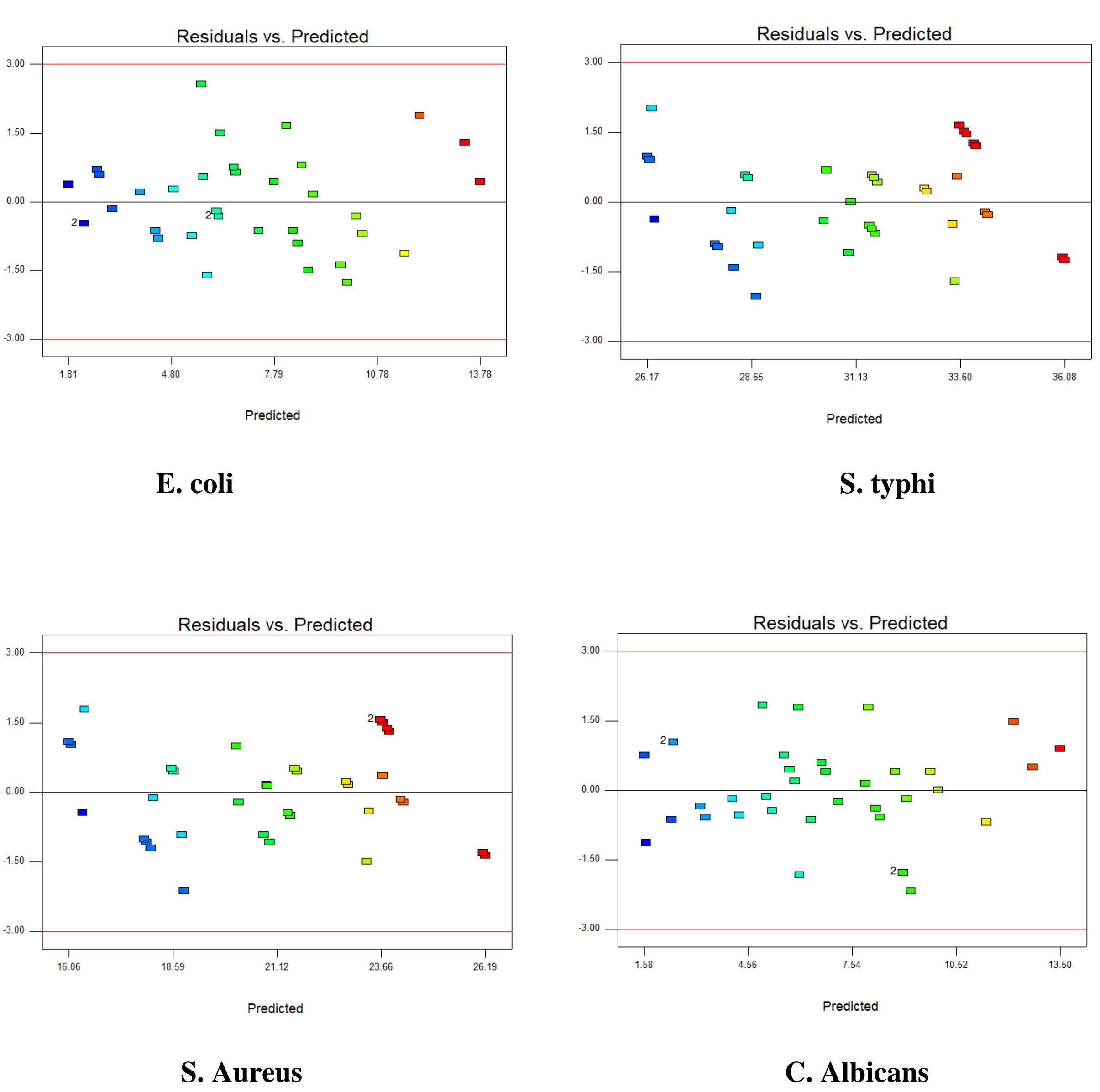


Figure 4. 2 Residuals vs Predicted

Figure 4.2. Indicate Studentized residuals versus predicted values to check for constant error. Residuals are normally distributed with no outliers or grouping in the data. If the model is correct and the assumptions are satisfied, the residuals should be structure-less. They should be unrelated to any other variable including the predicted response. A simple check is to plot the residuals versus the fitted (predicted) values. The plot shows random scatter which justifies no need for any alteration to minimize personal error. Therefore, the residual standard deviation can be used as the measure of the random variability of the process.

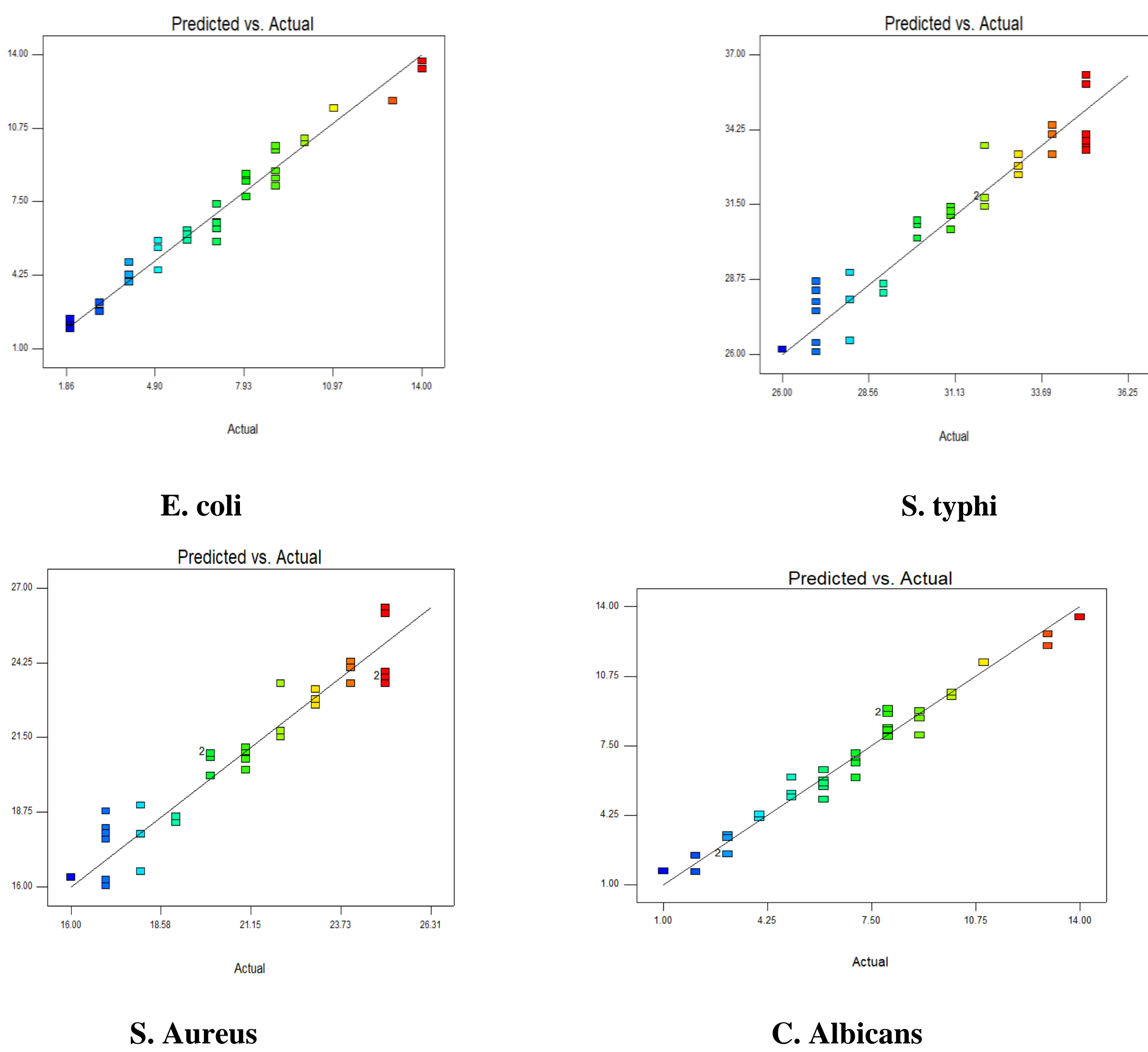


Figure 4. 3Predicted Vs Actual

The relationship between the model and experimental results of inhibition diameter for each microorganism is provided in Figure 4.3. In this Figure, the general factorial indicates that there is a good agreement between the model and experimental results since the residuals for the majority of the responses are close to the diagonal line.

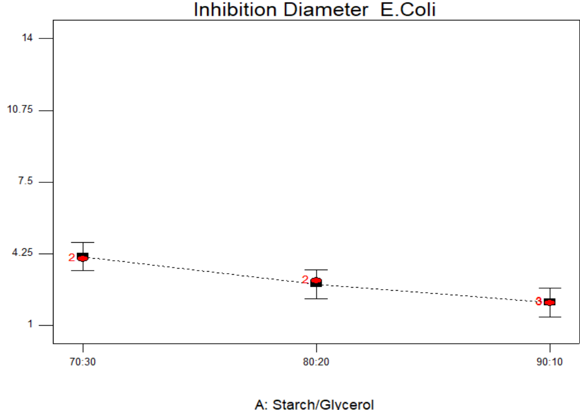
4.7. The Effect of Process Variables on Inhibition Zone

The activity of the prepared packaging film having three process variables with different combination formulations was tested on each organism. Full general factorial design method of statistical analysis was used to estimate the effect of three process variables on the responses.

Perturbation plots were drawn by using full factorial to investigate the effect of all the factors on the responses. Based on the analysis of variance, the activity of the packaging films was significantly affected by various interactions between the process variables. On the other hand, individual process variables that significantly affect the activity of the packaging films were starch/glycerol ratio (A), the concentration of antimicrobial agent (B), and thickness of the films (C).

4.7.1. Effect of Starch/ Glycerol Ratio on Inhibition Zone

The primary task in preparing the packaging film is to determine the correct ratio of starch/glycerol. As shown the Figure 4.4, the starch/glycerol ratio is one of the important factors that affect the activity of the packaging film. Hence, from the graph, the inhibition diameter of each organism is increased with increase in the ratio of starch/glycerol. The increase in the ratio of starch/glycerol causes decrease in the formation of strong hydrogen bonds (because of hydroxyl groups on starch chains) without decreasing the crystallite level. This, in turn, increases the release rate of the antimicrobial agent (CRUZ, 2012). A higher amount of glycerol gives a higher number of polar groups in the film, which could absorb more water from the surrounding atmosphere. (Nevena T. Nemet, Vladislava M. soso and Vera L. Lazic, 2010). In general, hydrophilic plasticizers, such as glycerol, enhanced water solubility dry matter in the film. It is probably because increasing the plasticizer content in the film increased the water-soluble dry content which intern increases the release amount of antimicrobial agent from packaging film to the medium. The relationship between water-soluble dry matter and hydrophilic plasticizer content is linear.



