Fabrication And Characterization Of *Lallemantia royleana* Seeds Mucilage (LSM) Based Film For Wound Healing.

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A thesis submitted in partial fulfillment of the requirements for the degree of MS Biomedical Sciences

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List of Abbreviations

LSM: *Lallemantia royleana* seeds mucilage

cm: Centimeter

DMSO: Dimethyle Sulfoxide

$FeCl_3$: Ferric Chloride

g: Grams

H_2SO_4: Sulfuric acid

HCL: Hydrochloric acid

Hrs: Hours

MIC: Minimum Inhibitory Concentration

ml: Milliliter

mm: Millimeter

MPa: Mega Pascal

N: Newton

N/mm: Newton Per Millimeter

NaCl: Sodium Chloride

NaOH: Sodium Hydroxide

NH_3: Ammonia

PBS: Phosphate Buffered Saline

RPM: Rotation Per Minute

SEM: Scanning Electron Microscopy

µg/ml: Microgram Per Milliliter

ABSTRACT

In the present study mucilage forming ability of *Lallemantia royleana* seeds was utilized for biomedical applications. Phytochemical screening was done to know about the chemical constituents of the mucilage. Antibacterial activity of *Lallementia royleana* seeds mucilage (LSM) showed good positive results when tested against. LSM showed good positive results when tested against *Bordetella pertussis* (*B. pertussis*), *Salmonella typhimurium* (*S. typhimurium*), *Shigella dysenteriae* (*S. dysenteriae*) and *Klebsiella Pneumoniae* (*K. pneumonia*). Among all extracts distilled water extract show best results while Hexane extract show poor activity as compared to standard antibiotics. LSM was used to fabricate biodegradable film for wound healing by combining homeostatic effect of sodium alginate, plasticizing property of glycerol and therapeutic capabilities of LSM. Furthermore, various tests were performed for characterizations of the film. Mechanical testing was done to evaluate physical properties. Results showed that LSM concentration greatly affects the properties of the film. Film with 70% LSM has more brittle nature as compared to films with 50% and 60%concentration. The tensile properties decrease with decreasing LSM concentration. While the percentage elongation increases as the concentration was decreased to 50%. Scanning electron microscopy was done for surface characterization. Film with 70% LSM was more porus and brittle as compared to film with 60% and 50% LSM. To test the hydrophilicity and in vitro degradation properties films were immersed in PBS solution and were checked at regular intervals for. It was found that increasing the LSM concentration greatly increases the water absorption ability of the film. It was found that film with the highest concentration of LSM lose weight much quicker as compared to film with lowest LSM.

Key Words: *Lallementia royleana, wound dressing material, phytochemical screening, antibacterial activity and biocompatibility.*

CHAPTER 1: INTRODUCTION

1.1 Background

Natural products are used since old human civilization. Since prehistoric time, plant products have been the successful remedies because of their greater acceptability in societies of human beings, good compatibility with the human body and to cure different diseases because of the synergistic and lower side effects combinations (Rates 2001).

For a thousand years therapeutic plants were used as the primary source in health care all over the world. But the influence of medicinal plants got decreased approximately about one fourth in the 20th century, because researchers preferred to use synthetic products for treating ailments. Now again the trend is going in reverse and the researchers are again getting in favor of medicinal plants because they contain natural ingredients that are chemically safe, and less harmful when compared to the synthetic products (Hamayun et al. 2006).

Lallemantia royleana that is also among one of the medicinal plants is more important for therapeutic purpose. Seeds of *Lallemantia royleana* called as Balangu seeds or tukhm-e-balanga belongs to the Labiatae family has botanical name *Lallemantia royleana* Benth (Naghibi, Mosaddegh, and Motamed 2005). This flowering plant is from kingdom Plantae with Order Lamiales and Family Labiata. Lallemantia is the genus name. The local name of this plant is Tukhm-e-balango. Sindhi population call it as Sindhi Nazboo while in English it is called as Black psyllium seeds. Mostly its seeds and leaves were mentioned in previous literature as therapeutic agents. Seeds of *Lallemantia royleana* are used as a source of fiber, polysaccharides, protein and oil. An important aspect of its physiological fiber behavior is that it produces gel when come in contact with water and high viscosity. polysaccharides. Balangu seeds have ability to absorb water when come in contact with water and produce a turbid, tasteless and sticky liquid on additional dilution (Mishra et al. 2015). *Lallemantia royleana* seeds mucilage has antibacterial potentials against *Escherichia coli, Enterobacter cloaceae, Pseudomonas aeruginosa and Staphylococcus aureus , Bacillus subtilis, and Pseudomonas aeruginosa* (Sharifi-Rad et al. 2015). It also posses good anti-fungal activity. *Lallemantia royleana* extract showed good activity against *Aspergillus niger* and *Candida albicans* with MIC 3.1 at concentration of 2.5 μg/mL,.

Lallemantia royleana showed excellent antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, and Pseudomonas aeruginosa* with MIC 5.6, 4.8 and 3.5 µg/ml respectively. This proves that *lallemantia royleana* has antifungal and antibacterial potential that make it more attractive for pharmaceutical and food industries (Sharifi-Rad et al. 2015).

Figure 1.1: Plant of *Lallemantia royleana*

Figure 1. 2: Seeds of *Lallemantia royleana*

Figure 1. 2: *L. royleana* seeds produce mucilage when immersed in water.

Wound possesses an important place in everyday pathology. Wounding results in demages of the tissues and thus effect the environment that surrounds the tissue (Elnar and Ailey 2009). Wound healing is the reverse process that enables the damaged tissue to repair itself after injury or any trauma. In undamaged and healthy skin a protrective barrier is formed by the epidermis (outer layer of the skin) to keep the skin safe. When the epidermis is broken a series of biochemical reactions starts to align the damage skin. This process of healing is divided in four phases called as haemostasis (blood clotting), inflammation, proliferation (tissue growth) and maturation (tissue remodelling) (Stadelmann, Digenis, and Tobin 1998).

Hydrogel and films used for various biomedical applications are hydrophilic network that has unique property to swell in water maintain structural integratity without being dissolute (Pasqui, De Cagna, and Barbucci 2012) and (Bartolo et al. 2012).

Wound dressing are made up of different types of polymers such as polylactic acid, collagen, chitosan and sodium alginate, etc. An ideal wound dressing material is the one with good biocompatibility and therapeutic properties. It can absord the wound fluid, easly removable and posses good mechanical properties (Abdelrahman and Newton 2011).

Wound management depends on patient's health condition, chemical and physical properties of biomaterials. The wound healing materials should be properly tested and characterized. Classification of wound dressing matrials are based on physical appearance, chemical composition, their primary functions in wound healing, and nature of biomaterial (Boateng et al. 2008)

Now a days there is a huge variety of healing products including solutions, ointments, creams, dressings, etc., but unfortunately there is no ideal dressing that can provide all the requirements such as biocompatibility, maintaining good mechanical strength, biodegradation to non-toxic substances within the time frame, ability to support proliferation of cells and possess therapeutic properties. There is a need to produce an ideal dressing that can satisfy all these conditions or requirements and efficiently deliver therapeutic agents at a controlled rate and is made from easily available, inexpensive materials and accelerate the healing process. Among all these products polymeric materials are more attractive for wound healing because of lesser manufacturing cost and good healing power (Wild et al. 2010) and (Boateng et al. 2008), but there are some type of wounds, for example infected wounds for which the polymeric dressing is insufficient to heal that wound ideally because most of do not have any

antimicrobial effect (e.g. Antiseptic and Antibacterial properties). To solve this problem, various dressing materials were develop that are incorporated with synthetic drugs that inhibit the microbes in wounds (Kim et al. 2008) and (Sripriya, Kumar, and Sehgal 2004). These dressing materials though provide good clinical results, causes the development of resistant strains by continuous admistration (Abdelrahman and Newton 2011).

LSM has antibacterial, anti fungal and anti inflammatory properties. In the present study, we implement the hypothesis that LSM incorporated in Sodium alginate and glycerol solution has the ability to diffuse in wound and because of its therapeutic properties that promotes the healing and can be used as an alternative to synthetic drugs for infected wound healing. To make it an ideal dressing material the chemical constituents and therapeutic properties of the mucilage is investigated first. In the present study the relevant Physical properties (thickness, Scanning electron microscopy, mechanical properties, swelling behavior and in vitro degradation) of the *Lallementia royleana* seed mucilage (LSM) based films are also investigated.

1.2 Objectives Of The Study

- Extraction of mucilage from seeds of *Lallemantia royleana* plant
- Phytochemical screening of *Lallemantia royleana* seed mucilage (LSM)
- Antibacterial activity of LSM
- Fabrication of LSM based film.
- Characterization of film for wound healing applications
    1. Mechanical properties
    2. Scanning electron microscopy of the film
    3. Thickness
    4. Swelling index
    5. In vitro degradation analysis

CHAPTER 2: LITERATURE REVIEW

2.1 Wounds

The wound is a break in the natural integrity of the skin and other body tissues. A wound is caused by the disruption of anatomic structure of normal skin. The wound may be caused by external factors, for example Physical trauma, mechanical injuries and surgical cuts. It may be caused by chemical damages, for example irritants and allergens that breaks the natural integrity of the skin or by medical conditions, for example, microbial infections, bedsores and diabetes, etc. Wound can be classified in many ways. Figure 2.1 shows the major classification of wounds (Boateng et al. 2008).

