Evaluating Antibacterial And Wound Healing Activity Of *Rumex acetosella* Leaves

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Declaration

I certify that this research work titled “Evaluation of anti-bacterial and wound healing activity of Rumex acetosella leaves” is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources has been properly acknowledged.

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Copyright Statement

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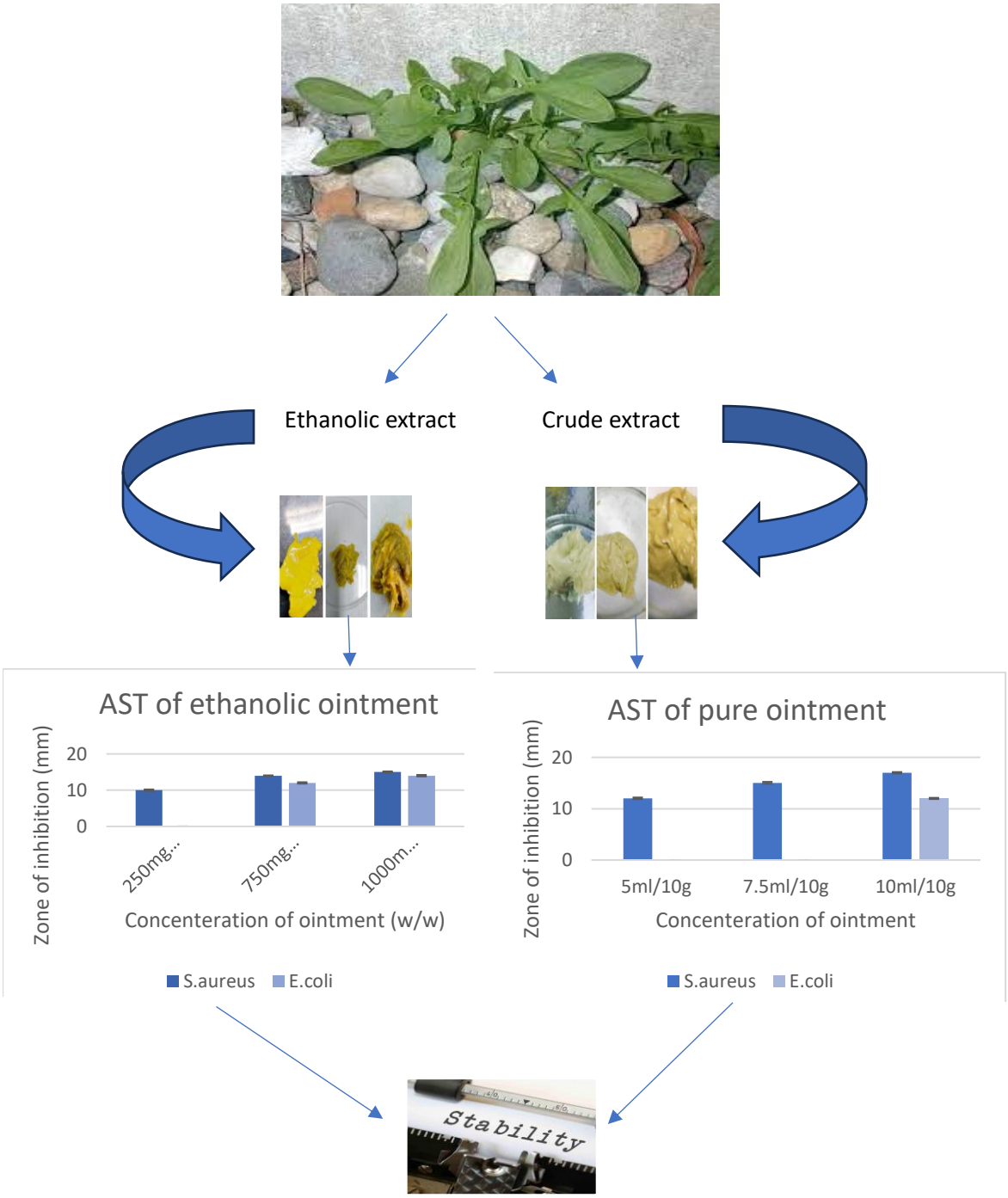
Abstract

Despite marvelous advances in the science and technology field, the significance of herbal remedies is irrefutable. The plant Rumex acetosella has been traditionally used as a wound-healing remedy for years. The objective of our study was to develop and evaluate a herbal product ensuring its biomedical application. In the current study, the herbal ointments were formulated using the pure and ethanolic extract of Rumex acetosella plant leaves. The ointment base was formulated using hard paraffin, soft paraffin, cetostearyl alcohol, and parabens as preservatives through the fusion method while the ointments were formulated by the incorporation of the drug into the base using the levigation method. The formulated ointments were subjected to different stability parameters such as physical appearance, temperature, pH, spreadability, LOD, washability, and non-irritancy tests. Moreover, the ointments were screened for having anti-bacterial potential against *S.aureus* and *E.coli.* All the formulations showed significant anti-bacterial efficacy against both stains but the ethanolic formulations were found to be more stable than pure extract formulations. All the formulations were acidic in nature, possessed notable spreadability, and less LOD. The patch test revealed that the formulations were non-irritant as they didn't cause any allergic reaction or burning sensation to the skin. Among all the formulations, 7.5% w/w of ethanolic extract was more stable under extreme temperatures i.e., 4°C, 25°C, 37°C and above 40°C with a stable pH of 4.8.

Keywords: Extract, levigation method, Spreadability, LOD, pH, Patch test

Graphical Abstract



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List of Acronyms and Abbreviations

LOD……Loss On Drying

EE……...Ethanolic Extract

PE……Pure Extract

MHA……...Mueller-Hilton Agar

E. coli…….…….Escherichia coli

S. aureus……..Staphylococcus aureus

R. acetosella……Rumex acetosella

gm……Gram

mg……Milligram

Chapter 1

1.0 Introduction

The major purpose of animal skin is to control microbial colonization on the outer surface and protect the internal tissues from microbial populations. A wound is elucidated as a of the outer skin and its function [1]. A variety of microbes can easily colonize wound infections formed on the skin. Studies have suggested that bacterial strains vary with the change in the geographical location of the patient. The pyogenic bacterial group is responsible for causing inflammation of the skin. A wide variety of skin infections are found, including necrotizing infections and pyoderma minor infections. Skin can easily be infected with different microorganisms such as fungi, bacteria, and other parasites. Mostly gram-positive and gram-negative bacterial strains are found in skin wound infections [2].

According to a rough estimate, about 8 million individuals suffer from wound infections globally, while the therapeutic drugs for wound care may reach up to 15 to 22 billion dollars in 2024 [3]. Usually, wound infections resolve easily, but chronic wounds persist for a long time and may cause severe harm if not treated properly. The basic focus of wound management is to remove or hinders the pathogenic bacterial populations growth and enhance the healing process [4]. Wound healing generally initiates soon after the injury. The process may continue for a long time, depending upon the severity of the wound [5].

