Isolation, Phytochemical Analysis and Antibacterial Activity of *Rumex acetosella* Plant

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MASTER'S THESIS WORK

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Declaration

I certify that this research work titled “Isolation, phytochemical analysis and antibacterial activity of Rumex acetosella” 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

Acknowledgments

I am thankful to my Creator Allah Subhana-Watala to have guided me throughout this work at every step and for every new thought which You set up in my mind to improve it. Indeed, I could have done nothing without Your priceless help and guidance. Whosoever helped me throughout my thesis, whether my parents or any other individual, was Your will, so indeed none be worthy of praise but You.

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Dedication

I dedicate the outcome of my efforts to my parents, my siblings and especially my uncle Jehanzaib Khan and my sister-in-law Samhia Jawad for their encouragement and support.

Abstract

Human beings are always prone to infectious diseases. Human pathogens are of various types. These could be a virus, bacteria, fungi, protozoans, and other parasites and cause various infections. Several deaths occur due to these infections every year. Despite various drugs available in market these pathogens still are potential threat due to their variability and increasing antibiotic resistance. It is a need of time to develop new drugs and plant isolates to cope with these infections. The role of medicinal plants has been known for centuries. The present paper analyzes the biologically active compounds present in the plant leaves, anti-bacterial activity, and cytotoxicity of the ethanolic and pure extracts of a traditional plant, *Rumex acetosella.* Preliminary phytochemical screening, UV-VIS spectroscopy, and GCMS was done to determine the chemical makeup and biologically active constituents present in the plant leaves. Antibacterial activity was tested through the well diffusion method and broth dilution method to determine the MICs. The RBC hemolysis assay was performed to test the cytotoxicity of the extracts at different concentrations. The present analysis revealed the existence of pharmacologically active constituents such as 1,3,5-Triazine, 2,4,6-tris(cyanomethyl)-, 4-[4-Aminopentylamino]-2-methoxy-9-methylacridine and Undecanoic acid, 10-bromo, etc. in *Rumex acetosella* leaves which are responsible for different therapeutic effects. Ethanolic extract showed antibacterial potential against *S. aureus* with MIC 800ug/ml but less sensitivity towards *E. coli*. Pure extract was potent against both *S. aureus* and *E. coli* with MICs 10ul and 20ul respectively. RBC hemolysis revealed that ethanolic extract was less toxic as compared to pure extract. In conclusion, *R. acetosella* extracts exhibited significant antibacterial potential and less toxicity.

Keywords: Pathogen, MIC, GC-MS, RBC hemolysis, Phytochemical, Cytotoxicity

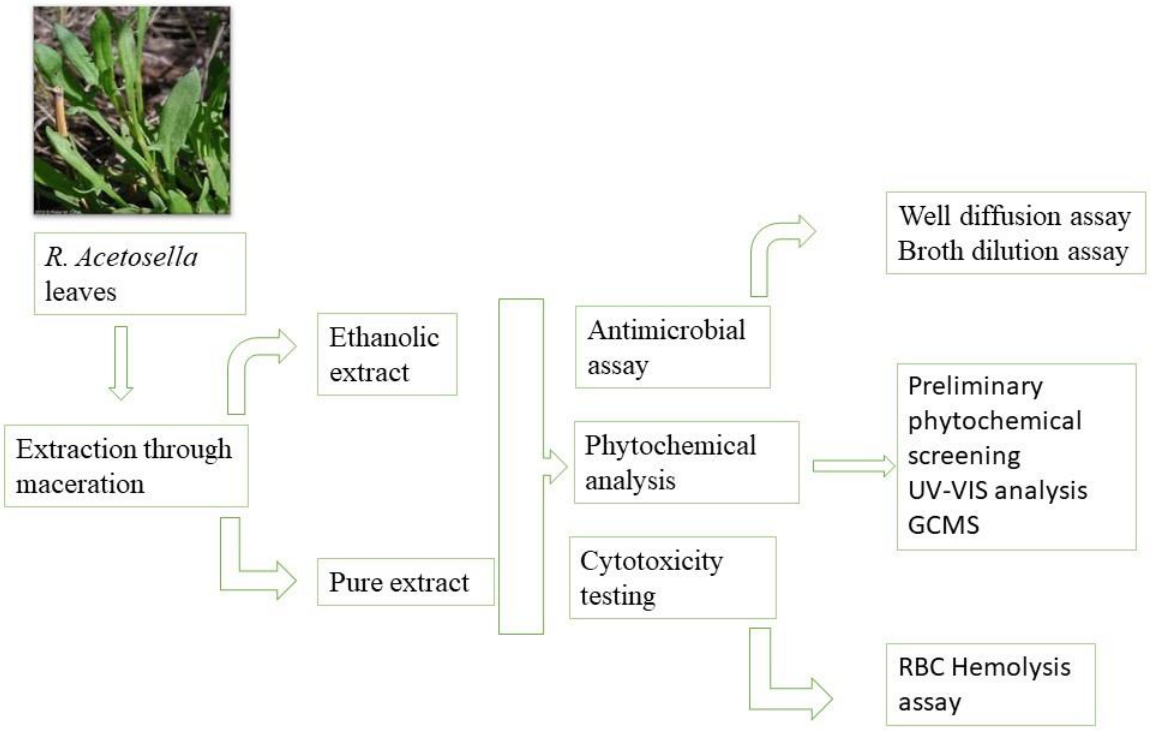

Figure 1. Graphical abstract of the study

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List of Acronyms and Abbreviations

