Addis Ababa Institute of Technology (AAiT)

School of Graduate Studies

Department of Chemical Engineering

Development and Evaluation of Antimicrobial Aloe Based Packaging Films

A Thesis Submitted to the School of Graduate Studies of Addis Ababa Institute of Technology in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemical Engineering (Food Engineering Stream)

By: Gashaw Asefa

Advisor: Dr.Eng. Shimelis Admassu (Associate Professor)

Addis Ababa, Ethiopia

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Abstract

This study was aimed at the development of antimicrobial packaging films, which is one of the most promising active packaging systems, from aloe and papaya leaves extracts with gelatine and glycerol. Three different extracts of papaya leaves were analyzed for their antimicrobial activities. Mechanically obtained papaya leaf extract showed the best result (12.10mm inhibition zone on S.typhi). Based on antimicrobial activity, film forming ability, transparency, and colour; *Aloe debrana* extract was found to be more appropriate for the development of antimicrobial packaging films than *Aloe trichosantha* extract. The antimicrobial activity of *Aloe debrana* extract was increased by the incorporation of papaya leaf extract up to 30%, above which it did not bring significant influence on most test organisms. As a result 70% *Aloe debrana* extract and 30% papaya leaf extract standard solution was prepared. Various concentrations of gelatine and glycerol were added to the standard solution for packaging films development. Films were evaluated for their antimicrobial activities, physicochemical, and mechanical properties. The antimicrobial activities of aloe based films were tested on *E. coli*, *S. typhi*, *S.aureus*, *C. albicans*, and *F. xylarioides*. All films exhibited inhibitory zones on the test microorganisms used in this study. A wide inhibition zone (6.52cm^2) was observed on *S. typhi* growth whereas the least (4.20 cm^2) was on *C.albicans.* Films were soluble in water with highest solubility (90.49%) for $P_{1,1}$ (film with 1g glycerol and 1g gelatine) and lowest (44.57) for $P_{0,2}$(film developed by adding only gelatine). Film solubility significantly increased as the concentration of glycerol increased and decreased as the concentration of gelatine increased. Film $P_{0.5,2}$(with 0.5g glycerol and 2g gelatine) has showed maximum tensile strength (65MPa) where as the lowest (20MPa) was obtained by film $P_{0.5,1}$(0.5g glycerol and 1g gelatine). Increasing gelatine concentrations significantly increases the tensile strength but glycerol has an opposite effect on the tensile strength. All the films were highly flexible and stretchable. Film $P_{1,2}$(1g glycerol and 2g gelatine) showed the maximum elongation (180%) and the minimum elongation (89%) was obtained for $P_{0.5,1.}$ This study demonstrated the technological feasibility of developing antimicrobial packaging films from aloe and papaya leaves extracts.

Keywords: Aloe extracts, Antimicrobial packaging film, Mechanical properties, Papaya extracts, Pathogenic microorganisms, Physicochemical properties

Table of Contents

List of tables

List of figures

List of abbreviations

ADE	*Aloe debrana* extract
AM	Antimicrobial
AP	Active packaging
ATE	*Aloe trichosantha* extract
ATCC	American type culture collection
BGA	Brilliant green agar
CFU	Colony forming units
EB	Elongation at break
EHNRI	Ethiopian health and nutrition research institute
FFS	Film-forming solution
FS	Film solubility
HTST	High Temperature Short Time
MA	MacConkey agar
MARC	Melkassa agricultural research center
MHA	Mueller Hinton agar
MSA	Manitol salt agar
NB	Nutrient broth
PDA	Potato dextrose agar
PLALE	Papaya leaf alcoholic extract
PLE	Papaya leaf extract
PLAQE	Papaya leaf aqueous extract
SDA	Sabouraud dextrose agar
TS	Tensile strength

Chapter one

Introduction

1.1. Background

The packaging industry of developing countries is a market with a value of US$ 15.4 billion. This represents around 27% of the packaging materials that have been exported worldwide in the past five years (2005-2009) and includes glass, paper, plastic, and wood. Import of plastic materials is the highest in terms of value ($ 9.5 billion), followed by paper ($ 4.0 billion), then by glass ($ 1.6 billion), and last is wood materials for packaging at a comparatively low level ($ 0.3 billion) (Nerlita *et al*., 2011).

Packaging can either be flexible or rigid with the former fast replacing the more traditional rigid form, owing to cost and flexibility advantage. Flexible packaging includes materials such as film, foil or paper sheeting. Rigid packaging includes glass, rigid metal, and wood. As to the share of the total packaging market, paper (34%) tops the list, followed by rigid plastic (27%), metal (15%), glass (11%), flexible packaging (10%), and others (3%). The flexible packaging market is expected to grow by around 3.2% annually over the next five years with food accounting for 75-80% of the demand (Nerlita *et al*., 2011).

The packaging industry may be classified in relation to the type of end user, namely: individual consumers, institutional and industrial users with the latter further sub categorized by industry type, namely, food, beverage and petrochemicals. Food accounts for 50% of the global consumer packaging industry valued at US$ 380 billion as of 2009. If the beverage sector is to be added, that will even increase to 69%. That the food and beverage market account for more than half of the packaging market is a worldwide phenomenon. In developing countries the growing demand from the food and beverage market has been instrumental in stimulating the overall growth in the packaging industry (Global Industry Analysts, 2010).

The three leading food packaging industries are located in the U.S., Europe and Asia. Regarding biodegradable packaging the market has been developing rapidly over the last decade and, although exact figures of this development are not accessible, a number of estimations denote an annual growth greater than 20% (European Commission, 2008). According to the European Bioplastics association, the global production of biodegradable

bioplastics reached an annual capacity of 400 thousand tonnes in 2009 (Observatory NANO, 2010).

The global demand for bioplastics will rise more than 400% mainly due to high crude oil and gas prices. Other important factors include: consumer demand for more environmentally sustainable products; the increased production of certain bioplastics; and political and regulatory pressure to reduce non-degradable plastics. Europe accounts for approximately 40% of global demand for bioplastics, driven by consumer demand, regulatory pressures for greener packaging options, and a growing composting infrastructure (Observatory NANO, 2010).

Food packaging has developed strongly during recent years, mainly due to increased demands on product safety, shelf-life extension, cost-efficiency, environmental friendliness, and consumer convenience. Novel packaging technologies have great commercial potential to ensure the quality and safety of food with fewer or no additives and preservatives, thus reducing food wastage, food poisoning and allergic reactions (Raija, 2003).

In order to control microbial growth in foods and improve their shelf-life and safety, different antimicrobial agents are mixed with the initial food formulations or they are applied to food surface by dusting, dipping or spraying. Incorporation of antimicrobial agents directly into food is appropriate when there is a risk of microbial growth at both surface and internal parts of final food product. However, when the main cause of spoilage of food is microbial growth at the food surface, the use of this method causes addition of excessive amounts of chemical additives into food materials. In addition, the antimicrobial compound directly added into the food cannot selectively target the food surface where spoilage reactions occur more intensively. Thus, this traditional strategy does not fit to the current trend of food technology to develop healthier processed foods by using minimum amounts of chemical additives (Min and Krochta, 2005)

The application of antimicrobial agents on food surface by different methods also has limited beneficial effects since this causes reduction of surface concentration of the antimicrobial due to its diffusion from food surface to interior parts (Min and Krochta, 2005). Another disadvantage of direct application of antimicrobials (chemical or natural) to food materials is neutralization of the added agent due to its possible complex interactions with the food components (Appendini and Hotchkiss, 2002). Therefore, AM packaging is an alternative method to overcome the above limitations since the agent slowly release from the film onto

the food surface during the storage, hence, maintains its critical concentration necessary for inhibiting the microbial growth as stated by Buonocore *et al*., (2004). Floros *et al*., 1997 reviewed the products and patents in the area of AP and identified antimicrobial packaging as one of the most promising versions of an AP system.

To prepare antimicrobial films, different synthetic antimicrobial chemicals have been incorporated into packaging materials including organic or inorganic acids, metals, alcohols, ammonium compounds or amines (Appendini and Hotchkiss, 2002;Suppakul et al., 2003). However, the increasing consumer health concern and growing demand for healthy food and environmental concerns for synthetic polymers have stimulated the use of natural polymers and bio preservatives such as plant extracts, antimicrobial enzymes and bacteriocins (Suppakul et al., 2003, Labuza and Breene, 1989).

The polymers and materials used for food-packaging today consist of a variety of petroleum-derived plastic materials, metals, glass, paper board, or combinations. With the exception of paper and board, all major packaging materials are based on non-renewable materials, implying that at some point, more alternative packaging materials based on renewable resources have to be found to avoid problems concerning waste disposal (Weber *et al*., 2002).

Even though most of the packaging films used today to preserve foods are of synthetic origin, it is worth to say that in recent years, bio-based materials such as carbohydrates and proteins have, gradually if not extensively, been tested and experimented to develop biodegradable films which had been proven to have more versatile properties (Perez-Mateos *et al*., 2009). This has undoubtedly set off and given rise to the diverse utilization of packaging films made of bio-based materials.

Most of the industries in Ethiopia are food processing industries. Ethiopia supplies agricultural products to the world market. The competiveness of these produces, in the world market, depends on the availability of proper packaging materials. The country is rich in natural resources which are underutilization, to be used as a raw material, for active packaging material development. Aloe is one of the underutilized plants in the country. The gel in aloe leaves contains various functional chemicals. There is, hence, scope to study techno-economically viable alternatives for the development of antimicrobial packaging films from aloe and other medicinal plants which grows in the country.

1.2. Statement of the problem

To meet the growing demand of recyclable or natural packaging materials and consumer demands for safer and better quality foods, new and novel food grade packaging materials or technologies have been and continue to be developed. The large food losses from farm to plate are attributed to poor handling, distribution, storage, and purchase/consumption behaviour. Huge resources that could otherwise be spent on more productive activities go into producing and transporting goods that only go to waste. Losses at almost every stage of the food chain may be reduced by using appropriate packaging materials and technologies.

Postharvest loss, food insecurity, and food poisoning are serious problems of the world in general and Ethiopia in particular. This problem is not only the result of low productivity but also poor post harvest handling and managements that lead to product losses. Almost all agricultural products are vulnerable to post harvest deterioration. Food preservation and packaging technology applied in the country did not play a great role in solving the food self insufficiency problem largely in the country. More than 30% of fruits and vegetables produced in the country are subjected to loss (Shimelis, 2004). The food sector does not have enough cold storage to preserve fruits and vegetables during transportation for export and local market. Moreover lack of proper packaging material hinder the market of agricultural produce the world market competitiveness of the country.

Agricultural Development Led Industrialization is the fundamental building block of industrial development in Ethiopia. Some of the strategic pillars of the growth and transformation plan like maintaining agriculture as major source of economic growth and creating conditions for the industry to play key role in the economy must be supported with the development of packaging technology in the country. In a country where production is much lower than the national demand and supplemented with greater level of post- harvest loss, shows how much effort is needed in the area of generating packaging technology that minimizes this loss. This could be in the form of technologies, which inhibit the growth of pests, proper storage facilities, appropriate packaging materials and transportation that are required to minimize losses and that could increase the shelf life of the food crops (Shimelis, 2004).

Moreover, an increase in the cost of petroleum, awareness of environmental issues, and international regulatory pressure banning non degradable plastics in packaging applications has promoted the development of biodegradable packaging materials. Production of edible

films causes less waste and pollution, however, their physicochemical and mechanical properties are generally poorer than synthetic films (Kester and Fennema, 1986). Extensive research is needed on the development of new materials, methods of films formation, methods to improve film properties and the potential applications.

According to the toxicologists and nutritionists, the side effects of some synthetic antimicrobials are problematic. For this reason, the search for antimicrobials from natural source has received much interest to replace synthetic ones (Jutaporn, 2009). To formulate edible films or coatings with functional properties, film-forming base materials as well as the bioactive ingredients must be carefully chosen. With regard to the health concerns of the consumers, it is highly interested in the use of bio-preservatives in antimicrobial packaging.

Ethiopia is rich in biodiversity and there are many plants in the country with great potential of functional qualities but not yet utilized. In this thesis work aloe and papaya leaf are selected as major raw materials for the development of antimicrobial packaging films. The application of antimicrobial aloe based packaging films in preserving highly perishable foods such as fruits and vegetables, meat and meat products, dairy products etc. can contribute to the development of agricultural sector and agro processing industries in Ethiopia.

1.3. Objectives

General objective

The general objective of this study was to develop and evaluate antimicrobial aloe based packaging films.

Specific objectives

- Study the antimicrobial activities of aloe gel and papaya leaf extracts and their combination
- Study the proximate composition of aloe gel and papaya leaf extracts
- Study the effect of addition of gelatin and glycerol on the Physico-chemical (transparency, solubility, and swelling), and mechanical (tensile strength and elongation) properties of the packaging films developed.
- Evaluate antimicrobial (antibacterial and antifungal) activities of the packaging films developed
- Evaluate the technological feasibility of aloe based packaging films production.

1.4. Scope of the study

Aloe and papaya extracts were selected for their natural antimicrobial substances. The films were developed by casting the film-forming solution prepared from the extracts. The antimicrobial property of the aloe gel in the film forming solution was enhanced by incorporation of papaya leaf extract. The mechanical and physicochemical properties of the packaging film were improved by the application of gelatine and glycerol. Different compositions of the film forming solutions were studied and compared for their suitability in food packaging.

1.5. Significance of the study

The application of antimicrobial aloe based packaging films will reduce post harvest and processed food losses, Maintain safety and quality of packed food, Can help the country to compete in the global food market, It will reduce food-borne microbial outbreaks, avoid environmental pollution by replacing petroleum based packages, Can be used as a secondary data for future work.

Chapter Two

Literature Review

2.1. Packaging in food industries

Packaging has a significant role in the food supply chain and it is an integral part of both the food processes and the whole food supply chain. Food packaging has to perform several tasks as well as fulfilling many demands and requirements. Traditionally, a food package makes distribution easier. It has protected food from environmental conditions, such as light, oxygen, moisture, microbes, mechanical stresses and dust. Other basic tasks have been to ensure adequate labeling for providing information e.g., to the customer, and a proper convenience to the consumer, e.g., easy opening, reclosable lids and a suitable dosing mechanism. Basic requirements are good marketing properties, reasonable price, technical feasibility (e.g., suitability for automatic packaging machines, sealability), 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 (Raija, 2003).

Package design and construction play a significant role in determining the shelf life of a food product. The right selection of packaging materials and technologies maintains product quality and freshness during distribution and storage. Materials that have traditionally been used in food packaging include glass, metals (aluminum, foils and laminates, tinplate, and tin-free steel), paper and paperboards, and plastics. Moreover, a wider variety of plastics have been introduced in both rigid and flexible forms. Today's food packages often combine several materials to exploit each material's functional or aesthetic properties (Kenneth and Betty, 2007).

2.2. Novel food packaging techniques

Food packaging has developed strongly during recent years, mainly due to increased demands on product safety, keeping quality, cost-efficiency, environmental friendly, and consumer convenience. Although traditional packaging covers the basic needs of food containment, advances in food packaging are both anticipated and expected. Society is becoming increasingly complex and innovative packaging is the result of consumers' demand for packaging that is more advanced and creative than what is currently offered. Active, intelligent, and smart packagings are the result of innovating thinking in packaging.

Active packaging is accurately defined as “packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system” (Robertson, 2006). This phrase emphasizes the importance of deliberately including a substance with the intention of enhancing the food product preservation and safe handling.

Intelligent packaging can be defined as “packaging that contains an external or internal indicator to provide information about aspects of the history of the package and/or the quality of the food” (Robertson, 2006). Intelligent packaging is an extension of the communication function of traditional packaging, and communicates information to the consumer based on its ability to sense, detect, or record external or internal changes in the product's environment.

Smart package devices can be defined as small, economical labels or tags that are attached onto primary packaging (for example, bottles), or onto secondary packaging (for example, shipping containers), to facilitate communication throughout the supply chain so that appropriate actions may be taken to achieve desired benefits in food quality and safety enhancement. Two basic types of smart package devices are existed: data carriers that are used to store and transmit data, and package indicators that are used to monitor the external environment and, whenever appropriate, issue warnings. A communication channel between the external environment and other components is provided in the system. Multiple smart package devices are employed in a packaging system at several strategic locations throughout the supply chain (Otles and Yalcin, 2008).

All these novel packaging technologies have great commercial potential to ensure the quality and safety of food with fewer or no additives and preservatives, thus reducing food wastage, food poisoning and allergic reactions (Raija, 2003).

2.3. Active packaging systems

Active packaging is one of the innovative food packaging concepts and has been introduced in response to the continuous changes in consumer demands and market trends. Active packaging systems can be distinguished into: active scavenging systems (absorbers) and active releasing systems (emitters).

Active scavenging systems (absorbers): Absorbing (scavenging) systems remove undesired compounds such as oxygen, carbon dioxide, ethylene, excessive water, taints and other specific compounds.