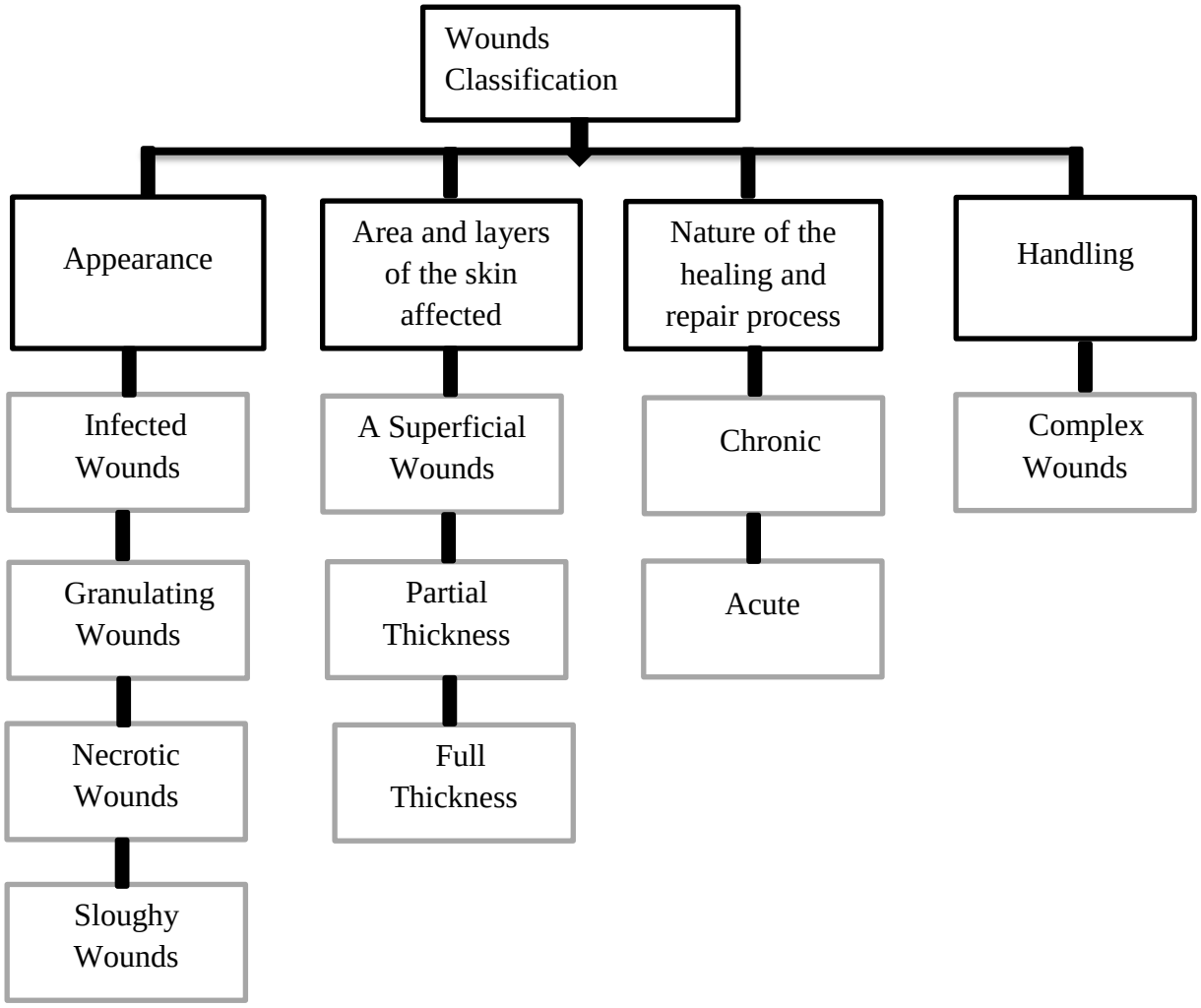


Figure 2.1: Classification Of wounds

2.2 Wound Healing

The reverse process of wounding is healing that involves the alignment of the disrupted skin or any tissue of the body. The Process of healing is classified into four main stages. First stage is hemostasis then inflammation and proliferation and last is maturation and remodeling. In hemostasis, coagulation of blood occurs due the formation of fibrin clot and infiltration of white blood cells or leukocytes also occurs (Boateng et al. 2008). In the second phase, bacteria and debris are removed by the process of phagocytosis. Macrophages release chemotactic agents that cause the migration of fibroblasts towards the wound area (Li, Chen, and Kirsner 2007). The proliferative phase immediately occurs after migration (Boateng et al. 2008). Proliferative activity of many elements such as fibroblasts, endothelial cells, epithelial cells and angiogenesis which is formation of new blood vessels also occurs during proliferation phase (Azizi and Osgouie 2010). In the remodeling or maturation phase, granulation tissue replaces the fibrin clot rich in collagen type III. Epithelium strengthens and wound contraction occurs. Wound size is reduced by the activity of myofibroblasts (Li, Chen, and Kirsner 2007).

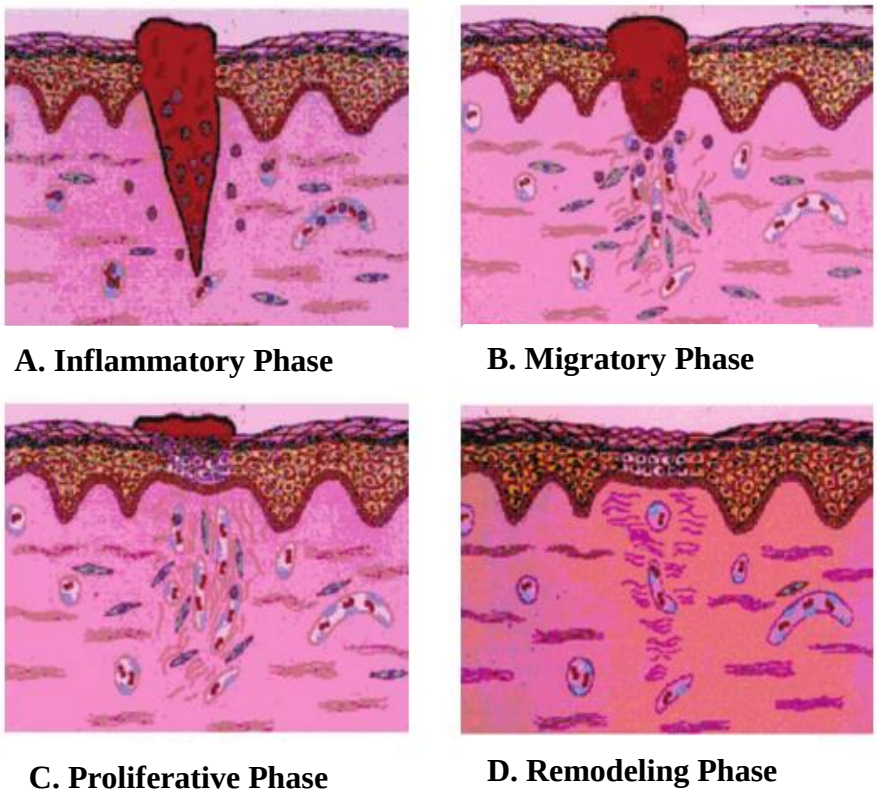


Figure 2.2: Wound healing Phases (Boateng et al. 2008).

2.3 Wound Dressing Materials

Dressings can be in the form of bandages, gauze, cotton, hydrocolloids, alginates, hydrogels, or films, etc. The main function of a dressing material is to keep the wound area dry and prevent in from contamination (Boateng et al. 2008). Management of wound depends on many factors such as type of wound, patient's physical condition, healing process of the wound, and physical and chemical characteristics of dressings. Dressings can be made from different types of polymers such as polyvinyl alcohol, polylactic acid, chitosan, sodium alginate, collagen, etc. Biological as well as synthetic polymers can be used to form different shapes suitable for wound healing such as hydrocolloids, alginates, hydrogels, and thin films. Hydrogels are of synthetic and natural origin. But natural one are more attractive for healing of various types of wounds. Natural hydrogels possess special properties such as malleability, elasticity, smoothness, and provide a moist environment that protects the damage tissue from desiccating (Huang and Fu 2010), (Bartolo et al. 2012) and (Weller and Sussman 2006). Films that are biodegradable and made up of natural products are widely used for various biomedical applications for example; wound healing, drug delivery and tissue engineering (Guo et al. 2011) and (Thu, Zulfakar, and Ng 2012). Such products of natural origins are now extensively in use because they are biodegradable, biocompatible and possess properties that are very similar to that of human skin and tissues (Sionkowska 2011)(Huang and Fu 2010).

2.4 Thin Films for Wound Healing

Thin films are used to treat superficial, lightly exudating or epithelializing wounds. They possess various properties for example, It is impermeable to bacteria, permeable to water vapour and air, and is easily removed. Polymers of both biological and synthetic origins are good candidates for wound dressings. Synthetic polymers have low protein adsorption and cell adhesion property and leeches into the physiological system and releasing harmful substances. On the other hand, biodegradable polymers are biocompatible and degradable (Shoichet 2010). The films are used both in dry state and wet state. Dry films are use for the treatment of infected skin surface because it absorb the wound fluid. They are mostly use for bleeding because of haemostatic property. Ion exchange between the film and wound fluids results in the formation of hydrophilic gel that keeps the environment moist. Wet films are use as hydrogels they are used for the treatment of dry wounds. These films are first immersed in appropriate solution, so that it absorb the fluid and becomes hydrated. Then it is

applied to the dry wound where it releases the absorbed water and thus creats a moist environment (Abdelrahman and Newton 2011).

Now a days alginate based films are used for healing of different types of wounds. Alginate provides best homeostatic properties. It forms hydrogel and get dissolved when comes in contact with wound exudate thus creating moist environment and keeps the wound safe and facilitating the healing process. Alginate gels are modified by incorporating various antibiotics that promotes the healing (Kim et al. 2008).

In recent years, wound dressing products have grown from simple gauze to smart dressings with considerable technical and commercial impact. Today, wound dressings are manufactured from a large variety of different materials and applied in all phases of wound healing. Commercially available wound dressing products were investigated and compared in vitro. The dressings are tested and assessed based on physical and chemical properties, depending on the wound type and wound healing stages. Dressings can be classified based on the nature of material used, their function in the wound, and their physical appearances (Boateng et al. 2008).

Now a days there is a huge variety of healing products including solutions, ointments, creams, dressings etc. Among all these products polymeric materials are consider more attractive for wound healing because of lesser manufacturing cost and good healing power (Wild et al. 2010) (Boateng et al. 2008). But there are some type of wounds, for example infected wounds for which the polymeric dressing is insufficient to heal that wound ideally because most of do not have any antimicrobial effect (e.g. Antiseptic and Antibacterial properties). To solve this problem, various dressing materials were develop that are incorporated with synthetic drugs that inhibit the microbes in wounds (Kim et al. 2008) and (Sripriya, Kumar, and Sehgal 2004). These dressing materials though provide good clinical results, causes the development of resistant strains by continuous admistration (Abdelrahman and Newton 2011). To solve these problems a biodegradable film is fabricated from natural source and is investigated for using it as a dressing material. This area covers many topics which are required in the production of the biologically active thin polymeric films for wound healing.

2.5 *Lallemantia royleana*

Lallemantia royleana that is also among one of the medicinal plant is more important for therapeutic purpose. Seeds of *Lallemantia royleana* called as Balangu seeds or tukhm e balanga belongs to the Labiatae family has botanical name Lallemantia royleana Benth (Naghibi, Mosaddegh, and Motamed 2005). This flowering plant is from kingdom Plantae with Order Lamiales and Family Labiata.. Lallemantia is the genus name. The local name of this plant is Tukhm- e- balango Sindhi population call it as Sindhi Nazboo while in English it is called as Black psyllium seeds. Mostly its seeds and leaves were mentioned in previous literature as therapeutic agents.

2.5.1 Morphological Features of *Lallemantia royleana*

Morphologically Lallemantia is an un-branched, or mostly branched from the very base. Their Stem is erect, 5 to 30 cm in length, leaves are simple and about, 15-20 x 7-15 mm in size that are veined. The Inflorescence starts from the base of the stem. It has pink calyx about 6 to 7 mm in size and is tubular, pale lilac corolla and is blue to whitish pink and about 7 to 8 mm, corolla is longer than calyx. The seeds are about 2.5 x 1 mm in size they are dark brown to black, and are triquetrous, having small scar attachment. When the seeds are immersed in water or any other liquid, they from clear mucilage.