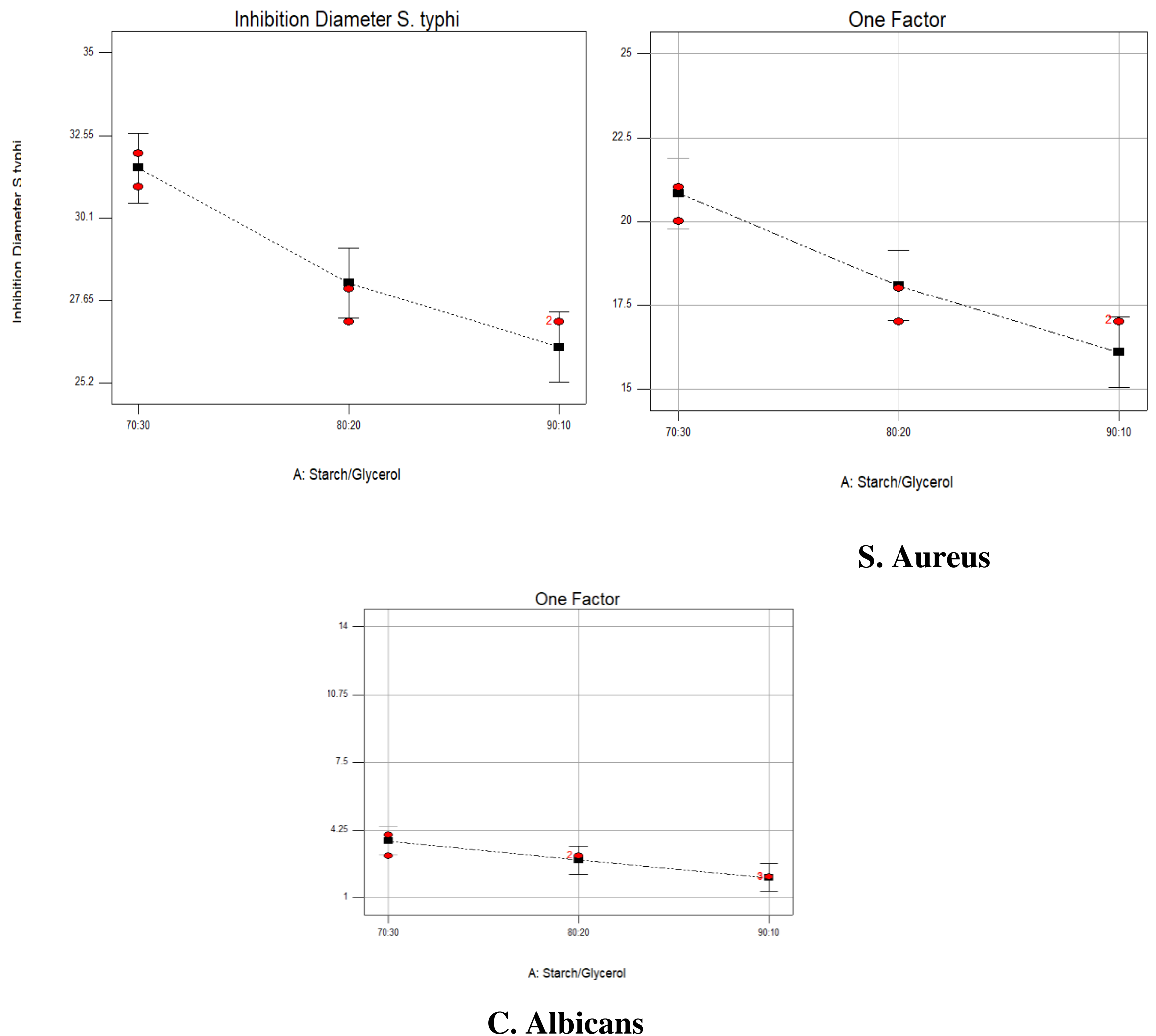
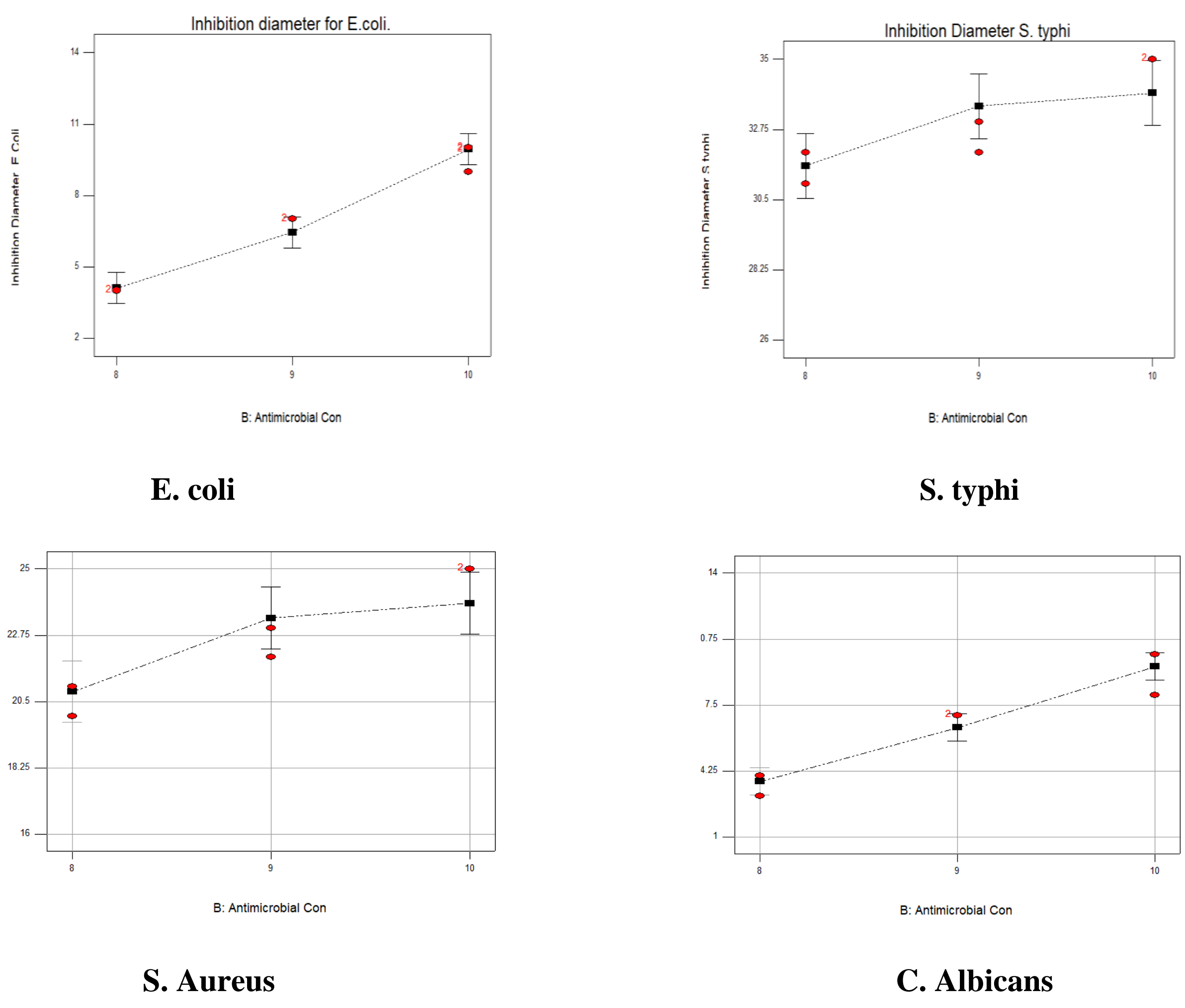


Figure 4. 4 Effect of Starch/ Glycerol Ratio on Inhibition Zone each Organism.

4.7.2 Effect of Antimicrobial Concentration on Inhibition Zone

The concentration of antimicrobial agents has a highly significant effect on the activity of the packaging film. In this study, there are three different concentrations of 8 g/mL, 9 g/mL, and 10 g/mL were taken. Their effect on the response (inhibition diameter) is illustrated graphically in Figure 4.5 below, as the concentration of anti-microbial in the packaging film increased, from (8 g/mL, to 10 g/mL) the inhibition diameter also increased for each microorganism (*E. coli, S. typhi, S. aureus*, and *Candida albicans)*. Therefore, the highest inhibition diameter was found at 10 g/mL. All the packaging films inhibited the growth of all the test microorganisms used. The greatest zone of inhibition (35 mm) was observed against *S. typhi*, and the least zone

of inhibition (1mm) was observed against *C. Albicans*. In the work of (Mesaret Zerihun, et al., 2016). The greatest zone of inhibition was observed against *S. typhi*. All the packaging films have almost similar antimicrobial activity at the same concentration of nettle extracted. Significance differences between the antimicrobial activities of the packaging films were observed at *S. aureus*, and *S. typhi.* This difference may have resulted from the concentration of the extracts. This means the concentration of antimicrobial is increased the inhibition diameter of also highly increase for all organisms. From the previous study, the sensitivity to tannins and other phenolic compounds varies greatly among organisms (BASARABA, 1964). In addition, (Jutaporn, 2011). reported that some organisms including *C. Albicans* and *E. coli* are capable of growing on tannins as a source of carbon. The difference in sensitivity may also be associated with the difference in cell wall structure and function.



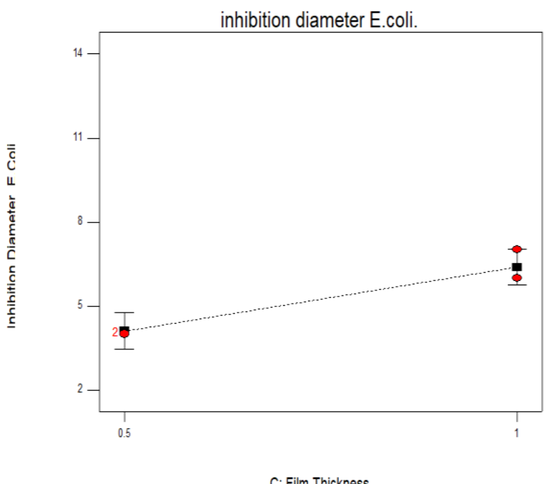
E. coli **S. typhi**

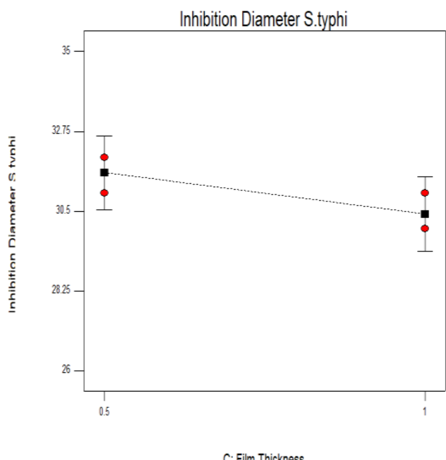
S. Aureus **C. Albicans**

Figure 4. 5 Effect of Antimicrobial Concentration on Inhibition Zone each Organism.

4.7.3. Effect of Film Thickness on Inhibition Zone

Figure 4.6 shows the effect of film thickness on the inhibition zone of microorganisms. In this study, for *S. typhi* and *S. aureus* bacteria, the thickness of the film is increased from 0.5 mm to 1 mm the inhibition diameter is decreased from 31.25 mm to 30.25 mm but for *E. coli* and *C. Albicans* the film thickness increases from 0.5 mm to 1 mm the inhibition diameter also increases from 4 mm to 7 mm. When the thickness large enough the physical and mechanical properties of film packaging were changed. The thin layer exhibited high water solubility, sorption, and poor mechanical properties whereas the thick films were found that low water solubility and sorption with good mechanical strength. Hence, the release rate of the antimicrobial agent from the film to the medium is increased. This is probably because of the increased surface area of the packaging films (Mesaret Zerihun, et al., 2016).





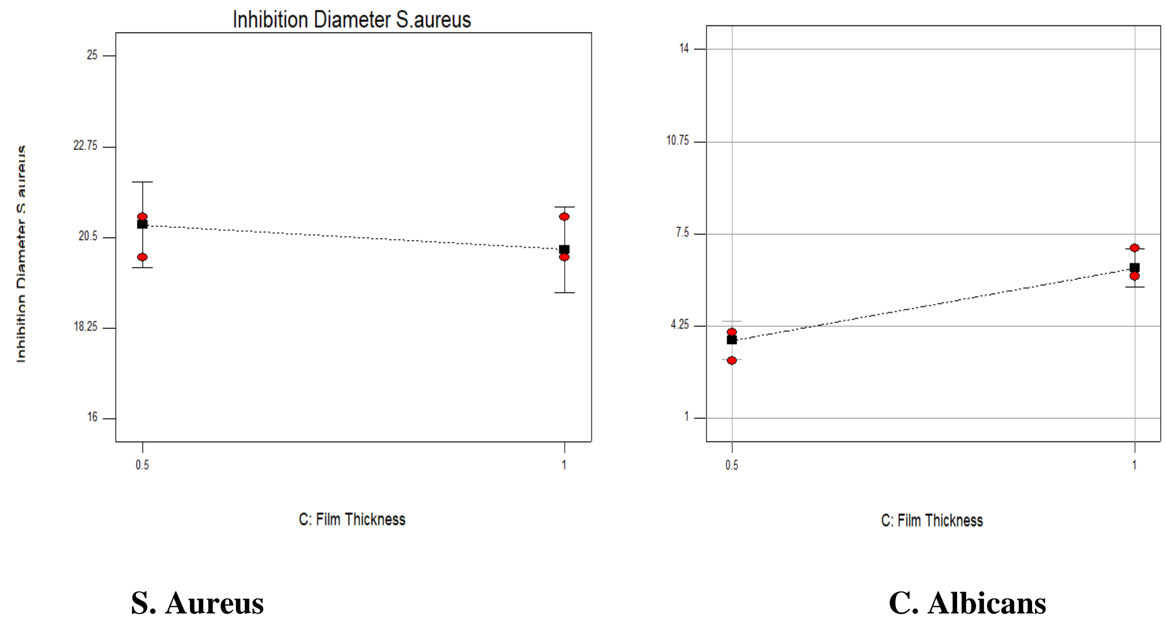
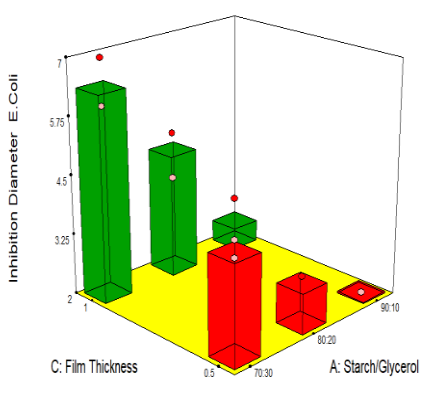


Figure 4. 6 Effect of film thickness on Inhibition Zone each Organism

4.8. The Interaction Effect of Process Variables on Inhibition Zone

The residual diagnosis reveals no statistical problem, so now let's generate response plots. For the graphical interpretation of the interaction, the use of interaction and 3D plots for the regression model is highly recommended for the significant effects factor on the inhibition diameter of each microorganism (Prabha, 2017). The interaction effect of the process variables on the Inhibition Zone shows in Figure 4.7. The interaction effect between factor Starch/glycerol Ratio (A) and Antimicrobial Concentration (B) was not significant Because p-value 0.4851 and the interaction effect between Starch/glycerol Ratio (A) and Film thickens (C) was significant with p-value $\leq$ 0.001, the interaction effect between Antimicrobial Concentration (B) and Film thickens (C) also significant with p-value $\leq$0.001.