Active releasing systems (emitters): Releasing systems actively add or emit compounds to the packaged food or into the head-space of the package such as carbon dioxide, antioxidants and antimicrobials. Active packaging and antimicrobial packaging in particular, plays a very important role in the protection of food products (Robertson, 1993) and the cost saving potentials of active packaging systems have been demonstrated by Hotchkiss, (1997).

2.3.1. Antimicrobial packaging

Antimicrobial packaging is a promising form of active food packaging. Since microbial contamination of foods occurs primarily at the surface, due to post processing and handling. Attempts have been made to improve safety and delay spoilage by use of antimicrobial sprays or dips. However, direct surface application of antibacterial substances onto food have limited benefits because the active substances are neutralized on contact or diffuse rapidly from the surface into the food mass (Stefania and Loredana, 2002).

Antimicrobial packaging systems are able to kill or inhibit spoilage and pathogenic microorganisms that can potentially contaminate food products (Hotchkiss, 1997). The inhibition of microbial activity is achieved by slow release of antimicrobial agents from the packaging system onto the food surface (Han, 2000). When a packaging system acquires antimicrobial activity, the packaging system limits or prevents microbial growth by extending the lag period and reducing the growth rate or decrease live counts of microorganisms. The goals of an antimicrobial system are safety assurance, quality maintenance and shelf-life extension (Raija, 2003).

Several antimicrobial compounds occur naturally in plants (Banks *et al*., 1986) and are known to retard the growth or kill food-borne pathogens (Beuchat and Golden, 1989). Essential oils (Beuchat, 1994) and juices (Beuchat and Doyle, 1995) of plants are known to have antilisterial activity. The extracts of plants are of growing interest both in the industry and scientific research because of their antibacterial, antifungal, antiviral and anti-parasitical activities that make them useful as natural additives in foods, cosmetic and pharmaceutical industries (Jutaporn, 2009). Edible films and coatings are especially valuable in controlling the surface microbial contamination because of the capability to serve as additive carriers and then release the active compounds on the food surfaces where they are actually needed for impeding microbial growth (Krochta & De Mulder- Johnston, 1997).

2.4. Edible films

Edible films are thin layer of material which can be eaten by the consumer and provide a barrier to moisture, oxygen and solute movement for the food. The material can complete food coating or can be disposed as a continuous layer between food components. Edible films can be formed as food coatings and free-standing films, and have potential to be used with food as gas or aroma barrier (Kester and Fennema, 1986). However, the technical information is still needed to develop films for food application (Donhowe and Fennema, 1993). The edible films and coatings have received a considerable attention in the recent years because of their advantage over the synthetic films. The advantages of edible films over other traditional synthetic films are summarized below:

- They can be consumed with the package products. This is obviously of critical importance since it represents the environmentally ideal package.
- The films are produced exclusively from renewable, edible ingredients and therefore are anticipated to degrade more readily than polymeric materials.
- The films can enhance the organoleptic properties of packaged foods provided that various components (flavorings, colorings, sweeteners are incorporated)
- The films can supplement the nutrition value of foods.
- The films can be applied inside heterogeneous foods at the interfaces between different layers of components. They can be tailored to prevent deteriorative inter-component moisture and solute migration in foods.
- The films can function as carriers for antimicrobial and antioxidant agents. In a similar application they also can be used at the surface of food to control the diffusion rate of preservative substances from the surface to the interior of the food.
- The films can be very conveniently used for micro encapsulation of food flavoring and leavening agents to efficiently control their addition and release into the interior of foods.
- Another possible application for edible films could be their use in multilayer food packaging materials together with non edible films. In this case, the edible films would be the internal layers in direct contact with food materials.

Production of edible films causes less waste and pollution, however, their permeability and mechanical properties are generally poorer than synthetic films (Kester and Fennema, 1986). Extensive research is needed on the development of new materials, methods of films

formation, methods to improve film properties and the potential applications.To formulate edible films or coatings with functional properties, film-forming base materials as well as the bioactive ingredients must be carefully chosen. With regard to the health concerns of the consumers, it is highly interested in the use of bio-preservatives in antimicrobial packaging. Edible films are typically comprised of either a protein or a polysaccharide or any of the above in combination with lipids, a plasticizer and, if necessary, a cross-linking agent.

2.4.1. Protein films

The proteins well studied for edible packaging are whey protein, casein, corn zein, soy protein, wheat gluten, gelatine, and collagen (Krochta and De Mulder-Johnston, 1997). With their availability, different molecular properties and chemical functions, proteins are very suitable sources to obtain edible packaging films. The edible films composed of proteins generally have good gas barrier properties and suitable mechanical and optical properties. However, they are highly sensitive to moisture and show poor water vapour barrier properties (Ryu *et al*., 2002).

2.4.2. Polysaccharide films

Due to their hydrophilic nature, similar to protein based films, carbohydrate films generally show limited water vapour barrier properties. However, they have good mechanical and optical properties. The polysaccharides and their derivatives used as edible films and coatings include starch, cellulose ethers, pectin, alginate, carragenan, and chitosan (Cigdem, 2005).

2.4.3. Lipid films

Lipid compounds prevent the transport of moisture. Thus, films made from lipids have good water vapour barrier properties. However, they are quite fragile, unstable and relatively inflexible. Also, lipids usually form opaque films (Ryu *et al*., 2002).Wax and acetylated monoglycerides are examples of lipid films.

2.4.4. Composite films

Different functional characteristics of several film elements can also be combined to form a single film or coating. Different kinds of polysaccharides, proteins, and lipids have been used, either alone or in mixtures, to obtain composite films. For example, lipids incorporated into low-methoxy pectinate films to improve resistance to water vapour permeation (Kester

and Fennema, 1986). Emulsion coatings containing methyl cellulose, vegetable oil, bee wax, and glycerol monooleate are also good examples of composite films (Lindstrom *et al*., 1992).

In composite films the combination of two or more materials can improve gas exchange, adherence to coated products, or moisture vapour permeability properties. Composite films have been reported to have improved mechanical, transport and physical properties compared to those of single component based films (Chiono *et al*., 2008).

Polysaccharides and proteins are bio-based materials with unique intra- and intermolecular interactions. These characteristics can be exploited in producing biodegradable films with improved properties (Perez-Mateos *et al*., 2009). The barrier and mechanical properties of whey-based protein films were improved by adding either sodium alginate or pectin (Parris *et al*., 1995) or methylcellulose (Erdohan and Turhan, 2005). Gelatin-based films were improved by adding small amounts of polysaccharides, such as gellan and k-carrageenan (Pranoto *et al*., 2007) or glucomannan (Xiao *et al.,* 2001). Pectin-based films were improved by adding small amounts of either fish skin gelatine or wheat gluten (Liu *et al*., 2007).

The antimicrobial activity of plant extracts has formed the basis of many applications, including raw and processed potential as natural agents for food preservation, pharmaceuticals, alternative medicine and natural therapies. In order to prolong the storage stability of foods, synthetic antimicrobial are mainly used in industrial processing. But according to the toxicologists and nutritionists, the side effects of some synthetic antimicrobials are problematic. For this reason, the search for antimicrobials from natural source has received much interesting to replace synthetic ones. Beside, these naturally occurring antimicrobial can be formulated as functional foods and can help to prevent spoilage of food products (Jutaporn, 2009).

According to (Sai *et al*, 2011) papaya leaf extract incorporated aloe gel coating has been identified as a suitable method to extend the shelf life of papaya fruits. In the following sections properties of aloe and papaya leaves related to their ability to be used as antimicrobial packaging film development will be reviewed.

2.5. Applications of aloe in food industries

Aloe vera is the most commercialised aloe species and processing of the leaf pulp has become a large worldwide industry. In the food industry, it has been used as a source of functional foods and as an ingredient in other food products, for the production of gel-containing health drinks and beverages. In the cosmetic and toiletry industry, it has been used as base material for the production of creams, lotions, soaps, shampoos, facial cleansers and other products. In the pharmaceutical industry, it has been used for the manufacture of tablets and capsules (Eshun and He, 2004).

In addition, the aloe leaf gel has recently been reported using as edible coating to extend the shelf life of fruits. Valverde, (2005) applied aloe gel on table grapes to preserve the quality by inhibiting bacteria, mould and yeast growth. Freeze dried powder of aloe gel was dissolved in water and then applied on nectarine to modulate the quality (Ahmed *et al*., 2009).

Recently, some species of aloe have been used in a wide range of skin and hair care products, and also form the basis of health drinks and tonics. The slimy gel inside the leaves consists of a complex mixture of polysaccharides, amino acids, minerals, trace elements and other biologically active substances, such as enzymes. Except for analyses of some chemical compounds undertaken by Professor Ermias Dagne and his group at the Chemistry Department in Addis Ababa University, little is known about the chemistry of the Ethiopian species. There are obviously several interesting aspects of the Ethiopian Aloe species that need further study, and the endemic Ethiopian aloes represent an economic potential (Sebsebe and Inger, 2010).

This study focuses on two of the endemics aloe species *Aloe debrana* and *Aloe trichosantha,* which are abundantly found around Addis Ababa, to use them as raw materials for antimicrobial packaging film production. Ethiopian jute fiber production factory used these plants for the treatment of fiber to give antimicrobial property for exporting coffee without any deterioration.

2.6. Aloe debrana and Aloe trichosantha

2.6.1. Aloe debrana

Aloe debrana is succulent herb, suckering from base to form small groups, mostly stemless but some old plants develop thick, prostrate stems. The species commonly grows in areas of grassland on thin soil overlying basalt, usually on gentle slopes between 2000 and 2700 m in Shoa, Gojam and Wollo floristic regions. It is so far not known anywhere else. The main flowering period is in the dry season, from December to February (Sebsebe and Inger, 2010).

Figure 2.1 *Aloe debrana:* (a) from top of Blue Nile Gorge, Shewa floristic region; (b) distribution in Ethiopia. Adapted from Sebsebe and Inger, (2010).

2.6.2. Aloe trichosantha

The species is subdivided into two subspecies (subsp. Trichosantha and subsp. longiflora) based on the density and length of the teeth along the leaf margin and the length of the flower. The differences between the subspecies are small, but consistent enough to justify sub specific rank.

The subspecific epithet ‘longiflora’ refers to the relatively long (longi) flowers (flora), at least compared to the other subspecies. It was described with the type material collected west of Daletti in Harerge by Gilbert and Sebsebe in 1997. This subspecies is widespread in Ethiopia and grows abundantly in open deciduous bushland on volcanic rocks and alluvial soils between 1000 and 1950 m. It is not known anywhere else. The flowering period is almost throughout the year, with records from August to May (Sebsebe and Inger, 2010).

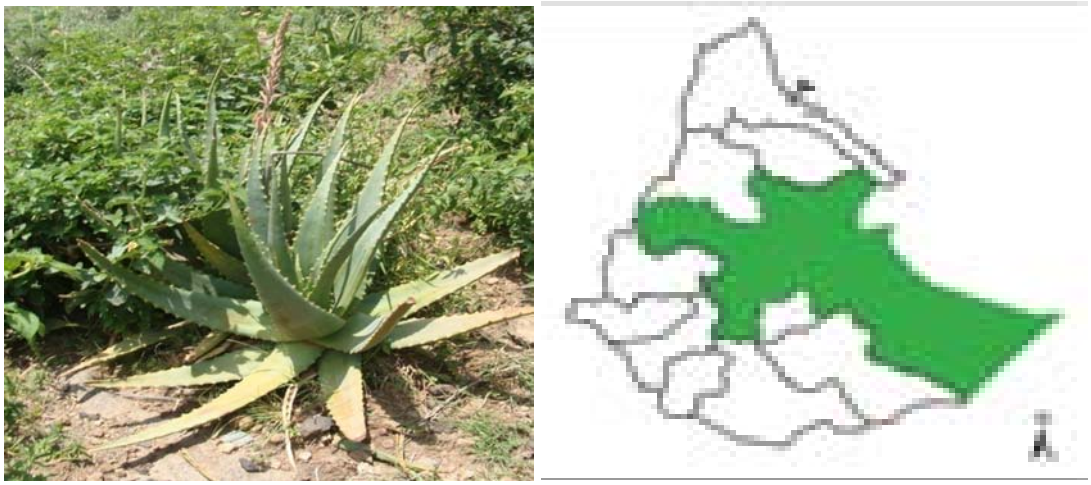

Figure 2.2: *Aloe trichosantha*, (a) from west of Daletti in Harerge, collected by Gilbert and Sebsebe in 1997; (b) distribution in Ethiopia. Aadapted from Sebsebe and Inger, (2010).

2.6.3. Chemical composition of aloe leaf

Aloes have in common green fleshy leaves covered by a thick cuticle or rind and an inner clear pulp. The rind of the Aloe leaf accounts for approximately 20– 30% by weight of the whole plant leaf, and the pulp represents approximately 65–80% (Femenia *et al*., 1999).

The leaf pulp of the Aloe plant, the major part of the leaf by volume, is the innermost portion of the leaf and is composed of large thin-walled parenchyma cells that contain aloe gel. Aloe gel is the clear mucilaginous aqueous extract of the leaf pulp (Yaron, 1993). The raw pulp of *Aloe vera* contains approximately 98.5% water, while the mucilage or gel consists of about 99.5% water (Eshun and He, 2004). The remaining 0.5 – 1% solid material consists of a range of compounds including water-soluble and fat-soluble vitamins, minerals, enzymes, polysaccharides, phenolic compounds and organic acids (Boudreau and Beland, 2006).

Many compounds with diverse structures have been isolated from both the central parenchyma tissue of aloe leaves and the exudate arising from the cells adjacent to the vascular bundles. The bitter yellow exudate contains 1,8 dihydroxyanthraquinone derivatives and their glycosides, which are mainly used for their cathartic effects (Vazquez *et al*., 1996). The aloe parenchyma tissue or pulp has been shown to contain proteins, lipids, amino acids, vitamins, enzymes, inorganic compounds and small organic compounds in addition to the different carbohydrates. Some evidence of chemotaxonomic variation in the polysaccharide composition of aloes exists (Reynolds, 2004).

The large fluctuations in polysaccharide composition of aloe gel as found in the literature has been explained by the fact that the mannosyl residues are contained in a reserve polysaccharide with a significant seasonal influence, as well as large variations between cultivars in terms of the quantities of mannose-containing polysaccharides within the parenchyma cells (Femenia *et al*., 1999).

2.6.4. Antimicrobial properties of aloe leaf

There are several scientific studies supporting antibacterial, antifungal and anti-inflammatory activity of substances found in aloe, such as: anthraquinones, flavonoids, saponins, tannins, polysaccharides, and essential oils. It has been claimed that the polysaccharides in aloe gel have therapeutic properties such as immunostimulation, anti-inflammatory effects, wound healing, promotion of radiation damage repair, anti-bacterial, anti-viral, anti-fungal, anti-diabetic and anti-neoplastic activities, stimulation of hematopoiesis and anti-oxidant effects (Mortan, 1961).

The ethanol, methanol and acetone extracts of aloe gel were studied for their antimicrobial activity against four Gram-positive and Gram-negative bacteria using agar well diffusion method. The extracts showed varied levels of antimicrobial activity against the tested pathogens. With the broad spectral antimicrobial effect of aloe gel, it could be further recommended in the treatment of various bacterial diseases (Rubina *et al*., 2009).

Antifungal activity of leaf pulp and liquid fraction of *Aloe vera* was evaluated on the mycellium development of *Rhizoctonia solani*, *Fusarium oxysporum*, and *Colletotrichum coccodes*, that showed an inhibitory effect of the pulp of *Aloe vera* on *Fusarium oxysporum* at 10^4 µl l^{-1} and the liquid fraction reduced the rate of colony growth at a concentration of 10^5 µl 1^{-1} in *Rhizoctonia solani*, *F. oxysporum*, and *C. coccodes* (Jasso *et al*., 2005). From this it is evident that leaf pulp and liquid fraction of *Aloe vera* act against plant pathogenic fungi.

The antifungal activity of *Aloe vera* gel was also tested by Yolanta Saks and Rivka Barkai-Golan , (1995) on four common postharvest fruit pathogens: *Penicillium digitatum, Penicillium expansum, Botrytis cinerea,* and *AIternaria alternata.* The natural gel suppressed both germination and mycelial growth with *P. digitatum* and *A. altemata* being the most sensitive species.

2.6.5. Aloe gel extraction

Potential use of aloe products often involves some type of processing, e.g. heating, dehydration and grinding. Processing may cause irreversible modifications to the polysaccharides, affecting their original structure which may promote important changes in the proposed physiological and pharmaceutical properties of these constituents. Processing of aloe gel derived from the leaf pulp of the plant, has become a big industry worldwide due to the application in the food industry (Ramachandra and Srinivasa, 2008).

The production process of aloe products involve crushing, grinding or pressing of the entire leaf of the aloe plant to produce an aloe juice, followed by various steps of filtration and stabilization of the juice. The resulting solution is then incorporated in or mixed with other solutions or agents to produce a pharmaceutical, cosmetic or food product (Ramachandra and Srinivasa, 2008).In view of the known wide spectrum of biological activities possessed by the leaves of the aloe plant and its wide spread use, it has become imperative that the leaf be processed with the aim of retaining essential bioactive components.