There are two types of cutaneous wounds, including chronic wounds and acute wounds. The immune system of an organism plays a distinguish role in acute wound healing. After an injury, immune cells and related factors activate the inflammatory process. The activation helps in cleansing skin wound and enhance tissue healing. Moreover, any dysregulation in the working of the immune system in the course of healing may lead to delayed healing and long-lasting inflammation. Persistent inflammation can convert acute wounds into chronic wounds. The chronic wound shows the presence of elevated levels of pro-inflammatory macrophages and inflammatory mediators, including IL-1β and TNF-α. Moreover, high levels of ROS and metalloproteinases are also found in the microenvironment of the wound. Bacterial biofilms play an important role in complicating the condition of chronic wounds and prolonging the inflammatory phase of wound healing. Microbial biofilms and persistent inflammation inhibit healing [6].

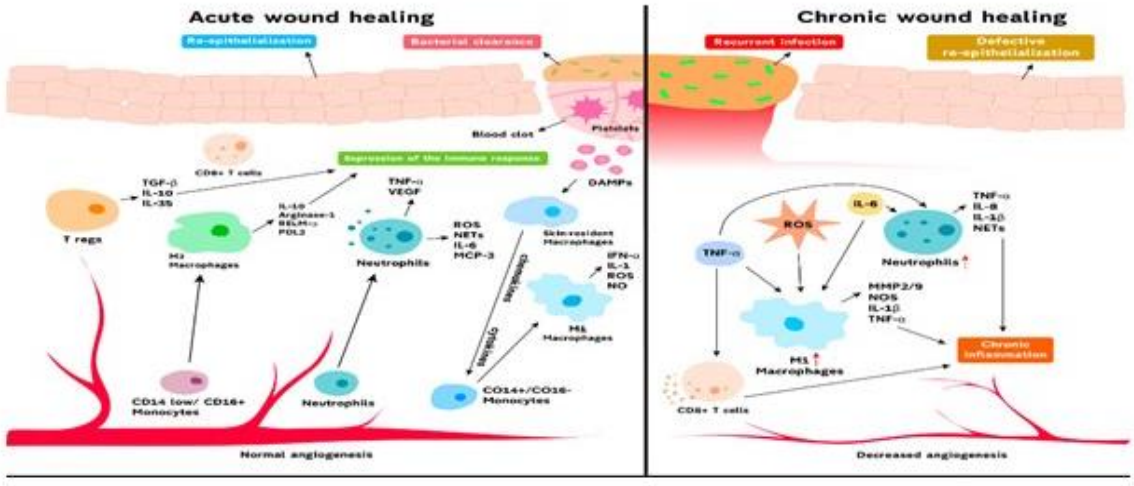

Figure 1. Role of the immune system in wound healing [7]

Mostly, bacteria are the major cause of wound infection. During the initial stage of infection (within a week), gram-positive bacteria such as S. aureus frequently colonize the wound. During the second week, gram-negative bacterial strains, including *E.coli*, *A. baumannii* and *P. aeruginosa* starts forming colony inside the wound. They can initiate sepsis after entering blood vessels and lymphatic vessels. The wound severity is known to be linked with microbial biofilm formation [8]. Staphylococcus aureus is a frequently occurring human pathogen. It contains many virulent components, including exfoliative toxins, exoenzymes, biofilms, surface proteins, and exotoxins. All these factors play a role in causing different infections. Bacteria attach to tissues and penetrate the host's immune system, causing toxicity. The major pathogenic factor is the pore-forming toxin hemolysin. Hemolysin disrupts red blood cells by rupturing their membrane [9].

A broad range of wound healing therapies is in work, including cyanoacrylate adhesives, anti-microbial agents, phototherapy (with low-power lasers), corticosteroids, immune-modulatory and immunosuppressive agents. All these therapies show a drawback that they cause side effects such as scar formation, irritation, and allergic reactions. Drugs used for wound infection may lead to resistant bacterial strains [10].

People living in the 21st century with a techno-savvy environment are facing multiple disorders. The answer to their problems is the use of basic herbs. Medicinal herbs provide effective remedies with minor side effects. Herbal treatment is usually effective for all age groups. Herbs can be used in different forms, but ointment is best for skin problems. The ointment is semisolid and shows

multiple characteristics such as antiseptics, keratolytic, astringents, emollients, and antipruritic.[11].

South Asian countries like China, India, and Pakistan are also working on alternative solutions for drug formations from medicinal plants due to low costs and easy availability. Flora of Pakistan is rich in plants and herbs with medicinal usage but very few of them have been studied (Baig et al., 2011). Multiple species of the genus Rumex are found in Pakistan. These species are famous for their therapeutic effects against wound healing, skin diseases, and bacterial infection [12].

Genus Rumex is famous for the production of secondary phenolic contents. Phenolic compounds are very advantageous because they are anti-inflammatory, anti-aging, antiproliferative, and antioxidant agents. The production of these biologically active components makes *Rumex* species a famous target for therapeutic study. The famous medicinal plants belonging to the genus Rumex include *R. Acetosella, R. Maritimus, R. Crispus, and R. obtusifolius.* All these plants show abundant phenolic compounds in their leaves. *R.acetosella* produces a high quantity of luteolin and apigenin (flavones derivative) in its leaves [13]. These components show anti-inflammatory and antioxidant effects, which are helpful in the recovery of wound infections [14].

Plant species of the genus Rumex also contains anthraquinones and phenylpropanoids, which show therapeutic properties. Anthraquinones include emodin, nepodin, physcion, epodin, etc. These components exhibit many medicinal properties such as antifungal, anticarcinogenic, antiarthritic, and antibacterial activity. The Flavonoid group is also found in multiple plants of the Rumex genus [14].

Rumex acetosella, commonly named sheep sorrel. It is a famous perennial herb found in Asia and Europe and about seventy countries around the globe. Leaves of *R. acetosella* are a rich source of vitamin C which leads to its usage in American and Europe cultures, where they are consumed as food and herbal medicine. Leaves also contain oxalic acid; therefore, the excessive usage of *Rumex acetosella* may be responsible for toxicity in sheep and horses. *R.acetosella* is an important component of traditional medicines because its leaves contain many nutritional properties and vitamins such as vitamins K, D, C, A, and B. Leaves are consumed in teas and various extracts to treat bacterial infections and scurvy [15].

The herbal ointment is formed in two major steps. The first step is base formation, while the second step includes mixing herbs in the base. The process begins with the formation of an ointment base by accurately weighing hard paraffin and grating it. Place it in the water bath to melt. All

ingredients need to be added to melted paraffin. A homogenized mixture is used to form an ointment base. Secondly, herbal ointment is formed by mixing desired herbs (medicinal plants) in the base. Levigation or fusion methods can be used to make the formulation of ointment [16].

Stability is a crucial factor in herbal drug development. It involves multiple factors (chemical and physical stability) and affects the storage date of herbal products. Stability can be described as the storage ability of a product in a container while retaining the prescribed standards, including pH, color, texture [17].

The stability of herbal ointment is essential to study because it is important for product production and to understand its storage conditions. A stability study also helps to determine the shelf life of the formulation. All the ingredients of the product (inactive or active) have the ability to disturb the product's stability. Multiple factors can alter the chemical nature of ingredients such as humidity, light, moisture, and chemical properties of ingredients. Herbal products can undergo changes in their pH, moisture content, and consistency. Stability study certifies product safety and quality [18].