RBCs……Red Blood Cells

MIC…….Minimum Inhibitory Concentration

MBC…….Minimum Bactericidal Concentration

MHA……Mueller-Hilton Agar

E. coli……..…….Escherichia coli

S. aureus…….Staphylococcus aureus

R. acetosella…….Rumex acetosella

TLC……..…..Thin Layer Chromatography

ul……Micro Liter

ug……Microgram

CHAPTER 1

1.0. Introduction

A human pathogen is a microbe that causes diseases in human beings. Pathogen could be a virus, bacteria, fungi, or parasites. The history of human health has been greatly compromised by infectious diseases caused by bacterial infections [3]. Most of the bacterial species are harmless but fewer than hundred are pathogenic species which causes infectious diseases in human beings[4]. About six million people in the United States are influenced by acute and chronic wound at an expense of 25 billion US Dollars. Infections emerge in 5.6-26% of wounds due to destruction of epidermis and local microbial colonization (moisture, temperature, and nutritional conditions). Burns, surgical sites, and traumatic wounds are vulnerable to infections [5]. Several pathogens, including Staphylococcus aureus (S. aureus), Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), coagulase-negative Staphylococci, and many other aerobic and anaerobic microbes, have been commonly found in wound infections [6].

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 with 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 [7].

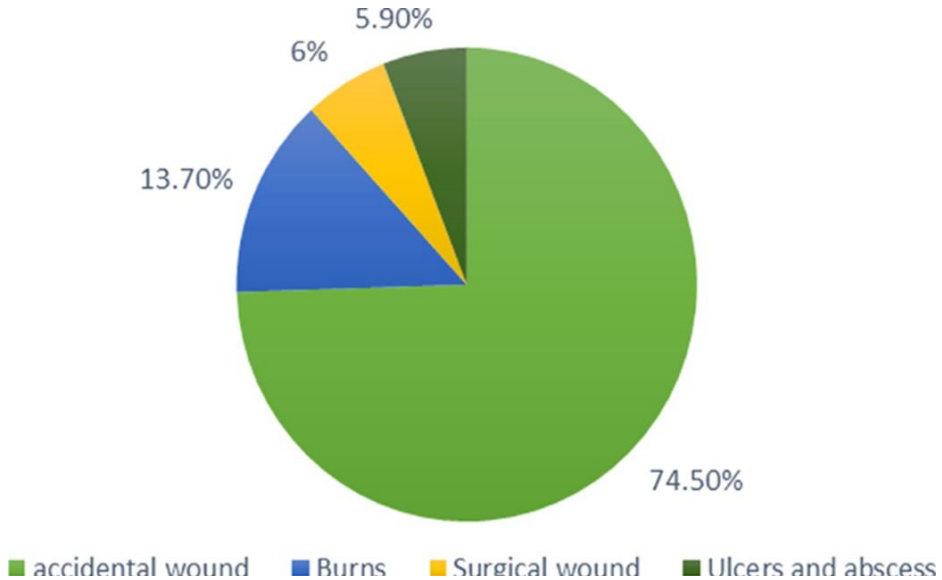

Figure 2. Prevalence of *S. aureus* isolated from wounds [2]

The utilization and search for plant-derived medications and nutritional supplements has increased in recent years. Ethnopharmacologists, botanists, microbiologists, and natural-products chemists are scouring the planet for phytochemicals that could be used to treat infectious diseases. Plants have a broad range of phytochemicals, including tannins, terpenoids, alkaloids, and flavonoids, all of them show antibacterial activities in vitro [8]. Herbal medicine is a plant substance that can be taken in the form of an extract or diluted. When conventional medication is becoming ineffective due to rising drug resistance, usage increases. As a result, lots of well-known herbs are employed around the world to treat infections caused by bacteria. Despite the fact that traditional medications or their precursors are produced from plants, there is a significant distinction between delivering a pure chemical and administering the exact same chemical in a plant form [9]. Phytochemicals have been discovered as substitute to traditional antibiotics in managing antibiotic resistance [10].