Electron microscopic studies have shown the microstructure of Lallemantia. The pollen of Lallementia has some features that resemble with the subfamily ‘Nepetoideae’ pollen. The exine of Lallemantia is reticulate-faveolated in the *L. canescens* and microreticulate in *L.iberica* and *L. peltata*. In the same way the seeds of Lallemantia are black with triangular oblong shape, having V – Shaped of areolas. Warts of lallementia are in separated and regular pattern in L. peltata, become separated and irregular in the L. iberica, specie and separated and irregular or in some cases arranged in groups of 2 to 4 in the specie L. canescens. There are two types of trichome, acicular, and capitate that is present on the stems, calyx, and leaves and also on bracts. The pollen and the seeds of Lallemantia are capable to provide phylogenetic indications basically on species level (Din?? et al. 2009)

2.5.2 Distribution Throughout The World

Lallemantia has further five species that are distributed throughout the world (Abbas et al. 2012).

1. ***Lallemantia baldshuanica*** are grown in Iran, Afghanistan, Turkmenistan, Tajikistan, Kyrgyzstan
2. ***Lallemantia canescens*** mostly cultivated in Turkey, Caucasus, Iran,
3. ***Lallemantia iberica*** is cultivated in Iran,Turkey, Turkmenistan, Iraq, Caucasus, Syria, Palestine , Lebanon,
4. ***Lallemantia peltata*** found in Iran, Turkey, Caucasus , Turkmenistan
5. ***Lallemantia royleana*** (Benth.) is grown in Central Asia ,Western Siberia, Pakistan,, Xinjiang, Kashmir, Iran, Palestine, Syria, Persian Gulf sheikdoms and Saudi Arabia.

2.5.3 Distribution In Pakistan

Balangu, is a mucilagenous common plant and is usually cultivated in different areas of Pakistan forexample Chishtian, Bahawalpur, Bhakkar, Hasilpur, and Layyah, etc. General performance of Balungoo seeds planted in the regions of Layyah was is reported good and much acceptable because of the use of fertilizer like DAP and also due to the timely rains in that region (Abbas et al. 2012).

2.5.4 Composition Of Lallementia *royleana*

The seeds of *lallemantia royleana* composed mainly of β-cubebene (2.1%)., carvacrol (1.6%), myrtenal (1.5%), terpinolene (1.1%), pinocarvone (20.0%), (*E*)-β-ocimene (4.1%), *trans*-pinocarvyl acetate (26.0%), *trans*-pinocarveol (1.6%), linalool (3.4%), verbenone (7.1%), dihydrocarvyl acetate (2.5%), 3-thujene-2-one (5.1%), β-pinene (1.5%), *trans*-carveol (5.3%), pulegone (4.4%) and *cis*-carveol (3.5%) (Sharifi-Rad et al. 2015).

2.5.5 Medicinal Importance

Microbes have the potentials to spread diseases and get resistance to the antibiotics. And thus cause a serious health problem worldwide. With the passage of time new drugs are developed, but on the other hand resistance to these drugs is also increasing. Moreover, people are getting suffered because their immunity suppressed and the development of new

resistant strains thus action should be taken to get knowledge about the mechanism of microbial resistance and also needs to develop new drugs. Plant derived compounds have potentials to inhibit the spread of bacteria and thus are valuable for the treatment of infections caused by the resistant microbes**.** Most of the antimicrobial drugs are of natural / plant origin.

Lallemantia royleana seeds possess good antifungal activity. *L.royleana* extract showed good activity against *Aspergillus niger* and *Candida albicans* with MIC 3.1 at concentration of 2.5 µg/mL,. *Lallemantia royleana* seeds showed excellent antibacterial activity against *Staphylococcus aureus, Bacillus subtilis,* and *Pseudomonas aeruginosa* with MIC 5.6, 4.8 and 3.5 µg/ml respectively. This proves that lallementia royleana has antifungal and antibacterial potential that make it more attractive for pharmaceutical and food industries.(Sharifi-Rad et al. 2015). Seeds extracts has greater capabilities to treat pneumonia and respiratory tract diseases (Lev and Amar 2002). The gel mixture of mucilage of balangu seeds and lidocaine can cause improve anaesthesia. The mucilage has good potentials of dermal penetration and hence improves the anesthetic effects of lidocaine (Atabaki and Hassanpour 2014).

Balangu seed gum is used in various food products as suspending agents. It is also used as an appropriate stabilizer in the formulation of chocolates and icecream etc (Bahramparvar, Haddad Khodaparast, and Razavi 2009). The seeds are used in for their cooling effect and antithirst properties (Amiri, Jabbarzadeh, and Akhondi 2012).

The seeds of lallementia royleana produce significant amount of mucilage that can be extracted and used in various pharmaceutical and food industries as natural disintigrants and food hydrocolloid (Abdulrasool, Naseer, and Rahi 2011) and (Bahramparvar, Haddad Khodaparast, and Razavi 2009).

Lallemantia royleana seeds gums are used as a natural excipient for drug coating. They are used as disintegrate because of its potential to absorb water so can cause tablet to break down easily. The mucilage also helps in the dissolution of the drug. The gum that coats the drug particles absorb water and thus improve drug solubility. In comparison with other disintegrants such as silica gel lallemantia seeds gums are proved to be most efficient disintegrants. Another important priority over aerosol is that most of them have carcinogenic effects and are expensive. LSM has high density and thus easily granulate tablets as compared to other low density products.(C-6 Kazim.pmd - C-6 Kazim.pdf n.d.). The

mucilage of lallementia seeds other important properties for example it can be used as a laxative, and healing etc. The mucilage of Balangu seed is stable and do not interact excipients. The mucilage of balangu seeds is excellent disintegrants in comparison with carmellose sodium. It integrates much faster without interact with the drug. They are used in formulation of various drugs because of its superdisintegrant (Malik et al. 2011).

L.royleana seeds gum is used as a natural remedy for improved local anesthetic effect of drugs. The anesthetic potency of lidocaine greatly increased by the addition of Balangu seed gum. Seeds gum shows almost same analgesic effect as that of lidocaine gel. But the anesthetic potency is increased when both the mucilage and lidocaine are mixed. The increased effect may be due to the dermal penetration of mucilage. The duration of anesthesia also increases that may be due to the sustained release of drug that is loaded in mucilage (Atabaki and Hassanpour 2014).

CHAPTER 3: MATERIALS AND METHODOLOGY

3.1 Sample collection And Preparation

Seeds of *Lallemantia royleana* were purchased from G10 Islamabad. After Identification by Plant Taxonomist Dr. Muhammad Qasim Hayat it was deposited to the Plant Biotechnology Laboratory Medicinal, Atta-ur-Rehman School of Applied Biosciences, NUST Islamabad, Pakistan. Pure cultures of pathogenic bacteria, i.e.; *Bordetella pertussis* (IARS-13), *Salmonella typhimurium* (IARS-12), *Shigella dysenteriae* (IARS-9), *Klebsiella pneumoniae* (IARS-11) were obtained from plant biotechnology Lab, ASAB- NUST.

3.1.1 Extraction Of *Lallemetia royleana* Seed Mucilage (LSM)

30g of seeds were washed three times in Ethanol to remove the dust, followed by filtration of ethanol from seeds. After filtration seeds were dried at 70 °C by placing it in the oven. The dried seeds were then soaked in 1 liter distilled water at 35 °C for 18 hours. After hydration the solution was boiled for two hours, followed by stirring with a rod paddle blender at 2000 RPM for 6 hours to scrap of the gum from seed surface. The mucilage solution formed was separated by centrifugation (800 RPM for 30 minutes). The solution was then passed from sieve to remove the seeds from mucilage and then from cheese cloth to obtain pure mucilage. In order to get pure mucilage, acetone was added to the solution. While adding acetone the solution was stirred continuously. The mucilage precipitated rapidly the precipitated mucilage was treated again with acetone and then passed from muslin cloth to get final pure mucilage. After formation of a homogenous mixture the P.H was adjusted to 9. The extracted LSM was dried in oven, weighted and stored in the fridge for further experiment (Mishra et al. 2015).

3.1.2 Sample Extraction

Three solvents were used for extraction, Methanol (polar), N-Hexane (Nonpolar) and distilled water for phytochemical screening and antibacterial analysis. 200ml solvent was poured in three separate flask and in each flask 4g of powder was added. The flasks were covered with aluminium foil and placed in the dark for three days. After three days the flasks were kept in shaker (Temperature 37°C, rpm 210/minute and Time 24 hours). After shaking all solutions were filtered using filter paper (what Mann #1). Filtrates were collected in clean beakers. All

three solutions were kept at rotary one after another for the evaporation of solvents. The temperature was set according to the boiling point of each solvent (Hexan 60°c, methanol 40°c and distilled water 100°c) at 210 RPM. The samples were kept in rotary until a thick and dense solution left. The solutions were then collected in sterilized Petri plates and placed in hot air oven till it completely dried. After complete drying, the powder was collected in clean Falcon tubes and stored.

3.2 Phytochemical Screening

3.2.1 Phenols

Three drops of ferric chloride solution were added to 10 ml of extract. Appearance of green colour indicated the presence of phenols (Arutselvi, Balasaravanan, Ponmurugan, Saranji, &Suresh, 2012).

3.2.2 Saponins

The ability of saponins to produce froth in aqueous solution was used as a screening test for saponins. 0.5 g of each plant extract was shaken with 20ml distilled water in a test tube, frothing which persisted on warming was taken as evidence for the presence of saponins (Egwaikhide and Gimba 2007).

3.2.3 Cardiac glycosides

10 ml extract was added in 4ml of glacial acetic acid. Add one drop of 5% $FeCl_3$, carefully added one drop of H_2SO_4. Brown ring formed at the junction of two liquids indicated deoxysugar characteristics of cardenolides (Kaur and Arora 2009).

3.2.4 Phytosteroles

5ml extract added with 2ml chloroform and 2ml acetic anhydride addition, followed by filtration, three drops of concentrated H_2SO_4 was added. Shake well and allowed to stand for 1minute. Greenish yellow color indicated a positive result (Anasane and Chaturvedi 2014).

3.2.5 Reducing Sugars

1ml extract added to 1ml distilled water, followed by the addition of 5 drops of Fehling's solution and heated on a water bath. Precipitate formation indicated a positive result (Ayoola et al., 2008)**.**

3.2.6 Volatile Oil

0.1ml dilutes NaOH was added to 2ml of extract followed by addition of 1ml dilute HCL. White precipitate formation indicated the presence of volatile oil (Jennifer Adline & Anchana Devi, 2014).

3.2.7 Diterpenes

5ml extract was added to 10ml distilled water, followed by the addition of a few drops of copper acetate. Emerald green precipitate indicated the presence of Diterpenes (Chethana G.S et al., 2013).