There are multiple factors that may affect stability. All herbal products face physical instability. It happens due to the presence of impurities in the product and certain active ingredients. Sometimes these ingredients react with storage containers. Herbal formulations can be degraded because of certain chemical reactions such as hydrolysis, emulsions breakdown, enzymatic deterioration, and oxidation. It also happens due to the reaction of formulation ingredients (addictive) with product constituents [19].

Herbal ointment formulation has multiple constituents, including alkaloids, glycosides, flavonoids, and tannins. Therefore, stability conditions are different for each product. One major factor is pH level which affects the stability and depends upon herbal extract. pH is checked for gingerols and catechins, and isoflavones. Herbal product usage has increased around the globe because of its effectiveness. Their stability study is important to certify the quality and efficacy. Moreover, it is good for patients and helps enhance people’s interest in herbal products [20].

1.1 Objectives:

- Translating traditional knowledge using the scientific way
- Formulation and evaluation of an herbal product ensuring its biomedical application
- Screening the ointment to inhibit the growth of harmful microorganisms such as bacteria to prevent wound infection

Chapter 2

2.0. Literature Review

2.1. Wound Infection

Wound infections usually occur due to colonized bacteria and certain microorganisms. The colonization of microorganisms results in a delay in wound healing, skin decay, or worse. Mostly, the major cause of wound infection is bacterial contamination. The contamination often originates from the external environment or skin. Skin is a protective barrier and consists of three major layers (Epidermis, inner dermis, and subcutaneous layer). Skin wound disrupts the epidermis barrier and causes the denaturation of lipids and proteins. All these conditions result in a productive and ideal environment for the colonization of bacteria. It triggers infections and the immune system that, leads to inflammation and retard the healing process [21].

2.2. Wound Types

A common definition of wound is "breakdown or loss of cellular structure and function of tissues" [22]. Skin wounds occur for various reasons, including injuries, surgery, burns, cuts, and pathological problems such as vascular diseases or diabetes. Skin damage is classified into two groups (chronic or acute wounds) based on causes and effects [23]. The basic difference between these two groups is the time of healing. In acute wounds, an organized repair process results in significant remodeling of functional and structural skin integrity. However, chronic wounds fail to process pathological attack and result in long-term inflammation, necrosis, and persistent microbial infection [5].

2.3. Wound Healing

2.3.1. Acute wound Healing

Acute wound healing involves four major steps (hemostasis, inflammation, proliferative phase, remodeling). In the case of an acute wound, hemostasis is the first action taken by the body that stops the bleeding and avoids blood loss. Second, is an inflammatory phase in which skin injury triggers an immune response to kill the pathogens invading the wound. It also activates the tissues to maintain anatomical integrity. The third step is the proliferative phase which includes granulation tissue formation, re-epithelialization, and neovascularization. The last step of healing is the remodeling phase in which a scar is formed in the place of granulation tissue. Immune cells regulating the immune response are removed from the epidermis by apoptosis or move back to the dermis. The immune system of the body plays a crucial role in wound healing. Basophils and

neutrophils initially respond to skin cuts or injuries. They trigger the assembly of other immune cells, including mast cells, macrophages, B cells, T cells, and Langerhans cells [24].

2.3.2. Chronic wound healing

An unbalanced immune response at the time of wound healing leads to the formation of chronic wounds. In this condition, the inflammatory phase is unable to resolve those resulting in a poor healing process. Long-term inflammation causes many other problems, such as the accumulation of a large number of pro-inflammatory macrophages instead of anti-inflammatory phenotypes. In addition, macrophages of chronic wounds show less capability to remove dead neutrophils. It results in elevated inflammatory conditions with abundant TNF-α, interleukin-1β, and many other inflammatory mediators. Further, these macrophages secrete different MMP (matrix metalloproteinases), such as MMP-9 and MMP-2. These proteinases degenerate the extracellular matrix and stop the proliferative stage of the wound-healing process [25].

Regulation of chronic wound healing is a big problem because of persistent inflammation in chronic wounds. A major reason for prolonged inflammation is the bacterial biofilms formed within and on the wound surface. The immune system of the host interacts with bacterial biofilms by activation of pro-inflammatory macrophages and neutrophils that initiate the accumulation of cytokines, including IL-6, TNF-α, and MMP [26].

2.4. Bacteria causing infection

Many microorganisms act as a causative agents for dermatological issues (skin infection) because skin tissues provide a productive environment for the proliferation and colonization of microorganisms such as fungi, bacteria, and viruses [27].

Some common bacterial species responsible for wound infection include *Klebsiella pneumonia, Pseudomonas aeruginosa, Staphylococcus aureus, Acinetobacter baumannii,* and *Enterococcus faecalis* [28].

2.4.1. *Staphylococcus aureus*

Staphylococcus aureus is responsible for multiple infections such as wound infections. Its pathogenicity depends on multiple virulence factors. About thirty percent of the human population is affected by *Staphylococcus aureus*. The population occurrence of *S. aureus* is about 10-30 % in 1000000 people annually. Studies have reported that the infection rate is high in the initial years of life, low rates during young age, while it rises gradually in older age. *S. aureus is an* opportunistic bacterium responsible for life-threatening and superficial infections. It is a causative

agent of several disorders, including SSTIs, toxin-mediated problems, and invasive infections. It can immediately generate resistance against drugs [29].

2.4.2. *Escherichia coli*

E.coli is a well-known gram-negative bacterium. Multiple strains of E.coli have evolved as human pathogens as well as show antibiotic resistance. Enteropathogenic and uropathogenic bacteria have become popular global health concerns due to their antibiotic-resistant activity (Alharbi et al., 2019). Multiple studies have reported that 25% of E.coli strains cause wound infection to become resistant to fluoroquinolones, tetracycline, and ampicillin. E.coli shows many factors responsible for virulence, such as cytotoxic necrotizing factor, hemolysin, drug resistance, and membrane protease [30].

2.5. Importance of Medicinal Plants

Plants are an important source of medicinal ingredients. Medicinal plants have been part of human traditions for centuries. The therapeutic use of plants has increased in the modern era because many novel products are manufactured commercially from these natural sources. Consumers believe that these plant medicinal products are more effective and safer to use as compared to chemotherapeutic drugs. MDR (Multiple Drug Resistance) has been reported in human pathogenic bacterial and fungal strains. One major cause of MDR is the indiscriminate usage of commercial drugs. Higher resistance against certain antibiotic groups has been reported in areas with excessive usage of these drugs. This condition demands the intervention of new drugs with modified action mechanisms. It has been proved from multiple experiments that medicinal plants can cure multiple diseases successfully. Therefore, plants can be used as a new source to combat MDR [31].

Plants contain different organic compounds that perform multiple bioactivities. These compounds can be separated from different parts of a plant including fruits, seeds, roots, stems, and leaves. The common antibacterial components secreted by plants are flavonoids and anthraquinones which show successful action against pathogenic bacteria [32].

2.6. Medicinal Importance of Genus RUMEX

Plants of the genus Rumex are famous as a traditional source of medicine. These plants can cure multiple and complex microbial diseases such as enteritis, dysentery, and bacterial causing skin infections. In the modern era, Rumex species, including *R. hydrolapathum R. obtusifolius, R. crispus, R. acetosella, and R. acetosa* are famous for their aerial and underground parts usage as

medicinal ingredients. These species are famous for treating multiple diseases due to their antimicrobial and anti-inflammatory components to cure dermatological infections [33].