Rumex acetosella L. (Polygonaceae) is found all over the world and has several ethnobotanical uses. The plant possesses various phytochemical groups, including anthraquinones and other phenolics, which may contribute to the development of plant-related activities. *Rumex acetosella* L. has been employed to treat diabetes traditionally. R. acetosella extracts and phytochemical components can function as antihyperglycemic agents. Alcohol-containing extracts of *R. acetosella* may be suggested helpful options in the management of type II diabetes [11]. Essiac, an American recipe, is a common cancer-fighting medication. In its fresh state, the entire plant is used as a refrigerant, diaphoretic, and diuretic. Furthermore, tea made from the leaves is used to cure scurvy, fevers, and inflammation. The juice of the leaves, on the other hand, is effective in the treatment of urinary and kidney problems. While the traditional usage for cancer, leaf paste also has application to various malignancies, cysts, and so on.

Agar well diffusion is an effective technique for determining the antimicrobial capacity of either extracts of plant or microbes. The agar plate's surface is inoculated like the disk-diffusion method, by dispersing a microbial culture over the agar surface. Then, a hole with 6 to 8 mm diameter is bored in aseptic conditions, and a volume (20-100 L) of the solution containing the or extract at the appropriate concentration is added into the well. The agar plates are then placed in the incubator under appropriate conditions based on the test pathogen. The antimicrobial ingredient diffuses across the agar medium, restricting the growth of the tested microbiological strain [12].

Macrodilution is the basic procedure for determining MIC (minimum inhibitory concentration). The MIC is the lowest antimicrobial agent concentration that totally restricts organism growth in

tubes or microdilution wells as seen by the unassisted eye [13]. The technique involves preparation of two-fold dilutions of the antimicrobial agent (e.g., 1, 2, 4, 8, 16, and 32 g/mL) in a liquid growth medium supplied in tubes with a minimum volume of 2 ml. The tubes are then injected with a microbial inoculum prepared in the same medium following dilution of standardized microbial suspension to 0.5 McFarland scale. Following well-mixing, the inoculation tubes are incubated under suitable conditions depending on the test microorganism [14].

The breakdown of RBCs by hemolysin is referred to as hemolysis. Although drug-induced hemolysis is uncommon, it is essential to test for hemolysis before delivering the drug by IV injection. Hemolysis can occur for two reasons. The one is either by direct drug action or through the actions of its metabolites. The second explanation is the individual's immunological reaction because of medication sensitivity [15].

The above discussion describes the importance of pathogenic microorganisms and the infectious disease caused by the agents. It is necessary to cope with these microbial pathogens to reduce the burden of diseases from the world. Phytochemicals have been exploited for the treatment of various diseases as these are natural chemical compounds. This research is conducted to explore the role of secondary metabolites, present in the leaves of the plant, *Rumex acetosella.* In this study its antimicrobial potential has been exploited on gram positive *(S aureus)* and gram negative *(E. coli)* strains. Along with it, its hemolytic properties are also measured.

1.1. Objective

As the *Rumex acetosella* was traditionally used for treating different kinds of infections but there was no scientific study on this plant. Therefore, the objectives of our study were

- To translate traditional knowledge into scientific knowledge
- To synthesize potential medicine to treat infections
- To calculate effective dosage form
- To investigate underlying mechanism of action
- To develop less toxic and cost-effective medicine

CHAPTER 2

2.0. Literature review

The dependence of human beings on drugs from allopathic, complementary, and traditional sources, has been long established. Among all the ailments microbial infections have always attained the central place in terms of associated mortality and morbidity. Though decoctions, extractions, and other pharmacological preparations have been used from the beginning of time, isolated chemical compounds have been utilized to treat a range of infections since 1928, following the discovery of penicillin [16].

2.1. Pathogens

A pathogen is a living thing that produce disease in its host, and the severity of the disease symptoms is known to as virulence. Pathogens are scientifically varied, including viruses, bacteria, and unicellular and multicellular eukaryotes. Pathogens, including bacteria, influence every living thing and are attacked by phages, which are specialist viruses [17]. The quantity of viruses and bacteria on the planet is wonderful, and they may be found in nearly any environment. A liter of surface seawater normally includes over ten billion bacteria and one hundred billion viruses. A normal human is composed of approximately thirty trillion cells, but has an equivalent amount of microbes, largely in the gut. The great amount of viruses and bacteria in our surrounding are harmless, and some may even be beneficial; nonetheless, a small number of them can have serious impact on our health [18]. Infectious diseases are ailments caused by infectious agents (pathogens) that enter the human body from the outside. Pathogens that are involved in infections include viruses, bacteria, fungus, parasites, and, in rare cases, prions. In recent years, there has been an upsurge in the frequency of documented epidemics of human diseases linked to the intake of raw fruits, vegetables, and unprocessed fruit juices. Global advancements in agronomic, manufacturing, storage, packaging, distribution, and promotional technologies have allowed the raw fruit and vegetable business to serve consumers with a diverse choice of high-quality products throughout the year in most countries. Likewise the same technology and techniques have also increased the danger of human diseases caused by harmful bacteria [19]. Infectious diseases continue to be one of the top causes of morbidity and mortality globally. contributing to more than fifty-two million (33%) yearly fatalities. Fifty percent of the global population is still vulnerable to infectious disease outbreaks.