3.2.8 Flavonoids

Dilute NH3 solution was added to 5ml filtrate of extract. Followed by addition of 3 drops of concentrated H_2SO_4. Yellow coloration indicated positive result for flavonoids (Arutselvi et al. 2012).

3.2.9 Tannins

4ml extract was added to 2 ml distilled water, followed by the addition few drops of $FeCl_3$. Dark green color indicated positive result (Egwaikhide and Gimba 2007).

3.2.10 Alkaloids

4ml of the extract was measured in a test tube to which picric acid solution was added. An orange coloration indicated the presence of alkaloids (Egwaikhide and Gimba 2007).

3.3 Anti-bacterial Activity

Initially LSM was tested in vitro against some pathogenic bacteria.

3.3.1 Bacterial Strains

Pure cultures of pathogenic bacteria, i.e.; *Bordetella pertussis* (*B. Pertussis* IARS-13), *Salmonella typhimurium* (*S. typhi* IARS-12), *Shigella dysenteriae* (*S. dysenteriae* IARS-9), *Klebsiella pneumoniae* (*K. pneumonia* IARS-11) were obtained from plant biotechnology Lab, ASAB- NUST.

3.3.2 Stock Solution And Dilutions Preparation

0.5 g extract of each sample per 10ml DMSO was prepared as a stock solution. Dilutions were done in the range of 5, 10, 15, 20, and 25. Total volume for each dilution was kept 1ml (Jennifer Adline & Anchana Devi 2014).

3.3.3 Nutrient Agar

Nutrient agar was prepared for the growth of required pathogenic bacteria for testing the antibacterial activity of plant extract. For preparation of medium 1.5g yeast, 3g of trypton, 3g of Nacl and 4.5g of nutrient agar was mixed in 300ml distilled water. The P.H was adjusted to 6.8. The mouth of the flask was covered with cotton plug and aluminum foil. Slowly shake the mixture so that the agar mixed well with water, after this autoclaving was done at 121 °c for 15 minutes. The flasks were transferred to laminar flow hood. The media was allowed to cool, after cooling, it was transferred into pre autoclaved petri plates. The plates were wrapped in food wrapper and stored at 4°c upside down for further use.

3.3.4 Discs Preparation

Discs of 6mm were in sequence and 15 µl of the extract was carefully placed on each disc from five different concentrations 5mg/ml, 10mg/ml, 15 mg/ml, 20 mg/ml, and 25 mg/ml. Erythromycin and Chloramphenicol 0.5mg/ml were used as positive control while distilled water was used as negative control.

3.3.5 Swabbing Of Bacteria

Sterilized cotton swabs were used for swabbing. A single colony was picked up from the 24 hours old pure bacterial culture and placed on pre labeled agar plate. The swab was moved in left, right direction until half of the plate was covered than the plate was rotated at 45 angles and again swabbing was done in the same way when half of the plate was covered again the plate was rotated at the angle of 45. Third time the same procedure was followed to cover half of the plate. For fourth time full plate was covered by swabbing the bacterial colony (Egwaikhide and Gimba 2007).

3.3.6 Disk Diffusion Assay

Pre prepared discs having plant extracts at different concentration were carefully placed on swabbed plates. All the plates were incubated at 37°c for 24 hours. After 24 hours zone of inhibitions were measured. 5mg/ml was kept as minimum inhibitory concentration. Whole experiment was carried out in triplicate (Sharifi-Rad et al. 2015).

3.4 Fabrication Of LSM Film

The alginate based LSM film was prepared by modifying method of Pavlath, Gossett, Camirand, and Robertson (1999). Three solutions were prepared for the fabrication of the films with different LSM concentrations. Initially 3g of LSM powder was mixed in 100ml distilled water. The solution was mixed well until a clear, uniform LSM solution formed. Sodium alginate (1 g) was dissolved in 100 ml of distilled water and rotary shaking was done concurrently. For flexibility 20% glycerol on the basis of the dry weight of alginate was added to sodium alginate solution. Glycerol alginate solution and LSM solution were mixed at the ratio of 30:70, 40:60 and 50:50 respectively. The solutions were mechanically stirred for 15 minutes at 1000 RPM. Centrifugation was done to remove the air bubbles. 20 ml of the suspension was poured on Teflon plates (64 $cm^{2)}$, placed on a uniform surface. After casting the plates were carefully placed at room temperature for complete drying. The unpeeled films were dipped in 45 ml of calcium chloride solution containing 1% Calcium ion and re-dried again in the oven for 4–6 hours. The dry films obtained were peeled off the Casting surface and stored in desiccator for evaluation (Pavlath et al. 1999).

3.5 Characterization Of Film

To evaluate the properties of biodegradable film, few tests were done. Physio-chemical characterization tests that were done include swelling index test to check the water absorbing capacity of the film. Mechanical properties were evaluated by a mechanical test using the mechanical testing machine. Scanning electron microscopy (SEM) was used to analyze the surface topography of the film. The film thickness of all the three films was tested to study the effect of LSM on it.

3.5.1 Mechanical Testing

LSM films were equilibrated at constant weight at 25°C temperature and 0% relative humidity before being tested. Mechanical properties were determined by the Universal Testing machine. The samples were cut in rectangular shaped strips (30mmX10mm). Each film was tested one by one. Each film was clamped between the two grips and force (N) / deformation (mm) was recorded. The initial distance between the grips was kept 20mm at a rate of 1millimimeter per second. The testing was done in triplicate for precise calculation. The following data were calculated using stress, strain curve, recorded by tensile testing machine using the ASTM manuals D882-91 (1996). **1)**. Maximum breaking force (N), **2)** Breaking factor (breaking force / film thickness, N/mm), **3)** Deformation at break (strain at rupture point, mm), **4)** Percentage elongation on break point (deformation / initial length of the sample X by 100, %), **5)** Tensile strength (maximum force / film cross-section, MPa) (Khazaei et al. 2014).

3.5.2 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) imaging technique is mostly used to investigate the surface microstructure of materials. It uses highly focused electron beams to clearly capture the surface topography and depth of the material with greater resolution (Merrett et al. 2002). Maximum magnification can be up to 100,000 X and even particles of 10Å can also be able to be viewed. Electrons beams are generated when signal pass through followed by production 2D image and thus information can be read out from those images. SEM can also be used to investigate the surface morphology of thin films. Micrographs of each film was captured by SEM. Accelerating voltage was kept 20kV. Each film was stuck on a stub made

of aluminum with adhesive tape, and the films were covered with gold. From the micrograph typographic visualization of all film surfaces was done. (Jang, Lim, and Song 2011)

3.5.3.1 Preparation Of Phosphate Buffered Saline (PBS An Artificial Wound Fluid)

PBS was prepared for testing the thickness, swelling index and degradation profile of the LSM film. In 500ml distilled water, add 5g Nacl, 0.125g KCl, 0.125g KH_2PO_4 and 0.71875g Na_2HPO_4 mix all ingredients in distilled water through a magnetic stir until a uniform solution formed. Poured in clean falcon tubes and autoclaved at 121 C for 15 minutes (Dulbecco, Vogt, and February 1954)

3.5.3.2 Film Thickness

The thickness was measured by using a digital micrometre (Model 102-301, Mitutoyo). The dry films were first cut in 2cm X 3cm strips and kept at room temperature and dirt free for an accurate measurement. The micrometre was calibrated to zero and the thickness was measured at three different points on the film (Khazaei et al. 2014). To examine the thickness of wet films, all were cut in strip of 2cm X 3cm and were dipped into 15ml of PBS solution for one week. PH was maintained. After specific interval of one week, all films were collected from the PBS solution, Films were dried with filter paper and the excess of water was removed and the thickness was then measured (Pereira et al. 2013).

3.5.4 Swelling Behaviour

To evaluate the water absorption capability of each film weighted samples of 2cm X 3 cm were dipped in 15 ml of PBS solution at 37ºC for a predetermined time interval, after specific interval of one hour each film was removed from the solution. An excess of water was removed from the surface and then weighted. The swelling was measured gravimetrically using the following equation:

$$\text{Swelling index (\%)} = \frac{hw - dw}{dw} \times 100$$

where *hw* is the weight of the sample after hydration, and *dw* is the weight of dry film before hydration(Pereira, Mendes, and Bártolo 2013).

3.5.5 Degradation Profile Of LSM Film

The alginate based LSM film was dipped in 10ml PBS solution and their weight was checked at regular interval of one weak to check the swelling and degradation that has been occurred (Ming et al. 2004). To assess the degradation profile all samples were cut in 3X2 inches strips and hold in mesh, 10ml of PBS solution was poured on the filmafter regular intervals of one week, the change in weight was calculated by digital microbalance (Ming et al. 2004). After a week each film was dried, weight and again fresh PBS solution was poured. The degradation tests were carried in triplet and the average value was determined. The test was done by checking the weight loss (WL), using the following formula:

$$\text{Weight Loss} = \frac{\text{Initial weight} - \text{final weight}}{\text{Initial weight}} \times 100$$

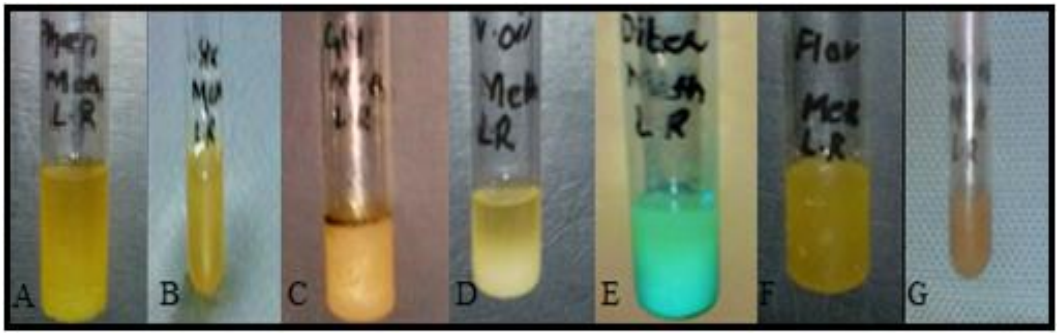
Where Initial weight is the weight of the samples dry film before degradation and final weight is the weight of the samples after hydration and degradation, respectively.