Rumex acetosella L commonly named sheep sorrel that is a perennial herb. It grows on fields, marshes, and banks during the spring season. It is a native species in Africa, Europe, and Asia. The herb's leaves can be served with salad because of its taste. It is famous for its antibacterial, anticancer, and anti-inflammatory effects [34]. In Pakistan, *Rumex acetosella* grows in northern hilly areas [35].

2.7. Herbal Ointment formulation

Herbal ointment is semisolid in nature. It changes into visco-elastic content upon applying shear stress. It contains herbal ingredients having medical importance and needs to be applied on external skin for therapeutic effects. It is mainly used as an emollient for human skin [11].

2.8. Preparation of Ointments by Fusion Method:

This method is used to mix ointment components by melting. It is helpful when ingredients include solid materials such as white beeswax, stearyl alcohol, cetyl alcohol, saturated fatty acid, and paraffin which needs to be softened. The melting process can be tried by two methods:

2.8.1 ***Method-I***

The elements are dissolved according to their melting points in decreasing order. The substance with a high melting point should be melted first while other substances come in the same order. The herbal ingredient is slowly added to the melted substances while stirring continuously. Keep the stir till these ingredients become homogenous in the mixture and cool down [36].

2.8.2. ***Method-2***

In this method, all ingredients are used in powdered form to dissolve them easily. A low temperature is required to dissolve substances as compared to method 1. Moreover, less time is required to complete the process. Melting time becomes short by grating all wax-related elements such as emulsifying waxes and beeswax etc. [36].

2.8.3 Levigation method

It is used to grind any insoluble ingredients to make a fine powder. It is also known as wet grinding. Levigation reduces the particle by using a mortar and pestle [11].

2.9. Evaluation test for ointment

2.9.1. Antimicrobial evaluation using the cup and plate method

The cup plate method is a widely used technique to examine the sensitivity of any compound against bacterial activity. In this method, microorganisms growing in agar are taken inside a petri dish, and a cylinder coated with antibiotics is diffused inside the agar layer. A zone is generated around the diffused cylinder. Ready a NA plate (nutrient agar) coated with test bacteria. Keep the depth at 4-5mm and let it solidify. Make four portions of the plate and make cavities (four) in all portions. Add antibiotic solution in three cavities, while one cavity needs to be filled with a standard solution. Measure the inhibition zone after incubating the plates [37].

2.9.2. Organoleptic properties

Organoleptic properties, including texture, odor, color, and appearance, need to be analyzed for all newly developed herbal ointments. The physical appearance of herbal ointment can only be described by its roughness and color. Physical factors such as odor and color can be judged through physical examination [11]. The texture can be examined by putting the base of the ointment in between the finger and thumb to analyze its greasiness and stiffness [38].

2.9.3. pH

pH is the major analyzing parameter for skin products. pH near 4 for products shows good chemical compatibility of skin with ointments. Digital pH meters can be used to analyze herbal ointment pH levels [36].

2.9.4. Loss on drying (LOD)

LOD is an extensively used parameter to examine the moisture quantity in any sample. Sometimes it is used to check the loss of volatile content in the sample. It can be analyzed by putting the ointment formulation in a water bath and letting it dry at 105^{o}C [11].

2.9.5. Washability

To check the washability of the ointment, apply the ointment on the skin and wash it with water. Oil in water formulation shows good washability [11].

2.9.6. Stability testing

A stability test plays an important role to check the working of ointment under stress conditions. It is important for the patient who is using the manufactured ointment. When any product become denatured, it results in the formation of toxic contents and loss of therapeutic activity. Therefore,

it is necessary to provide all information related to stability testing for the approval of the new product [39].

2.9.7. Real-Time stability testing

The test is usually performed for a long time period. It checks the degradation of the product under storage conditions for a specific time period. The test period depends upon product stability. Longer periods support the good stability of the product [40].

2.9.8. Accelerated stability testing

The test can be done by placing the product at a high temperature to keep the environment stressed. It helps to determine the heat level at which products become denatured. Such an environment can accelerate the degradation process of products. The noted data can be used to determine the shelf life of ointment. It can also help to understand the relative stability level of different products [41].

2.9.9. Spreadability

The spreadability test helps in the selection of a standard dose of herbal ointment on the skin. A decrease in spreadability shows that the ointment or cream is easy to apply on the skin. It can be done by placing a definite quantity of ointment sample between a pair of slides. Compress them to equal thickness for a fixed time. Spreadability was calculated by the time needed to separate these slides. A shorter time period shows better spreadability [11].

2.9.10. Test for non-irritancy

There is a chance of allergic reactions caused by the ointment base. Non-irritancy level of ointment is usually checked through patch tests with the involvement of human volunteers. A selective quantity of ointment needs to be pasted on the forearm or back of the hand for some time. The result should be noted daily [36].

Chapter 3

3.0. Materials and Methods

3.1. Materials:

The drug was obtained from the local area of Bagh, AJK. The materials for the ointment base which are soft and hard paraffin, propyl and methyl parabens, and cetostearyl alcohol were purchased from Global Laboratories Rawat Pvt. Ltd.

3.2. Methodology

3.2.1 Formulation of ointment base

To prepare the ointment formulation, the base was devised first.

Table 1: components of ointment base

Sr. #	Ingredients	Quantity
01	Soft Paraffin	8.5 gm
02	Hard Paraffin	1.0 gm
03	Cetostearyl Alcohol	0.5 gm
04	Methyl Paraben	0.02 gm
05	Propyl Paraben	0.18 gm
	Total	10 gm

The following ingredients were used in an appropriate proportion to formulate a base that can facilitate the adequate diffusion of drug components into it. White soft paraffin was taken as the major constituent of the ointment base while the hard paraffin was added to obtain the suitable consistency of the ointment. Methyl and propyl parabens were used as preservatives in a combined ratio of 0.2% and cetostearyl alcohol was used as an emulsion stabilizer.

The fusion method was used and for this purpose, all the ingredients of the base were kept in a Petri. After their fusion, the base was allowed to cool down by continuous stirring [42].

Figure 2: ointment base

3.2.2. Formulation of ointments by using pure Extract

The crude extract was prepared from the plant by using mortar and pestle. This pure extract was used in different concentrations to formulate the ointments. The ointments were formulated in 25%, 75%, and 100% v/w. To carry this out the amount of base was kept the same which was 10g in each sample in which a precisely measured drug was added. The Levigation method of preparing ointments was used to prepare the formulations [11].

Table 2 shows the composition of different ointment concentrations containing the pure extract.

Table 2: Ointment formulations using pure extract

Sr. #	Ingredients	50% (v/w)	75% (v/w)	100% (v/w)
01	Pure Extract	5ml	7.5ml	10ml
02	Ointment Base	10gm	10gm	10gm

3.2.3. Formulation of ointments by using ethanolic extract

Ethanolic extract was prepared by mixing the accurately measured dried leaves into the ethanol followed by a specific protocol. The ethanoic extract was used in different concentrations i.e., 2.5%, 7.5%, and 10% w/w. The amount of the base was kept the same which was 10g in each sample with varying concentrations of the extract. The formulations were prepared by using the levigation method which is the most common method of preparing ointment.