According to recent data, there were roughly fourteen million worldwide deaths among children under the age of five, 70% of which were caused by vaccine-preventable diseases, with 99% occurring in developing countries. Despite substantial improvements and worldwide education campaigns, infectious disease prevention and control face severe hurdles. Many infectious illnesses are prevalent in Pakistan, with very minor variations in case load and prevalence documented across decades [20]. About six million people in the United States are influenced by acute and chronic wound at an expense of $25 billion US Dollars. Infections erupt in 5.6-26% of wounds due to destruction of epidermis and local microbial colonization (moisture, temperature, and nutritional conditions). The top three wounds that are vulnerable to infection include burns, surgical sites, and traumatic wounds [21].

2.2. Pathogenic bacteria

Pathogenic bacteria cause infections. There are about 100 species that cause infectious diseases in human beings. Infection refers to the invasion of the host by microorganism. Bacteria causes a range of infections with different severity according to their pathogenicity. Previously, kingdom Monera was distinguished on the basis of gram staining. Gram negative bacteria that stain negatively are referred to as Gracilicutes, Gram positive bacteria that stain positively are referred to as Firmicutes, molicutes that stain neutrally, and mendocutes that stain variably. Bacteria are typically categorized as gram positive or gram negative on the basis of their staining [22]. There are six gram-positive bacteria that are harmful to humans. Staphylococcus and streptococcus are two types of cocci. Gram-positive bacteria cause serious and sometimes fatal illnesses. Gram negative bacteria use Escherichia coli as a model organism. Their pathogenicity is enhanced by their complex lipopolysaccharide structure, which functions as an endotoxin. It triggers toxic reactions in the body, resulting in septic shock [23].

2.2.1. *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium, and it is defined in bunches, referred to as "grape-like." These microbes can survive in saline on media, and yellow colonies. These organisms can develop facultatively, and at 18 to 40 degrees Celsius [24]. *Staphylococcus* and its resistant strains occurs on the epidermis and mucosa membranes, and humans are the organisms' primary source [25].

Infections involving *S. aureus* are prevalent in people, causing a variety of human illnesses, including bacteremia, infective endocarditis, and skin and soft tissue infections. These bacteria can

produce invasive infections and/or toxin-mediated diseases, based on the strains present and the location of infection. The pathophysiology of *S. aureus* infection differs based on the type. For escaping the host defense system, *S.aureus* uses different mechanism such as development of an antiphagocytic capsule, sequestering of host antibodies or antigen masking by Protein A, biofilm formation, intracellular survival, and preventing leukocyte chemotaxis . protein associated with bacterial cell wall facilitate bacterial anchoring to extracellular matrix proteins and fibronectin in infected endocarditis [25].

2.2.2. *Escherichia coli*

Escherichia coli is normally found in the human intestine but can also cause ailments in the intestines. *E. coli* is common bacteria in the human digestive tract, but it does not cause disease in it. *E. coli*, on the other hand, can cause urinary tract infections when discovered outside of the intestinal system [26]. Treatment is determined by both the strain and the disease. Antibiotics are not given as primary medications for *E. coli* diarrhea in most individuals due to side effects and the relation with antibiotic resistance. Antibiotics may be given patients with serious conditions (e.g., dysentery). Currently, rifaximin, azithromycin, and ciprofloxacin are suggested [27].

2.3. Antibiotic resistance and phytochemicals

The emergence of antibiotic resistance promotes the failure and rising costs of already available medications for treating infections and other complex disorders. Multi drug resistance (MDR) bacterial strains have been developed because of improper and excessive usage of antibiotics. *S. aureus* related infections are prevalent in 30% of the population and this bacterium has evolved as a significant multidrug resistant infection. Methicillin resistant *S. aureus* (MRSA) strain is now a major threat. Similarly, E. coli, a gram-negative pathogen, is also involved in infections mostly wound infections, and has also developed antibiotic resistance.

Phytochemicals have been discovered as substitutes to traditional antibiotics in managing antibiotic resistance. Several plants based biologically active phytoconstituents have been investigated for their potential to reverse antibiotics and kill bacteria. They can restrict the progression of virulence factor linked to resistance, including cell permeation, efflux pumping, DNA replication processes and other bacterial virulence-related processes like biofilm development and quorum detection. Additionally, these phytoconstituents can be combined with antibiotics to produce synergistic effect [28].