CHAPTER 4: RESULTS

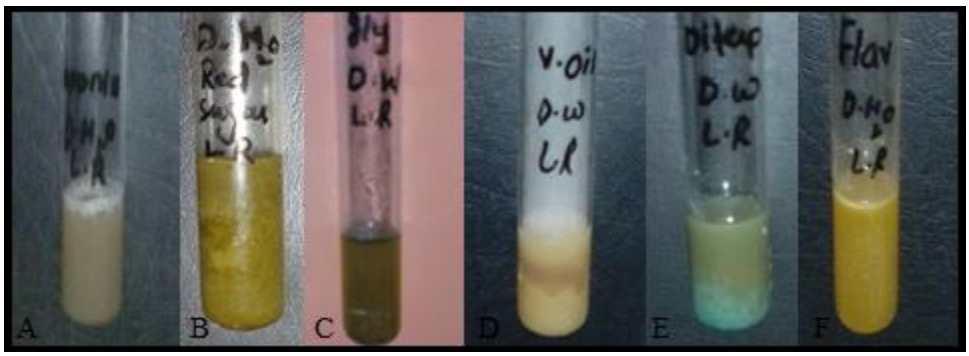
4.1 Phytochemical Screening

S.No	Phytochemical Tests	Methanol Extract	n-Hexane Extract	Distilled Water extract
1	Phenols	+	-	-
2	Saponins	-	-	+
3	Phytosteroles	+	-	-
4	Reducing Sugar	-	-	+
5	Cardiac Glycosides	+	-	+
6	Volatile oils	+	-	+
7	Diterpenes	+	-	+
8	Flavonoids	+	-	+
9	Tannins	-	+	-
10	Alkaloids	+	-	-

Table 4. 1: Qualitative Phytochemical Screening of LSM Extract



A: From left to right: pharmacological screening of Methanol extracts LSM showed positive results, for the presence of Phenole, Phytosterol, Cardiac glycosides, Volatile oil, Diterpenes, Flavonoids, and Alkaloids.

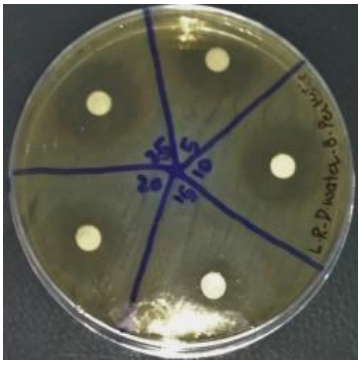
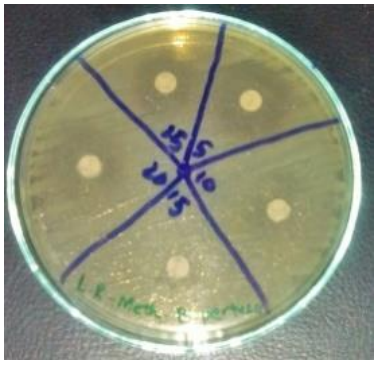


B: From left to right: phytochemical screening of Aqueous extract of LSM showed positive results, for the presence of Saponins, Reducing sugars, Cardiac glycosides, Volatile oil, Diterpenes, and Flavonoids.

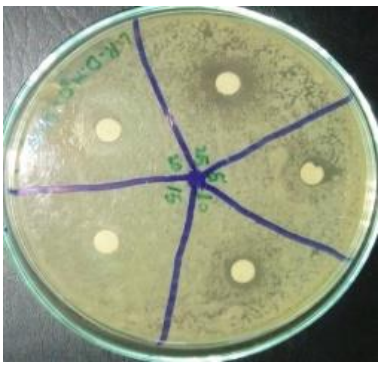
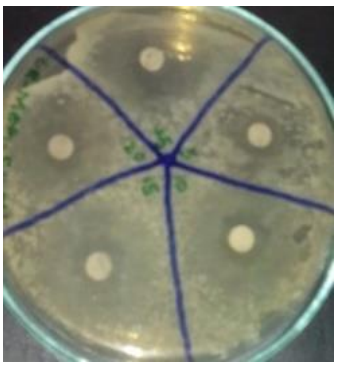
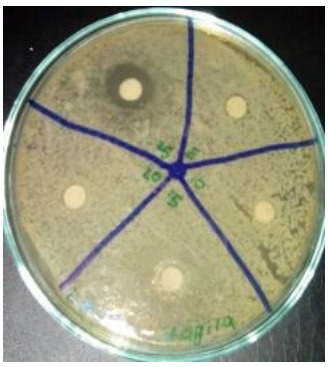
C: phytochemical screening of n-Hexane extract of LSM showed positive results, for the presence of Tannins.

Figure 4.1: Phytochemical Screening of LSM extracts

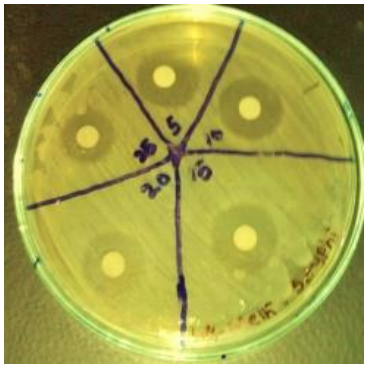
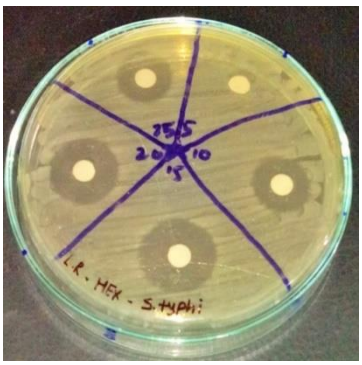
4.2 Antibacterial Activity

A  B  C 

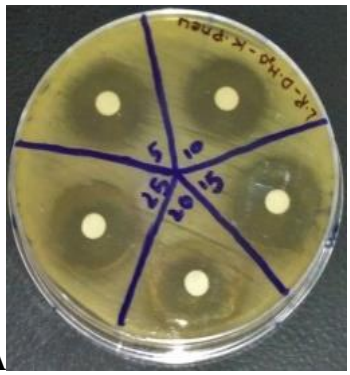
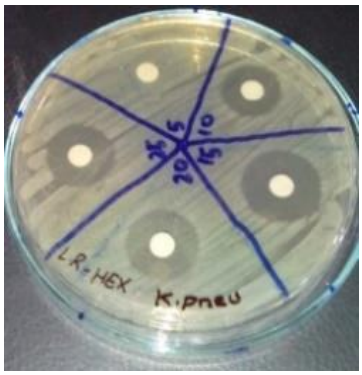
A: Antibacterial activity of A. Aqueous extract, B. Methanolic extract, C. N-Hexan extract of LSM at 5 different concentrations, i.e. 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml and 25mg/ml against *B. pertussis*. Liquid cultures were used to create a bacterial lawn. Discs were inoculated with 5 different concentrations.After overnight incubation at 37°c *B. pertussis* showed maximum sensitivity against aqueous extract and no inhibition against Hexane extract.

A  B  C 

B: Antibacterial activity of A. Aqueous extract, B Methanolic extract, C N-Hexan extract of LSM at 5 different concentrations, i.e. 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml and 25mg/ml against *S. dysenteriae*. Liquid cultures were used to create a bacterial lawn. Discs were inoculated with 5 different concentrations. After overnight incubation at 37°c *S. dysenteriae* showed maximum inhibition against methanol extract and was less sensitive to Hexane extract.

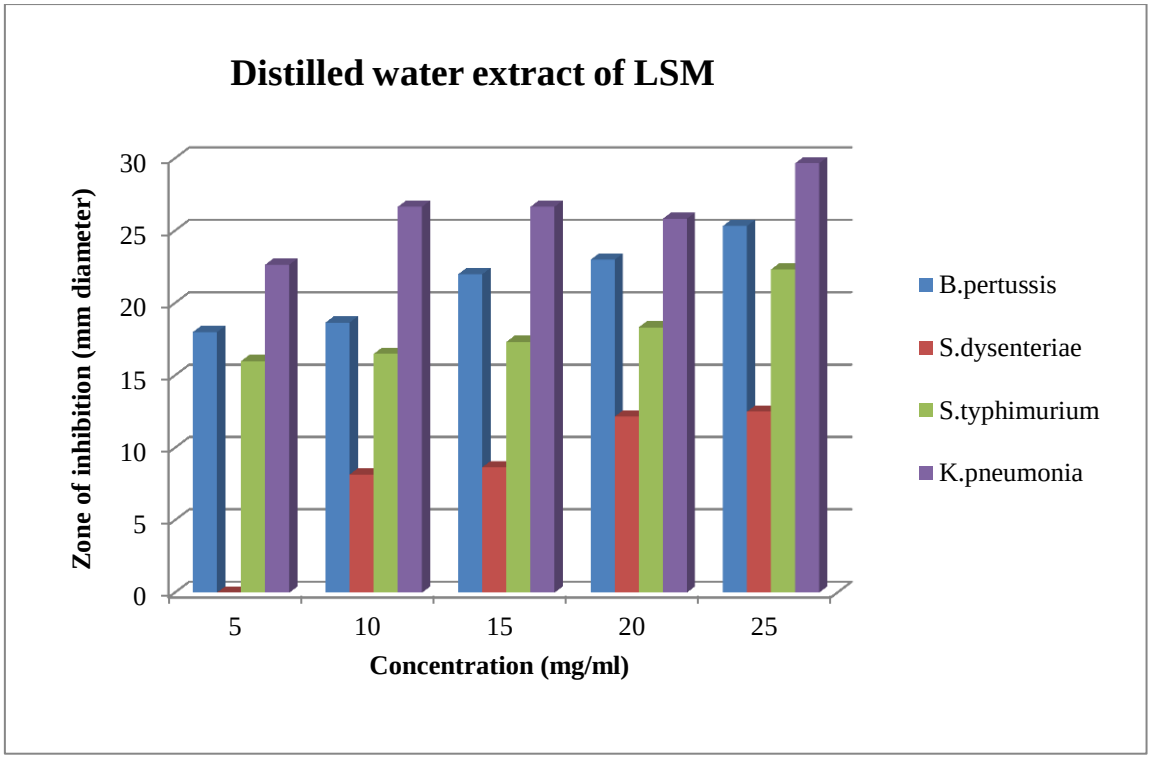
A  B  C 

C: Antibacterial activity of A. Aqueous extract, B Methanolic extract, C N-Hexan extract of LSM at 5 different concentrations, i.e. 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml and 25mg/ml against *S. typhimurium.* Liquid cultures were used to create a bacterial lawn. Discs were inoculated with 5 different concentrations. After overnight incubation at 37°c *S. typhimurium* showed maximum sensitivity to distilled water extract.