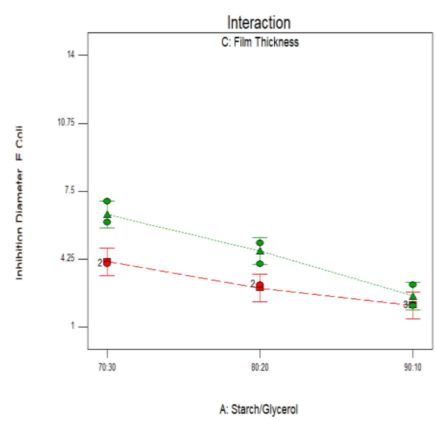
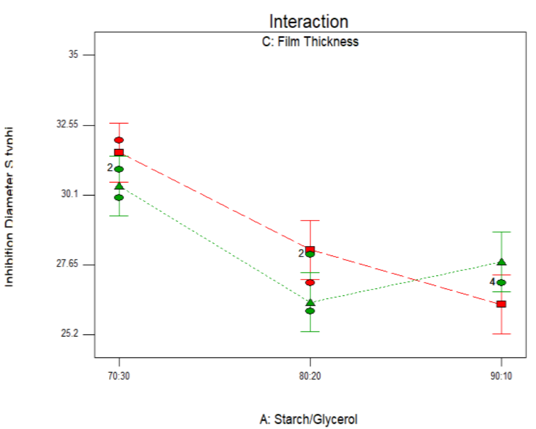


Figure 4. 7 Interaction effect of process variable starch/glycerol ratio with film thickness on different views (interaction, and 3D surface respectively) for response 1 inhibition diameter *E. coli.*



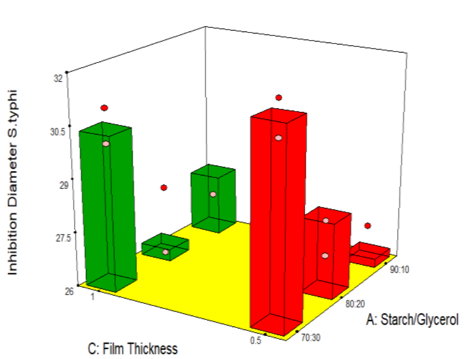
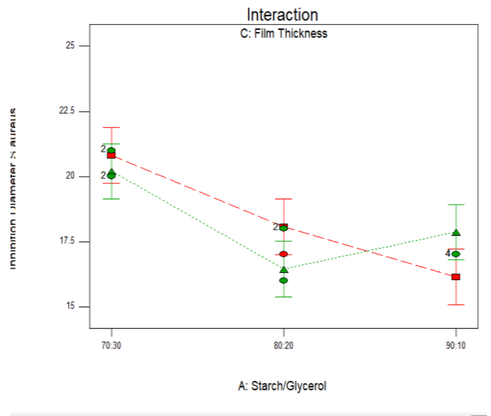


Figure 4. 8 Interaction effect of process variable starch/glycerol ratio with film thickness on different views (interaction, and 3D surface respectively) for response 2 inhibition diameter *S. typhi.*



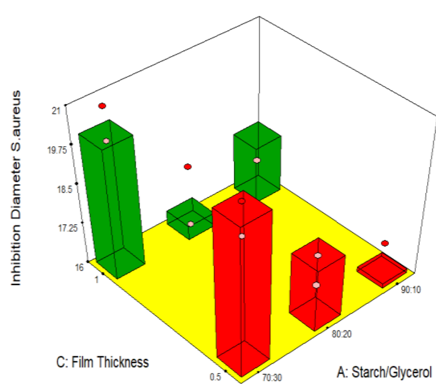
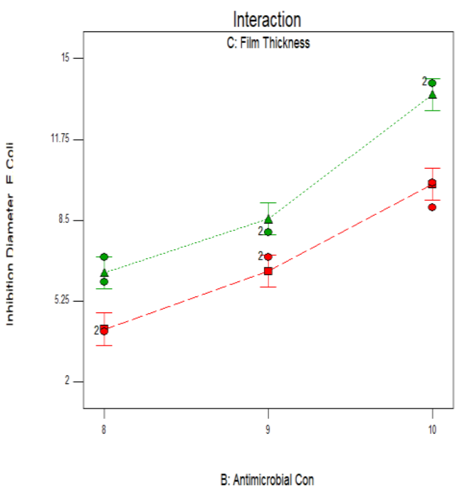


Figure 4. 9 Interaction effect of process variable starch/glycerol ratio with film thickness on different views (interaction, and 3D surface, respectively) for response 3 inhibition diameter *S. aureus.*



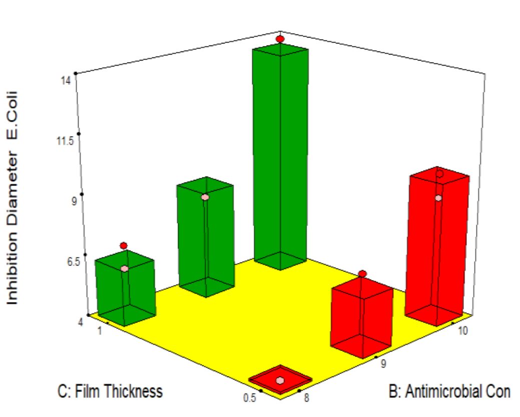
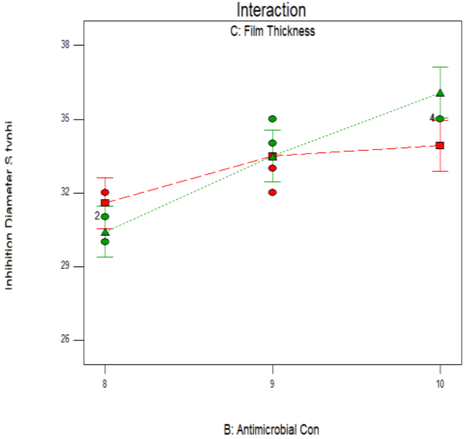


Figure 4. 10 Interaction effect of the process variable, antimicrobial concentration with film thickness on different views (interaction, and 3D surface respectively) for response 1 inhibition diameter *E. coli*.



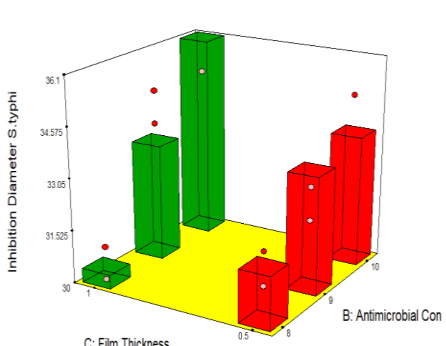


Figure 4. 11 Interaction effect of the process variable, antimicrobial concentration with film thickness on different views (interaction, and 3D surface, respectively) for response 2 inhibition diameter *S. typhi.*

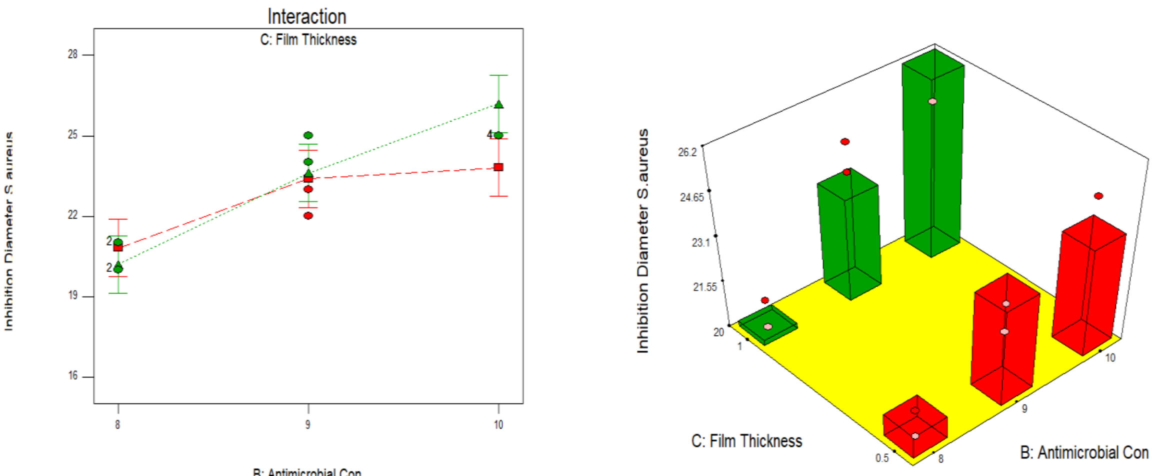


Figure 4. 12 Interaction effect of the process variable, antimicrobial concentration with film thickness on different views (interaction, and 3D surface, respectively) for response 3 inhibition diameter *S. Aureus*.

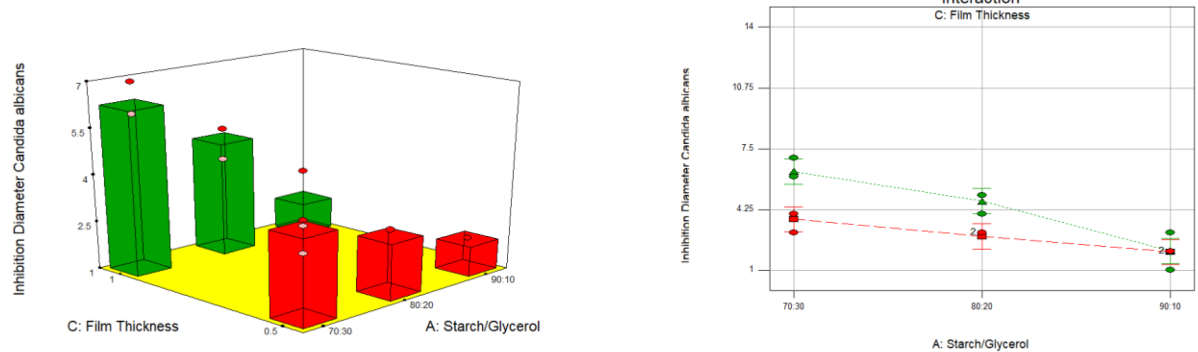


Figure 4. 13 Interaction effect of process variable, Starch/glycerol concentration with film thickness on different views (interaction, and 3D surface, respectively) for response 4 inhibition diameter *C. Albicans*.

The ANOVA results (Figure 4.8. up to 4.13.) show that the inhibition diameter was significantly (P< 0.001) affected by antimicrobial concentration and Starch/glycerol as well as the film thickness. As can be observed increasing antimicrobial concentration, the inhibition diameter has an increasing. Nettle level has high antimicrobial component, which is tannins and poly-phenol was used to prevent the micro-organism when the concentration level of nettle extract increased, higher inhibition diameter was achieved for *S. typhi, and S. aureus* (Astuti, 2014).