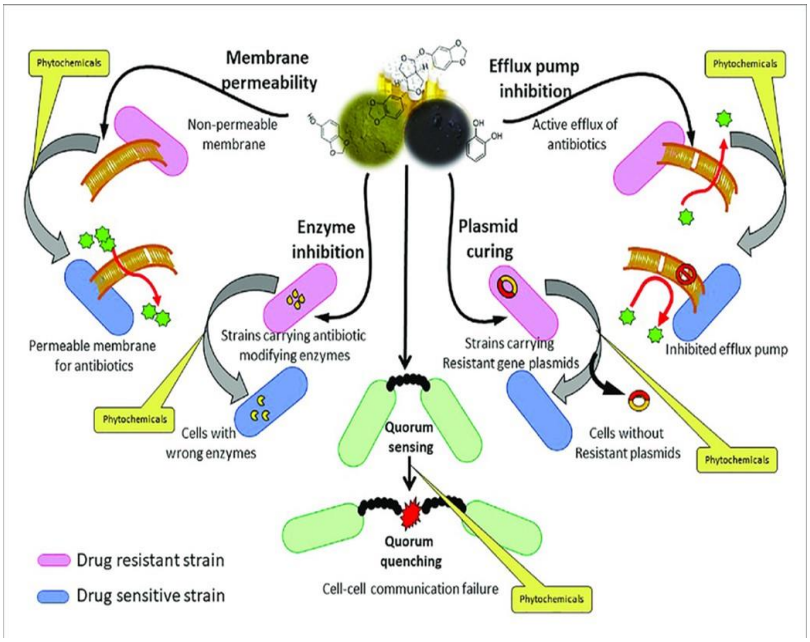

Figure 3. Phytochemicals involved in combating bacterial resistance [1]

2.4. Importance of Medicinal Plants

All human cultures have an ancient tradition of using plants as medicine. The usage of plants as a treatment model is increasing rapidly, and new products derived from these natural sources are entering the market daily. Consumers believe in safety and efficacy of these natural products other than traditional chemotherapeutic medicines [29].

Due to excessive usage of commercially available medications, multiple drug resistance (MDR) has been reported in human pathogenic bacterial species. Resistance is high for antibiotics in regions where they are misused. A prompt treatment with novel drugs with different mode of action is required for the treatment of these diseases. However, there are many scientific proofs that plants

are effective in treating different anomalies, therefore plants can be used to overcome multidrug resistance [30].

Plants produce complex secondary metabolites that have several biological activities. These secondary metabolites are found in many parts of the plants such as fruits, flowers, leaves, roots, stems. Anthraquinones and flavonoids present in plants have antibacterial potential towards many human pathogenic bacterial strains [29].

2.5. Medicinal Importance of Genus Rumex

Plants of the genus Rumex (family Polygonaceae) are traditionally used in medicine to cure multiple and complex microbial diseases such as enteritis, dysentery and bacteria causing skin infections.

Some rumex species such as are used for diseases treatments frequently. These species have anti-inflammatory and antimicrobial potential and are applied for curing dermatological infections [31]. Rumex species are easily available in India, China and Pakistan and applied as safe and cost-effective way to health conditions. Due to wide variety of climate zones, Pakistan has abundance of medicinal plants but only few are investigated so far [32].

2.6. *Rumex acetosella*

Rumex acetosella L. (Polygonaceace), is a perennial herb, commonly named sheep sorrel. It grows on fields, marshes and banks and is harvested during the spring season. It is a native species in Africa, Eurpoe and Asia. Leaves of this plant are consumed as salad because of the sour taste. Its anti-inflammatory, anticancer antibacterial activities are reported. In Pakistan, *Rumex acetosella* grows in northern hilly areas. Traditional applications of sheep sorrel include the treatments for gastrointestinal disorders, fevers, inflammation, and diarrhea [33].

Plants of Rumex genus have an abundance of secondary metabolites which have pharmacological activities. The leaves of R. acetosella have the highest number of flavonoids and phenolic content.

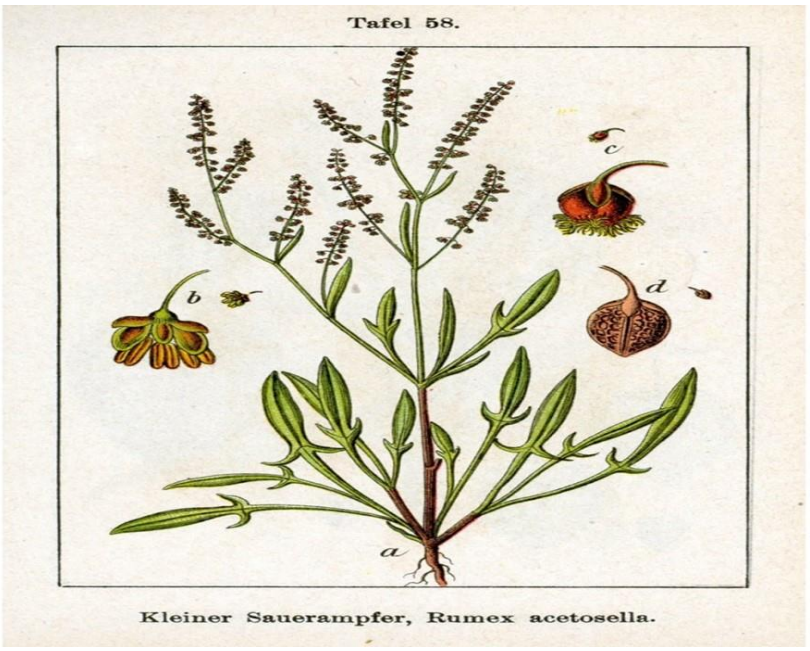

Figure 4. ***Rumex acetosella*** **plant**

Figure 5. ***Rumex acetosella*** **leaves**

2.7. Techniques for extract preparation and antimicrobial activity

2.7.1. Maceration

In this technique, grinded plant parts (leaves, stem, roots) are placed in a container and a solvent is added. The container is covered and placed in a dark place for 3-7 days at room temperature. The content is shaken from time to time for extraction. After the extraction, content is filtered followed by evaporation through rotary evaporator to concentrate the extract. This process is appropriate for plants which are prone to heat [34].