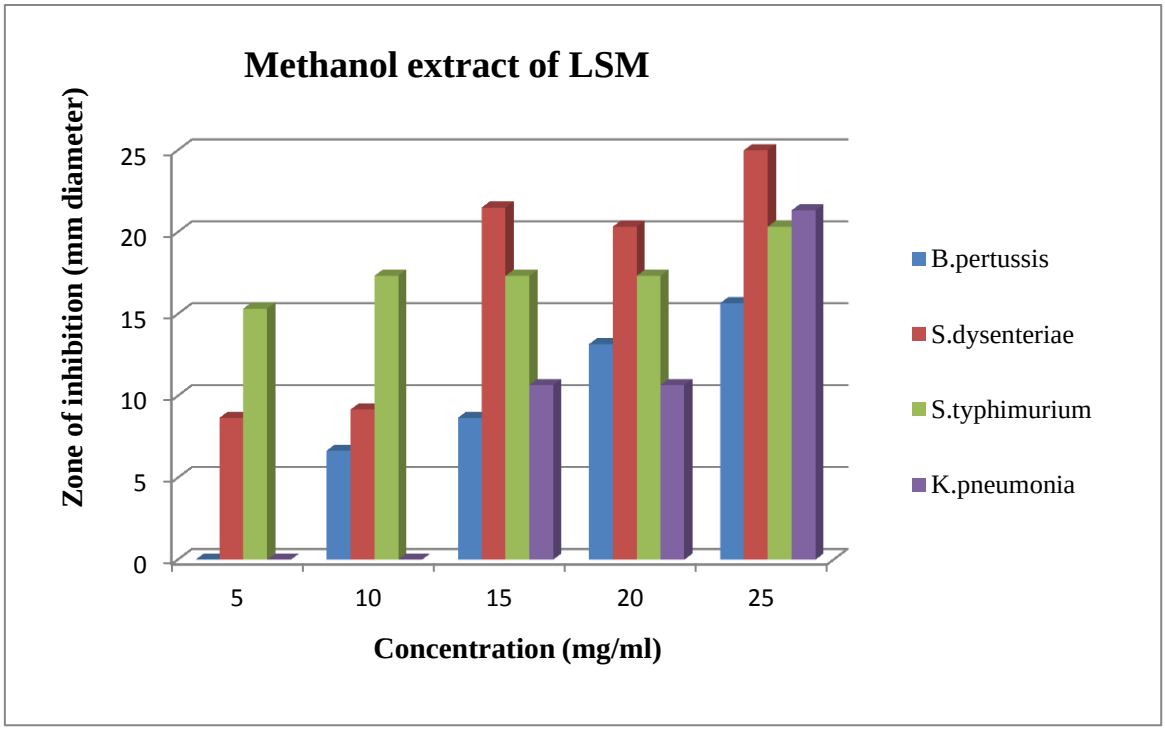
A  B  C 

D: Antibacterial activity of A. Aqueous extract, B Methanolic extract, C N-Hexan extract of LSM at 5 different concentrations, i.e. 5mg/ml, 10mg/ml, 15mg/ml, 20mg/ml and 25mg/ml against *K. pneumonia*. Liquid cultures were used to create a bacterial lawn. Discs were inoculated with 5 different concentrations. After overnight incubation at 37°c *K. Pneumonia* showed maximum Sensitivity to aqueous extract while showing minimum inhibition against methanol extract.

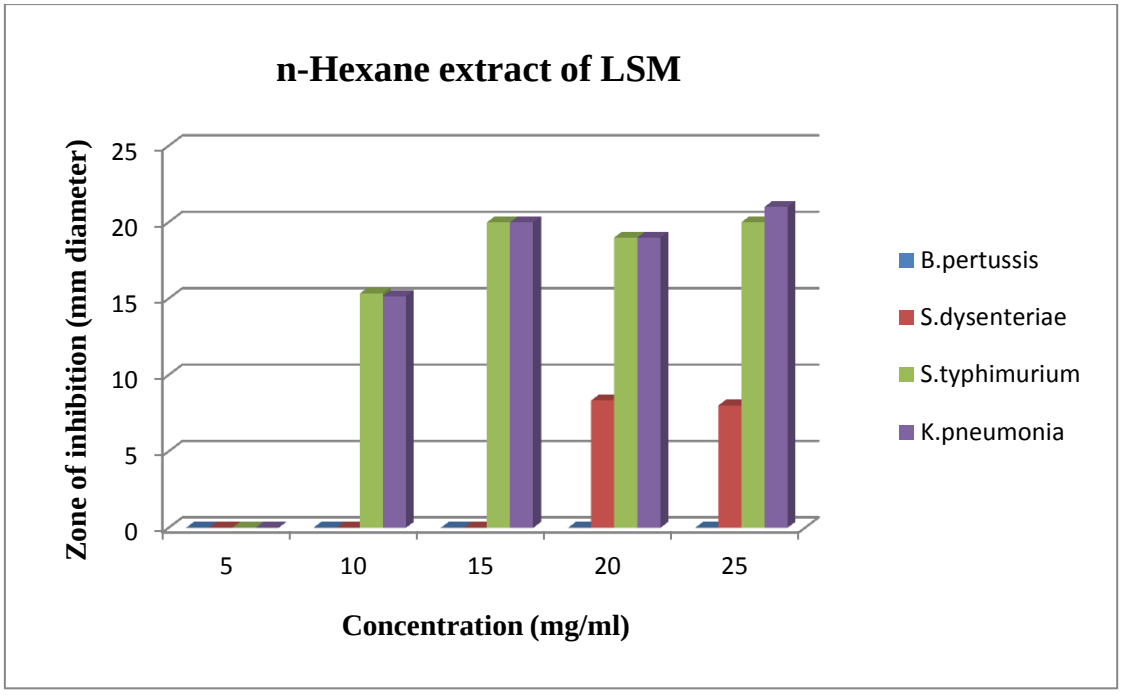
Figure 4. 2: Antibacterial Activity of LSM extracts against four pathogenic bacterial strains



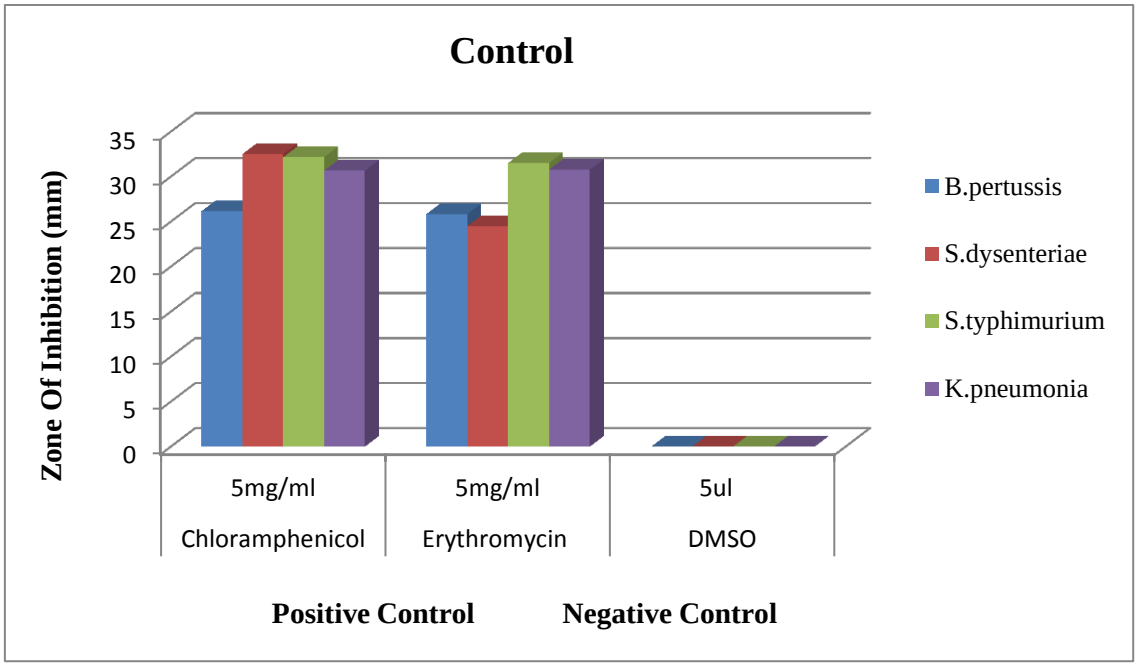
A: Antibacterial Activity of Aqueous extract of LSM against four pathogenic bacterial strains.



B: Antibacterial Activity of Methanol extract of LSM against four pathogenic bacterial strains.



C: Antibacterial Activity Of n-Hexane extract of LSM against four pathogenic bacterial strains



D: Antibacterial Activity Of positive control Standard antibiotics (Chloramphenicol and Erythromycin) and a negative control DMSO against four pathogenic bacterial strains.

Figure 4.3: Graphical representation of antibacterial activity of LSM extracts, standard antibiotics (Positive control and DMSO (negative control).

4.3 Fabrication Of LSM Based Wound Dressing Material

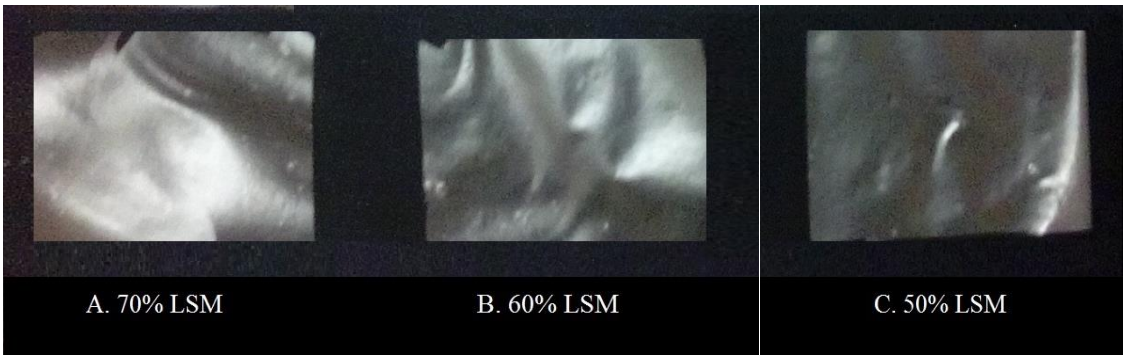


Figure 4.4: Biodegradable films fabricated from LSM with different LSM concentration (70%, 60% and 50%).

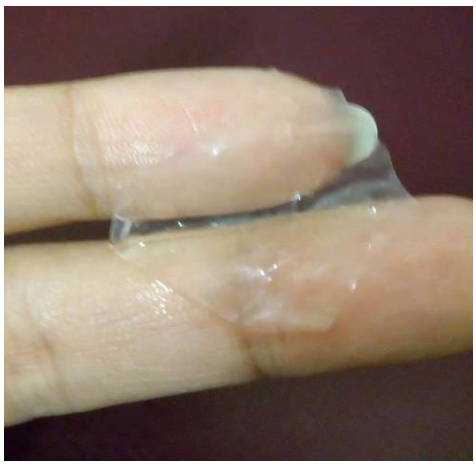


Figure 4.5: Transparency of the LSM 60% film in wet state and its appearance on the finger of a volunteer.