Table 3 shows the composition of ointments containing ethanolic extract.

Table 3: Ointment formulations using ethanolic extract

Sr. #	Ingredients	EF1 (w/w)	EF2 (w/w)	EF3 (w/w)
01	Ethanolic Extract	250mg	750mg	1000mg
02	Ointment Base	10gm	10gm	10gm

3.2.4. Antibacterial Susceptibility testing of the formulations:

All the formulations were screened to check the anti-bacterial efficacy against the test micro-organism through AST assay. The cup-plate method was used for the evaluation of antibacterial susceptibility testing of the ointments. The anti-bacterial potential was evaluated against two bacterial strains i.e., gram-positive and gram-negative (Escherichia coli and Staphylococcus aureus). Inoculum was prepared by the subculturing of both strains in Luria broth (LB) media and was allowed to incubate overnight at 37°C with continuous agitation. The optical density of the incubated culture was adjusted to 0.5 McFarland standard.

To prepare 500 ml of media, 19g of Muller Hilton Agar (MHA) was taken into a bottle and mixed with 500 ml of distilled water. The solution was then autoclaved to prevent any contamination. The media was set at 45°C followed by the seeding with 100µl of incubated bacterial culture of each strain having approximately 10^5 CFU. The seeded media was then poured into the sterile petri plates and the cups or wells of 6mm were bored into the plate and loaded with three-quarters of the different concentrations of the ointments. All the plates were allowed to remain at room temperature to facilitate adequate diffusion of ointment into the wells. The plates were incubated at 37°C overnight to check the inhibition zones. The entire experiment was performed in an aseptic environment.

3.2.5 Evaluation of the ointment:

The stability of the ointments was accessed by using different parameters such as color, odor, consistency, PH, centrifugation, accelerated temperature, etc. for a duration of 8 weeks.

3.2.5.1. Physical evaluation

The formulations were evaluated physically to access the organoleptic properties of the formulation for a period of 8 weeks, The texture, color, and odor of the formulations were observed to check the quality of the formulations [43].

3.2.5.2. Real-time and accelerated Temperature stability testing

In the real-time temperature stability testing, the ointments were kept at room temperature (25°C) for the interval of 8 weeks to observe the gradual changes in the formulation. For accelerated temperature stability testing the ointments were subjected to extreme temperature conditions i.e., 4°C, 37°C and above 40°C [43].

3.2.5.3 Phase separation Test

This test was done to check the phase separation of the formulations. For this purpose, 10g of each formulation was taken in a falcon tube and allowed to be centrifuged at 4000 RPM for 15 min [44].

3.2.5.4. PH Measurement

The PH of the ointments was measured by using a digital PH meter to check the acidic nature of the formulation.

3.2.5.5. Spreadability

To carry out this test, the slip and drag method was used. An accurately weighed 4g of the ointments was sandwiched between the two slides. The length of the slide was measured and a weight of 500g was placed on the slide to let the ointment spread uniformly on the surface of the slide. An additional 75g weight was tied on the upper slide to apply shear. The slide was dragged under the shear, and the time required to separate the slide was noted. The spreadability was measured by using the following formulae: [11].

$$S = M \times L / T$$

Whereas:

S= spreadability

T= Time required to pull the slides apart

L= Slide's length

M= Additional weight attached on the above slide

3.2.5.6. Loss On Drying (LOD)

To determine the volatile and moisture content present in the ointments, LOD was performed. For this, all the samples were placed in autoclaved petri plates at 105°C in a water bath for 15 minutes[37]. The initial weight of the sample was measured before drying, and the final weight was taken after drying. The experiment was repeated twice to get a constant weight after drying. The percent LOD was determined by using the formula as per USP :

$$\% \text{ LOD} = \frac{\text{weight of the sample (before drying) – the weight of the sample after drying}}{\text{Weight of the sample before drying}} \times 100$$

3.2.5.7. Washability And Non-Irritancy Test

To evaluate the quality control of the ointments these tests were performed. To access the washability of the formulations a specified amount was applied on a skin patch and allowed to set. After this skin patch was washed with water and ease of washability was observed.

For monitoring the topical sensitivity, a patch test was performed. For this purpose, a specific amount of formulation was applied to the bruised skin of a volunteer.

Chapter 4

4.0. Results

4.1. AST Results of Pure Extract Ointments

Among the formulations of pure extract, the 100% concentration i.e., 10ml/10gm showed significant activity against both gram-negative and gram-positive strains while the 75% and 50% concentrations possessed potential towards *S.aureus* but no activity against *E.coli*.

Table 4: Inhibition Zones of different concentrations of ointment containing pure extract

Concentration	*S.aureus*	*E.coli*
5ml/10gm	12mm ± 0.18	0mm
7.5ml/10gm	15mm ± 0.22	0mm
10ml/10gm	17mm ± 0.14	12mm ± 0.12