2.7.2. Well diffusion method

This method is used to determine antimicrobial activity of plant extracts. In this process inoculum is spread on agar plates and wells of 6mm were made. Specific concentration of plant extract is added in these holes and plates are incubated overnight at 37°C. plant extract will diffuse through the wells and restrict the growth of microbes[35].

2.7.3. Broth dilution method

One of the basic antimicrobial sensitivity testing procedures for determining MIC (Minimum Inhibitory Concentration) is macrodilution. The MIC is the lowest antimicrobial agent concentration that totally restricts organism growth in tubes or microdilution wells. The technique involves preparation of dilutions of the antibacterial compound in media supplied in tubes with a minimum volume of 2 ml. The tubes are then injected with a culture following dilution of standardized to 0.5 McFarland scale. The inoculation tubes are then incubated under suitable conditions depending on the test microorganism [14].

2.8. Hemolysis assay

Hemolysis is the bursting or lysis of red blood cells. Any agent that is responsible for the lysis of RBCs is called hemolysin. Hemolysis assay has two approaches. One is to check the cytotoxicity of the test compound and the second is to check the cytocompatibility of the drug delivery system. In this process, blood is incubated with the drug at the defined ph. After incubation, mixture is centrifuged at 5000rpm for 5minutes, and absorbance of the supernatant is measured at 550nm and compared with the positive control which should be detergent. For the desired results hemolysis should be negligible [36].

Drug induced cytotoxicity occurs by two methods, Toxic hemolysis, and Allergic hemolysis. In toxic hemolysis direct drug or its metabolites behaves as a toxin or hemolysin, while allergic hemolysis occurs due to immunological reaction in individuals who are sensitive to the drugs.

According to the U.S,FDA any compound which is intended to IV injection should be tested for its toxicity in vitro [37].

CHAPTER 3

3.0. Material and methods

3.1. Materials:

The plant *Rumex acetosella* leaves were gathered from district Bagh, AJK. The solvents and other chemicals were purchased from Sigma Aldrich®.

3.2. Methodology

3.2.1. Extract preparation

The leaves of the plants were used to prepare ethanolic and pure extracts as shown in figure 6. For ethanolic extract, accurately weighed 50 g of leaves were placed in a conical flask containing 100ml of 70% ethanol and kept at 25°C for 72 hours. The mixture was then centrifuged at 1000 RPM for 10 minutes to facilitate filtration. The Rotary evaporator was used to obtain the concentrated extract. The pure extract was prepared by grinding 100g of washed leaves using pestle and mortar. The crushed leaves were squeezed to get the pure extract. Both extracts were placed in the refrigerator.

3.2.2. Preliminary Phytochemical Analysis

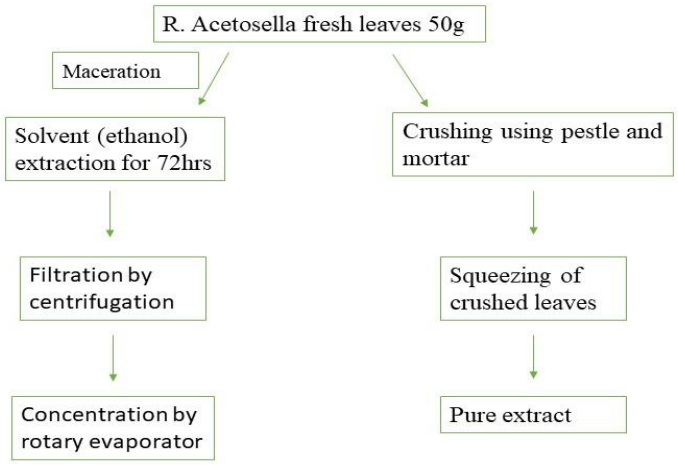

Figure 6. Extract preparation methodology

Different tests were performed to recognize the presence of the following constituents in ethanolic and pure extract:

3.2.2.1. Flavonoids

1ml of each extract was dissolved in 2ml of 1% NaOH solution to observe yellow color. A few drops of diluted sulphuric acid were added. Formation of colorless solution on addition of dilute sulphuric acid confirmed the flavonoids presence in the extracts.

3.2.2.2. Quinones

5ml of alcoholic KOH was added to 1ml of both extracts. The appearance of dark reddish coloration indicated the presence of quinones.