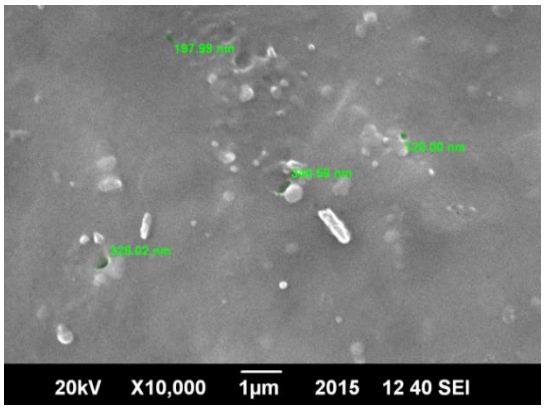
4.4 Characterization of Film

4.4.1 Mechanical Testing

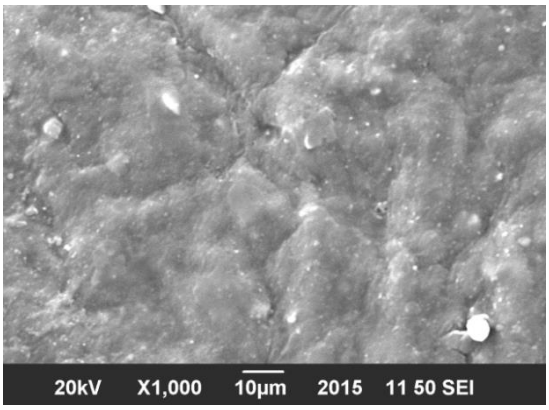
Parameters calculated From stress-strain curve	LSM Concentration		
	70%	**60%**	**50%**
Maximum breaking force (N)	27.166± 0.311	25.563± 0.648	23.635± 0.550
Breaking factor (N/mm)	222.672± 2.545	148.619± 3.768	116.426± 2.709
Deformation at break (mm)	4.484± 0.407	5.367± 0.424	5.496± 0.464
%elongation at break (%)	22.418± 2.033	26.834± 2.119	24.482± 2.321
Tensile Strength (Mpa)	22.267± 0.255	14.862± 0.377	11.643± 0.271

Table 4. 2: Various mechanical properties of LSM based film (All values are in term of average ± standard deviation).

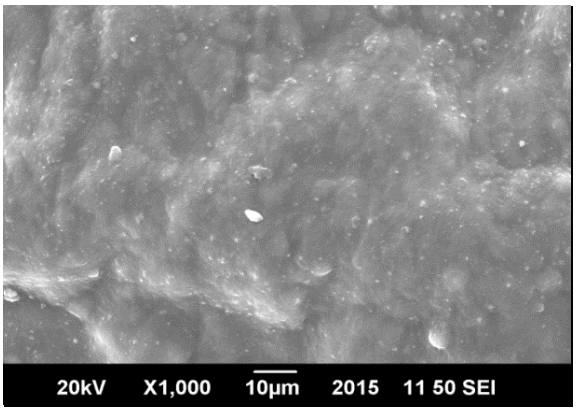
4.4.2 Scanning Electron Microscopy



A.Micrograph of 70% LSM based film



B. Micrograph of 60% LSM based film



C. Micrograph of 50% LSM based film

Figure 4.6: Microstructure examination of LSM based film with different mucilage (70%, 60% and 50%)

4.4.3 Thickness

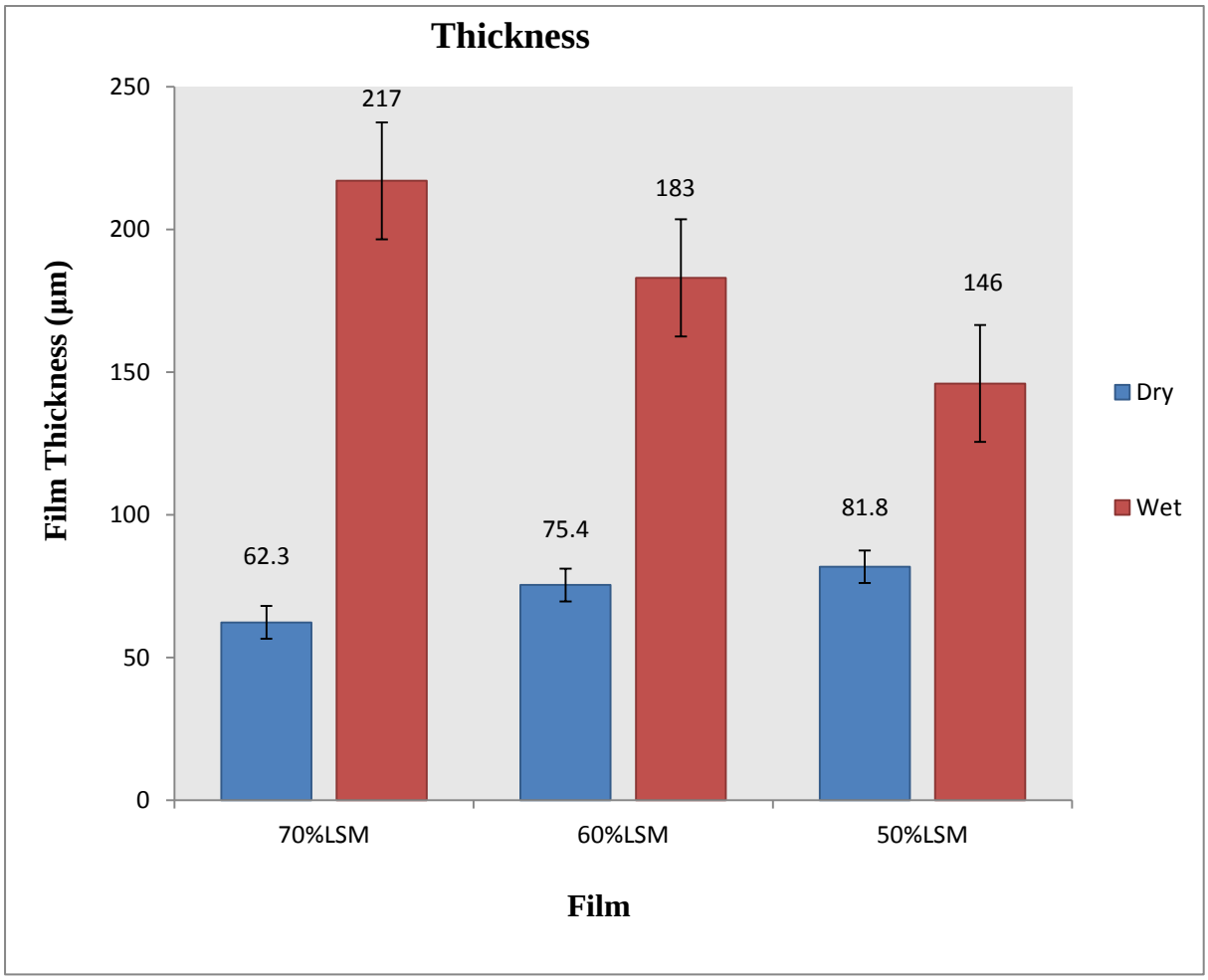


Figure 4.7: Effect of LSM content of thickness of the film

4.4.4 Swelling Index

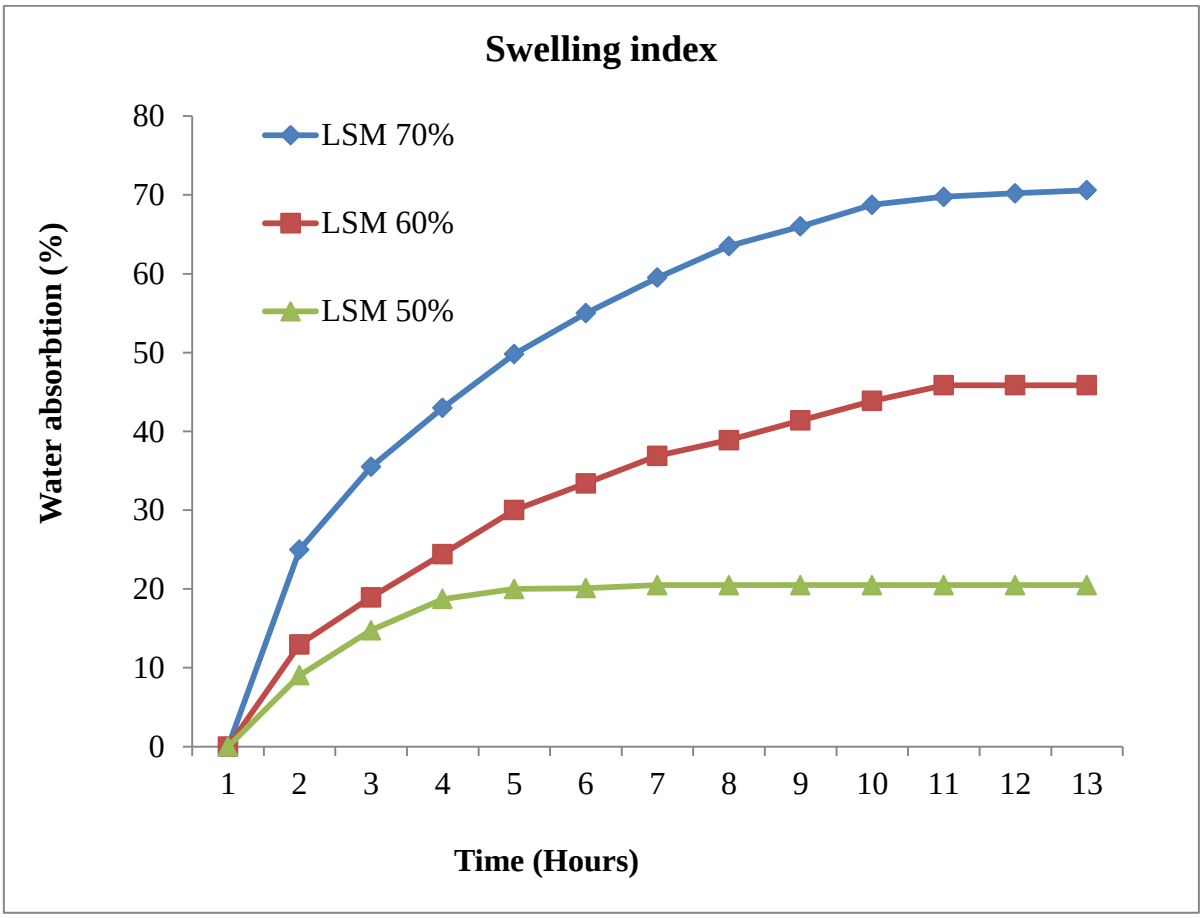


Figure 4.8: Swelling behaviour of the LSM base films immersed in PBS.