As shown in Figure 4.9. 3D plot at 70:30 starch/glycerol ratio with 0.5 mm film thickness increased the inhibition diameter for *S. typhi* and *S. Aureus* microorganism, on the other hand, 70:30 starch/glycerol ratio with 1 mm film thickness also inhibit the *S. typhi* and *S. Aureus* microorganism but compare to 70:30 starch/glycerol ratio with 0.5 mm film thickness is a little difference on inhibition of *S. typhi* and *S. Aureus* microorganism. This is probably because of the increased surface area of the packaging films (Muche, 2018). 80:20 starch/glycerol ratio with 0.5 mm film thickness inhibits the growth of *S. typhi* and *S. Aureus* micro-organism, 80:20 starch/glycerol ratio with 1 mm film thickness low inhibition diameter but compare to 80:20 starch/glycerol ratio with 0.5 mm film thickness very low inhibition diameter. 90:10 starch/glycerol ratio with 0.5 mm film thickness the inhibition diameter for *S. typhi* and *S. Aureus* is very low but 90:10 starch/glycerol ratio with 1 mm film thickness highly inhibited for *S. typhi* and *S. Aureus*.

In this study, the interaction plot shows that increase in inhibition diameter is greater when antimicrobial concentration is higher (10 g/mL) than the concentration of antimicrobial (9 g/mL) and (8 g/mL) in all microorganisms (response). The antimicrobial concentration is the main effect on the microorganism(response) and also the graph shows that antimicrobial concentration had a significant effect by itself on the response. Generally, the interaction effect of antimicrobial concentration and film thickness on the inhibition diameter of all microorganisms was significant.

The packaging films developed in this study inhibited all the test microorganisms used. Nettle leaf extracts were responsible for the films to have a wider antimicrobial spectrum. Consumers continue to demand foods that are minimally processed and posse's fresh like quality, while modern distribution systems require an adequate shelf life. Such type of antimicrobial packaging is a promising form of active packaging to improve the safety and shelf-life of food products. Therefore, these packaging films are vital to control the post-processing contamination of food products and to improve the safety and shelf-life of food products.

Numerical Optimization for independent Variables

In the Design-Expert software (DOE) numerical optimization, the possible goals are maximizing, minimizing, target, in range, and set to an exact value factor only (Saviano, 2015).

One of the main objectives for this study was to produce antimicrobial resistance packaging film and find the optimum process variable to maximize the percentage of inhibition diameter from the developed mathematical model equation.

Table 4.10 Optimization criteria for optimum inhibition diameter

Name	**Goal**	**Lower Limit**	**Upper Limit**
Starch/Glycerol	is in range	70:30	90:10
Antimicrobial Con	is in range	8	10
Film Thickness	is in range	0.5	1
Inhibition Diameter *C. Albicans*	maximize	1	14

Model validation or the experimental confirmation is that the final step within the optimization process using the 2FI model. To confirm the optimization results, an experiment was performed under predicted condition by the developed model. The experimental conditions with great desirability (0.932) were selected to be verified. The desirable optimization solution at optimum conditions is listed in table 4.13.

Table 4.11 The desirable optimization solution

Number	Starch/Glycerol	Antimicrobial Con	Film Thickness	Inhibition Diameter E. Coli	Desirability	
1	70:30	10	1	13.1111	0.932	Selected
2	80:20	10	1	11.7778	0.829	
3	90:10	10	1	9.6111	0.662	

The optimization result shows in Table 4.13 Starch/Glycerol with 70:30 ratio, Antimicrobial Concentration with 10g/ml and Film Thickness with 1mm were optimal for inhibited bacteria diameter. The above optimum operating parameters obtained sufficient for preventing microorganisms.

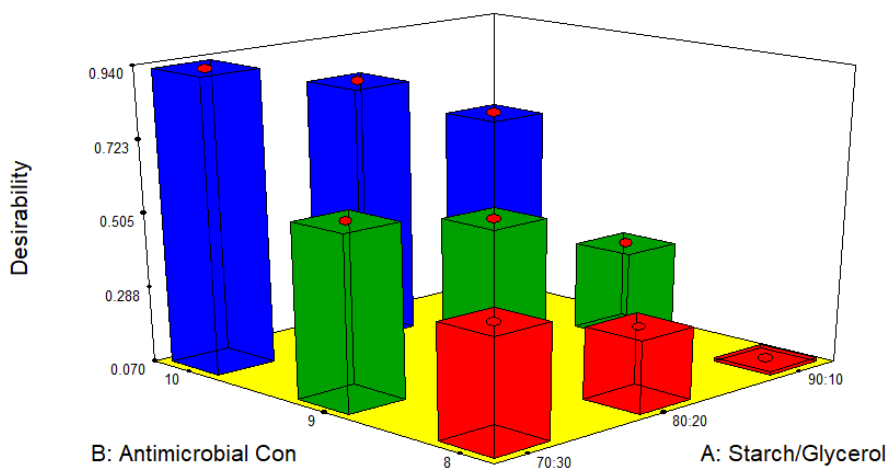
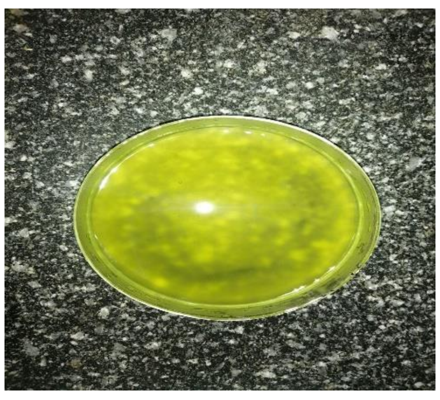


Figure 4. 14 Optimization of starch/glycerol and anti-microbial concentration plot.

4.9. Physiochemical & Mechanical Strength of AM Packaging Film

4.9.1. Colour of antimicrobial packaging film

Color attributes are of prime importance because they directly influence consumer acceptability. Visually, all the films developed from nettle leaf extract were light green as shown in Figure 4.15(A). Figure 4.15(B) demonstrates the color of the packaging films developed by excluding nettle leaf extract, to see the influence of nettle leaf extract on the color of the composite film.



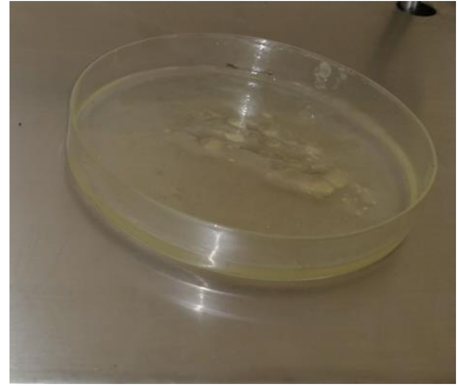


Figure 4.15(A)

Figure 4.15(B)

Figure 4. 15 Films developed in the presence of nettle leaf extract, and Films developed without nettle leaf extract.

The addition of nettle leaf extracts affected the color of the packaging films. This was due to the light green color of nettle leaf extract. The color of the composite film developed by excluding nettle leaf extract was white and somewhat transparent as illustrated in Figure 4.15(B).

4.9.2. Moisture of Antimicrobial Packaging Films

The moisture content in the film decreased when the nettle leaf extract was incorporated with the film as compared to the control film which was formed without antimicrobial agents. Nettle leaf are a high molecular weight of carbohydrate, and this might be led to the increase in the crystalline phase of a semi-crystalline material which was highly related to or associated with the decrease in its moisture content. Consequently, the increase in the crystalline fraction was observed with the addition of antimicrobial. Perhaps moisture or water molecules are used as the carrier to diffuse out the antimicrobial substances from the film matrices to obtain the inhibition action (Antonio Bevilacqua, 2017). The percentages of the moisture content decrease from 35% to 22% for 0.5mm film thickness and 30% to 20% for 1mm film thickness for the film with the increase in the amount of antimicrobial activity from 8 g/mL to 10 g/mL (Raimova, 2020).

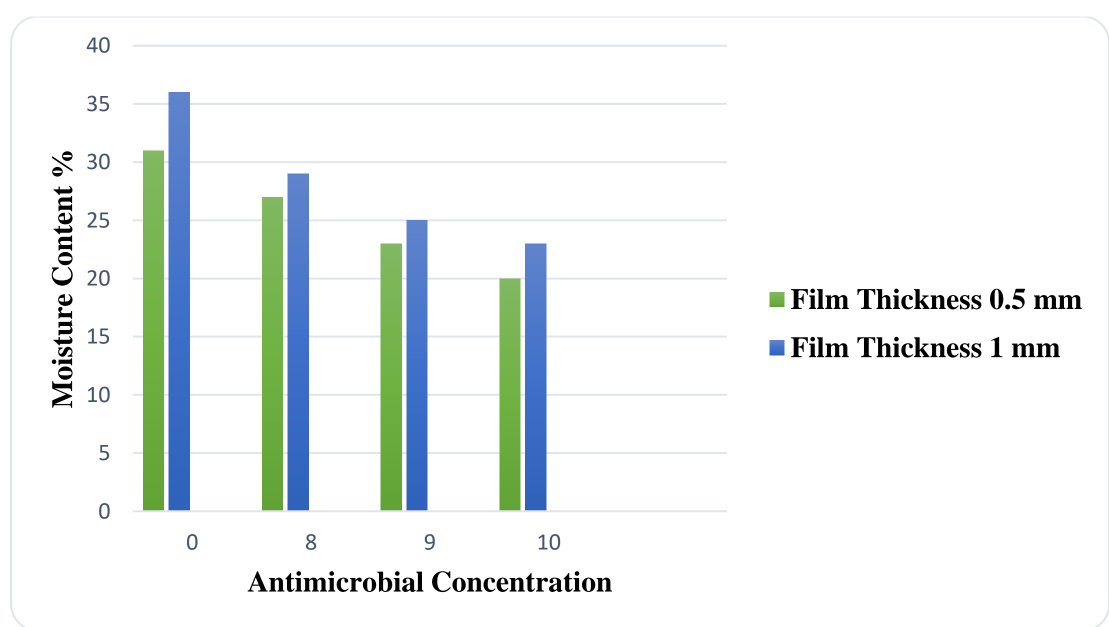


Figure 4. 16 Moisture Content Antimicrobial Concentration with Film Thickness.

When the starch/ glycerol ratio increased from 70:30 to 90:10 ratio, the moisture content of packaging films increased 12% to 19% for 0.5 mm film thickness and 20% to 25% for 1 mm film thickness. This may be related to glycerol increased the higher content of hydrophilic hydroxyl groups of polysaccharides which increased the water absorbability of starch-based antimicrobial packaging films (Da Roz, et al. , 2006), were reported that higher levels of plasticizer increased the films moisture affinity and these results could be attributed to the hydrophilicity of the plasticizers, with an accessible hydroxyl group.

The difference in moisture content could be related to the difference in the composition of the films. The increase in moisture content could also be related to the hydrophilicity of glycerol. Also, it could possible to observe that the moisture content of antimicrobial packaging films decreased as the concentration of antimicrobial crude extract increased. The films prepared with glycerol were more soluble than films without plasticizers. The effect of starch/glycerol and concentration of antimicrobial agent ratio on the moisture content has been demonstrated in the two Figures 4.15 and 4.16. These Figures were grouped into 0.5 mm and 1 mm film thickness and it indicates that the films with 1 mm thickness were exhibited high moisture content.