3.2.2.3. Saponins

The presence of saponins was confirmed by diluting the extracts with 20ml of water. After dilution extracts were shaken for 15 minutes. The appearance of 1cm foam layer indicated the presence of saponins.

3.2.2.4. Terpenoids

To 5ml of both extracts, 2ml of chloroform was added following the addition of concentrated H_2SO_4. Reddish brown colored interface assured the presence of terpenoids.

3.2.2.5. Tannins

Formation of white precipitates by addition of 1% gelatin solution in both extracts showed the presence of tannins.

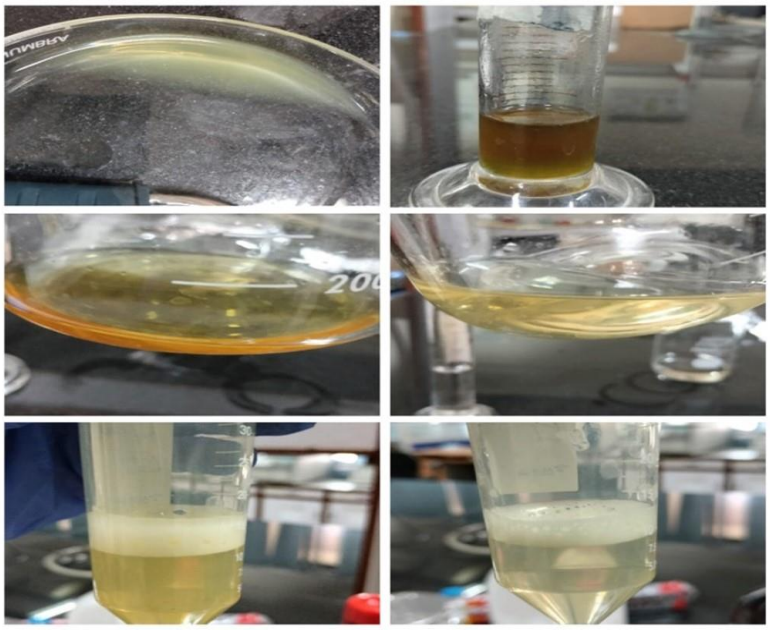

Figure 7. Qualitative tests for preliminary screening

3.2.3. Thin layer chromatography

To identify polar or non-polar nature of crude plant extracts, thin layer chromatography (TLC) was executed on (20×20cm) aluminuim sheets of silica gel plate 60 F254. On a TLC plate, all crude extract samples were spotted. The TLC plate was placed in a closed jar and a small volume of mobile phase was added in the container. TLC experiment was performed to investigate 100µg of the extract positioned in a band of 1 cm, utilizing three solvent systems of differing polarities. The solvent systems consumed as mobile phase were:

1. Hexane: Ethyl Acetate
2. Methanol: Chloroform
3. Ethyl Acetate: Chloroform

TLC plates were sprayed with KMNO4 to examine the bands.

Retention factor was measured using he formula

Retention factor (Rf) = Distance travelled by a solute / Distance travelled by a solvent

3.2.4. UV-VIS Analysis

UV-VIS Spectrophotometer was used to perform UV-VIS analysis of ethanolic and pure extract of the R. acetosella plant for the identification of phytoconstituents. The extracts were analyzed for the wavelength ranging from 200 -600nm under UV and visible light.

3.2.5. Gas chromatography and Mass Spectrometry (GC-MS) analysis

GC-MS analysis was conducted to recognize the active components and chemical makeup of the R. acetosella plant extract. GC-MS Shimadzu QP-2020 with column specs: Shimadzu SH-Rxi-5Sil MS and dimensions (L=30M, ID=0.25, DF=0.25) was used to perform GC-MS analysis of the extract. Methanol was used as solvent. The identification of constituents was done by comparing unknown peaks with known peaks present in NIST (National Institute of Standards and Technology) library.

3.2.6. Antibacterial Susceptibility testing (AST) of the Plant Extract

For performing antibacterial susceptibility test, two bacterial strains Staphylococcus aureus and Escherichia coli were used.

3.2.6.1. Concentrations of extracts

Well diffusion assay at the concentration ranges from 1400ug/ml, 1200ug/ml, 1000ug/ml, 800ug/ml for ethanolic extract and concentration ranges from 50ul, 40ul, 30ul, 20ul, 10ul for pure extract was performed.

3.2.6.2. Selection of Bacterial Strains

Two human pathogenic bacterial strains were selected and obtained from UG Plant laboratory, ASAB, NUST. These include *Escherichia coli* and *Staphylococcus aureus*. All these strains are pathogenic and cause serious infections in human beings.

3.2.6.3. Preparation of Fresh Culture

At first fresh culture of bacteria was grown in Lauria Broth media (LB media).