4.4.5 Degradation Profile

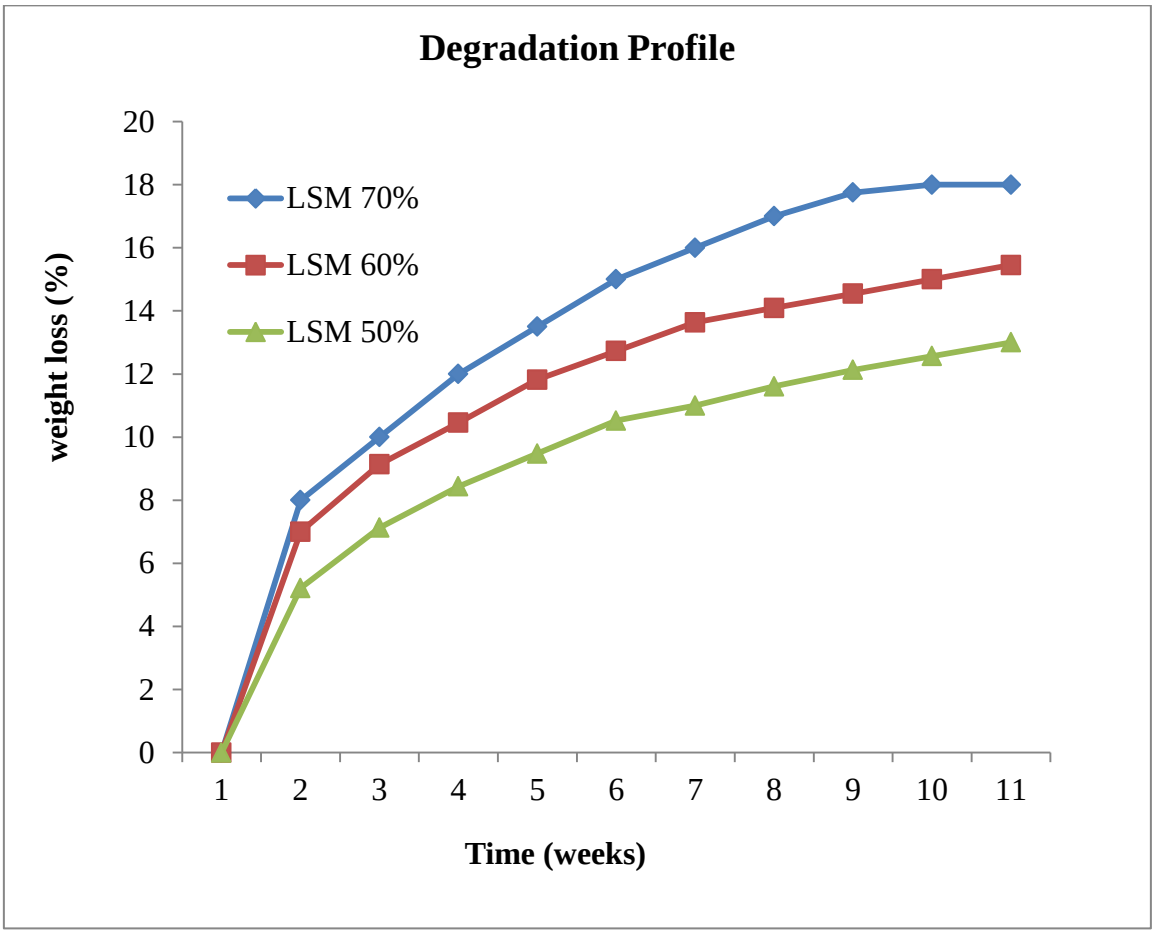


Figure 4.9: Degradation Profile of film with different LSM concentration (70%, 60% and 50%).

CHAPTER 5: DISCUSSION

Therapeutic plants are of great importance since prehistoric time for the health of communities (Rates 2001). Phytochemical screening of the LSM extract of the plant showed the presence of active ingredients that are important both medicinally and physiologically. Analysis of the three extracts of seed mucilage revealed the presence of compounds, such as phenols, saponins, phytosterols, reducing sugars, cardiac glycosides, volatile oil, diterpenes, tannins, flavonoids and alkaloids. The methanol extract showed positive results for the presence of Phytosteroles, cardiac glycosides, volatile oils, diterpenes, flavonoids, alkaloids, while the presence of tannins was confirmed only in Hexane extract. Distilled water extract of LSM shows positive results for the presence of Saponins, Reducing Sugar, Cardiac Glycosides, Volatile oils, Diterpenes, and Flavonoids.

The invitro antibacterial activity of *Lallemantia royleana* seeds mucilage extracts at five different concentrations 5mg/ml, 10 mg/ml, 15 mg/ml, 20 mg/ml, and 25 mg/ml agaisnt four human pathogenic bacteria show good result The bacterial strains included *Bordetella pertussis, Shigella dysenteriae, Salmonella typhimurium* and *Klebsiella Pneumoniae.* The aim was to explore the extract with best antibacterial potentials with particular minimum inhibitory concentration (MIC) values. Almost all extracts show positive results. But Distilled water extract showed best activity against all pathogenic strains. The highest activity was shown by distilled water extract at a concentration range of 25mg/ml with zone of inhibition 29.66 diameter against *K. pneumonia*. MIC for the distilled water extract was recorded at concentration 5mg/ml. For the hexane extract the MIC recorded was 10mg/ml with a zone of inhibition 15.166mm against *K. pneumonia*. The MIC for methanol extract was 5mg/ml with a zone of inhibition 5.66mm when tested against *B. pertussis*. *S. typharium* showed to be the most sensitive to all the extract of LSM. n.Hexan showed no activity against *B. pertussis.* When compared with the standard antibiotic activity distilled water extract show good results among all the three extracts. The activity was determined as dose dependent. Most of the samples showed greater zone of inhibition at a concentration range of 25mg/ml. Results showed that LSM has a good potential to inhibit the growth of pathogenic bacteria. It can be used to treat various bacterial infections. Following the formulations transparent, homogeneous and flexible films were formed, having a smooth surface, continuous and homogeneous, film with 70% LSM content was brittle while that of the 50 % LSM content was smoother and more flexible.

Results showed a great difference in three films having different LSM concentrations. The Films having 70% LSM content exhibit high breaking force and tensile strength. While deformation values were lower (Mali et al. 2005). As the LSM concentration was decreased the tensile strength starts decreasing this may due to the effect of high concentration of glycerol causing an increase mobility in sample having 50% LSM. Thus decreasing tensile strength may be due to modification in a structural network of film when high concentrations of plasticizer were added. Plasticizers addition to polymeric network facilitating the slipping and thus increasing flexibility decreased resistance (Sothornvit and Krochta 2001). Increasing the plasticizer content greatly affects the mechanical properties, flexibility and resilience. Film having 50% LSM content has low tensile strength and greater % elongation due to high flexibility. Film having 70% LSM content were rigid than the film having 50% LSM concentration.

To study the effect of LSM concentration changes in film's microstructure, the samples with 70%, 60% and 50% were used for observation. Film with 70% LSM content showed major pores that could allow water vapors to pass through. This may be due to low concentration of glycerol that more crakes in the films and are brittle. As the LSM concentration was decreased the film showed more homogenous and dense structure, but was more crumpled. This may be due to increase in glycerol concentration. The porosity becomes less in sample 2 having 60% LSM content as compared to sample 1 with 70% LSM content. Increasing the glycerol content causes an increase solubility of mucilage granules and thus improves the smoothness and homogeneity. Increasing the glycerol content greatly increases the opacity of the film this may be due to the fact that increasing the plasticizer content results in increasing the thickness.

In dry state the thickness varies from 62.3μm to 81.8μm while in wet state the values range between in 146μm to 217μm. Results showed an increase in film thickness when the LSM content was decreased in dry state this may be due to the effect of plasticizers that causes disruption and restructuring of intermolecular networks, thus creating free volumes those results in increasing thickness. In the wet state as the LSM concentration increases, it causes an increase in the thickness of the films, which may be due to increase in absorbing capacity of the films. All film showed hydrophilic behavior.

Swelling behaviour is important property that need to be studied for for designing a wound healing material because it determines the absorbing capacity of the film from wound fluids

(Gijpferich 1996). Results shows that increase in LSM concentration increase the water absorption capacity and thus the time taken to reach equilibrium. This may be due to the hydrophilic behaviour of the LSM which greatly improves the affinity for water and thus hydrophilicity (Pereira et al. 2013). It was also concluded from the results that film with low concentration of LSM (50%) absorb water very quickly and attain equilibrium after 4 hours, this may be due to increase in concentration of glycerol. Films with 60% and 70% LSM content absorb water rapidly when immersed in PBS, but after that these films showed a very slow absorption and thus do not reach equilibrium even after 24 hours. These results showed that both absorption properties of the film and the removal of wound fluid are totally dependent on the LSM concentration. Thus, on the bases of these results it is possible to fabricate a film with a specific water absorption capacity.

To evaluate the effect of LSM concentration on the degradation of the film in vitro degradation test was done. During the first week all films loss weight very quickly. But during following weeks the weight loss gradually slows down. The quick weight loss may be due the presence of plasticizer that leached out when exposed to an aqueous environment. As shown by fig 4.4.4 50% LSM have a very low influence on the degradation as compared to films with 60 and 70% LSM content. There is a significant weight loss observe in films with 60 and 70% LSM content during first to 6 weeks after this period the weight loss slowly stabilized reaching maximum of 17.5$\pm$ 0.4% and 15.454 $\pm$ 0.8% for film with 70% and 60% LSM content respectively. It is observed that the water absorbtion increases as the mucilage concentration in the film increases thus films with highest LSM concentration are more susceptible to degradation due to prolong penetration of water with in polymeric network that can cause leakage of the degradable cross linkages and thus rises the degradation of the film (Gijpferich 1996). Overall degradation occurs in two steps. In the first step the films start absorbing water and thus cause significant weight gain and swelling. The swelling period is very short after this the film starts losing weight gradually and thus starts degrading (Kenawy et al., 2007). These two steps make films a good remedy for healing wounds. For an ideal wound healing process any film must possess a good swelling behaviour so that it absorb adequate wound fluid and remain adhered to the skin surface for a longer time period.

CONCLUSION

The present study revealed the phytochemical constituents of *Lallemantia roylean* seed mucilage extract. On the bases of the present work it is concluded that LSM mucilage can be used for various clinical alignments. Results showed that LSM has a good potential to inhibit the growth of pathogenic bacteria. It can be used to treat infections like sore throat, gingivitis, pneumonia, whooping cough and dysentery. The antibacterial hydrogel films that were composed of alginate, LSM and glycerol can be used for many biomedical applications. Alginate provides the homeostatic properties, glycerol makes the film flexible while LSM provides therapeutic properties. The films fabricated were transparent and can be used as dressing material for wet and dry wound. Degradation and swelling behavior totally depend on LSM concentration. Films with the highest percentage of LSM possess a high percentage of weight loss and greater percentage of water absorption. The present study opened a new way of research to utilize the special properties that LSM are holding.

Future Directions

The activity and efficacy of the LSM needs further study to completely understand mechanism of action. These results suggested that LSM films should be further explored as dry and wet wound dressing material. These thin films are of great importance in the wound dressing market. The film fabricated can only be used for superficial wounds. Further study needs to be conducted to fabricate a film that can treat chronic and complex wounds because they are difficult to treat and the treatment is more expensive that is a big issue in health care. In this film only three ingredients were used for fabrication. This film can be further investigated as drug coating and encapsulation which can assist in further healing process.

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