According to Ramachandra and Srinivasa, (2008), the things that happens to make aloe products less desirable or cause it to become virtually non beneficial are stem from the harvesting of the leaves, processing and distribution of leaves. The freshly removed leaves must go directly into production or must be appropriately refrigerated to prevent a loss of biological activity, principally through the degradative decomposition of the gel matrix. The value of aloe further diminishes if the processing procedure applies too much heat for too long time. Extended heating effectively destroys aloe's mucopolysaccharide and consequently reduces its efficacy.

Aloe vera gel is the mucilaginous jelly obtained from parenchyma cells of the *Aloe vera* plant. When exposed to air, the gel rapidly oxidizes, decomposes and looses much of its biological activities. Different researchers have described different processing techniques of the gel with regards to its sterilization and stabilization, i.e., cold processing or heat treatment. However, the fundamental principle underlying these processing techniques remains almost the same. Regardless of the relative quality of the plant, the best results are obtained when leaves are processed immediately after harvesting. This is because degradative decomposition of the gel matrix begins due to natural enzymatic reactions (Ramachandra and Srinivasa, 2008).

The entire process involves washing the freshly harvested *Aloe vera* leaves in a suitable bactericide, followed by processing of the leaves to mechanically separate the gel matrix from the outer cortex. The separation of the gel from the leaf could be facilitated by the addition of cellulose dissolving compounds. The resultant liquid is then subjected to various steps of filtration, sterilization and stabilization. The stabilized liquid, thus, obtained could be concentrated to reduce the amount of water or, alternatively, almost all of the water removed to yield a powder (Ramachandra and Srinivasa, 2008).

In the heat treatment processing, sterilization is achieved by subjecting the aloe liquid obtained from the activated carbon treatment to pasteurization at high temperature. has reported the biological activity of *Aloe vera* gel essentially remains intact when the gel is heated at 65°C for periods less than 15 min. Extended periods or higher temperatures have resulted in greatly reduced activity levels. They, however, suggested that the best method of pasteurization is HTST (High Temperature Shot Time), followed by flash cooling to 5°C or below. In all these processing techniques, stabilization can be achieved by the addition of preservatives and other additives. The use of sodium benzoate, potassium sorbate, citric acid, vitamin E in synergism and the resultant efficacy, has been reported (Ramachandra and Srinivasa, 2008).

Traditional hand filleting is one of the basic methods for processing *Aloe vera*. In order to avoid contamination of internal fillet with the yellow sap, the traditional hand-filleting method of processing aloe leaves was developed. In this method, the lower 2.5cm of the leaf base (the white part attached to the large rosette stem of the plant), the tapering point (5.08-10.16cm) of the leaf top and the short, sharp spines located along the leaf margins are removed by a sharp knife, then the knife, is introduced into the mucilage layer below the green rind avoiding the vascular bundles and the top rind is removed. The bottom rind is similarly removed and the rind parts, to which a significant amount of mucilage remains attached, are discarded. The hand filleting method is very labour intensive. Owing to this fact, machines have been designed and employed with attempt to simulate the hand filleted techniques, but generally the product contains higher amounts of the anthraquinones laxatives than the traditional hand filleted approach (Ramachandra and Srinivasa, 2008).

2.7. Papaya (*Carica papaya*)

Papaya is a plant with a weak stem, large leaves and with fruits weighing between 0.5 to 2 kg within which there are several seeds. It grows in Kola (hot lowlands) and Weina Dega (midlands) agro-ecological zones of Ethiopia under rainfed condition or with irrigation. Papaya is a fast maturing fruit tree. It grows well in areas with a temperature of 25 to 40^{o}C. Currently, papaya is grown in homesteads and commercial farms (Yitebitu, 2004)

The plant leaves contain a number of medicinally important compounds. According to the study of Suresh *et al*. (2008), papaya contain Alkaloids, Anthroquinone, Flavonoids, Tannins, Saponin, Triterpenoids, and Steriods.

2.7.1. Antimicrobial properties of papaya leaf extract

The antibacterial activity of the leaf extracts of *Carica papaya,* against pathogenic bacteria like gram positive (*Bacillus subtilis, Pseudomonas aeruginosa* and *Staphylococcus aureus*) and gram negative (*Escherichia coli* and *Klebsiella pneumoniae)* bacteria by *invitro* agar well diffusion method was evaluated (Suresh *et al*., 2008). The plants aqueous leaf extracts showed pronounced inhibition than chloroform leaf extracts. Leaf extracts showed more inhibitory action on *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Klebsiella pneumonia* (Suresh *et al*., 2008).

According to Ebele, (2011) the mycelial growth of fungus (*Aspergillus niger, Botryodiplodia theobromae, Fusarium solani* and *Penicillium Sp)* in-vitro has been significantly reduced by crude extracts of papaya leaves at various concentrations. Oluduro and Olumide (2010) also reported that the aqueous extract of papaya leaves showed appreciable antimicrobial activities on *Salmonella typhi.* Antimicrobial activity increased with increasing concentration of the extract.

Bacterial strains (staphylococcus aureus MTCC 2940, Basillus subtilis MTCC 441, Escherichia coli MTCC 739 and pseudomonas aeruginosa MTCC 2453) were found to be sensitive to aqueous papaya leaf extract (Indranil *et a*l., 2011).The results obtained by the study of Dickson (2011) provide, pharmacological evidence to support the use of papaya leaves in the treatment of the diseases caused by the test organisms *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus* studied. The result also supports the use of water as the solvent of choice for the extraction of the active components, since the aqueous extracts were found to be more effective than the ethanol and chloroform extracts.

2.8. Packaging film production technology

2.8.1. Film-forming methods

Generally, formulations of edible packaging include at least one natural polymer capable of forming a sufficiently cohesive and continuous structural matrix. Functional properties of edible films and film coatings and their applications to food products depend on cohesion forces, including covalent bonds (e.g., disulfide bond cross-linking), ionic bonds, and H-bonding, between film forming polymer molecules. Cohesive strength depends upon biopolymer structure and chemistry (e.g., molecular weight, regularity, branching, and polarity), film-formation procedures (e.g., solution casting, extrusion), film-formation parameters (e.g., temperature, pressure, solvent type, dilution, application technique, drying technique), and the concentrations of plasticizers and additives (Theeranun and John, 2010). Hydrocolloid-based edible biopolymer films can be achieved by two basic technologies: the dry and wet processes.

2.8.1.1. Dry process

Some edible films, such as starch films, can be prepared using a dry-process, such as thermoplastic extrusion. This extrusion process is based on the thermoplastic properties of polymers when plasticized and heated above their glass transition temperature under low water content-conditions (Arvanitoyannis and Billiaderis, 1998).

2.8.1.2. Wet process

The wet-process mechanism is based on a film-forming dispersion or solution in which polymers are first dispersed or solubilised into a liquid phase, and then dried. Freeze drying is employed to obtain sponge-type scaffolds used in tissue engineering. The wet process is often preferred as it permits the application of films as coatings in a liquid form directly onto food products by dipping, brushing or spraying (Peressini *et al*., 2004).

The film-coating formulation and conditions must be optimized to obtain a sufficient cohesiveness of the film matrix and adequate adhesion and coverage on the food surface in order for the coating to perform its intended functions. Although the thickness of the coatings depends primarily on the application technique and viscosity of the casting solutions, the adhesiveness of the coatings on the surface of food product is essentially governed by chemical affinity between the coatings and their supporting surfaces (Baldwin, 2007). For example, hydrophilic film-forming solutions have poor adhesion on hydrophobic product

surfaces. To enhance the adhesion in these cases, surface active agents such as emulsifiers can be coated on the food or added to the film-forming solution. Solution casting is often used as a method to evaluate the film-forming potential of bio based materials.

The activity of antimicrobial agents used for antimicrobial packaging can change during and/or after film formation. For example, the chemical stability of incorporated antimicrobial agents may reduce during converting operations. The adhesives and solvents used in the process may also affect the antimicrobial activity of the agents. There may be loss of volatile antimicrobial agents during casting and storage which causes reduction of antimicrobial activity (Suppakul *et al*., 2003). Thus, it is essential to select suitable film materials, processing methods and antimicrobials to obtain sufficient residual antimicrobial activity after film making.

2.8.2. Film-forming mechanisms

Polymeric solutions form films through a series of phases. When the polymer solution is cast on a surface, cohesion forces form a bond between the polymer molecules (Banker, 1996). When the cohesive strength of the polymer molecules is relatively high, continuous surfaces of the polymer material coalesce. Coalescence of an adjacent polymer molecule layer occurs through diffusion. Upon evaporation of water, gelation progresses and allows the polymer chains to align in close proximity to each other and to get deposited over a previous polymer layer. When there is adequate cohesive attraction between the molecules, sufficient diffusion, and complete evaporation of water, polymer chains align themselves to form films (Harris and Ghebre-Sellassie 1997).

2.8.3. Film application techniques

2.8.3.1. Dipping

This method lends itself to food products that require several applications of coating materials or require a uniform coating on irregular surface. After dipping, excess coating material is allowed to drain from the product and it is then dried or allowed to solidify. This method has been used to apply films of acetylated monoglycerides to meats, fish and poultry and to apply coating of wax to fruits and vegetables (Krochta, 2002).

2.8.3.2. Spraying

Films applied by spraying can be formed in a thinner, more uniform manner than those applied by dipping. Spraying, unlike dipping is more suitable for applying a film to only one side of the food to be covered. This is desirable when protection is needed on only one surface, e.g., when a pizza crust is exposed to a moist sauce. Spraying can also used to apply a thin second coating such as the cation solution needed to cross-link alginate or pectin coatings (Krochta, 2002).

2.8.3.3. Casting

For formation of a film the film forming biopolymers are first dissolved in the solvent. If heating and pH adjustment enhances film formation and/or properties, this is done next. Degassing is an important step to eliminate bubble formation in the final film or coating. Finally the edible film or coating is formed by applying the prepared formulation to the desired casting or product surface and allowing the solvent to evaporate. Providing heated air at low humidity and high velocity increases drying rates. Coating is simple and allows film thickness to be controlled accurately on smooth, flat surfaces (Krochta, 2002).

Casting can be accomplished by controlled - thickness spreading or by pouring. Controlled thickness - spreading requires a spreader with a product reservoir and adjustable gate, the height of which can be set accurately and with good reproducibility. The spreader is simply drawn over the receiving surface, depositing a layer of the film- forming solution of the desired thickness, which is subsequently dried. Alternatively, the film- forming solution can be poured in a confined area of a level receiving surface and subsequently dried (Krochta, 2002). Various drying methods have been developed for solution casting to produce self-supporting films including air drying, hot surface, infrared, and microwave techniques (Theeranun and John, 2010).

2.9. Film-forming ability of aloe gel

To formulate edible films or coatings with functional properties, film-forming base materials as well as the bioactive ingredients must be carefully chosen. With several decades of studies, proteins, polysaccharides and lipids are the major types of base materials used in fabricating edible films and coatings, and the selection of a specific one or combination of different materials is determined by the requirements of each application (Tzoumaki *et al*., 2009). As

for the selection of bioactive compounds such as antimicrobial agents, it should be noted that the bioactive compounds for edible coating or films must also be edible materials.

Although aloe gel can be used as edible coatings, the suitability of aloe gel used for formulating edible films should be carefully re-evaluated. The moisture content of the aloe leaf gel is usually greater than 98% and the mixed polysaccharides (including glucomannan, galactan, arabinan and pectic substances) make up the most parts of the gel solid matter (Femenia *et al*., 1999). The relatively low solid content and brittle characteristic of mixed polysaccharide film make the aloe leaf gel itself technically infeasible for using as the supporting base material for edible films. Combination of different biomaterials to form composite or blend is a useful solution to enhance the mechanical and/or functional properties of bioactive packaging materials (Rivero *et al*., 2009). To overcome the lack of film-forming capability of the aloe leaf gel, it is advisable to incorporate other suitable filmogenic materials with the aloe leaf gel to form edible films. Moreover, composite edible coatings or films can combine the advantages of each component.

2.10. Film-forming ability of gelatine

Gelatine has been successfully used to form films that are transparent, flexible, water-resistant, and impermeable to oxygen (Hebert and Holloway, 1992). As a common edible film forming material, the applications of gelatine used for edible films or coatings were reported in many literatures. Gelatine has been used, alone or in combination with other materials, to serve as additive carrier for various foods (Krochta & De Mulder-Johnston, 1997). Gelatine and chitosan were fabricated into composite or bi-layer films (Rivero *et al*., 2009). Gelatine has been blended with soy protein isolate to improve the physicochemical properties of edible composite films (Cao *et al*., 2007). Gelatine films have been formed as coatings on meats to reduce oxygen, moisture and oil transport (Gennadios *et al.,* 1994).

Reza *et al*. (2009) had reported that the use of keratin- chitosan-gelatin films has avoided the defects of using chitosan, gelatine or keratin alone. Protein and polysaccharide are both hydrophilic biopolymers and have been combined to form composite edible films (Liu *et al*., 2007; Rivero *et al*., 2009). Most parts of aloe gel solid matter are mixed polysaccharides, thus it is reasonable for gelatine and the aloe leaf gel to be formulated together to form composite films or coatings (Cheng-Pei *et al*., 2010).

2.11. Functions of Plasticizers in film production

Films prepared from pure polymers tend to be brittle and often crack upon drying. Addition of food-grade plasticizers to film-forming solution alleviates this problem (McHugh and Krochta, 1994). When a plasticizer is added, the molecular rigidity of a polymer is relieved by reducing the intermolecular forces along the polymer chain. Plasticizer molecules interpose themselves between the individual polymer chains, thus breaking down polymer-polymer interactions, making it easier for the polymer chains to move past each other. The plasticizer improves flexibility and reduces brittleness of the film. Polyethylene glycol, glycerol, propylene glycol, and sorbitol are the most commonly used plasticizers in edible film production (Aydinli and Tutas, 2000).

Several researchers have conducted studies to evaluate the efficiency of different plasticizers in protein-based films, and have developed empirical models to describe the observed phenomena. Sothornvit and Krochta, (2001) found glycerol to be the most efficient plasticizer in a whey protein film matrix. According to Bourtoom, (2008) increasing the plasticizer concentration will decrease tensile strength and the type and concentration of plasticizers can affect the film solubility. Increasing the plasticizer concentration results in higher solubility.

The amount of plasticizer added can cause adverse effects on film properties such as increasing mass transfer through the films. Hence, plasticizers must be used with caution. When the plasticizer concentration exceeds its compatibility limit in the polymer, it causes phase separation and physical exclusion of the plasticizer. This leads to development of a white residue on edible films which have been referred to as “blooming” (Aulton *et al*., 1981).

Polymeric films should be uniform and free from defects for their applications. Uniformity of the films is critical for their functionalities. During the film-forming process, shrinkage of the films due to evaporation of water or rapid drying often causes defects such as cracks or curling in the films (Obara and McGinity, 1995). Addition of plasticizers such as glycerol or sorbitol is often used to reduce such defects.

2.12. Effectiveness of aloe based packaging films

A novel edible coating based on *Aloe vera* gel has been used as postharvest treatment to maintain sweet cherry quality and safety. During cold storage, uncoated fruit showed increases in respiration rate, rapid weight loss and colour changes, accelerated softening and ripening, stem browning and increased microbial populations. On the contrary, sweet cherry treated with *Aloe vera* gel significantly delayed the above parameters related to postharvest quality losses, and storability could be extended. The sensory analyses revealed beneficial effects in terms of delaying stem browning and dehydration, maintenance of fruit visual aspect without any detrimental effect on taste, aroma or flavours (Martinez-Romero *et al*., 2006).

Edible antimicrobial aloe/gelatin composite films with different ratio of freeze‐dried aloe leaf gel powder and gelatine showed significance difference in mechanical and antimicrobial properties. The mechanical properties were increased with the increasing amount of gelatine used in the composite formulation. *Citrobacter freundii, Escherichia coli, Enterobacter aerogens, Serratia marcescens, Staphylococcus aureus and Bacillus cereus* were used in antimicrobial activity test. The antimicrobial activities of the composite films increased as the amount of aloe gel powder used in the composite films increased (Cheng-Pei *et al*., 2010).

2.13. Applications of antimicrobial packaging films to food products

The major potential food applications of antimicrobial packaging films include meat, fish, poultry, bread, cheese, fruits and vegetables (Brody and Budny, 1995). Edible films and coatings can serve as carriers for a wide range of food additives, including antimicrobials, which can reduce microbial growth on meat and poultry surfaces to improve product safety and extend product shelf life (Milda and Kerry, 2009).

Use of edible films and coatings has been studied as a good alternative for preservation of intact and fresh-cut fruits and vegetables, since such films can create semi permeable barriers to gases and water vapour, maintaining quality of the product. Edible films and coatings have also been studied as potential carriers of additives to help preserve, or even improve quality of produce. Overall, the purpose of using edible films and coatings for fruits and vegetables is to retard transfer of gas, vapour and volatile, thus providing food with a modified atmosphere that decreases respiration and senescence, reduces aroma loss, retains moisture and delays colour changes throughout storage (Milda and Kerry, 2009).