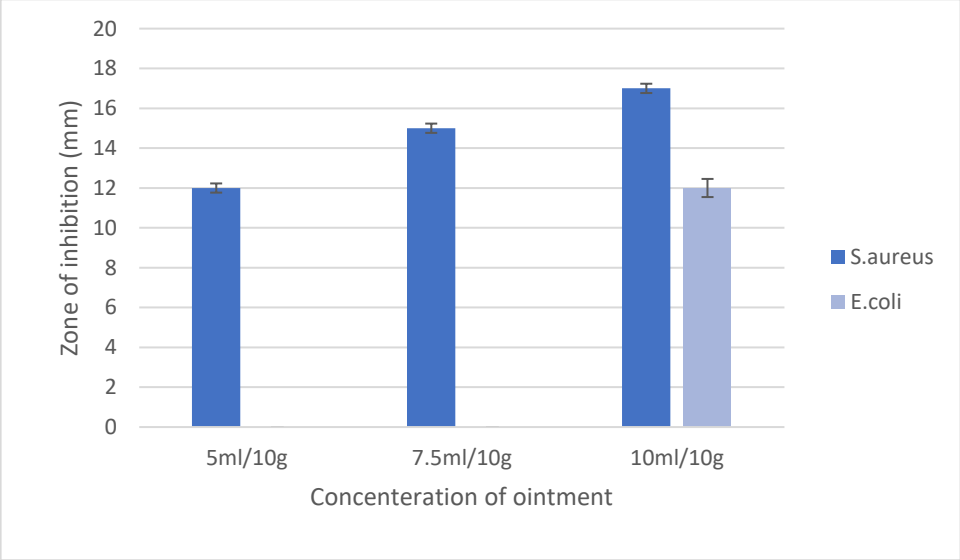

Figure 3: Dose dependent activity of ointments containing pure extract

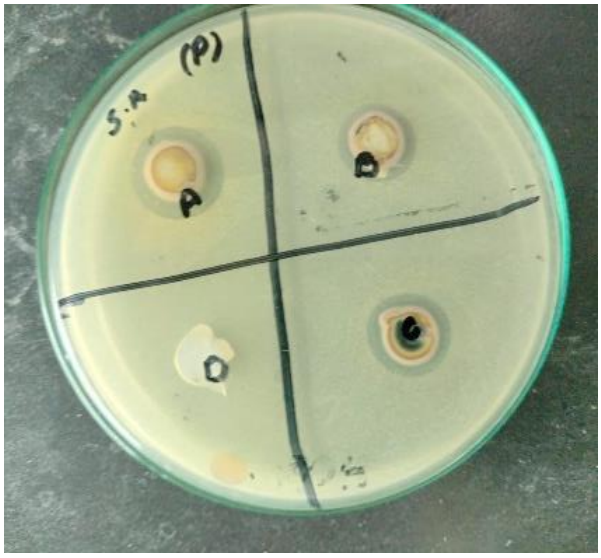

Figure 4: zones of pure extract ointments against *S.aureus*

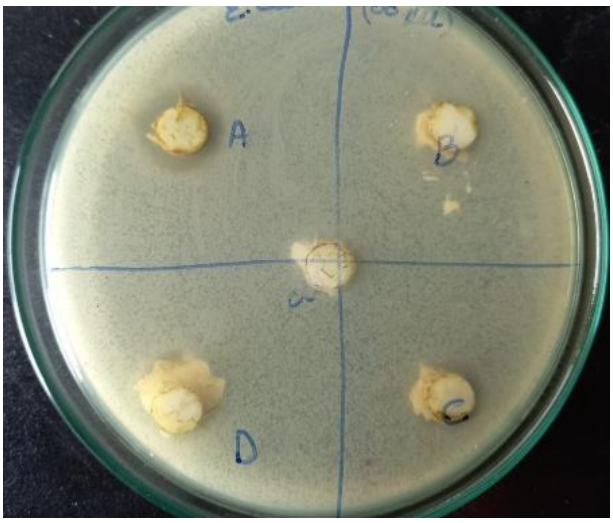

Figure 5: Zones of pure extract ointments against *E.coli*

4.2. AST Results of Ethanolic Extract Ointments

All the concentrations of ointment formulated by using the ethanolic extract i.e., 2.5%, 7.5%, and 10% showed remarkable activity against both strains except for 2.5% w/w which didn't show activity against *E.coli.*

Table 5: Inhibition Zones of different concentrations of ointment containing ethanolic extract

Concentration	*S.aureus*	*E.coli*
250mg/10gm	10mm ± 0.20	0mm
750mg/10gm	14mm ± 0.08	12mm ± 0.20
1000mg/10gm	15mm ± 0.18	14mm ± 0.20

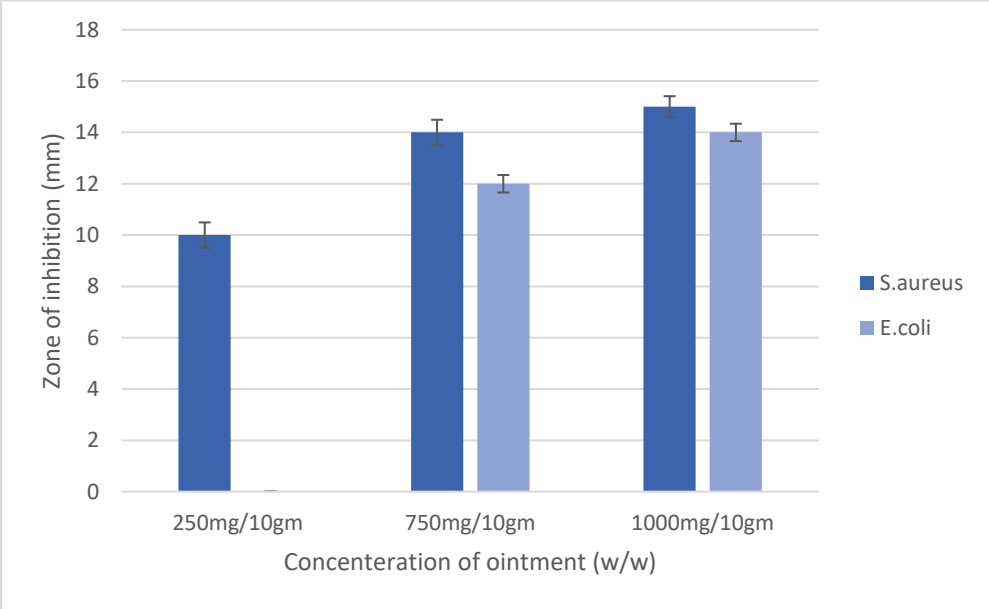

Figure 6: Dose dependent activity of ointments containing pure extract

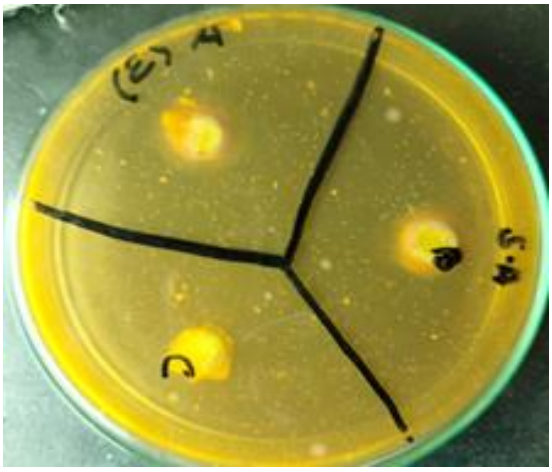

Figure 7: inhibition zones of ethanolic extract ointments against *S.aureus*

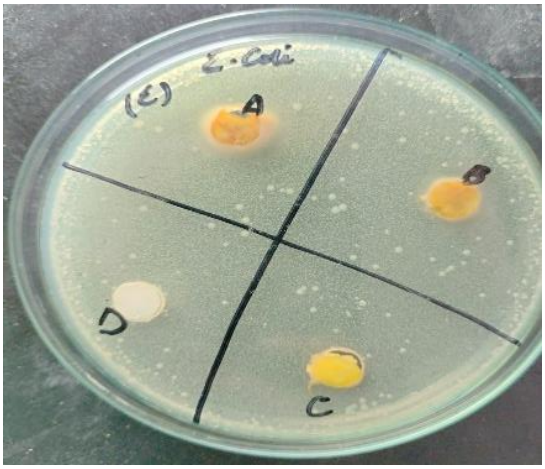

Figure 8: inhibition zones of pure extract ointments against *E.coli*

4.3. Physical evaluation of pure extract ointments

The physical parameters such as color, odor, and texture of the formulations were observed for a duration of 2 months i.e., 8 weeks. The 50% concentration of pure extract showed a tea green color, light characteristic odor, and slightly thick texture throughout the testing period. The formulation with 7.5% concentration showed a tea green color, characteristic odor, and smooth texture while the 10% concentration showed a tea green color, strong characteristic odor, and smooth and silky texture throughout the evaluation period.