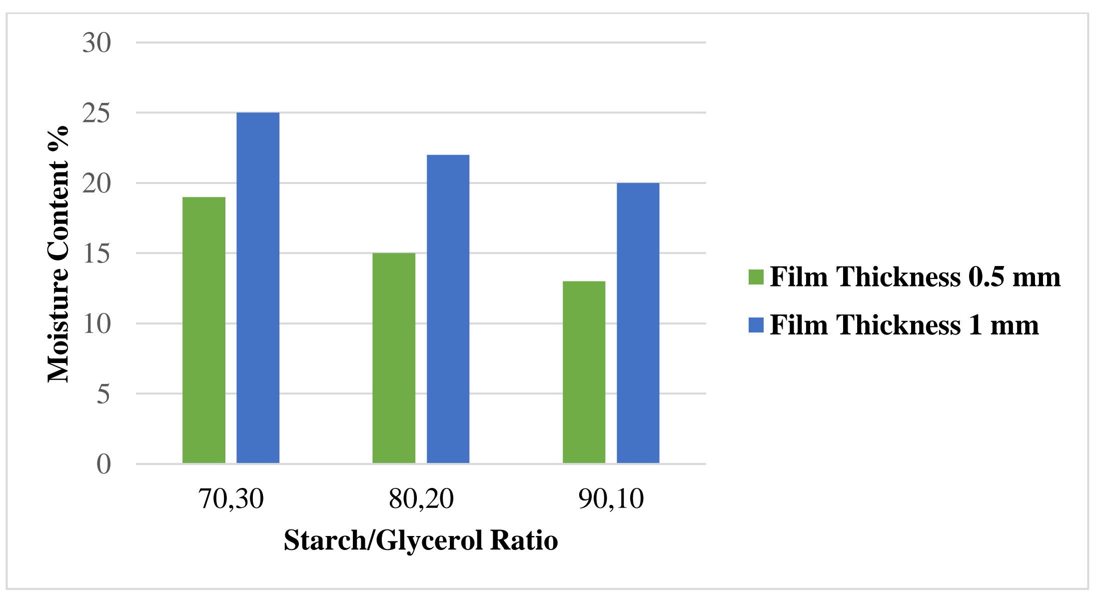


Figure 4. 17 Moisture Content Starch/Glycerol with Film Thickness

4.9.3 Swelling and Solubility of Starch-Based Films

The swelling capacity of the films increased from 12% to 19% for 0.5 mm film thickness and 20% to 25% for 1 mm film thickness, when the glycerol ratio increased, because glycerol is hydrophilic polymers, shows a high affinity towards water (Gashaw & Shimeles, 2013). The effect of glycerol, antimicrobial concentration, and film thickness on the swelling of the films was shown in Figures 4.17 and 4.18 below. As it indicated the swelling capacity of the films was increased from 12% to 19% for 0.5 mm film thickness and 20% to 25% for 1 mm film thickness as glycerol content (10% to 30%) and film thickness increased (0.5 mm to 1 mm). But swelling capacity decreased from (30% to 20%) with 0.5 mm film thickness as the antimicrobial concentration increased from (8 g/mL to 10 g/mL because the higher concentration increased the crystallite of the films. From the Figures presented below, it could possible to understood that the film with higher thickness had higher swelling capacity compared to thin films.

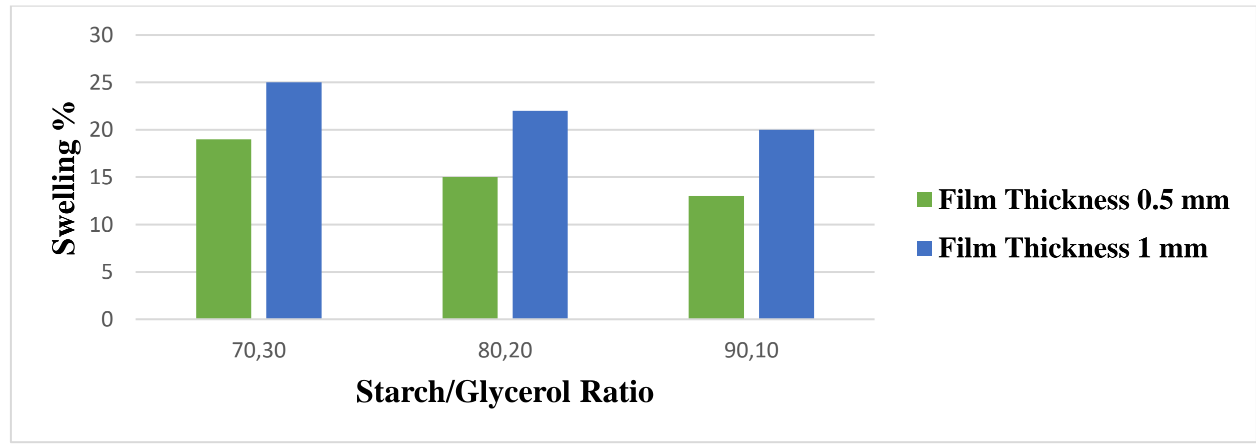


Figure 4. 18 Swelling of the film for Starch/glycerol with film thickness.

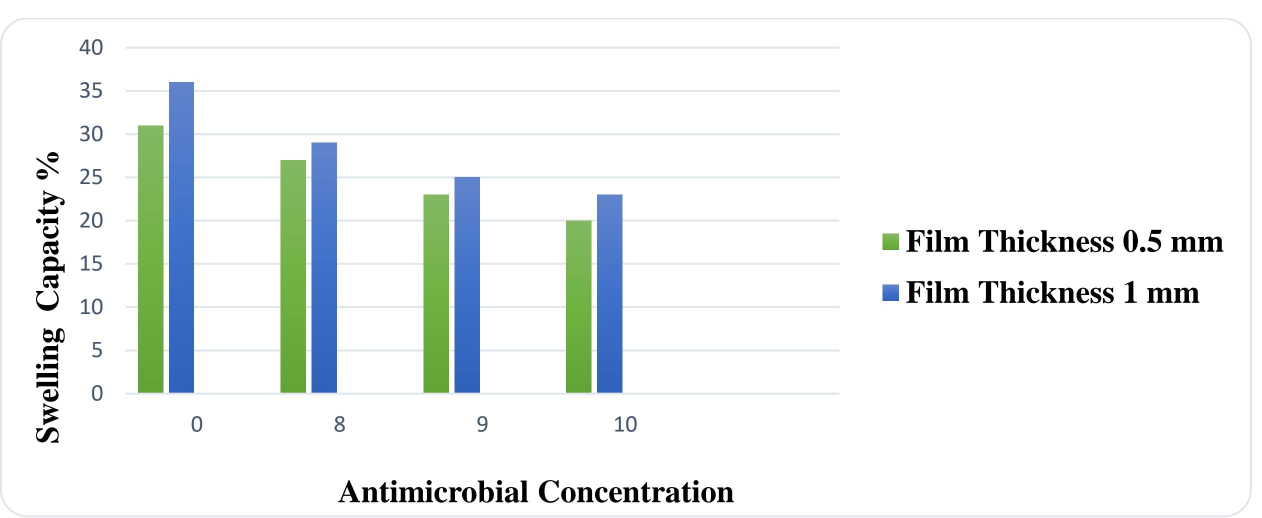


Figure 4. 19 Swelling of the film for Antimicrobial Concentration with film thickness.

The solubility of the films was influenced by the number of antimicrobial agents used in each of them. As can be seen in Figure 4.20 the films containing a higher amount of the antimicrobial agent had a higher solubility. On the other, the films containing a small amount of antimicrobial agent had a lower solubility. It is probably because since nettle leaf extract has fat and oil, increasing the extract amount, might be increasing the lipid content in the film (Gashaw & Shimeles, 2013). The control films had the smallest solubility (20%) than all the other starch based-films. Based on results it can be recommended that films having a low solubility are favour for the packaging purpose.

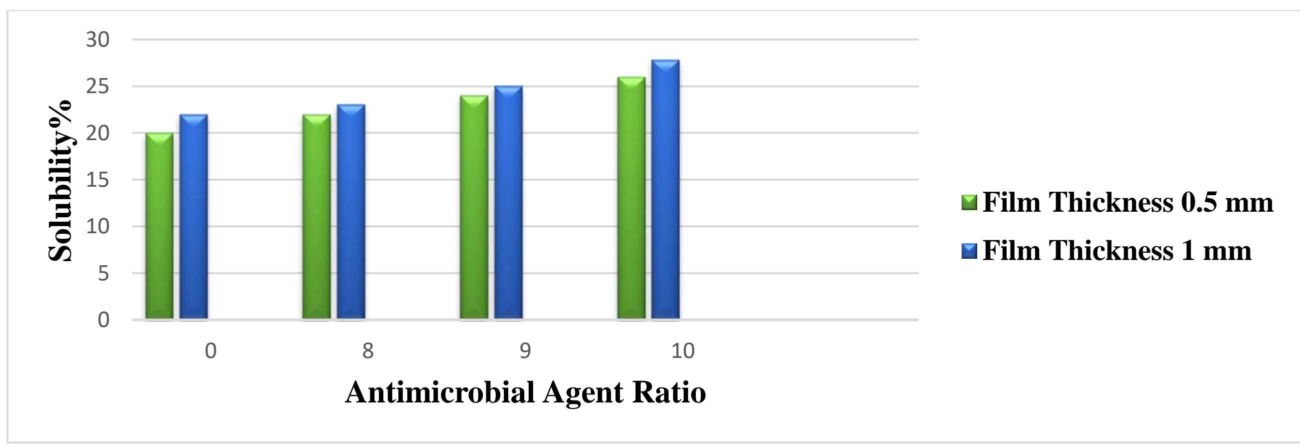


Figure 4. 20 Solubility of the film for Antimicrobial Concentration with film thickness.

Figure 4.20. It indicates of glycerol, in all concentrations, increased the water solubility of starch films. It is probably because increasing the plasticizer content in the film increased the water-soluble dry content. The relationship between water-soluble dry matter and hydrophilic plasticizer content is linear (Castro, 2012).

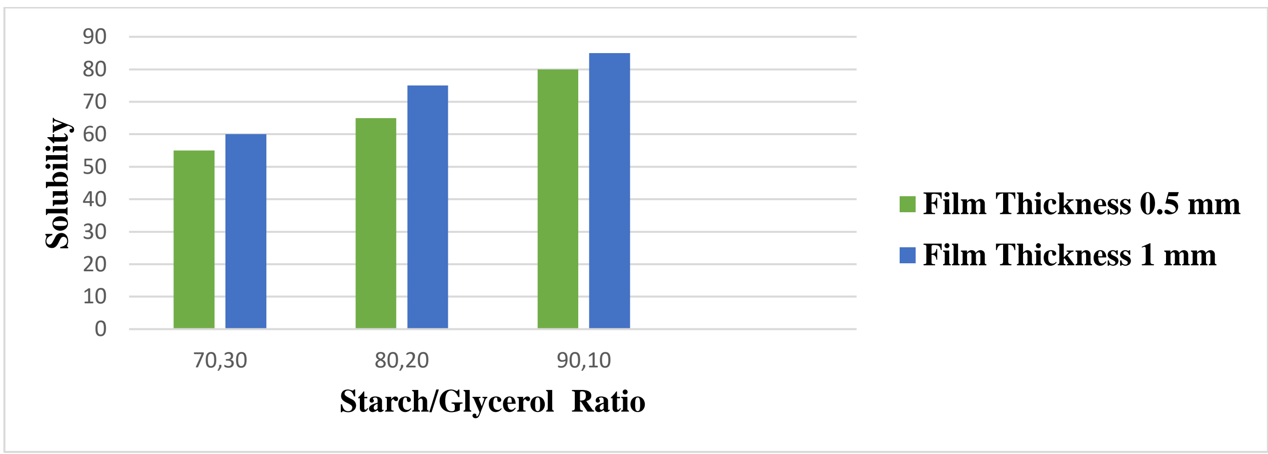


Figure 4. 21 Solubility of the film for Starch/glycerol ratio with film thickness.

4.10. Mechanical Strength Analysis

4.10.1. Tensile strength of the antimicrobial packaging film

The tensile strength of the bio-film is an important mechanical property of the packaging biofilm during application. The effect of glycerol concentration (10, 20, and 30 v/wt. %) on the tensile strength of the bio-film is shown in Figure 4.22.

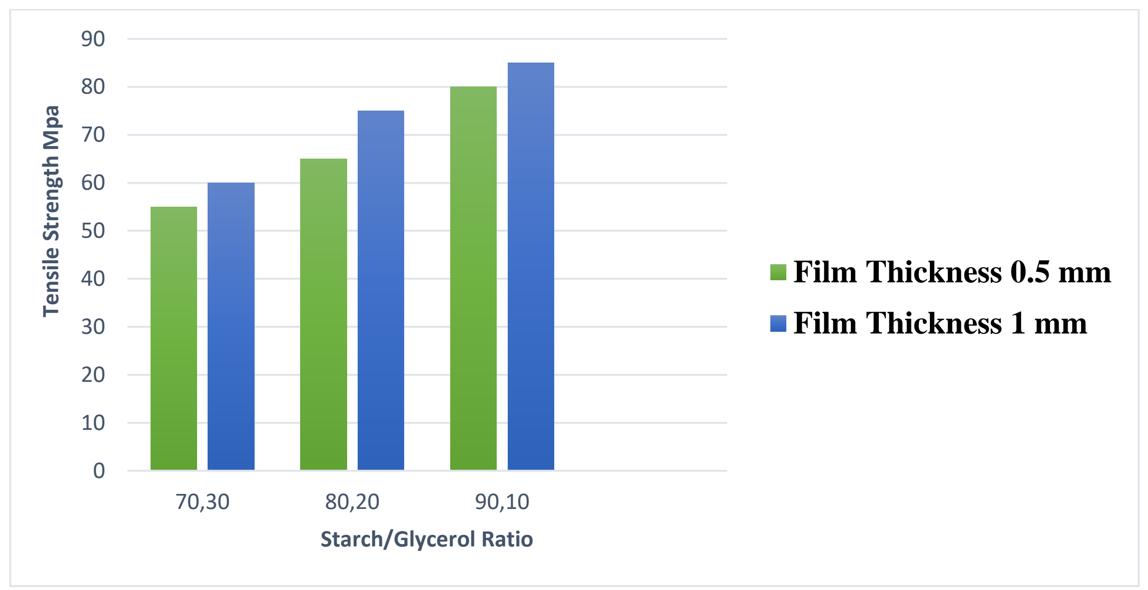


Figure 4. 22 The effect glycerol/starch ratio, and film thickness on tensile strength.