Chapter Three

Materials and Methods

3.1. Sample collection and experimental location.

The research materials for this study were aloe leaves, papaya leaves, gelatine and glycerol. The fresh leaves of *Aloe debrana* and *Aloe trichosantha* were collected from Chancho, a place from Addis Ababa 40km to north and near Nazreth on the way from Mojo to Nazreth; respectively. They were identified by a botanist at Addis Ababa University National Herbarium. Fresh papaya leaves (*Carica papaya*) were obtained from Melkassa Agricultural Research Center (MARC). All of the leaves were packed and transported to Addis Ababa University where they are processed in to extracts. Gelatine powder (bluluxr, blulux laboratories (P) Ltd. Product code: 121001) and glycerol (product code: 38454 L05, Batch noE10A/0110/1801/08) were purchased from Neway PLC, Addis Ababa.

Test cultures for the antimicrobial activity assay were selected from both bacterial and fungal strains, which are common food spoilage and pathogenic microorganisms. The bacterial strains used in this investigation were *Escherichia coli* (American type culture collection (ATCC) 25922*)*, *Staphylococcus aureous* (ATCC25923) and *Salmonella typhi* (ATCC6539). These test organisms were obtained from microbiology department of Ethiopian Health and Nutrition Research Institute (EHNRI).The fungal pure cultures (yeast and mold) taken for the antimicrobial assay were *Candida albicans* (ATCC62376) and *Fusarium xylarioides.* These test cultures were obtained from Biology Department of Addis Ababa University, Science Faculty.

The study was conducted at Addis Ababa University, Ethiopia. Processing of the extracts, development of the packaging films and evaluation of the packaging film for their physicochemical and mechanical properties were performed at AAiT laboratories. The antimicrobial activities of the extracts and the packaging films developed were performed at the Science Facility, Biology Department laboratories of Addis Ababa University. The chemicals, media, and equipments that were used to achieve the objectives of this study are listed in Appendix I.

Framework of the experiments: The research was conducted to develop and evaluate aloe based antimicrobial packaging films from aloe and papaya leaves extracts. The overall framework of experiments of the thesis is shown in Figure 3.1.

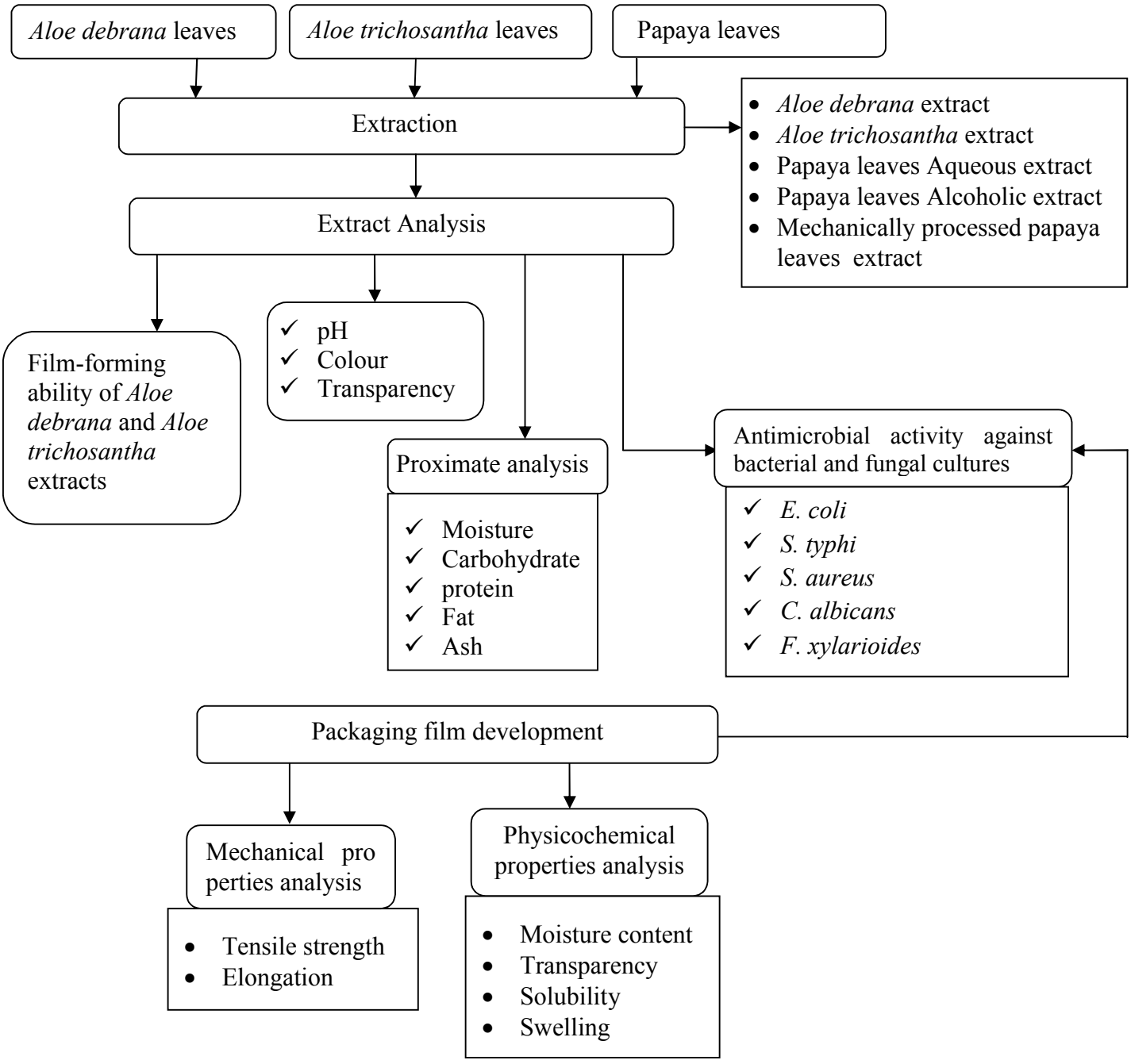

Figure 3.1: Overall framework of experiments in this research.

3.2. Aloe and Papaya leaves extraction methods

3.2.1. Aloe gel extraction

Freshly harvested leaves of both *Aloe debrana* and *Aloe trichosantha* were washed with clean water. the extraction was performed according to the method described by Ramachandra and Srinivasa, (2008). The lower 2.54 cm of the leaf base, the tapering point (5.08 -10.16 cm) of the leaves top and the short, sharp spines located along the leaves margins were first removed by a sharp knife (Appendix V (a)). Then the knife is introduced into the mucilage layer below the green rind avoiding the vascular bundles and the top rind is removed as shown in Appendix V (b). The colorless hydro parenchyma obtained was homogenized in to solution using Food blender (Philips HR2021, made in china) and the solution was filtered using cheesecloth. The clear aloe extract is susceptible to enzymatic degradation. Therefore, it was pasteurized at 70^0C for 45 minutes (Sai *et al*., 2011). Finally the pasteurized extract was stored at 4^0C until used for further analysis.

3.2.2. Papaya leaves extraction

Three different extracts from papaya leaves were prepared according to the method described by Mashiar *et al*. (2009). This was performed to choose the best extract with strong antimicrobial activity among the extracts for the film-forming solution. Papaya leaves were cleaned with water, alcohol, and with distilled water to avoid dirt materials adhered to the leaves. The leaves were shredded by knife and extracted as described below.

Aqueous extraction: About one hundred grams of fresh leaves of *Carica papaya* were weighed out and crushed directly by grinder and dipped into 400ml water into a conical flask and left for 4 days with occasional shaking. The extract was filtered off using filter paper (Whattman no. 1) into a clean conical flask and 80% aqueous solvent was evaporated at 100°C. The filtered extract was stored under refrigeration for further analysis (Mashiar *et al*., 2009).

Alcoholic extraction: About one hundred grams of fresh papaya leaves were shredded and extracted by 400ml of 97% ethanol in a similar manner to the aqueous extraction. The extract was filtered, concentrated by evaporation of 80% the solution, and stored refrigerated until further analysis (Mashiar *et al*., 2009).

Mechanical leaves extraction: Fresh leaves of *Carica papaya* were crushed and extracted directly by screw press without adding any solvent, and leaves extract was collected in a clean beaker, filtered and stored in similar manner to that of the water and alcohol extracts for subsequent tests (Mashiar *et al*., 2009).

3.2.3. Packaging film development

Aloe debrana extract (ADE) and mechanically obtained papaya leaves extract (PLE) were used for the development of antimicrobial aloe based packaging films. These two extracts were selected from the analysis of antimicrobial activity and film-forming ability of the extracts explained in section 3.4 of this document. The proportion of aloe debrana extract to papaya leaves extract was set at 7:3. This proportion was selected from the analysis of "effect of papaya leaves extract on the antimicrobial activity of *Aloe debrana* extract" explained in the analysis section of this study (section 3.4). Accordingly about 70% aloe debrana extract and 30% of papaya leaves extract standard solution was prepared. For stabilization of the aloe gel, (2.0 g/L) ascorbic acid and (4.5 g/L) citric acid were added. Films were developed by varying gelatine and glycerol concentrations on 100ml of the prepared standard solution (70% ADE + 30% PLE) based on the experimental design given in Table 3.1.

Table 3.1: Experimental design used to study the effect of gelatine and glycerol concentration on physicochemical and mechanical properties of aloe based packaging films

Gelatine (g)	Glycerol (g)		
	0	**0.5**	**1**
1	$P_{0,1}$	$P_{0.5,1}$	$P_{1,1}$
2	$P_{0,2}$	$P_{0.5,2}$	$P_{1,2}$

The ranges for glycerol concentration were adjusted between zero and one gram based on the preliminary tests that were carried out in the range 0 to 5 grams per 100ml the standard solution. The amount of gelatine to be added was according to Cheng-Pei *et al*. (2005). They studied the film properties by varying the proportion of gelatin powder and aloe gel powder.

The films development techniques were adapted from Caroline *et al.,* (2011). Initially, gelatine was hydrated with the standard solution (70% ADE+30% PLA) at room temperature in a beaker, and solubilised later in a water bath at 50^0C. After complete solubilization, glycerol was added

and the solution was kept in water bath under slight agitation for 30 min. Finally, the film-forming solution (350 ml) was conveniently applied on leveled rectangular (15x20 cm) plastic tray. The first batch of films was dehydrated at room temperature and the second batch of films was dehydrated by using tray dryer (TAURO B105EC, N11/2006). Films were peeled off when dried and stored in desiccators for further analysis.

3.3. Analysis methods

The antimicrobial activities of all the extracts (*Aloe debrana* extract, *Aloe trichosantha* extract, papaya leaves aqueous extract, papaya leaves alcoholic extract, and mechanically obtained papaya leaves extract) were evaluated to compare and select the extracts with better performance. The extracts (*Aloe debrana* extract, *Aloe trichosantha* extract, and mechanically obtained papaya leaves extract) were selected from antimicrobial activity assay were also subjected to colour and pH determination. *Aloe debrana* and *Aloe trichosantha* extracts were further compared by their film forming ability. The effect of incorporation of mechanically obtained papaya leaves extract on the antimicrobial activity of *Alo*e *debrana* extract was also determined in the following sections. The developed aloe based packaging films were characterized for their physicochemical properties (transparency, solubility, and swelling), antimicrobial activities, and mechanical properties (tensile strength and elongation at break).

3.3.1. Characterization of the extracts

3.3.1.1. Antimicrobial activities of the extracts

The antimicrobial activity of all extracts *Aloe debrana* extract (ADE), *Aloe trichosantha* extract (ATE), papaya leaves aqueous extract (PLAQE), papaya leaves alcoholic extract (PLALE), mechanically obtained papaya leaves extract (PLE) against *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Candida albicans* and *Fusarium xylarioides* were evaluated. This activity was performed for (1) to make sure that whether the film-forming solutions have antimicrobial activity and (2) to compare and select the best film-forming solution. The comparison was performed between *Aloe debrana* and *Aloe trichosantha* extracts and all papaya leaves extracts were compared with each other, to select only one film-forming solution from aloe and papaya extract group. Composite film-forming solution was prepared from the selected aloe and papaya extracts.

Disk diffusion technique was used to evaluate the antimicrobial activities of each extract on *Escherichia coli, Staphylococcus aurous, Salmonella typhi and Candida albicans.* The poison food technique was used to evaluate the degree of antimicrobial activities of all the extracts on *Fusarium xylarioides.*

Preparation of bacterial inocula: Stock cultures were maintained at 4^0C on slants of selective media for each test organism (see appendix II for agar slant preparation). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of nutrient broth (NB) that were incubated for 24 hrs at 37^0C. The cultures were diluted with fresh nutrient broth to achieve optical densities corresponding to approximately 1.5 X 10^8 CFU/ml (based on 0.5 McFarland standards). The preparation of 0.5 McFarland standard is given in appendix III. Potato dextrose broth was used for the preparation of *Candida albicans* inocula. The inoculated test tubes of potato dextrose broth were incubated at 27^0C for 72 hrs.

Disk diffusion technique: The antimicrobial activity test for bacterial strains and *Candida albicans* was employed by disc diffusion method described by Ergene *et al*., (2006). In vitro antimicrobial activity was screened by using Muller Hinton agar (MHA). The MHA plates were prepared by pouring 65 ml of molten media into 15cm diameter sterile Petri plates. The plates were allowed to solidify for 5 minutes and inocula suspension was swabbed uniformly using sterile cotton swab. The inocula were allowed to dry for 5 minutes. The different extracts were loaded on 5 mm diameter sterile discs (30µl/disk). The loaded discs were placed on the surface of medium and the compound was allowed to diffuse for 5 minutes and the plates were kept for incubation at 37^0C for 24 hrs. Disks that were loaded with distilled water were used as a control. The same procedure was followed for the *Candida albicans*, except that potato dextrose agar plates were used and incubated at 27^0C for 72 hrs. The preparation of potato dextrose broth is explained in Appendix I. The diameters of zone of inhibitions in mm were recorded using transparent ruler after growth of the test organisms. The experiments were performed in triplicates and average diameters of zone of inhibitions were recorded.

Poison food technique: The antifungal activities of all the extracts on *Fusarium xylarioides* were determined by poisoned food technique (Das *et al*., 2009). Five-day old fungal cultures were punched aseptically with a sterile cork borer of 5 mm diameter. The fungal culture discs were then put on the gelled potato dextrose agar plate. The agar plates were prepared by

impregnating 3 ml plant extract in to 60 ml potato dextrose agar at a temperature of 45 - 50°C (poisoned plate) and 3 ml distilled water in to 60 ml agar (non poisoned plate) as a control. The plates were then incubated at 27°C for three days. Colony diameter was recorded by measuring the two opposite circumference of the colony growth. Percentage inhibition of mycelia growth was evaluated by comparing the colony diameter of poisoned plate (with plant extract) and nonpoisoned plate (control) and calculated using the formula described by Das *et al*., (2009).

$$\%\ \text{Mycelial inhibition} = \frac{MG_c - MG_t}{MG_c} x100 \qquad \text{Eq. (1)}$$

Where: MG_c is mycelia growth for control

MG_t is the mycelia growth for treated

3.3.1.2. Film-forming abilities of *Aloe debrana* and *Aloe trichosantha* extracts

The film-forming ability of the two aloe extracts were studied according to the method described by Caroline *et al*. (2011). Both of the extracts were heated at 50^{o}C for 30 min and poured on to rectangular plastic tray. The extracts were then allowed to dry at room temperature and peeled for observation.

3.3.1.3. Colour and transparency of the extracts

The colours of the three extracts (*Aloe debrana* extract, *Aloe trichosantha* extract, and papaya leaves extract) were analyzed by visual inspection. Their transparencies at 600 nm were measured by Spectrophotometer (6405 UV/Vis spectrophotometer, manufactured in UK). The transparency (T_{600}) was calculated from the following equation:

$$\text{Transparency}\ (\%T) = 100x\frac{P}{P_o} \qquad \text{Eq. (2)}$$

Where: P is the intensity of the light radiant power that has passed through the material (transmitted radiation), and P_o is the intensity of the radiation before it passes through the material (incident radiation).

3.3.1.4. pH value of the extracts

The pH values of *Aloe debrana* extract, *Aloe trichosantha* extract, and mechanically obtained papaya leaves extract were measured by pH meter at room temperature.

3.3.1.5. Effects of incorporation of papaya leaves extract on the antimicrobial activities of *Aloe debrana* extract

From the analyses of antimicrobial activity and film forming ability, the two samples remain to be used for the film-forming solution were *Aloe debrana* extract (ADE) and mechanically obtained papaya leaves extract (PLE). Therefore, only the effect of addition of PLE on the antimicrobial activity of ADE was studied because they are going to be used together. The effect of incorporation of PLE on the antimicrobial activity of ADE was evaluated by varying the amount of papaya leaves extract from (10 %- 50%) with 10% interval (Table 3.2) and performing the antimicrobial activity assay against the test cultures (*Escherichia coli, Staphylococcus aureus, Salmonella typhi, Candida albicans* and *Fusarium xylarioides*), using the methods explained above (section 3.3.1.1).