Table 6: physical parameters of the ointments of pure extract

	Initially	**After a week**	**After 4 weeks**	**After 8 weeks**
5ml/10g				
Color	Tea green	Tea green	Tea green	Tea green
Odor	Light characteristic	Light Characteristic	Light characteristic	Light Characteristic
Texture	Thick	Thick	Thick	Thick
7.5ml/10g				
Color	Tea green	Tea green	Tea green	Tea green
Odor	Characteristics	Characteristics	Characteristics	Characteristics
Texture	Smooth	Smooth	Smooth	Smooth
10ml/10g				
Color	Tea green	Tea green	Tea green	Tea green
Odor	Strong characteristic	Strong characteristic	Strong characteristic	Strong characteristic
Texture	Smooth, silky	Smooth, silky	Smooth, silky	Smooth, silky

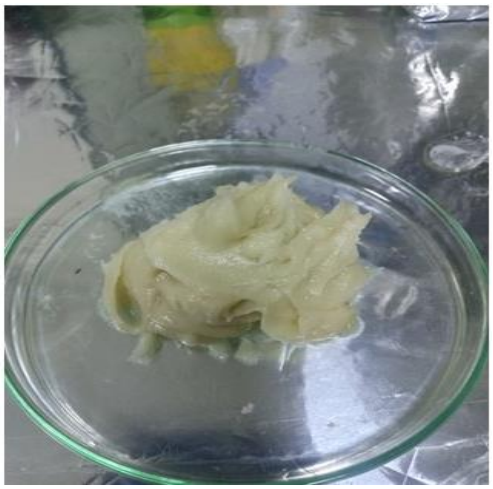

Figure 9: Physical appearance of 50% formulation

Figure 11: Physical appearance of 75% formulation

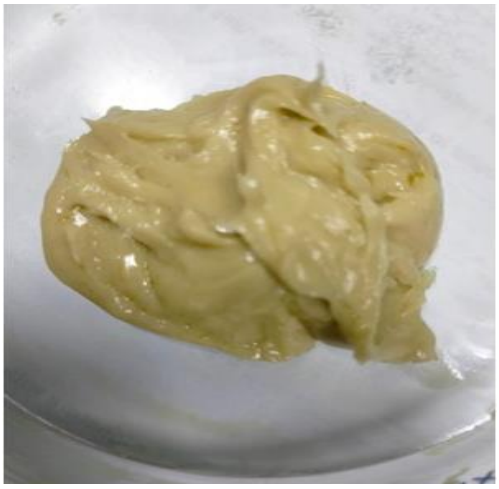

Figure 10: Physical appearance of 100% formulation

4.4. Physical evaluation of ethanolic extract ointments

The ethanolic extract formulation having 2.5% concentration showed bright yellow color, light characteristic odor, and slightly rough texture and it remained stable throughout 8 weeks. The 7.5% concentration showed yellow color, characteristic odor, and smooth texture whereas the 10% concentration showed dark yellow color, strong characteristic odor, and smooth and silky texture throughout the screening duration.

Table 7: Physical parameters of the ointments of ethanolic extract

	Initially	**After a week**	**After 4 weeks**	**After 8 weeks**
250mg/10g				
Color	Bright yellow	Bright yellow	Bright yellow	Bright yellow
Odor	Light characteristic	Light characteristic	Light characteristic	Light characteristic
Texture	Slightly rough	Slightly rough	Slightly rough	Slightly rough
750mg/10g				
Color	Yellowish	Yellowish	Yellowish	Yellowish
Odor	Characteristics	Characteristics	Characteristics	Characteristics
Texture	Smooth	Smooth	Smooth	Smooth
1000mg/10g				
Color	Yellowish	Yellowish	Yellowish	Yellowish
Odor	Strong characteristic	Strong characteristic	Strong characteristic	Strong characteristic
Texture	Smooth, silky	Smooth, silky	Smooth, silky	Smooth, silky

Figure 13: Physical appearance of 5% formulation

Figure 12: Physical appearance of 7.5% formulation

Figure 14: Physical appearance of 10% formulation

4.5. Phase Separation Test

A phase separation test was conducted to check the stability of the ointments. Among the pure extract formulations, only 10% concentration showed instability while all the formulations of ethanolic extract were found to be physically stable.

Table 8: Phase separation results of both extract ointments

	After a week	After 4 weeks	After 8 weeks
PE formulations			
5ml/10g	No Phase separation	No Phase separation	No Phase separation
7.5ml/10g	No phase separation	No phase separation	No phase separation
10ml/10g	No phase separation	Droplets appear	Droplets appear
EE formulations			
250mg/10g	No Phase separation	No Phase separation	No Phase separation
750mg/10g	No phase separation	No phase separation	No phase separation
1000mg/10g	No phase separation	No phase separation	No phase separation

PE* pure extract

EE* ethanolic extract

4.6. Accelerated Temperature Stability Testing

4.6.1. For pure extract formulations

The formulations were exposed to extreme temperatures to evaluate their stability. The pure extract formulation of 5ml/10g was stable at all temperatures for the entire testing period. The formulation of 7.5ml/10g was stable at 4°C and 37°C for the testing period but showed instability at temperatures above 40°C while the formulation having 10ml/10g concentration was stable at 4°C. At 37°C and above 40°C it was stable initially but after one week it was unstable.

Table 9: Temp. stability testing of pure extract ointments

	Initially	**After a week**	**After 4 weeks**	**After 8 weeks**
5ml/10g				
4°C	Stable	Stable	Stable	Stable
37°C	Stable	Stable	Stable	Stable
Above 40°C	Stable	Stable	Stable	Stable
7.5ml/10g				
4°C	Stable	Stable	Stable	Stable
37°C	Stable	Stable	Stable	Stable
Above 40°C	Stable	Unstable	Unstable	Unstable
10ml/10g				
4°C	Stable	Stable	Stable	Stable
37°C	Unstable	Unstable	Unstable	Unstable
Above 40°C	Stable	Unstable	Unstable	Unstable

4.6.2. For ethanolic extract formulations

The formulation of ethanolic extract was stable at all temperatures but 1000mg/10g showed to be unstable above 40°C after 8 weeks.

Table 10: Temp. stability testing of ointments containing ethanolic extract

	Initially	**After a week**	**After 4 weeks**	**After 8 weeks**
250mg/10g				
4°C	Stable	Stable	Stable	Stable
37°C	Stable	Stable	Stable	Stable
Above 40°C	Stable	Stable	Stable	Stable
750mg/10g				
4°C	Stable	Stable	Stable	Stable
37°C	Stable	Stable	Stable	Stable
Above 40°C	Stable	Stable	Stable	Stable
1000mg/10g				
4°C	Stable	Stable	Stable	Stable

37°C	Unstable	Stable	Stable	Stable
Above 40°C	Stable	Stable	Stable	Unstable

4.7. PH Stability

All the formulations were acidic in nature. Some formulations showed stable PH throughout the evaluation period such as 5ml/10g of pure extract showed a stable PH of 4.0, 250mg/10g of ethanolic extract showed a stable PH of 5.1 while 750mg/10g showed 4.8 PH which remained same throughout testing time while PH of 7.5ml/10g of pure extract changed from 3.7 to 3.5 and of 10ml/10g changed from 3.4 to 3.0. On the other hand, PH of 1000mg/10g varied from 4.3 to 4.2.

Table 11: PH of both extracts' ointments

	Initially	**After a week**	**After 4 weeks**	**After 8 weeks**
Formulation of PE				
5ml/10g	4.0	4.0	4.0	4.0
7.5ml/10g	3.7	3.7	3.7	3.5
10ml/10g	3.4	3.4	3.3	3.0
Formulation of EE				
250mg/10g	5.1	5.1	5.1	5.1
750mg/10g	4.8	4.8	4.8	4.8
1000mg/10g	4.3	4.3	4.2	4.2

PE* (Pure Extract), EE* (Ethanolic Extract)

4.8. Spreadability

From the slip and drag method, the spreadability of the pure extract ointments having concentrations 5ml/10g, 7.5ml/10g, and 10ml/10g was 80.35, 86.53, and 93.75 respectively while the spreadability of the ethanolic extract ointments having concentrations 250mg/10g, 750mg/10g and 1000mg/10g 74.01, 78.12 and 80.35 respectively.