The presence of plasticizer at a lower concentration of 90:10 (starch/glycerol ratio), higher concentration of antimicrobial concentration (10 g), and film thickness (1 mm) demonstrated a high tensile strength value of 27.8 MPa. The possible reason for the high tensile strength at low plasticizer concentration is the domination of strong hydrogen bonds produced by a starch–starch intermolecular interaction over starch–plasticizer attraction (Muhammed L. et al., 2015). When the glycerol/starch ratio is increased, the hydrogen bond is weakened. This may be related to the amount of starch decreased which results in decreasing the amylose and amylopectin in the film. The pure amylose structure is very stable, with strong molecular orientation, forming films denser, higher glass transition temperature, tensile strength and modulus of elasticity values, and lower elongation values than low amylose starch films.

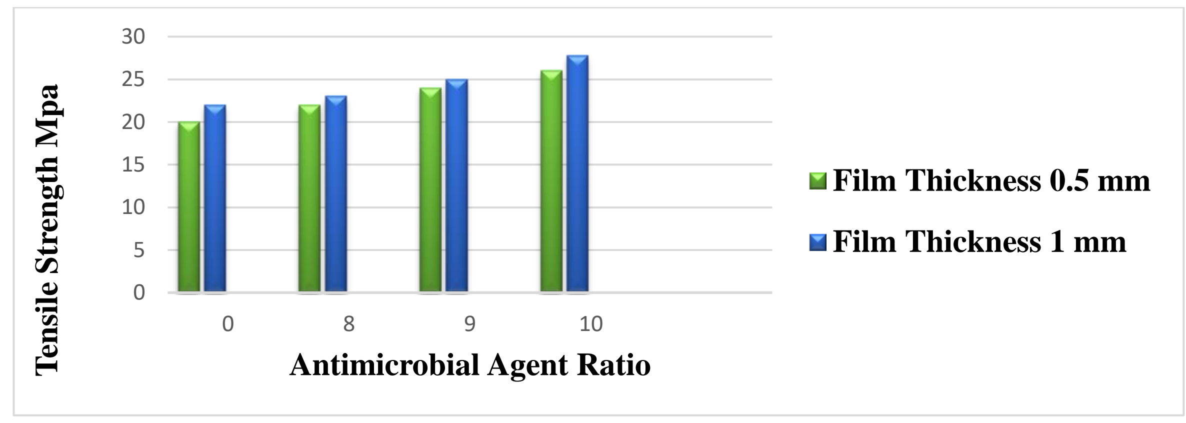


Figure 4. 23 The effect of antimicrobial concentration, and film thickness on tensile strength.

Also, the presence of plasticizer at a higher concentration of 70:30 (starch/ glycerol ratio), lower concentration of antimicrobial concentration (8 g/mL), and film thickness (0.5 mm) demonstrated a low tensile strength value of 24.2 MPa. Therefore, these results also describe that high antimicrobial concentration and film thickness had a positive effect on the tensile strength of the antimicrobial packaging films.

4.10.2. Elongation Break

The percentage elongation (E) values of the film with the incorporated nettle leaf extracted were the measure of the flexibility of the film and were affected by the starch/glycerol to film thickness. An increase in the percentage elongation with an increase in starch content is due to

the reduction in the number of intermolecular cross-links and increase in the inter-molecular distance (Gashaw & Shimeles, 2013). Structurally, starch contains amylopectin which is a branched polymer.

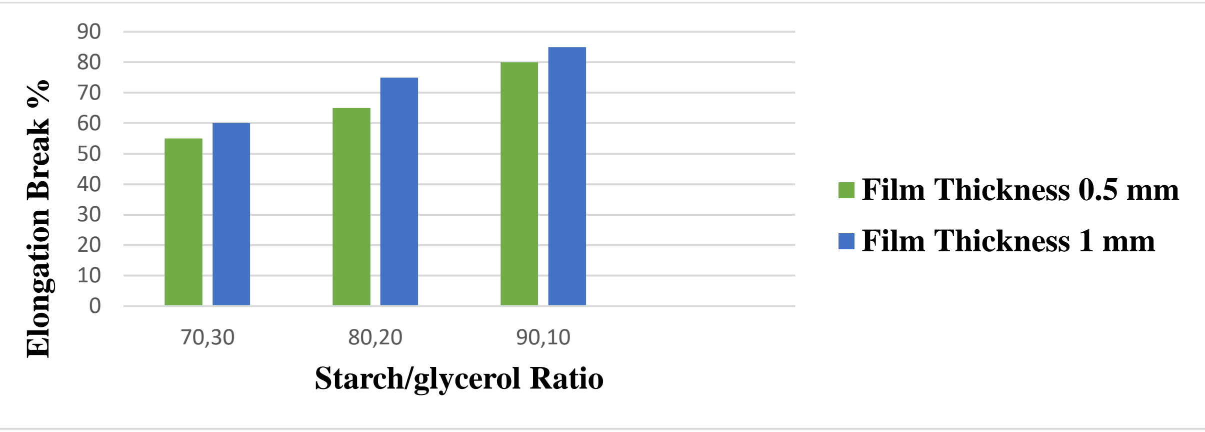


Figure 4. 24 The effect Starch/glycerol and film thickness on Elongation Break.

As a result, the incorporation of nettle leaf into the starch-based film led to improvement in both tensile strength and young's modulus due to the reinforcement effect.

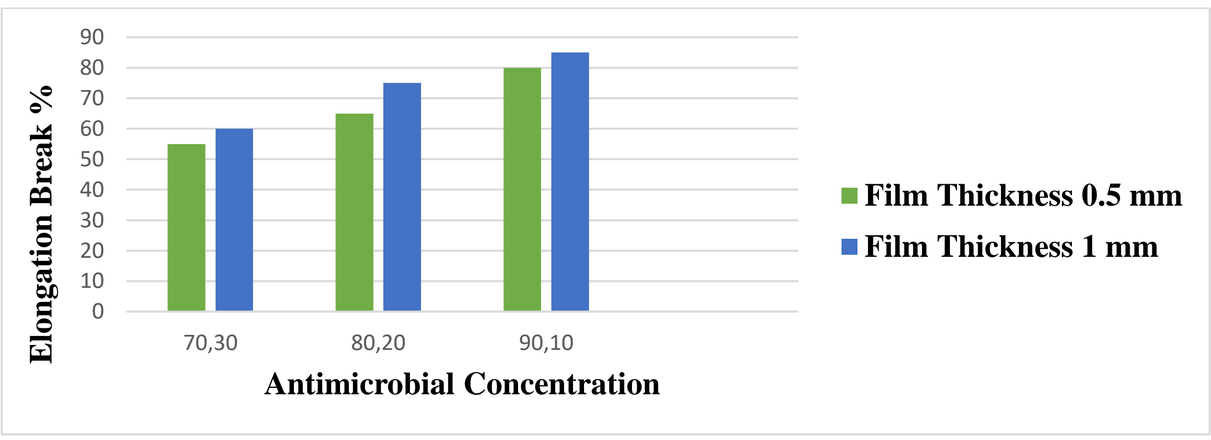


Figure 4. 25 The effect antimicrobial concentration and film thickness on Elongation Break.

5. CONCLUSION AND RECOMMENDATION

5.1. Conclusion

During this globalization era, food industry services have become unbounded to geographical boundaries. However, by their nature foods are susceptible to different microbes and physical damages. Hence, retaining the quality of the food (physical, biological, and chemical); requires proper handling, transportation, and protection from possible damages. To do so, proper packaging plays a vital role. As a result, food packaging has become an important issue in the food industry. Plastics are the majorly used packaging materials in the industry. However, it is a non-biodegradable, non-renewable, and unevenly distributed material. Besides, it lacks some important features such as antimicrobial activity in the food packaging.

Thus, to overcome these limitations this study was designed. In the study, antimicrobial packaging film was produced using a starch extracted from cassava roots, an antimicrobial agent extracted from nettle leaf, and glycerol as a plasticizer. Both raw materials were prepared (collection, cleaning, and size reduction). Then, the starch from cassava and the liquid from the nettle leaf are extracted. The extract from the nettle leaf was characterized and its antimicrobial activities are tested on four microbes: Staphylococcus aureus, Salmonella typhi, and E. coli and Candida. To know the amount of nettle extract required to inhibit microbial growth, the minimum nettle extract concentration required to hinder the microbes' growth was determined. Thereafter, the agar (MH broth and MHA for bacteria and SDA for antifungal activity) was prepared for microbial growth using the broth dilution method. Using the prepared cassava, nettle leaf extract and glycerol, a packaging film with different thickness (0.5 mm and 1 mm) were prepared. Thereafter, the antimicrobial activity and physical and mechanical properties of the film were determined. In designing the experiments, three factors namely starch/glycerol ratio (A), antimicrobial concentration (B), and film thickness (C) and mixed levels (three levels for A and B and two levels for C) and inhibition diameter of each microbe were used as a response. Based on the above experiment design, the experiments we conducted and the following results were obtained.

- The phytochemical test shows that the nettle leaf mainly contains Saponins, phenols, and tannins. This shows that the nettle leaf exhibit antimicrobial activity.

- The minimum concentration test (MIC) shows that all microbes cease growing around 10 mg/mL concentration of nettle leaf extract. This indicates as the nettle leaf extract can hinder microbial growth at low concentrations and as it is a potential microbial agent.
- Physical property test shows that the physical properties (moisture content, solubility, and swelling capacity) decrease as the extract concentration increases. This proves the packaging material becomes more resistant to microbial attacks as the concentration increases.
- Mechanical property test prevails that the mechanical properties (tensile strength and elongation break) increase with an increase in nettle extract concentration. This also proves as the film becomes more tensile and robust with an increase in nettle extract concentration.
- The antimicrobial test presents as inhibition diameter of the microbes increased with an increase of the concentration of antimicrobial agents.
- The ANOVA analysis shows that all three factors and the AC and BC interactions are significant for all microbes. The parameters also show that the model is well fitted as the predicted values are closer to the actual values.
- The optimization result shows that Starch/glycerol ratio 70:30, Antimicrobial Concentration 10 g/mL, and film thickness 1mm are optimum parameters for producing antimicrobial packaging film.
- Based on the above facts, the developed packaging film is a potential alternative to commercially available food packaging materials.

Therefore, by doing additional studies for its practicability, nettle leaf extract and cassava starch-based films can be used as food packaging to elongate the shelf-life and maintain the quality of perishable food products.

5.2 Recommendation

Based on the analysis carried out in this study the following recommendation was provided.

- Quantitative analysis of the phytochemicals present in the nettle leaf must be determined.
- The effect of processing on the nettle leaf extracts' antimicrobial activities must be studied.
- The effectiveness of these antimicrobial packaging films on different foods during storage and transportation must be studied.
- The migration kinetics of the antimicrobial component from the film to the food must be studied.
- Profitability analysis of the film must be done.

References

Antolak, H. (2017). Antibacterial and antiadhesive activities of extracts from edible plants against soft drink spoilage by Asaia spp. *J. Food Protection*, 5-34.

Antonio Bevilacqua, M. R. (2017). *The Microbiological Quality of Food.* Foggia, Italy.

Astuti, P. &. (2014). Antimicrobial Edible Film from Banana Peels as Food Packaging Abstract. 65–70.