Table 3.2: Experimental design to study on the effect of papaya leaves extract on antimicrobial activity of *Aloe debrana* extract

Sample	**Proportion in the film-forming solution**					
ADE(ml)	100	90	80	70	60	50
PLE(ml)	0	10	20	30	40	50

3.3.1.6. Composition analysis

The proximate composition (moisture content, crude protein, total carbohydrate, total ash, and crude fat of the *Aloe debrana* extract and papaya leaves extract were determined at JIJE LABOGLASS Pvt. Limited company. The determination of proximate composition helps to adjust the amount of gelatin to be added in the film- forming solution.

Moisture determination: Moisture contents of the extracts were determined by oven drying method after placing the samples in the oven and drying them to constant weigh (AOAC, 2000 official method 925.10). Then, the moisture content was estimated by weight difference using the formula:

$$\%\text{Moisture} = \frac{W_2 * 100}{W_1} \qquad \text{Eq. (3)}$$

Where: W_1= weight of wet sample

W_2 = loss of weight

Crude protein: Protein content was determined by Kjeldahl method according to (AOAC, 2000) using the official method (920.87). All nitrogen was converted to ammonia by digestion with a mixture of concentrated sulfuric acid and concentrated orthophosphoric acid. The ammonia released after alkalinization with sodium hydroxide is steam distilled into boric acid and titrated with sulfuric acid. The crude protein content was estimated using the formula:

$$\text{Total Nitrogen}(\%) = \text{N X } \frac{(V_1 - V_2)}{W} \text{X } 14.007 \text{ X } 100 \qquad \text{Eq. (4)}$$

Where: V_2=Volume in ml of standard sulfuric acid solution used for the test material titration

V_1=Volume in ml of standard sulfuric acid solution used in the titration for the blank determination.

N = normality of standard sulfuric acid.

W = weight in grams of the sample material.

The universal conversion factor was used to convert percent N to percent crude protein (dry basis). Most proteins contain 16%N, so the conversion factors is 6.25 (100/16 = 6.25).

Crude Fat: The fat content was determined by AOAC, (2000) official method 933.05. The fat in the sample was extracted by petroleum ether using soxhlet apparatus. Samples were added in to extraction thimbles. The thimbles with the sample content were placed into soxhelt extraction chamber. The fat was washed by petroleum ether into the extraction flasks. The content in the extraction flasks was removed from the extraction chamber and placed in the drying oven and dried. Finally the fat content was calculated using the formula

$$\text{Weight of fat } (W_F) = W_1 - W_2 \qquad \text{Eq. (5)}$$

$$\text{Crude Fat content } (\%) = \frac{W_F \left(100 - \text{moisture}(\%) \right)}{W_3} \qquad \text{Eq. (6)}$$

Where: W_1 = weight of extraction flask after extraction.

W_2= weight of extraction flask before extraction.

W_3 = dried sample obtained after determination of moisture

Total Ash: The ash content was determined using AOAC, (2000) the official method 923.03. Dishes containing samples were placed in the muffle furnace and the temperature was increased slowly up to 550^0C so that the organic matter will be burnet and removed in the form of smoke.

The percentage of total ash (dry basis) was calculated using the following formula:

$$\text{Total Ash}(\%) = \left[\frac{W_2 - W}{W_1 - W}\right] * 100 \qquad \text{Eq. (7)}$$

Where: W = weight in grams of empty dish

W_1 = weight in grams of the dish plus the dried sample material

W_2 = weight in grams of the dish plus ash

Carbohydrates: The total carbohydrate (%) of the sample by mass including crude fiber was obtained as follows:

$$\text{Total carbohydrate (\%)} = 100 - [P + F + A + M] \qquad \text{Eq. (8)}$$

Where: P= the mass percent of protein F= the mass percent of fat

A= the mass percent of ash M = the mass percent of moisture

3.3.2. Evaluation of aloe based packaging films

3.3.2.1. Moisture content and thickness of packaging films

The moisture contents of composite films were determined by Moisture Analyzer (MB45, manufactured in Switzerland). A digital micrometer was used to measure the films thickness prior to tests. The results were expressed as an average of 5 random measurements and standard deviation.

3.3.2.2. Physicochemical properties of packaging films

i) Transparency of films

The transparencies of the films were determined using spectrophotometer (6405 UV/Vis spectrophotometer, manufactured in UK). The film samples were cut into rectangles and placed in the internal side of the spectrophotometer cell. The transmittance of films was determined at 600nm as described by Eraricar *et al*. (2009).

ii) Solubility of films

The film solubility (FS) was determined according to the methodology of (Bourtoom, 2009). Film strips with dimension of (2 cm x 2 cm) were immersed in distilled water (50 ml) for 24 h with slow mechanical stirring using shaker (Excella E24, incubator shaker) at room temperature. Samples were then removed from the solution by filtration and dried in electrical oven (105^0C for 24 h). The initial dry mass was determined from the sample moisture content (determined by gravimetric analysis), and the difference in weight used to calculate the water soluble matter as a percentage of the initial weight. Then film solubility was calculated by the following equation.

$$FS = \frac{W_1 - W_2}{W_1} \times 100 \qquad \text{Eq. (9)}$$

Where W_1 is the initial weight of the film and W_2 is final weight of the film after immersion.

iii) Swelling properties

Water absorption capacities of films were determined according to Bourtoom, (2009) by soaking them in phosphate buffered saline (PBS) at room temperature. The preparation of phosphate buffered saline is given in appendix IV. Weighed film strips of the composite films were placed in the PBS media for 30 minutes. The wet weights of the films were determined by first blotting the surface of the composite films with filter paper to remove excess water, and the films weighed immediately. The percentage of water absorption in the medium (Wsw) was calculated from the equation:

$$Wsw = \frac{W_f - W_o}{W_o} \times 100 \qquad \text{Eq. (10)}$$

Where W_f represents the final weight of the film after 30 minutes of absorption and W_o is the initial weight of the film. `

3.3.2.3. Antimicrobial activities of packaging films

Testing of the antimicrobial activities of the films was carried out using the agar diffusion method according to Cheng-Pei *et al*. (2005). 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 inocula containing approximately 1.5 X 10^8 CFU/ml and 10^4 - 10^5 CFU/ml of test bacteria and fungus; respectively. The plates were incubated at 37ºC

for 48 hours and at 27^{o}C 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 (Cheng-Pei *et al*., 2010). Similar dimension of thick synthetic plastic was used as a control. Observations were made of the diameter of the inhibitory zone surrounding film discs including contact area of film with agar surface.

3.3.2.4. Mechanical properties of the packaging films

Tensile measurements for tensile strength (TS) and elongation at break (EB) of films were determined by using the method described by Cheng-Pei *et al*., (2010) with a tensile testing machine (Computer–electrohydraulic universal testing machine, model WAW600, made in China). The initial grip separation was 50 mm and the crosshead speed was set at 30mm min $^{-1}$. For each test run, the dimensions (thickness and width) of the film strip were input to the coupled personal computer; and TS and EB were automatically calculated by the computer software installed in the computer by the manufacture of tensile testing machine. Mean with standard deviation of two samples for each type of film were reported.

3.4. Experimental design and data analysis

Factorial experimental design was used to the study of effect of incorporation of papaya leaves extract on the antimicrobial activities of *Aloe debrana* extract and the effect of gelatine and glycerol on physicochemical and mechanical properties of the aloe based films. Data obtained from experiments were analyzed by one way ANOVA (Analysis of Variance) using JMP statistical analysis software version 5.0. Significance was accepted at 0.05 level of probability ($p<0.05$). Mean separation was performed by "Each Pair Student's t" for multiple comparisons of means.

Chapter Four

Results and Discussion

4.1. Characteristics of the extracts

4.1.1. Antimicrobial activities of the extracts

To develop antimicrobial packaging film, it was a must for the film forming solution, to have antimicrobial components. Due to this, the study was started with the evaluation of the antimicrobial activity of the extracts. It was aimed on the selection of the best extracts from the aloe extracts and papaya extracts to obtain aloe–papaya composite extract as an important part of the film-forming solution. The combined use of aloe and papaya extracts in the film-forming solution was an attempt to give a wide antimicrobial spectrum that could inhibit the growth of several food spoilage and poisonous microorganisms. According to Sai *et al*., (2011) report, the use of the combination of aloe and papaya extract coating on papaya fruits had shown maximum effect on retarding the changes in pH, titrable acidity, total soluble solids, colour development, and softening of fruit tissue. A packaging material with a wide antimicrobial spectrum would be necessary and desirable for universal use to improve the storage stability of a variety of foods.

It is generally believed that the principal food spoilage microorganisms are mixed microorganism population; including Gram (+) bacteria, Gram (-) bacteria, molds and yeasts. Taking this in to consideration it was tried to take the test cultures from all group of microorganisms listed above. The ability to inhibit all types of organisms is favorable to be an effective antimicrobial food packaging.

This study has showed that aloe and papaya extracts have antimicrobial properties against bacterial and fungal cultures (Table 4.1 and Table 4.2). This implies that the extracts contain active compounds that have antimicrobial properties. The work of Suresh *et al*., (2008) has showed that papaya leaves extract contains alkaloids, tannins and flavonoids which have been shown to posses' antimicrobial activities. Concerning aloe gel, the study of Arunkumar and Muthuselvam, (2009) has indicated that tannins, Saponins, flavonoids and terpenoids are the components of the extract which are known to have antimicrobial activities.

In this this study, the antimicrobial activity of aloe gel has showed great variation from season to season. Aloe gel harvested in August and September (rainy months) has showed less antimicrobial activity than aloe gel harvested from December to March (dry seasons). From this phenomenon it can be concluded that, during the rainy season the aloe absorbs more water from the soil which can make the active ingredients in the gel below the minimum inhibitory concentration. The seasonal variation of aloe leaves composition has been previously reported by Femenia *et al*., (1999). But papaya leaves extract did not show significant seasonal difference in antimicrobial activity. This creates best opportunity to produce the antimicrobial packaging film throughout the year. It may be possible to evaporate part of the water from the aloe gel harvested during the rainy season so that the active ingredients will become more concentrated. But this has high risk of losing the biological activities of active ingredients.

The results of the antimicrobial activities of the extracts against *Escherichia coli, Salmonella typhi*, *Staphylococcus aureus* and *Candida albicans* are presented in Table 4.1. The inhibitory activity was measured based on the average diameter of the clear inhibition zone. If there was no clear zone surrounding the extract loaded disks, it was assumed that there was no inhibitory effect. Antifungal activity against *F. xylarioides* is presented in Table 4.2. It was evaluated by comparing mycelia growth of the treated culture with the control.

Table 4.1: Comparison of antimicrobial activity of the extracts against *E .coli, S. typhi, S. aureus,* and *C. albicans*

Samples	Inhibition zone (mm)			
	E.coli	*S.typhi*	*S. aureus*	*C. albicans*
Aloe debrana extract	11.96 ± 0.50^{a}	9.66 ± 0.28^{c}	10.36 ± 0.32^{ab}	9.16 ± 0.35^{a}
Aloe trichosantha extract	11.70 ± 0.72^{a}	9.56 ± 0.40^{c}	10.40 ± 0.52^{bc}	8.8 ± 0.34^{a}
Papaya leaves aqueous extract	8.83 ± 0.76^{c}	10.73 ± 0.25^{b}	9.66 ± 0.57^{c}	7.26 ± 0.30^{c}
Papaya leaves alcoholic extract	0	0	0	0
Papaya leaves extract	10.66 ± 0.28^{b}	12.10 ± 0.36^{a}	11.26 ± 0.46^{a}	7.90 ± 0.36^{b}
Control	ND	ND	ND	ND

All values are means of triplicates ± standard deviation.
Means with different superscript letters within a column are significantly different ($p < 0.05$)
ND means not detected

Table 4.2: Comparison of antimicrobial activity of the extracts against *Fusarium xylarioides*

Extracts	ADE	ATE	PLAQE	PLALE	PLE
Inhibition (%)	36.66 ± 1.52^{c}	35.33 ± 3.21^{c}	42.33 ± 2.08^{b}	0.00^{d}	56.00 ± 2.04^{a}

All values are means of triplicates ± standard deviation
Means with different superscript letters within a row are significantly different ($p < 0.05$)
ADE - *Aloe debrana* extract; ATE - *Aloe trichosantha* extract; PLAQE - papaya leaves Aqueous extract; PLALE - papaya leaves alcoholic extract; PLE - Papaya leaves extract

From the result in Table 4.1, *Aloe debrana* extract and *Aloe trichsantha* extract have shown almost similar antimicrobial activities. Among the three papayas extracts mechanically obtained papaya leaves extract showed higher antimicrobial activity followed by aqueous extract while alcoholic extract of papaya leaves did not show inhibitory action. Similar result was reported by Dickson *et al*., (2011). Dickson *et al*., (2011) had conducted Sensitivity study on *E. coli, S. typhi*, and *S. aureus* to crude aqueous and alcoholic extracts and they found that water is the better extractive solvent for the active components than alcohol. The difference in antimicrobial activities between mechanically obtained papaya leaves extract and aqueous extract of the papaya leaves is mainly due to difference in concentration of the active components. Because the solvent for active ingredients in both papaya aqueous extract and mechanically obtained papaya leaves extract is water. The highest inhibition zone (12.16 mm) was recorded by mechanically obtained papaya leaves extract on *S. typhi*. Generally *E. coli* and *C. albicans* were more sensitive to aloe extracts whereas *S. typhi*, *S. aureus* and *F. xylarioides* were more sensitive to papaya extracts except papaya alcoholic extract (Table 4.1 and Table 4.2).

The results obtained in this research study showed better inhibition effect than those reported by Saks and Barkai-Golan, (1995) who found antifungal activity of the pulp against: *P. digitatum*, *A. alternata*, *B. cinerea*, and *P. expansum*, and those of Fujita *et al*., (1978) who obtained antifungal activity of the pulp from *Aloe arborescens* Miller *spp.natalensis* Berger, against the human fungal pathogen *T. mentagrophytes*, and those of Reynolds and Dweck, (1999) who observed control activities against 18 human pathogen microorganisms. The results also indicated the need of addition of papaya leaves extract on aloe gel extract to make the film-forming solution posses wide antimicrobial spectrum. Being wide antimicrobial spectrum of the packaging film helps to inhibit different microorganisms that will prevail on the food surfaces.

The mechanism of action responsible for antimicrobial activity of phenolic compounds present in herbaceous and woody plants has not been fully defined, although activity has been attributed to inhibition of extracellular enzymes, deprivation of substrates required for growth, inhibition of oxidative phosphorylation or iron deprivation (Scalbert, 1991). Sikkema *et al*., (1995) reported that the antibacterial properties of woody plant extracts are associated with its lipophilic components, leading to change in membrane potential and increase in permeability of the cytoplasm membrane for protons and potassium ions, including depletion of the intracellular ATP pool. The bitter taste, pungent and repulsive smell in some plants; have been found to have repressive ability over the metabolic activities of a wide range of microorganisms (Mitscher *et al.,* 1992).

Mechanically obtained papaya leaves extract showed higher inhibition than other papaya extracts. As a result, the following analyses were only performed on this extract among papaya leaves extracts. The analysis for papaya leaves aqueous alcoholic extracts was completed here. *Aloe debrana* and *Aloe trichosatha* extracts were more or less similar in their antimicrobial activities. It was impossible to recommend one of the two aloe extracts as a candidate for the film-forming solution based on their antimicrobial activity test only. There for, film-forming abilities of *Aloe debrana* and *Aloe trichosatha* extracts were also evaluated as additional comparison parameter.

4.1.2. Film-forming ability of *Aloe debrana* and *Aloe trichosantha* extracts

The result of the study showed that aloe gels are poor in producing films from aloe extracts alone. The films were highly brittle with poor physical appearance. It was difficult to separate the films from the support. Both of the films were not attractive for film development. *Aloe debrana* gel has relatively better film-forming ability than *Aloe trichosantha* gel.

Cheng-Pei *et al*., (2010) also reported that films made of aloe gel powder were brittle and not easy to handle. The moisture content of the aloe leaves gel is usually greater than 98% and the mixed polysaccharides (including glucomannan, galactan, arabinan and pectic substances) make up the most parts of the gel solid matter (Femenia *et al*., 1999). The relatively low solid content and brittle characteristic of mixed polysaccharide film make the aloe leaves gel technically not feasible for using as the supporting base material for edible films. Combination of different biomaterials to form composite or blend is a useful solution to enhance the mechanical and/or

functional properties of bioactive packaging materials (Rivero *et al*., 2009). To overcome the lack of film-forming capability of the aloe leaves gel, it is advisable to incorporate other suitable film-forming materials with the aloe leaves gel to form edible films. Moreover, composite edible coatings or films can combine the advantages of each component.

This study used gelatine as a film-forming material. As a common edible film-forming material, the applications of gelatine used for edible films or coatings are reported in the literature. Gelatine has been successfully used to form films that are transparent, flexible, water-resistant, and impermeable to oxygen (Hebert and Holloway, 1992).