Table 12: Spreadability of ointments of pure and ethanolic extracts

Pure ointment conc.	Spreadability (g.cm/s)	Ethanolic ointment Conc.	Spreadability (g.cm/s)
5ml/10g	80.35 ± 1.93	250mg/10g	74.01 ± 0.79
7.5ml/10g	86.53 ± 1.09	750mg/10g	78.12 ± 0.88
10ml/10g	93.75 ± 1.27	1000mg/10g	80.35 ± 0.93

4.9. Loss on Drying

The pure extract ointments having concentrations of 5ml/10g, 7.5ml/10g and 10ml/10g have 0.7%, 1.0%, and 104% LOD respectively while the ethanolic concentrations of 250mg/10g, 750mg/10g, and 1000mg/10g possessed 0.3%, 0.5% and0.8% LOD respectively.

Table 13: % LOD of both extract ointments

Concentration	Loss on drying
Ointments of pure extract	
5ml/10g	0.7%
7.5ml/10g	1.0%
10ml/10g	1.4%
Ointment of ethanolic extract	
250mg/10g	0.3%
750mg/10g	0.5%
1000mg/10g	0.8%

4.10. Washability and non-Irritancy tests

The ointments containing ethanolic extract are greasier than pure extract ointments therefore they possess less washability when compared with the washability of ointments containing pure extract. The patch test revealed that all the formulations were non-irritant because they didn't cause any allergic reaction or burning sensation to the skin.

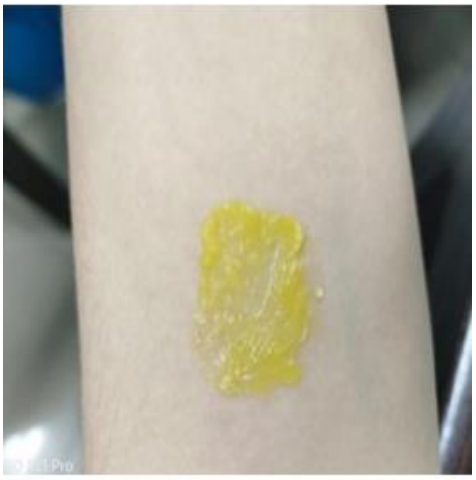

Figure 15: Ointment applied on a skin patch

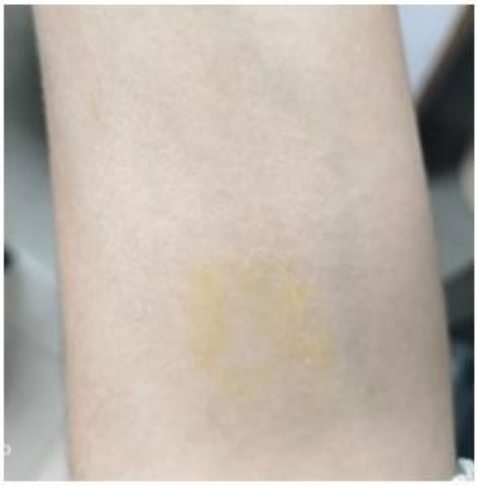

Figure 16: 15 minutes after patch test

Figure 17: Control

Chapter 5

5.0. Discussion

Wound infections usually occur due to colonized bacteria and certain microorganisms. The colonization of microorganisms results in a delay in wound healing, skin decay, or worse. Mostly, the major cause of wound infection is bacterial contamination[4]. A broad range of wound healing therapies is in work, including cyanoacrylate adhesives, anti-microbial agents, and phototherapy (with low-power lasers). All these therapies show a drawback that they cause side effects such as scar formation, irritation, and allergic reactions. Drugs used for wound infection may lead to resistant bacterial strains[10].

Rumex acetosella L is commonly named sheep sorrel that is a perennial herb. It is famous for its antibacterial, anticancer, and anti-inflammatory effects[45]. In Pakistan, *Rumex acetosella* grows in northern hilly areas[46]. In this study, we have formulated an herbal ointment to treat wounds. The base was formulated using white petrolatum, hard paraffin, cetostearyl alcohol, and parabens as preservatives through the fusion method on a water bath while the drug was added by using the levigation method[11]. Anti-bacterial activity of the formulations was screened against the gram-positive *S.aureus* and gram-negative *E.coli*. Although all the pure extract formulations also showed significant bactericidal efficacy towards S.aureus stains and only 100% showed activity against E.coli but hemolysis assay showed that the pure extract of this plant is cytotoxic at higher concentrations while the ethanolic extract is less cytotoxic. Therefore, the ethanolic extract formulations are more effective as wound healing therapy specifically 7.5% of ethanolic extract formulation.

The stability evaluation of the herbal ointments is crucial because the herbal compounds in the formulation are prone to degradation[18]. Different stability parameters were evaluated for the formulations of pure and ethanolic extracts such as temp, PH, spreadability, LOD, phase separation, washability, and non-irritancy test[11]. All the formulations were found to be physically stable after 2 months. Only one formulation of pure extract that was 100% v/w showed phase separation while all other formulations of pure and ethanolic extracts were stable after centrifugation at 4000 RPM[47]. All the formulations were stable at room temperature while the accelerated temp stability was also evaluated, and the 75% and 100% formulations of pure extracts showed instability at high temperatures while the ethanolic extracts ointments were comparatively stable.

The spreadability of the ointments was significant and it was evaluated that by increasing the drug concentration spreadability was increased and it was revealed that the spreadability of the pure extract ointments was greater than the ethanolic extract ointments. The PH of an ointment plays a crucial role in wound healing. The acidic buffers having PH 4 were found to promote faster healing of wounds as compared to those buffers having PH 6. All the ointments of ethanolic and pure extract were observed acidic in nature, and it was also revealed that by increasing the drug concentration the PH tends to decrease. The washability and skin sensitivity tests were performed to check the quality of the formulations. The ethanolic formulations were greasy as compared to the pure extract formulations so the washability of the ethanolic formulations was less than the pure extract formulations. The patch test didn't show any kind of redness, itching, and burning sensation so it was observed that all the formulations were non-irritant.

Conclusion

The current study revealed the possibility to develop an ointment with ethanolic and pure extract of *Rumex acetosella* leaves and evaluate them for antibacterial activity. In vitro, analysis confirmed the wound-healing efficiency of the ointment formulations. The pure and ethanolic formulations were screened for different stability parameters. The ethanoic formulation having 7.5% w/w concentration was found to be more stable under different temperatures i.e., 4 °C, 25°C, and 37 °C and showed remarkable anti-bacterial activity against both gram-positive and gram-negative strains.

Future Prospectives

Although the plant has been traditionally used for years but the precise mechanism of action needs to be explored. Moreover, the current study focused on the stability evaluation and anti-bacterial potential of the ointments but the in-vivo evaluation of the ointments on an animal model needed to be investigated.

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