Baher, Z.F., Mirza, M., Ghorbanli, M., & Bagher Rezaii, M.,. (2002). The influence of water stress on plant height, herbal and essential oil yield and composition in Satureja hortensis L. Flavour Fragr J, 17:. 275–277.

Baker and Pariz Maluta . (2014). *Most food patogenic organism relation ship in E.coli*.

Baldwin, E. (1994). Edible coatings for fresh fruits and vegetables: past, present and future In:edible coatings and films to improve food quality. 25-26.

Baldwin, E.A, Nisperos-Carriedo, M.O & Baker, R.A. . (1995). *Use of edible coatings to preserve quality of lightly and (slightly) processed products, Critical Reviews in Food Science and Nutrition*, 24-35.

Barreto, P. L., Pires, A. T., Soldi, V. (2003). Polymer Degradability Stabilities. 147–152.

Basaraba, J. (1964). Effects Of Vegetable Tannins On Glucose Oxidation. 787-793.

Bhargavi, G. (2017). Review on the Extraction Methods of Crude oil. 3-5.

Brody, A. (2005). Packaging Food Technology.

Burt. (2004). Essential oils: Their antibacterial properties and potential applications in foods. *Int J Food Microbiol*, 223-253.

Carbonell, J. G. (2017). Development of multi-layer films containing natural antimicrobial agents. 25-26.

Castro, N. (2012). *Development and characterize of edible film based on cassava starch-glycerol blends to incorporate nutriceuticals.* new,jersey.

Coma V., Sebti I., Pardon P., Deschamps A. and Pichavant F. H. (2001). Antimicrobial edible packaging based on cellulosic ethers, fatty acidsmand nisin incorporation to inhibit Listeria innocua and Staphylococcus aureus. *Journal food science* , 470 - 475.

Cruz R., Baptista P., Cunha S., Pereira J.A., Casal S. (. (2012). Caretonoids of Lettue (Lactuca sativa L.) grown on soil enriched with spent coffee grounds. *Journal of Food Composition and Analysis*, 111-119.

CRUZ, E. N. (2012). Development and characterization of edible films based on cassava. 45-73.

Da Róz, A.L, Carvalho, A.F., Gandini, A. & Curvelo, A.S. (2006). The effect of plasticizers on thermoplastic starch compositions obtained by melt processing, Carbohydrate Polymers. 417-424.

Daniel Ikhane, Kolawole Banwo, and Ogunremi Omotade. (2014). Phytochemical and Antimicrobial Activities of Methanolic Extract of Paullinia pinnata Leaf on Some Selected Bacterial Pathogens. *Journal of Herbs, Spices & Medicinal Plants*, 62-64.

Dugo, G. & Di Giacomo, A. (2002). Antidiabetic effects of sage (Salvia officinalis L.) leaf in normal and streptozotocin-induced diabetic rats. 40-44.

Eker, B. (2016). PACKAGING MATERIALS AND effect on quality of life . *Aysel Içöz*, (p. 272). Turkey,istanbul.

Eldhose, B. N. (2013). Evaluation of Phytochemical Constituents and In vitro Antioxidant Activities of Plumbago indica Root Extracts. 157–161.

Eraricar Salleh, I. I. (2009). Structural Characterization and Physical. *World Academy of Science, Engineering and Technology*, 1-2.

Eraricar Salleh, Ida Idayu Muhamad, and Nozieanna Khairuddin. (2009). *Structural Characterization and Physical Properties of Antimicrobial (AM)*, 431-433.

Ferede, B. (2104). isolation, identification, antimicrobial susceptibility test and public awareness of salmonella on raw goat meat at dire dawa municipal abattoir, eastern ethiopia. 10-12.

fhrh. (345). thjreyj. *rhwh*, 11e.

Fornari, T., Vicente, G., Vázquez, E., García-Risco, M.R., & Reglero, G. (2012). Isolation of essential oil from different plants and herbs by supercritical fluid extraction. 34–48.

G, K. (2003). An introduction to Urtica (botanical aspects). 1-11.

Garcia, M.A., Martino, M.N. & Zaritzky, N.E. (2000). Lipid Addition to Improve Barrier Properties of Edible Starch-based Films and Coatings. *journal of food science* , 941-944.

Gashaw & Shimeles. (2013). Development and Characterization of Antimicrobial Packaging Films. *Journal of Food Processing & Technology*, 5-8.

Gennadios, A. a. (1997). Application of edible coatings on meats, poultry and sea foods: A review. 337-350.

Gisila, T. (2018). Extraction, Kinetics Study and Characterization of Moringa. 23-28.

Guil-Guerrero J.L., Rebolloso-Fuentes M.M., TorijaIsasa M.E. (2003). Fatty acids and carotenoids from stinging nettle (Urtica dioica L.). *Journal of Food Composition and Analysis*, 111-119.

Guil-Guerrero J.L., Rebolloso-Fuentes M.M., TorijaIsasa M.E. (2003). Fatty acids and carotenoids from stinging nettle (Urtica dioica L.). *Journal of Food Composition and Analysis, Vol. 16,*, 111 – 119.

Gülçin, I.; Küfrevioglu, O.I.; Oktay, M.; Büyükokuroglu, M.E. (2004). Antioxidant, antimicrobial, antiulcer and analgesic activities of nettle (Urtica dioica L.). *J. Ethnopharmacol*, 205–215.

Hojnik M., Škerget M., Knez Ž. (2007). Isolation of chlorophylls from stinging nettle (Urtica dioica L.). *Separation and Purification Technology, Vol. 57, Issue 1,*, 37-46.

Hughes R.E., Ellery P., Harry T., Jenkins V., Jones E. (1980). The dietary potential of the common nettle . *Journal of the Science of Food and Agriculture, Vol. 31, Issue 12*, 1279 – 1285.

J., A. R. (1980). Studies of chemical substances present in Urtica dioica leaf. Part I Trace element. *Herba Polonica, Vol. 26, No. 3*, 177-180.

Jay Loessner & Golden. (2005). *Food Born bacteria*, 25.

Jutaporn, C. (2011). Antimicrobial activity and characteristics of edible films incorporated. 39-54.

Kang, J.-Y., Chun, B.-S., Lee, M.-C., Choi, J.-S., Choi, I.S., & Hong, Y.-K. (2016). Antiinflammatory activity and chemical composition of essential oil extracted with supercritical CO2 from the brown seaweed Undaria pinnatifida. J Essent Oil Bear plants. 46–51.

Kenneth Marsh, P. A. (2007). Food Packaging—Roles, Materials,and Environmental Issues. *scientific Status Summary—Food Packaging* . ., 40-45.

Koubaa, M., Roselló-Soto, E., Šic Žlabur, J., Režek Jambrak, A., Brnčić, M., Grimi, N.,. (2015). Current and new insights in the sustainable and green recovery of nutritionally valuable compounds from Stevia rebaudiana Bertoni. 6835–6846.

Kukri´c, Z., & Topali´c-Trivunovi´c, L. (2012). Characterization of antioxidant and antimicrobial activities of nettle leaf (Urtica dioica L.). 1–12.

Kuorwel, K. (2011). Incorporation of natural antimicrobial agents in to starch based material for food packaging. *Journal of food science*, 76.

M. Jawaid, M. R. Ishak & J. Sahari M. L. Sanyang, S. M. Sapuam. (2015). Effect of plasticizer type and concentration on physical properties of biodegradable films based on sugar palm (arenga pinnata) starch for food packaging. *Food Science and Technology* , 326-328.

Mahmoudi, R., Amini, K., Fakhri, O., & Alem, M. (2012). Aroma profile and antimicrobial properties of alcoholic and aqueous extracts from root, leaf and stalk of nettle (Urtica dioica L.). *J. Microbiol. Biotechnol. Food Science*, 220–224.

Mau-Change Chen, Gene Horng-Chin Yeh. (1996). Antimicrobial And Physicochemical Properties Of Methylcellulose And Chitosan. *Journal of Food Processing and Preservation*, 379-390.

McHugh, T.H., Aujard, J.-F., Krochta, J.M. (1994). Plasticized whey protein edible films: water vapor permeability properties. *Journal of Food Science*, 419-423.

Mesaret Zerihun, Teshome Worku and Sushil R. Sakkalkar . (2016). Development and Characterization of Antimicrobial Packaging Films. *Journal Of Ready To Eat Food*, 22-23.

Muche, M. (2018). Production and Characterization of Starch Based Antimicrobial Packaging Film through Incorporation of Plumbago Zeylanica (Amira) Root Crude Extract. 40-45.

Muhammed L. Sanyang,Salit M. Sapuan, and Mohammad Jawaid. (2015). Effect of Plasticizer Type and Concentration on Tensile, Thermal and Barrier Properties of Biodegradable Films Based on Sugar Palm (Arenga pinnata) Starch. *polymers*, 1109 - 1113.

Myllärinen, P., Partanen, R., Seppälä, J., & Forssell, P. (2002). Effect of glycerol on behaviour of amylose and amylopectin films. Carbohydrate Polymers. 355-361.

Nevena T. Nemet, Vladislava M. soso and Vera L. Lazic. (2010). Effect Of Glycerol Content And Ph Value Of Film-Forming Solution On The Functional Properties Of Protein-Based Edible Films. 61-62.

Nurul, N. (2010). Biodegradable biocomposite starch based films blended with chitosan and gelatin.

Pecorino-Issartier, S., Ginies, C., Cravotto, G., & Chemat, F. (2013). A comparison of essential oils obtained from lavandin via different extraction processes: Ultrasound, microwave, turbohydrodistillation, steam and hydrodistillation. J Chromatogr A,. 41–47.

Prabha, P. H. (2017). Modelling And Optimization Of Cassava Starch Based Film For Food Packaging Applications. 672-678.

R. Velasco Lezama,R. Tapia Aguilar and T. E. Sánchez Siles. (2020). Antibacterial Activity Of Three Urtica Species. *International Journal of Pharmaceutical Sciences and Research*, 1293-1297.

R.Ahvenainen, V. (2012). *Novel Food Packaging Techniques.* Filand.

Raheem, D. (2012). *Application of plastics and paper as food packaging materials – An overview*, 178-179.

Rahmanian,m.pakizeh. (2011). hazard mater. In application of expermental design approach and artifial neural network (ANN) for determination of potential micellar enhanced ultrafiltration process. 68-72.

Raimova, K. (2020). Comprehensive Study Of The Chemical Composition Of Urtica Dioica L. *Journal of Critical Reviews*, 750-756.

Rajpal, G. (2007). Color and Mechanical Properities of Chistosan film during storage. 1-2.

Rojas, J., & Buitrago, A. (2015). Essential oils and their products as antimicrobial agents Progress and prospects. In: Duarte, M.C.T., Rai, M. (eds.), Therapeutic Medicinal Plants Boca Raton, FL: CRC Press,. 253–278.

Rounds, J. M., Hedberg, C. W., Meyer, S., Boxrud, D. J. and Smith, K. E. (2010). The bacterium of swine plague. Am. Month. 15-25.

S. L. Ezeoha1 and J. N. Ezenwanne. (2013). *Production of Biodegradable Plastic Packaging Film from Cassava Starch*, 15-16.

Saviano, A. (2015). Design of expert applied to pharmaceutical and analytical quality. . 1-7.

Shale. (1999). Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. *J. Ethnopharmacol*, 347–354.

Shaza Sitrallah and Joumaa Merza. (2018). Chemical Composition of Essential Oil Extracted from Urtica pilulifera and Evaluation Its Biological Activity. *Food Science and Quality Management*, 45-47.

Singh, R. H. (2001). *Introduction to Food Engineering.* London, UK:: Academic Press.

Sukri, N. A. (2012). Effect of Different Types of Solvent On Extraction of Phenolic Compounds From Cosmos caudatus. 16-21.

Upton R., Graff A., Jolliffe G., Langer R., Williamson E. (2011). American Herbal Pharmocopoeia: Botanical Pharmacognosy – Microscopic Characterization of Botanical Medicines,. 800.

Van Soest, J.G., & Essers, P. (1997). Influence of Amylose-Amylopectin Ratio on Properties of Extruded Starch Plastic Sheets. Journal of Macromolecular Science. 1665-1689.

Vogl C.R., H. A. (2003). Production and processing of organically grown fiber nettle (Urtca dioica L.) and its potential use in the textile industry. *American Journal of Alternative Agriculture, Vol 18 No 3,*, 119-128.

zewdu, z. (2020). plastprintpack. *4th International Trade Show* (pp. 6-8). addis abeba: ethiopian custom.