Protein and polysaccharide are both hydrophilic biopolymers and have been combined to form composite edible films (Liu *et al*., 2007; Rivero *et al*., 2009), thus it is reasonable for gelatine and the aloe leaves gel to be formulated together to form composite films or coatings.

4.1.3. Colour and transparency of aloe and papaya leaves extracts

The color of papaya leaves extract was dark brown, while aloe extracts were translucent white. *Aloe trichosantha* gel has somewhat light green appearance where as *Aloe debrana* gel has whitish appearance. The transparencies of these extracts are given in Table 4.3.

Table 4.3: Transparencies of *Aloe debrana*, *Aloe trichosantha* , and papaya leaves extracts

Extracts	Transparency (% transmittance)
Aloe debrana extract	63.83 ± 0.76^{a}
Aloe trichosantha extract	58.16 ± 1.04^{b}
Mechanically obtained papaya leaves extract	0.86 ± 0.05^{c}

All values are means of triplicates ± standard deviation

Aloe debrana gel is better to produce more transparent packaging films due to higher transparency. Papaya leaves extract is almost opaque with transparency value (0.86%); it absorbs 99.14 % of the light directed to it. Different pigments like phenols are responsible for larger absorbance.

Colours of the film-forming solution were affected by the incorporation of papaya leaves extract into the *Aloe debrana* gel extract. The effect of papaya leaves extract on the colour of the film-forming solution is illustrated by Figure. 4.2.

Figure 4.1: The effect of papaya leaves extract addition on the colour of aloe debrana extract. (T= *Aloe trichosantha*, d=*Aloe debrana,* p= papaya leaves extract), dp1, dp2, dp3, and dp4 =10%, 20%, 30%, and 40% papaya leaves extract in *Aloe debrana* extract; respectively.

The film-forming solution without papaya leaves extract appeared clear and translucent. The film-forming solution with increasing level of papaya leaves extract was become brownish dark.

4.1.4. pH of the extracts

The pH values at room temperature of *Aloe debrana* gel extract, *Aloe trichosantha* gel extract and papaya leaves extract are given in Table 4.4.

Table 4.4: The pH value of *Aloe debrana* gel extract, *Aloe trichosantha* gel extract and papaya leaves extract

Samples	pH value
Aloe debrana gel extract	4.3±0.00
Aloe trichsantha gel extract	4.3±0.00
Papaya leaves extract	4.7±0.00

All values are means of triplicates ± standard deviation

Aloe debrana and *Aloe trichosantha* were similar in their pH values (4.3) but the pH value of papaya leaves extract was relatively higher (4.7) than the two aloe extracts. All of the samples have pH values in acidic range; these can contribute to the antimicrobial effectiveness of the extracts.

The pH of the extracts can also affect many properties of the packaging film. Nevena *et al*., (2010) reported the pH value had significant influence on light transmission, colour, and film solubility.

4.1.5. The effect of papaya leaves extract on the Antimicrobial activities of *Aloe debrana* extract

It was quite easy to produce the packaging film from aloe gel without incorporation of papaya leaves extract. But the film will not have wide antimicrobial spectrum. The amount of the papaya extract has significance influence on physicochemical and mechanical properties of the packaging film. The packaging film with low papaya extract has attractive features. Therefore it was found important to study the amount of papaya leaves extract to be added in to the *Aloe debrana* extract for significance antimicrobial activity enhancement, with little influence on other properties. The effect of papaya leaves extract on the antimicrobial activity of the *Aloe debrana* extract is given in Table 4.5.

Table 4.5: The effect of papaya leaves extract (PLE) on antimicrobial activity of *Aloe debrana* extract

	Inhibition zone (mm)				Inhibition (%)
PLE (%)	*E.coli*	*S.typhi*	*S. aureus*	*C. albicans*	*F.xylarioides*
0	11.96±0.50^{a}	9.66±0.28^{d}	9.70±0.26^{c}	9.30±0.43^{b}	36.66±1.52^{d}
10	12.06±0.30^{a}	11.40±0.40^{c}	11.13±0.30^{b}	9.40±0.20^{b}	40.66±1.43^{c}
20	12.33±0.29^{a}	12.73±0.11^{b}	12.56±0.26^{a}	9.83±0.05^{a}	51.33±3.05^{b}
30	12.43±0.15^{a}	12.96±0.20^{b}	12.50±0.26^{a}	9.86±0.11^{a}	54.00±2.00^{ab}
40	12.43±0.25^{a}	13.43±0.21^{a}	12.63±0.28^{a}	9.90±0.10^{a}	55.33±1.52^{a}
50	12.52±0.30^{a}	13.60±0.20^{a}	12.60±0.20^{a}	9.93±0.23^{a}	56.00±1.80^{a}

All values are means of triplicates ± standard deviation
Means with different superscript letters within a column are significantly different ($p < 0.05$)

In this study, antimicrobial activity of *Aloe debrana* gel extract was significantly increased ($p<0.05$) with increasing level of incorporation of papaya leaves extract. But the influence on *E. coli* was not significance. Generally increasing the papaya leaves extract proportion beyond 30% did not bring significant effect on the antimicrobial activity of the solution (Table 4.5) for most test organisms. Therefore, it is appropriate to use about 30% papaya leaves extract in the film-forming solution so that the mechanical and physicochemical properties will not be much affected.

4.1.6. Proximate composition of *Aloe debrana* extract and papaya leaves extract

All properties of the packaging films are the result of the constituents present in the film. The results are given in Table 4.6.

Table 4.6: Proximate composition of *Aloe debrana* extract and papaya leaves extract

Sample	Moisture content (%)	Carbohydrate content (%)	Protein content (%)	Fat content (%)	Ash content (%)
ADE*	97.27±0.08	1.45±0.02	0.04±0.01	0.62±0.01	0.63±0.03
PLJ**	98.64±0.06	0.47±0.04	0.23±0.03	0.39±0.02	0.27±0.01

All values are means of duplicates ± standard deviation
ADE* = aloe debrana extract, PLJ** = mechanically obtained papaya leaves extract

The moisture content of the ADE was 97.27 ± 0.08 %. The result was similar with other report in the literature (Femenia *et al*., 1999). The contents of crude protein, ash, fat and carbohydrate were 0.04 ± 0.02%, 0.63 ± 0.03%, and 0.62 ± 0.01% and 1.45 ± 0.02%; respectively. With the exception of crude fat and protein content, the results were in agreement to previous published results by Femenia *et al.,* (1999). The difference in cultivar or climate could be the reasons for the inconsistence. Although the aloe leaves gel has been used as edible coating after blending with water (Valverde *et al*., 2005), the results of this study reveal that the moisture of aloe leaves gel is 97.27 ± 0.08% and it is difficult to form edible film by itself. However, it is feasible to use aloe in combination with other film-forming materials to formulate composite films.

The moisture content of papaya leaves extract was 98.64 ± 0.06% which is greater than the moisture content of *Aloe debrana* extract. The incorporation of papaya leaves extract in aloe gel could further increase the moisture content of the film-forming solution. This also indicates that there should be film-forming material which can construct the body of the film. Gelatine was used as a film-forming material to compensate that weakness. The amount of gelatine in the film forming solution was adjusted based on the dry mass of the film-forming solution. The contents of crude protein, carbohydrate, ash, crude fat were 0.23 ± 0.03%, 0.47 ± 0.04%, 0.27 ± 0.01%, and 0.39 ± 0.02%; respectively. These all have their own effect on mechanical, physicochemical and antimicrobial properties of the packaging film. Further study is needed to evaluate the effect of each component and to identify the antimicrobial substances present in these medicinal plants.

4.2. Evaluation of aloe based packaging films

Before starting the discussions of the properties of the packaging films obtained, it may be proper to explain some ideas about the film-forming processes and conditions. Solvent removal technique was used to produce the packaging films. Several different film-forming techniques are available including solvent removal, thermal gelation and solidification of melt. Cha *et al.,* (2003) reported that, Solvent removal is typically used to produce hydrocolloid edible films like aloe based packaging films. In this process, a continuous structure is formed and stabilized by chemical and physical interactions between molecules.

When the cohesive strength of the polymer molecules is relatively high, continuous surfaces of the polymer material coalesce. Coalescence of an adjacent polymer molecule layer occurs through diffusion. Upon evaporation of water, gelation progresses and allows the polymer chains to align in close proximity to each other and to get deposited over a previous polymer layer (Harris and Ghebre-Sellassie, 1997). When there is adequate cohesive attraction between the molecules, sufficient diffusion, and complete evaporation of water, polymer chains align themselves to form films (Harris and Ghebre-Sellassie 1997).

Film forming process was highly environmental sensitive. The most influencing conditions were the composition of film forming solution, temperature and duration of heating of the film-forming solution, the drying condition, the support used etc. There is a chance of getting plastic films and non plastic films from deviations of these parameters.

Starch, protein and lipid based films have their own optimum temperatures of heating and cooling for gelation to progress. For example starch should be heated about 85^{o}c for its gelatinization to produce starch based films (Eraricar *et al*., 2009). Protein films could be developed by heating the protein solution at about 40^{o}C (Bower *et al*., 2006). But the film-forming material of this study was not pure substance. Rather it was composite of protein, carbohydrate, fat and other minor components available in all raw materials used. There was no previous study about the optimum temperature of heating for such type of film-forming solution before casting.

This study has tried to screen out relatively better temperature of heating for the film-forming solution in the range 40-90^{o}C and has found that 50^{0}C is relatively better temperature. It does

not mean that 50^0C is the optimum temperature. Films produced above 55^oC were not attractive. They look like burnt and did not have elastic property. This may be the result of denaturation of the protein molecules and became unable to develop bond with each other and other molecules. Films developed bellow 45^0C were full of morphological defects such as inclusion of air bubbles shape defects etc. This may resulted from limited interaction between different molecules.

The drying condition was another important parameter which could influence the quality of films. Milda and Kerry, (2009) reported that drying can either enhance or damage properties of the coating.

In this study, the packaging films were developed under normal atmospheric conditions and using tray dryer (TAURO B105EC, N11/2006). It was difficult to obtain films by drying the film-forming solution at normal atmospheric condition. The film forming solution took longer time (more than a week) to dry and it has started to ferment instead of drying. Films produced by the tray dryer were homogenous and flexible with good appearance. But determining of optimum drying conditions is still a big issue. The properties of the packaging films obtained are discussed in the following sections.

4.2.1. Moisture Content and thickness

Moisture content of a packaging film at a given air temperature and humidity is an important property of a packaging film that can limit its application in food packaging. The films were developed by varying the proportions of glycerol and gelatine based on the experimental design given earlier. Among the six different treatments, only three were effective to build a standalone film. The first two treatments ($P_{0,1}$) (with zero glycerol and 1g gelatin) in the film-forming solution and ($P_{0,2}$) (with zero glycerol and 2g gelatin) in the film forming solution resulted in brittle films which are difficult to handle. On the other hand the fifth treatment (1g glycerol and 1g gelatine in the film forming solution ($P_{l,1}$)) was unable to give stand alone film. It was very difficult to separate the film ($P_{l,1}$) from the support. Films with composition 0.5g glycerol and 1g gelatine in the film-forming solution ($P_{0.5,1}$), 0.5g glycerol and 2g gelatine in the film forming solution ($P_{0.5,2}$) , and 1g glycerol and 2g gelatine in the film forming solution ($P_{1,2}$) were the only treatments that produce stand alone films to which most of the analyses performed. $P_{0,1}$,$P_{0,2}$ and $P_{l,1}$ were not suitable for most analyses. The moisture contents and thickness of the packaging films are shown in Table 4.7.

Table 4.7: Moisture content and thickness of the packaging films

Sample code	Moisture content (%)	Thickness(mm)
$P_{0,1}$	8.20 ± 0.33^{e}	0.23 ± 0.07^{d}
$P_{0,2}$	7.15 ± 0.29^{f}	0.42 ± 0.03^{c}
$P_{0.5,1}$	10.19 ± 0.39^{c}	0.34 ± 0.12^{cd}
$P_{0.5,2}$	9.26 ± 0.21^{d}	0.54 ± 0.04^{b}
$P_{1,1}$	12.83 ± 0.36^{a}	0.40 ± 0.08^{c}
$P_{1,2}$	11.25 ± 0.58^{b}	0.68 ± 0.13^{a}

All values for moisture content are means of triplicates ± standard deviation
All values for thickness are means of five measurements ± standard deviation.
Means with different superscript letters within a column are significantly different ($p<0.05$)
$P_{0,1}$= no glycerol+1g gelatine, $P_{0,2}$ = no glycerol +2g gelatine, $P_{0.5,1}$= 0.5g glycerol +1g gelatine, $P_{0.5,2}$= 0.5g glycerol+2g gelatine, $P_{1,1}$= 1g glycerol+1g gelatine, $P_{1,2}$= 1g glycerol+2g gelatin

The moisture content of the packaging films significantly increased ($p<0.05$) as the percentage of glycerol in the film-forming solution increased and decreased ($p<0.05$) as the percentage of gelatine increased. The lowest moisture content of 7.15 % was obtained in $P_{0,2}$, while the highest moisture content of 12.83% was detected in $P_{1,1}$ (Table 4.7). The glycerol content in both $P_{0,1}$ and $P_{0,2}$ was 0 g but the gelatine content increased from $P_{0,1}$ to $P_{0,2}$ .Therefore aloe to gelatine proportion in $P_{0,2}$ is lower than in $P_{0,1}$. The moisture content of $P_{0,1}$ (8.20%) is greater than the moisture content of $P_{0,2}$ (7.15%). It is because that the major component of aloe gel powder is mixed polysaccharides including glucomannan, galactan, arabinan and pectic substances (Femenia *et al*., 1999), which exert higher affinity to water molecules for more hydroxyl groups existing in the polysaccharide structure than in gelatine structure. Liu *et al*., (2007) reported that higher content of hydrophilic hydroxyl groups of polysaccharide increased the water absorbability of gelatine/polysaccharide blend films.

The moisture content of edible films depends on the hydrophilic group density of the film. That is why $P_{1,1}$ with the highest proportion of glycerol has the highest moisture content (12.83%). Since glycerol is more hydrophilic than gelatine and aloe gel. Compared to previous study conducted by Cheng-Pei *et al*., (2010), the films have higher moisture content. The difference in moisture content could be related to the difference in composition of the films. In this study papaya leaves extract and glycerol were added, in addition to gelatine and aloe gel. The increase in moisture content could also be related to the hydrophillicity of glycerol and papaya leaves

extract. $P_{1,2}$ has the highest thickness (0.68 mm), and $P_{0,1}$ has the lowest thickness(0.23 mm). Addition of both gelatine and glycerol has significantly ($p < 0.05$) influenced the thickness of the films. It is obvious that the film dry matter increases by the addition of these solutes which resulted in larger thickness.

4.2.2. Physicochemical Properties of aloe based packaging films

4.2.2.1. Colour and Transparency

Colour attributes are of prime importance because they directly influence consumer acceptability. Visually, all the films developed from papaya leaves extract and *Aloe debrana* extract were light brown as shown in Figure 4.3. Fig 4.4 demonstrates the colour of the packaging films developed by excluding papaya leaves extract, to see the influence of papaya leaves extract on the colour of the composite film.

Figure 4.2: Films developed in the presence of papaya leaves extract.

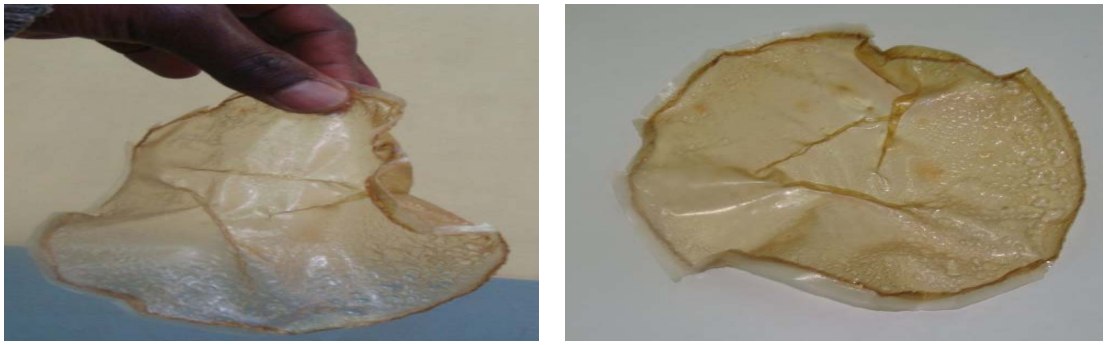

Figure 4.3: Films developed without papaya leaves extract.

Addition of papaya leaves extract affected the colour of the packaging films. This was due to the red-brownish colour of papaya leaves extract. The colour of the composite film developed by excluding papaya leaves extract was white and somewhat transparent as illustrated in Figure 4.4.

Transparency is also one of the common optical properties of light permeable materials. Development of transparent packaging materials which allow product visibility is a general trend and requirement in packaging films. It was difficult to analyze the transparencies of films with treatment codes ($P_{0,1}$, $P_{0,2}$&$P_{1,1}$) because of their fragile nature. The transparencies of the three stand alone films are given in Table 4.8.

Table 4.8: Transparencies of the packaging films

Sample code	Transparency (**%** transmittance)
$P_{0.5,1}$	32.67 ± 0.57^{b}
$P_{0.5,2}$	28.00 ± 1.00^{c}
$P_{1,2}$	36.67 ± 1.52^{a}

All values are means of triplicates ± standard deviation
Means with different superscript letters within a column are significantly different ($p < 0.05$)
$P_{0.5,1}$= 0.5g glycerol +1g gelatine, $P_{0.5,2}$= 0.5g glycerol+2g gelatine, $P_{1,2}$= 1g glycerol+2g gelatin

Transparency was significantly affected by the concentration of glycerol and gelatine. %T of film slightly increased with increasing glycerol content.$P_{1,2}$ was more transparent than the other films. Generally these packaging films may not be used as see-through packaging or coating materials, since their transparency is affected by the papaya leaves extract.

4.2.2.2. Solubility of aloe based packaging films

Film solubility (FS) is a parameter of biodegradability of films and it was expressed as the percentage of film dry matter solubilized in distilled water. Solubility in water is an important property of edible films, since potential applications may require water insolubility to enhance product integrity and water resistance. However, in some cases water solubility of the film before consumption of the product might be beneficial (Perez-Gago *et al*., 1999). The FS of tested samples is presented in Table 4.9.

Table 4.9: Solubility of the packaging films

Sample code	FS (%)
$P_{0,1}$	50.82 ± 2.88^{e}
$P_{0,2}$	44.57 ± 1.05^{f}
$P_{0.5,1}$	70.37 ± 2.55^{c}
$P_{0.5,2}$	61.18 ± 1.40^{d}
$P_{1,1}$	90.49 ± 0.76^{a}
$P_{1,2}$	85.70 ± 1.20^{b}

All values are means of triplicates ±standard deviation
Means in the same column with different superscript letters are significantly different ($P < 0.05$)
$P_{0,1}$= no glycerol+1g gelatine, $P_{0,2}$ = no glycerol +2g gelatine, $P_{0.5,1}$= 0.5g glycerol +1g gelatine, $P_{0.5,2}$= 0.5g glycerol+2g gelatine, $P_{1,1}$= 1g glycerol+1g gelatine, $P_{1,2}$= 1g glycerol+2g gelatin

Generally, the results indicate that films were highly soluble. The highest solubility was 90.49% of film composed of 1g glycerol and 1g gelatine ($P_{1,1}$) while the lowest solubility was 44.57% of film with composition of 0g glycerol and 2g gelatine ($P_{0,2}$). Films with even more solubility have been reported earlier. Mahamadou *et al*., (2007) reported 97.98% film solubility for whey protein isolate films. Film solubility increased significantly ($p<0.05$) as the content of glycerol increased and decreased ($p<0.05$) as the content of gelatine increased. Cuq, (2002) reported that, in general, hydrophilic plasticizers, such as glycerol, enhance water solubility. 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 (Hernandez-Munoz *et al*., 2004).

The decrease in film solubility as gelatine content increased is due to the low hydrophilic nature of gelatine compared to aloe gel and glycerol. Addition of more gelatine increases the insoluble portion in the film. Gelatine itself is soluble but the degree of solubility compared to aloe gel and glycerol is low. Furthermore, the increase of film solubility might be related to the hydrophillicity of papaya leaves extract. Jutaporn *et al*., (2011) reported that an increase in phayom wood extract in edible films led to an increase in film solubility (FS). It could be hastily concluded that papaya leaves extract enhance film solubility in water. This water solubility behavior could not be generalized, and understanding the film solubility remains a complex subject.

Generally, higher solubility would indicate lower water resistance. However, high solubility may have an advantage for some applications (Perez-Gago *et al*., 1999). Low water solubility is important when films are in contact with water during processing and storage. The opposite is desirable when the intent is to design a package with pre-measured dry food amounts to be dissolved in water or in hot food. Gelatine films have been reported to be soluble in water. Completely biodegradable transparent films for foodstuff packaging, for example, have already been successfully produced from physically hardened gelatine films (Schrieber and Gareis, 2007). One of the major drawbacks in the use of gelatine films in technical applications is their water absorption tendency. The aloe based films produced by this study have similar problems. Any improvement in water resistance of these films is highly important.

4.2.2.3. Swelling property of aloe based packaging films

Aloe polysaccharides, gelatine and glycerol, all being hydrophilic polymers, show high affinity towards water, Hence, upon hydration, aloe based packaging films absorb water and swell and/or solubilize. All films showed swelling behavior. But it was not possible to take swelling data since the films did not retain their integrity and showed high degree of dissolving in water. The reason behind the soluble nature of aloe based films was the high hydrophilic nature of the components in the film. Such types of films are good candidates to develop edible films and packaging materials which require complete solubility upon hydration. To develop water resistant films incorporation of hydrophobic components could be mandatory.

4.2.3. Antimicrobial activities of aloe based packaging films

Antimicrobial activities of films were evaluated using the agar diffusion method. The inhibitory activity was measured based on the clear zone surrounding the square film strips as revealed in Figure 4.5. Measurement of clear zone area has included area of film strips. Antimicrobial activities of the composite films on *Escherichia coli, Staphylococcus aureus, Salmonella typhi, Candida albicans* and *Fusarium xylarioides* are shown in Table 4.10.

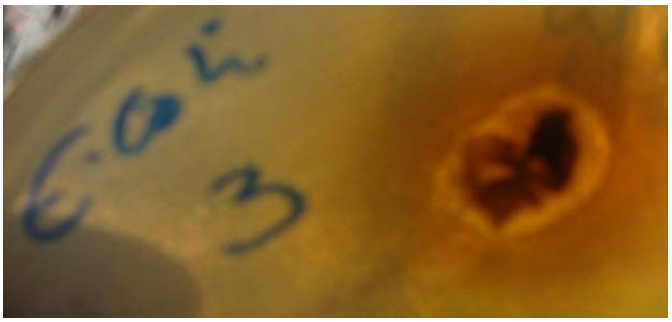

Figure 4.4: picture of inhibitory zone of aloe based packaging film against *E.coli*.

Table 4.10: Antimicrobial activity of the packaging films on *E. coli*, *S. aureus*, *S.typhi*, *C. albicans* and *F. xylarioides*

Sample code	Average area of inhibitory zone (cm^2)				
	Salmonella typhi	*Escherichia coli*	*Staphylococcus aureus*	*Candida albicans*	*Fusarium xylarioides*
$P_{0,1}$	6.01 ± 0.22^{b}	4.34 ± 0.12^{a}	5.15 ± 0.71^{a}	4.40 ± 0.20^{ab}	6.20 ± 0.12^{a}
$P_{0,2}$	5.83 ± 0.14^{b}	4.27 ± 0.13^{a}	5.61 ± 0.37^{a}	4.20 ± 0.20^{b}	6.01 ± 0.43^{a}
$P_{0.5,1}$	6.24 ± 0.24^{ab}	4.48 ± 0.33^{a}	5.77 ± 0.81^{a}	4.51 ± 0.08^{ab}	6.38 ± 0.13^{a}
$P_{0.5,2}$	6.09 ± 0.37^{ab}	4.62 ± 0.22^{a}	5.22 ± 0.56^{a}	4.48 ± 0.32^{ab}	6.35 ± 0.66^{a}
$P_{1,1}$	6.52 ± 0.28^{a}	4.63 ± 0.20^{a}	5.53 ± 0.61^{a}	4.61 ± 0.22^{a}	6.39 ± 0.11^{a}
$P_{1,2}$	6.23 ± 0.24^{ab}	4.40 ± 0.20^{a}	5.95 ± 0.27^{a}	4.47 ± 0.24^{ab}	6.25 ± 0.24^{a}
Control	ND	ND	ND	ND	ND

All values are means of triplicates ±standard deviation
Means in the same column with different superscript letters are significantly different ($P < 0.05$).
ND means not detected
$P_{0,1}$= no glycerol+1g gelatine, $P_{0,2}$ = no glycerol +2g gelatine, $P_{0.5,1}$= 0.5g glycerol +1g gelatine, $P_{0.5,2}$= 0.5g glycerol+2g gelatine, $P_{1,1}$= 1g glycerol+1g gelatine, $P_{1,2}$= 1g glycerol+2g gelatin

All the packaging films inhibited the growth of all the test microorganisms used. The greatest zone of inhibition (6.52 cm^2) was observed at P1,1 against *S. typhi*, and the least zone of inhibition (4.20 cm^2) was observed at $P_{0,2}$ against *C. albicans*. All the packaging films have almost similar antimicrobial activity since they contain the same amount of *Aloe debrana* extract (ADE) and papaya leaves extract (PLE). Significance differences between the antimicrobial activities of the packaging films were observed specially on *S. typhi* and *C. albicans.* This difference may be resulted from two possible reasons. The first reason could be the interference

of gelatine and glycerol on the antimicrobial activity of ADE and PLE against *S. typhi* and *C. albicans.* The other reason could be the difference in solubility of the packaging films. More soluble films could release the antimicrobial component quickly and large diameter around the films. The antimicrobial component releasing rate of each film requires careful investigation.

In this study different test organisms have showed different sensitivity to similar antimicrobial films. Film $P_{0,1}$ has 6.01±0.22 mm inhibition zone on *S. typhi* but it has 4.34±0.12 mm inhibition zone on *E. coli* (Table 4.5). Basarada, (1966) also reported that the sensitivity to tannins and other phenolic compounds varies greatly among organisms. In addition Jutaporn *et al*., (2011) reported that some organisms including *E. Coli* and *P. Fluorescens* are capable of growing on tannins as a source of carbon. The difference in sensitivity may also be associated with difference in cell wall structure and function.

The antimicrobial activities of the packaging films developed in this study were better than antimicrobial films developed by Cheng-Pei *et al*., (2010). Cheng-Pei *et al*., (2010) had developed antimicrobial films from aloe gel powder and gelatine using distilled water as a solvent. The antimicrobial activities of these films were not comparable to films developed in this study by their antimicrobial activity. This deference may be resulted from one or all of the following reasons. The first one is the presence of papaya leaves extract in the films developed in this study has increased their antimicrobial activity. The second difference may be the difference between the aloe species. Cheng-Pei *et al*., (2010) developed films from *Aloe barbadensis.* But the films in this study were developed from *Aloe debrana* (endemic aloe species to Ethiopia). In addition to this, seasonal and geographical variation could also bring difference in antimicrobial activities of the aloes used. Moreover, the film-forming conditions may result in different antimicrobial activity.

The packaging films developed in this study inhibited all the test microorganisms used. *Aloe debrana* and papaya leaves extracts were responsible for the films to have 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 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 safety and shelf-life of food products.

4.2.4. Mechanical Properties of aloe based packaging films

The determination of the mechanical properties involves not only scientific but also technological and practical aspects. Because biopolymer materials, such as films, may be subjected to various kinds of stress during use (Cagri *et al*., 2001). Tensile strength (TS) is the maximum tensile stress sustained by the sample during the tension test. Elongation at break (EB) is an indication of a film's flexibility and stretchability (extensibility). It is expressed as the percentage of change of the original length of the specimen between the grips of a film to stretch. The mechanical properties including TS and EB of the packaging films are shown in Table 4.11. The mechanical analysis of films ($P_{0,1}$, $P_{0,2}$, $P_{1,1}$) were not conducted because the films were too fragile and could not be mounted to the grips of tensile testing machine.

Table 4.11: Mechanical properties of aloe based packaging films

Sample code	Tensile strength (MPa)	Elongation at break (%)
$P_{0.5,1}$	20 ± 0.00^{c}	89 ± 15.55^{b}
$P_{0.5,2}$	65 ± 7.07^{a}	170 ± 14.14^{a}
$P_{1,2}$	45 ± 7.07^{b}	180 ± 0.00^{a}

All values are means of duplicates ±standard deviation
Means in the same column with different superscript letters are significantly different ($P < 0.05$).
$P_{0.5,1}$= 0.5g glycerol +1g gelatine, $P_{0.5,2}$= 0.5g glycerol+2g gelatine, $P_{1,2}$= 1g glycerol+2g gelatin

Film $P_{0.5,2}$ has maximum tensile strength (65 MPa) followed by film $P_{1,2}$ (45 MPa) and film $P_{0.5,1}$ (20 MPa). The results showed that the TS of aloe based packaging films were significantly ($p<0.05$) affected by the addition of gelatine and glycerol. The proper amount of gelatin and glycerol in the film forming solution was crucial to develop functional films.

The difference in composition between $P_{1,1}$ and $P_{1,2}$ was their gelatine content only. In $P_{1,1}$ the gelatine content was 1%(minimum) and in $P_{1,2}$ it was 2% (maximum). $P_{1,1}$ was sticky and fragile film but $P_{1,2}$ was flexible and attractive film. The difference in composition between $P_{0.5,1}$ and $P_{1,1}$ was their glycerol content only. $P_{0.5,1}$ was flexible and attractive film but $P_{1,1}$ was sticky and fragile film. From this we can be understood that gelatine is better film-forming material. But increasing the amount of glycerol beyond certain limit could result in sticky and fragile film. Films developed without both gelatine and glycerol were difficult even to handle. Films without glycerol but with 1 and 2g gelatine ($P_{0,1}$ and $P_{0.2}$) were also brittle. On the other hand, the film

with 1g gelatine and 1g glycerol ($P_{1,1}$) was fragile and sticky. It was very difficult even to separate it from the support without destructive damage.

The TS of composite films increased from (20 to 65 MPa), as the percentage of gelatine increased from 1% ($P_{0.5,1}$) to 2 % ($P_{0.5,2}$). But glycerol produced an opposite effect on the TS. The tensile strength decreased from 65 MPa to 45 MPa, as the percentage of glycerol increased from 0.5% to 1%. The effect of plasticizer (glycerol in this study) on reduction of TS is well known and its explanation was reported by some researchers (Cuq *et al*., 1997). Tensile strength was found to decrease as the plasticizer concentration was increased. Plasticizers weaken the intermolecular forces between the chains of adjacent macromolecules, increasing the free volume and causing a reduction of mechanical resistant (Sobral *et al*., 2001).

Elongation at break was significantly ($p<0.05$) increased by increasing gelatine content in the film-forming solution. Cheng-Pei *et al.*, (2010) reported similar results on the effect of gelatine on TS and EB of composite films.

Maximum elongation was 180% at $P_{1,2}$ and the minimum elongation was 89% at $P_{0.5,1.}$ Similar results were reported by Junianto *et al*., (2012) from Tilapia's skin gelatine edible films with addition of plasticizer sorbitol at 10% of gelatine and 5% of sorbitol concentration. The increase in EB was noticeable when gelatine content increased ($p <0.05$). But the effect of glycerol was not as such significant ($p >0.05$). Such effect of glycerol on elongation at break was reported by Sazedul *et al*., (2011) when gelatines with 0.80 and 1.20% degree of hydrolysis were used. Sazedul *et al*., (2011) concluded that mechanical properties of gelatine-based film were largely affected by the chain length of gelatine (i.e. degree of hydrolysis). At high degree of hydrolysis, shorter gelatine molecules with the higher mobility of chain ends might perform plasticizing effect by preventing protein-protein interaction but preferably formed H-bonds with water. This will result in the breakage of films before stretched too much. Considering the results of mechanical properties, the obtained films have showed interesting results to be used as a food packaging material.

Chapter Five

Suggested Processing Technology for Aloe Based Packaging Film Production

5.1. Aloe based packaging film production

It is quite easy to produce active packaging films from aloe gel, papaya leaf juice and gelatine using glycerol as plasticizer and ascorbic acid as stabilizing agent. The technology is not sophisticated. The production can easily be achieved at small scale industries with the following simple processing procedures and equipments. The production starts with the preparation of each ingredient individually for the packaging film development.

Preparation of stabilized aloe extract: aloe gel will first be extracted from fresh clean aloe leaves by hand- filleting method. Aloe gel extraction can also be performed mechanically, but the purity is not comparable to that of hand filleted gel. Filleting is term used to describe separation of gel from the leaf parts. Next, the gel obtained will be homogenized by blender and filtered using cheesecloth to avoid the fibbers that could be otherwise a cause for film defects. Finally the clear aloe juice will be pasteurized at 70^{o}c for about 45 minutes to stop enzymatic degradation and stabilized by ascorbic acid and citric acid (2 and 4) grams per liter of aloe extract respectively.

Preparation of papaya leaf extract: the juice from fresh, clean papaya leaves can be extracted by mechanical pressing using filter press. Fibbers, sticks, chlorophyll and any suspended materials should be separated by filtration. The remaining film-forming ingredients are gelatine and glycerol. These are ready to use materials for the preparation of film forming solution if they are purchased from supermarket.

Preparation of film forming solution (FFS): the first operation in the preparation of FFS is the mixing of stabilized aloe juice with the papaya leaf juice. This is then followed by solubilisation of gelatine powder by the mixture extract first at room temperature and later in a water bath at 50^{0}c. After complete solubilisation of gelatine, glycerol will be added and the Solution will be kept in water bath under soft agitation for 30 min.