Zhang, M., Tang, J., Mujumdar, A.S., & Wang, S. (2006). Trends in microwave-related drying of fruits and vegetables. *Trends Food Sci Technol*, 524-534.

Zinash Assefa and Shimelis Admassu. (2013). *Development and Characterization of Antimicrobial Packaging Films*, 3-4.

Žnidarčič D., Ban D., Šircelj H. (2011). Carotenoid and chlorophyll composition of commonly consumed leafy vegetables in Mediterranean countries. *Food Chemistry, Vol. 129, Issue 3*, 1164-1168.

Zoran Z. Kukrića*, Ljiljana N. Topalić-Trivunovića, Biljana M. Kukavicab,. (2012). Characterization Of Antioxidant And Antimicrobial, 260-266.

Appendix A

Appendix A1 McFarland standard preparation

McFarland standards are suspensions of either barium sulphate or latex particles that allow visual comparison of bacterial density (Fig. 1). A 0.5 McFarland standard is equivalent to a bacterial suspension containing between 1 x108 and 2 x 108 CFU/ml of *E. coli*. A 0.5 McFarland standard could be prepared as describe below according to EUCAST Version 1.0, Dec 2009.

1. Add a 0.5-ml aliquot of a 0.048 mol/liter BaCl2 (1.175% wt/vol BaCl2 • 2H20) to 99.5 ml of 0.18 mol/liter H2SO4 (1% vol/vol) with constant stirring to maintain a suspension.
2. Verify the correct density of the turbidity standard by measuring absorbance using spectrophotometer with a 1-cm light path and matched cuvette. The absorbance at 625 nm should be 0.08 to 0.13 for the 0.5 McFarland standard.
3. Transfer the barium sulfate suspension in 4- to 6-ml aliquots into screw-cap tubes of
4. the same size as those used in standardizing the bacterial inoculums.
5. Tightly seal the tubes and store in the dark at room temperature
6. Mix the standard thoroughly on a vortex mixer immediately before use.

Appendix A2 Media preparation for Inhibition Zone Assay and agar slant preparation.

The medium is prepared differently for slants and Petri dishes. Sterilization is done with the agar in the tubes; Petri dishes are pre-sterilized before sterilized agar is poured into them. Measure the amount of water needed and put it in a pot. Heat it on a stove until it is almost boiling. The formulations used for preparing media for the inhibition zone assay and agar slant is given in the Table below. All the ingredients were dispersed in distilled water and autoclaved at 121 °C for 15 minutes prior to use.

$$\text{For Muller Hinton agar, 38 grams} = \text{ in 1000 ml of distilled water}$$

$$? = \text{ X ml of distilled water}$$

$$\text{For Muller Hinton broth, 21 grams} = \text{ in 1000 ml of distilled water}$$

$$? = \text{X ml of distilled water}$$

$$\text{For Sabroud dextrose agar, 62 grams} = \text{ in 1000 ml of dist}illed\ water$$

$$? = X\ ml\ of\ distilled\ water$$

Appendix Table 1 Formula for media used in inhibition zone agar preparation

Test organism	Selective Medium	Formulation (g/L of water
	Muller Hinton agar	38
	Potato Dextrose agar	39
S. Aureus	Manitol salt agar	43
E. Coli	MacConkey Agar	51.5
S. Typhi	Nutrient Broth	13
Candida albician	Saboraud Dextrox Agar	65

Appendix A3 Sabouraud Dextrose Agar or SDA

Sabouraud Dextrose Agar or SDA was formulated by Raymond Sabouraud in 1892. Sabouraud Dextrose Agar is used for the cultivation of fungi (yeasts, moulds), particularly useful for the fungi associated with skin infections. This medium is also employed to determine microbial contamination in food, cosmetics, and clinical specimens. The pH is adjusted to approximately 5.6 in order to enhance the growth of fungi, especially dermatophytes, and to slightly inhibit bacterial growth in clinical specimens. Antibacterial agents can also be added to augment the antibacterial effect. Chloramphenicol, gentamicin, and tetracycline are selective agents added to inhibit bacterial overgrowth of competing microorganisms while permitting the successful isolation of fungi and yeasts.

Appendixes B Regression Analysis for Each Response

Appendixes B1: Results of regression analysis for Response 1

Results of regression analysis for Response 1 Inhibition Diameter *E. coli*

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	6.94	1	0.11	6.71	7.18
Block 1	-0.22	1			
Block 2	0.22				
A [1]	-1.21	1	0.14	-1.50	-0.92
A [2]	0.014	1	0.080	-0.15	0.18
B [1]	3.33	1	0.14	3.05	3.62
B [2]	0.14	1	0.080	-0.027	0.31
C-Film Thickness	0.94	1	0.11	0.71	1.18
A [1]B[1]	0.12	1	0.17	-0.23	0.48
A [2]B[1]	0.042	1	0.098	-0.16	0.25
A [1]B[2]	-0.17	1	0.098	-0.37	0.037
A [2]B[2]	0.028	1	0.057	-0.090	0.15
A [1]C	-0.46	1	0.14	-0.75	-0.17
A [2]C	-0.069	1	0.080	-0.24	0.097
B [1]C	0.33	1	0.14	0.045	0.62
B [2]C	0.14	1	0.080	-0.027	0.31

Appendixes B2 Results of regression analysis for Response 2

Results of regression analysis for Response 2 Inhibition Diameter *S. Typhi*

Term	Coefficient Estimate	df	Standard Error	95% CI low	95% CI High
Intercept	31.08	1	0.19	30.69	31.47
Block 1	-0.028	1			
Block 2	0.028				
A [1]	-1.87	1	0.23	-2.35	-1.4
A [2]	0.21	1	0.13	-0.067	0.48
B [1]	2.42	1	0.23	1.94	2.89
B [2]	-0.25	1	0.13	-0.53	0.026
C-Film Thickness	0.53	1	0.19	0.14	0.92
A [1]B[1]	0.062	1	0.28	-0.52	0.65
A [2]B[1]	-0.35	1	0.16	-0.69	-0.016
A [1]B[2]	-0.062	1	0.16	-0.4	0.28
A [2]B[2]	0.021	1	0.094	-0.17	0.22
A [1]C	0.71	1	0.23	0.23	1.19

A [2]C	0.35	1	0.13	0.071	0.62
B [1]C	0.83	1	0.23	0.36	1.31
B [2]C	0.056	1	0.13	-0.22	0.33

Appendixes B3 Results of regression analysis for Response 3

Results of regression analysis for Response 3 Inhibition Diameter *S. Aureus.*

Term	Coefficient Estimate	df	Standard Error	95%CI Low	95% CI High
Intercept	21.03	1	0.19	20.63	21.42
Block 1	0.028	1			
Block 2	-0.028				
A [1]	-1.79	1	0.23	-2.28	-1.31
A [2]	0.18	1	0.13	-0.1	0.46
B [1]	2.5	1	0.23	2.01	2.99
B [2]	-0.28	1	0.13	-0.56	2.54E-03
C-Film Thickness	0.58	1	0.19	0.19	0.98
A [1]B[1]	-0.063	1	0.29	-0.66	0.53
A [2]B[1]	-0.31	1	0.17	-0.66	0.031
A [1]B[2]	-0.021	1	0.17	-0.36	0.32
A [2]B[2]	6.94E-03	1	0.095	-0.19	0.21
A [1]C	0.62	1	0.23	0.14	1.11
A [2]C	0.38	1	0.13	0.095	0.66
B [1]C	0.75	1	0.23	0.26	1.24
B [2]C	0.083	1	0.13	-0.2	0.36

Appendixes B4: Results of regression analysis for Response 4

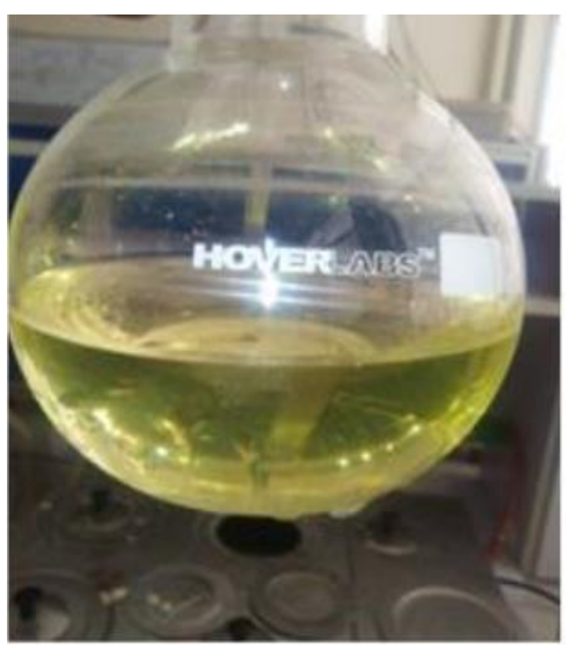
Results of regression analysis for Response 4 Inhibition Diameter *C. Albicans.*

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	6.78	1	0.12	6.52	7.03
Block 1	-0.38	1			
Block 2	0.38				
A [1]	-1.2	1	0.14	-1.52	-0.89
A [2]	-0.06	1	0.08	-0.24	0.11
B [1]	3.29	1	0.14	2.97	3.6
B [2]	0.09	1	0.08	-0.08	2.70E-01
C-Film Thickness	0.88	1	0.12	0.63	1.14
A [1]B[1]	0.18	1	0.18	-0.19	0.56

A [2]B[1]	0.02	1	0.1	-0.19	0.24
A [1]B[2]	-0.1	1	0.1	-0.32	0.11
A [2]B[2]	6.00E-03	1	0.06	-0.12	0.13
A [1]C	-0.62	1	0.14	-0.93	-0.31
A [2]C	-0.09	1	0.08	-0.27	0.08
B [1]C	0.29	1	0.14	-0.02	0.6
B [2]C	0.15	1	0.08	-0.02	0.33

Appendix C Tannins and saponins Indicators

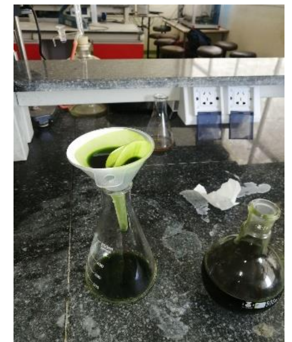
Dark green color indicates tannin



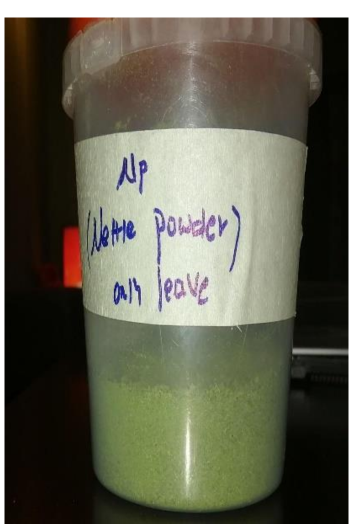
Light Creamy color indicates Saponin

Appendix D Comparison of other literature

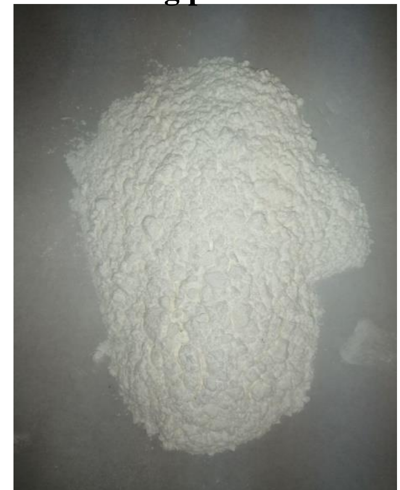
Concentration of Plumbago root Extract(mg/mL)	Visible growth of microorganisms			
	S. typhi	*E. coli*	*S. Aureus*	*Candida albicans*
6	Yes	Yes	Yes	No
10	No	Yes	Yes	No
12	No	No	No	No
Control without Antimicrobial Agent	Yes	Yes	Yes	Yes



Cassava starch filtration **Filtration process of nettle leaf**



Cassava starch heating process **Film making process** **Nettle Leaf Powder**



Soxhlet Extraction Process **Cassava starch powder** **Nettle Leaf Extracted**

Air Drier

Sedimentation process of cassava starch juice

Cassava Tuber

UV-Spectrophotometer

Muslin cloth (for filtration of cassava juice)

Evaporation

Appendix E. Pictures taken during this research work.

Cassava Starch after sedimentation

Sieving materials

Film without AM Agent

Film with AM Agent