Film drying: the film forming solution will be conveniently applied on a suitable support and dried at room temperature or suitable dryers. The following flow diagram clearly describes the processes for the production of aloe based packaging films.

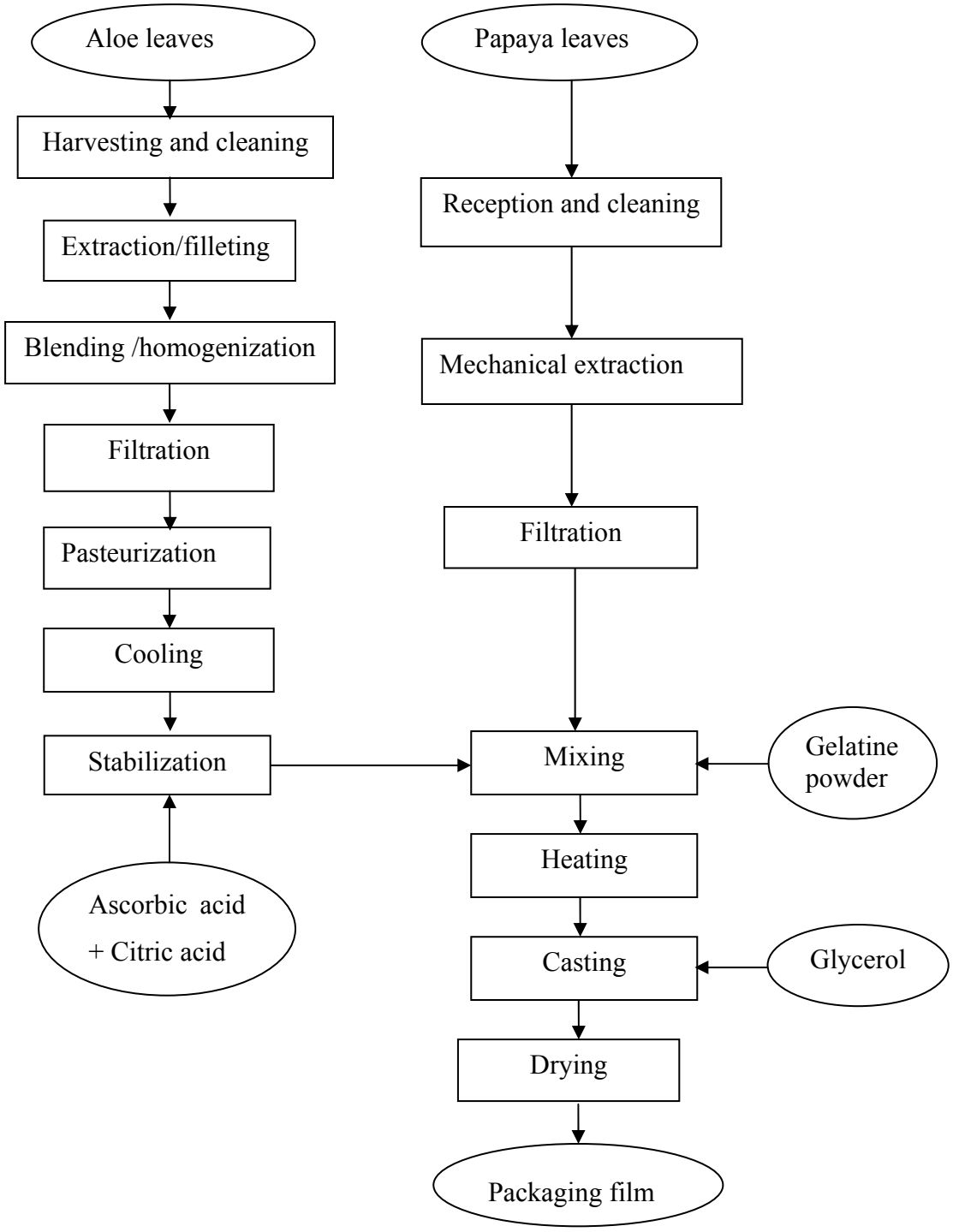

Figure 5.1: Flow diagram of aloe based packaging film production.

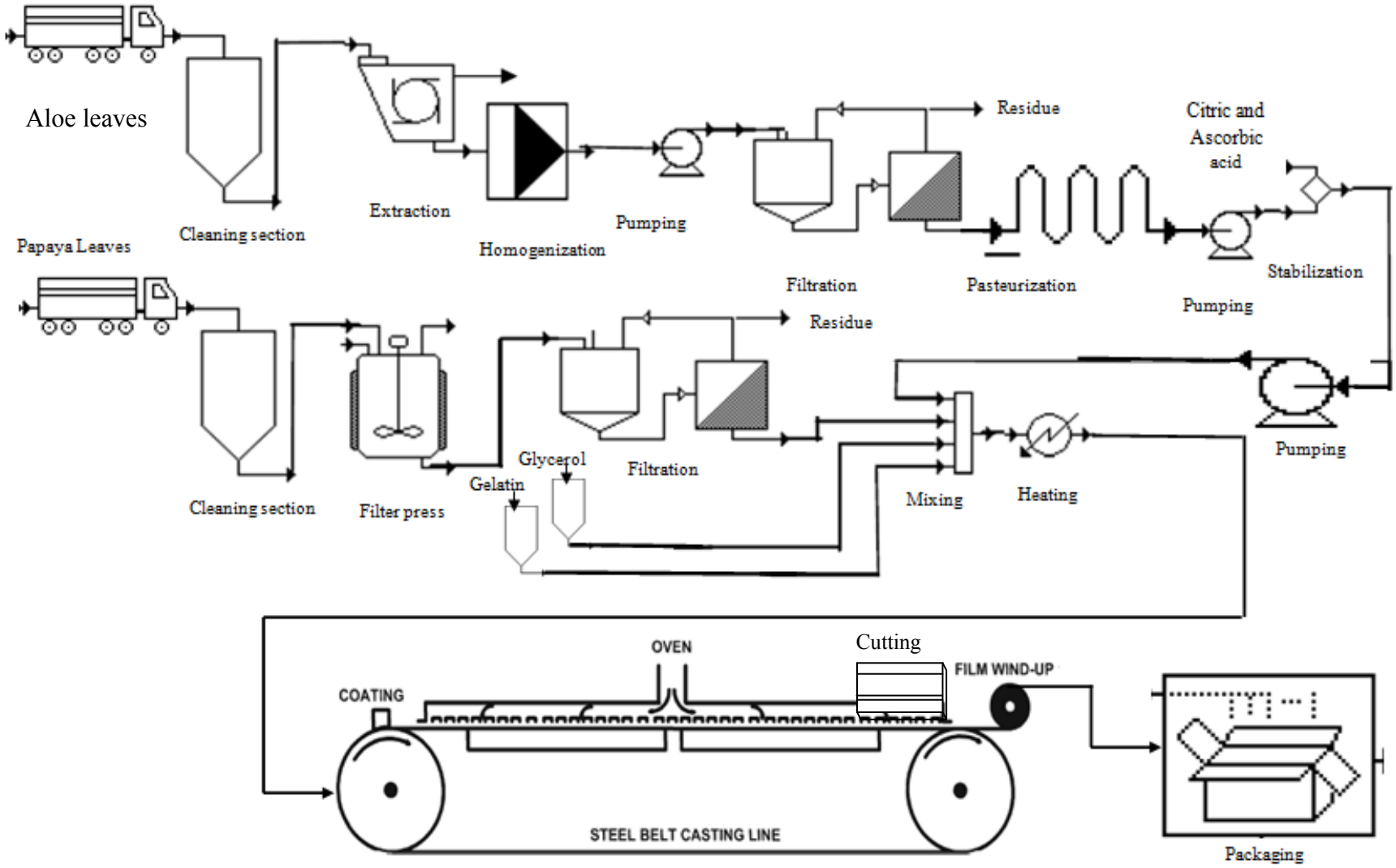

Figure 5.2: Equipment layout for aloe based packaging film production

Chapter Six

Conclusions and Recommendation

6.1. Conclusions

Edible films can be produced from materials with film forming ability. This study indicated that aloe, gelatine and papaya leaves can be used to formulate edible films. The film-forming ability of the aloe gel was highly improved by the addition of gelatine and glycerol. Addition of more plasticizer (glycerol) made the films more hydrophilic, sticky, and difficult to handle. Films formed without glycerol were highly brittle. The colours of the packaging films developed were red-brownish; it was highly influenced by the colour of papaya leaf extract. Aloes based packaging films exhibited high solubility in water and, hence, have potential for developing edible packaging material intended for easy solubility.

The antimicrobial system formed by aloe based packaging films, showed better antimicrobial activity on Gram (+) bacteria, Gram (-) bacteria and fungus. The antimicrobial property of the film-forming solution has been improved by the addition of papaya leaf extract. The packaging film developed has wide antimicrobial spectrum. *S. typhi* was the most inhibited microorganism by the developed packaging films.

The packaging films developed were strong enough. Tensile strength and elongation at break were significantly increased by the addition of gelatine. On the contrary glycerol has reduction effect on the tensile strength of the packaging films.

Packaging films containing aloe will not only protect foods from microbial deterioration as shown in this study but also might possess the potential to enhance the consumer health. Because the aloe leaf gel has contain functional ingredients. According to the research findings, aloe based packaging films can be used as a packaging material for exporting high value crops. Alternatively aloe based packaging films can be used as antimicrobial packaging materials for perishable food products, by using hydrophobic plasticizers. Generally aloe based packaging films have the potential to prolong the shelf-life of foods and reduce the burden of petroleum products on the environment.

6.2. Recommendation

Development of packaging films from aloe, papaya, and gelatine using glycerol as plasticizer is studied for the first time in this research in Ethiopia; therefore extensive researches are still needed on the following aspects:

- Qualitative and quantitative determination of antimicrobial substances in aloe and papaya leaf extracts
- Study the effect of processing on antimicrobial properties of aloe and papaya leaf extracts
- Study the barrier properties of these films
- Study the effect of hydrophobic plasticizers on the properties of these films
- Optimization of the film formation process
- Study the effectiveness of these films on different foods during storage
- Study migration kinetics of the antimicrobial component from the film to the food.

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Appendices

Appendix I: Chemicals, media, and equipments used for the study

Culture media: Mueller Hinton Agar (MHA) (HIMEDIA, India) and Potato dextrose agar (PDA) (OXOID, England) were purchased from Neway PLC, Addis Ababa. Nutrient broth (NB) (OXOID, England), Brilliant green agar (BGA), Manitol salt agar (MSA), Sabouraud dextrose agar (SDA), MacConkey Agar(MA) (OXOID, England), were obtained from Biology Department of Addis Ababa University (AAU).

Chemicals and reagents: Ethanol, ascorbic acid, citric acid, sodium chloride, sodium phosphate, potassium chloride, potassium phosphate, sulphuric acid, and barium chloride were obtained from Food and Nutrition Program and Biology Department in AAU Science Faculty.

Equipments purchased from *Mercato* (Addis Ababa market center).

Knives, bowels, cheesecloth, plastic jars, ruler, Food Blender (Philips HR2021, made in china), Different plastic containers

Equipments those were available in the laboratories of AAiT

Screw press, Thermostat, Tray dryer (TAURO B105EC, N11/2006), Tensile testing machine (computer- electro hydraulic universal testing machine, model AW600, made in china), Analytical balance, Oven, Moisture analyzer (MB45, OHAUS, Switzerland), Digital caliper and pH meter, Shaker (new brunswick scientific Exella E24 incubator shaker)

Equipments those were available in the laboratories of Biology Department and Food Science and Nutrition Program of science faculty in AAU.

Distiller, Autoclave, Petri dishes, Test tubes, Cotton swab, Filter paper (Whattman no. 1), Inoculating loop, Incubator, Micropipette, Spectrophotometer (6405 UV/Vis spectrophotometer, manufactured in UK)

Preparation of potato dextrose broth

Potato dextrose broth is general purpose broth for yeasts and molds. The low pH of this medium inhibits bacterial growth. Potato dextrose broth is the same formula as potato dextrose agar, but agar has been omitted.

Principles of the procedure: Potato dextrose broth is composed of potato infusion solids and dextrose that encourage luxuriant fungal growth.

200g cleaned, peeled, and sliced potatoes were boiled in 1L distilled water and filtered. 20g dextrose was dissolved by the filtrate and boiled. Finally the solution was autoclaved at 121^{o}c for 15 minutes. The product is sterile potato dextrose broth.

Appendix II: 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.

Table: Formula for Media Used in Inhibition Zone Assay agar preparation

Test organism	Selective Medium	Formula (gram/ L of water)
	Muller hinton agar	38
	Potato dextrose agar	39
Staphylococcus aureous	Manitol salt agar	43
Escherichia coli	MacConkey agar	51.5
	Nutrient broth	13
Candida albicans	Saboraud dextrose agar	65
Salmonella typhi	Briliant green agar	63

Agar slant preparation

- Transfer about 5 milliliters of the boiled medium in to test tubes
- Place all the caps loosely on the test tubes; the agar won't be sterilized if they are sealed tight and sterilize all the tubes for about 15 minutes at 121^{o}c.
- When the agar is still hot, tilt the rack holding the test tubes on a solid surface or a thick book, making sure the medium inside the tubes is at a slanted position.
- Allow the medium to cool and solidify at this angle, which increases the surface area of the agar.
- Inoculate the slant by transferring cells with an inoculating loop from a single-colony microorganism on a plate to the slant's surface. Move the loop across the surface of the slant and cap the tubes.
- Incubate the slant until there is evidence of growth, then put the tube in a refrigerator.

Appendix III: McFarland standard preparation

McFarland standards are suspensions of either barium sulfate 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 a 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 the same size as those used in standardizing the bacterial inoculums.
4. Tightly seal the tubes and store in the dark at room temperature
5. Mix the standard thoroughly on a vortex mixer immediately before use.

Appendix IV: Preparation of phosphate buffered saline

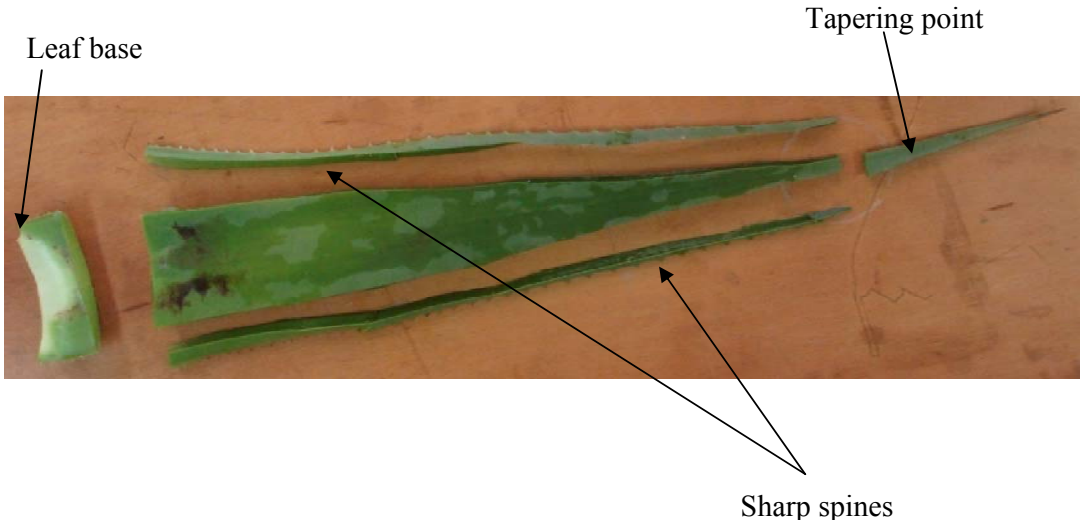
Phosphate buffered saline (abbreviated as PBS) is a buffer solution commonly used in biological research. It is a salty solution containing sodium chloride, sodium phosphate, and (in some formulations) potassium chloride and potassium phosphate. The buffer helps to maintain a constant pH. The osmolarity and ion concentrations of the solution usually match those of the human body (isotonic).

PBS has many uses because it is isotonic and non-toxic to cells. It can be used to dilute substances. It is used to rinse containers containing cells. PBS can be used as a diluent in methods to dry biomolecules, as water molecules within it will be structured around the substance (protein, for example) to be 'dried' and immobilized to a solid surface. The thin film of water that binds to the substance prevents denaturation or other conformational changes.

Preparation: There are many different ways to prepare PBS. Some formulations do not contain potassium, while others contain calcium or magnesium.

A 1 liter stock of PBS can be prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \cdot 2H_2O$ and 0.24 g KH_2PO_4 in 0.8 L of distilled water, and topping up to 1L. The pH is ~6.8, but when diluted it should change to 7.4. When making buffer solutions, it is good practice to always measure the pH directly using a pH meter. If necessary, pH can be adjusted using hydrochloric acid or sodium hydroxide. Dispense the solution into aliquots and sterilize them by autoclaving (20 min, 121°C, liquid cycle). Store at room temperature.

Appendix V: illustration of operations during aloe gel extraction



a) Sectioning of aloe leaf for gel extraction.

b) Aloe gel extraction by hand filleting method