3.2.6.4. Preparation of LB media

To make 400 ml LB media take a glass test bottle of 500 ml and add 4g NaCl, 4 g tryptone, 2 g Yeast extract, and 400 ml distilled water. We can also use premixed Lb agar powder if available in the laboratory. Loosely close the cap and cover it with aluminum foil. Then autoclave the media and allow it to cool at room temperature.

3.2.6.5. Inoculation of Pure Colony

Pour the required amount of LB media into the test tube. Select a pure colony and through a sterile tip or toothpick and inoculate it along with antibiotic in the test tube containing LB media. Cover the test tube with aluminum foil and incubate the bacterial culture at 37°C in the shaking incubator for 12-18 hours. After incubation growth can be observed by cloudy haze in the test tube.

3.2.6.6. Preparation of Mueller Hilton Agar

Mueller Hilton agar was used for the growth of bacteria on petri plates. 200 ml Mueller Hilton agar was prepared in a glass bottle by adding 7.6g agar in 200 ml distilled water. Adjust the pH to 7.4 and autoclave the media.

3.2.6.7. Pouring

Autoclaved media is poured in the petri plates in sterile conditions in the safety cabinet. First, make the environment sterile in the safety cabinet by illuminating the UV light and make it clean by 70% ethanol. Then pour the media in the petri plates to allow it to solidify. After solidification of the media in the petri plates incubate these plates at 37°C overnight in the incubator to determine the contamination if any.

3.2.6.8. Well diffusion method

For this purpose, 10ml of Mueller- Hilton Agar (MHA) was surged into autoclaved petri plates by subsequent addition of each bacterial strains. The wells of 6mm were bored and filled with different concentrations of extracts. The plates were kept in incubator overnight at 37°C and the zones were calculated. The entire experiment was performed in an aseptic environment.

3.2.6.9 Broth dilution for MIC determination

For the determining minimum inhibitory concentrations of the extracts, inoculum was prepared by subculturing bacterial strains overnight at 37°C. Culture was diluted to adjust to 0.5 McFarland scale and tubes containing liquid growth medium were inoculated with the culture. Different concentrations of ethanolic extract (800ug/ml, 1000ug/ml, 1200ug/ml, 1400ug/ml) and pure extract (10ul, 20ul, 30ul, 40ul, 50ul) were added in the tubes. A tube containing only growth medium was used as negative control while positive control was the tube containing bacterial culture and growth media. Tubes were under incubation overnight at 37°C. Optical density was measured by spectrophotometer and samples from the tubes were spread on the agar plates for CFU (Colony Forming Unit) measurement. Agar plates were placed in incubator for 24hrs. at 37°C and colonies were counted through colony counter. Both MIC and MBC were determined.

3.2.8. RBC hemolysis

Cytotoxicity testing of the drug was evaluated by RBC hemolysis assay. For this 4ml of human blood was taken in EDTA tube and mixed with 8ml of PBS buffer solution. The centrifugation was performed for mixture at 10000 RPM for 10 min at 4°C. The supernatant was removed, and RBCs were collected as pellets. The Pellet was then washed with PBS three times at 10000 RPM for 3 min. The RBCs obtained were diluted with PBS and different conc. of both extracts (ethanolic and pure) were added with 1ml of diluted RBCs in different reaction tubes. PBS with diluted RBCs was used as negative control and 1% triton X-100 with diluted RBCs was used as positive control. Reaction mixtures were incubated for 3 hrs. at 37°C. Following incubation, the reaction mixtures were vortexed, and centrifugation occur at 10000 RPM for 5 min. The absorbance of reaction mixtures was measured by UV spectrophotometer at 550 nm. The hemolysis percentage was obtained by using the formulae:

$$\% \text{ Hemolysis} = \frac{\text{Sample absorbance} - \text{Negative control absorbance}}{\text{Positive control absorbance} - \text{Negative control absorbance}} \times 100$$

CHAPTER 4

4.0. Results

4.1. Plant extraction yield

Ethanolic extract of 50g fresh leaves of Rumex acetosella yield 1g of the extract. A 30mg/ml stock solution was prepared from 1g of ethanolic extract in 70% ethanol. 100g of freshly grinded leaves yielded 45ml of pure extract.

4.2. Preliminary phytochemical testing

Phytochemical testing confirmed the presence of flavonoids, quinones, saponins and tannins in both extracts while terpenoids were absent in pure extract as shown in table 1.

Table 1.Presence of phytoconstituents in plant extracts

Sr. #	Constituents	Ethanolic Extract	Pure Extract
01	Flavonoids	+	+
02	Quinones	+	+
03	Saponins	+	+
04	Terpenoids	+	-
05	Tannins	+	+

These constituents have been reported to have certain pharmacological activities such as anti-microbial, anti-inflammatory, wound healing, antioxidant, and anti-cancer activities.

4.3. Thin layer chromatography

TLC is performed using different solvents from less polarity to high polarity using hexane, ethyl acetate, chloroform, and methanol. Rf values 0.6, 0.7, 0.8 indicated the presence of flavonoids, terpenoids, tannins and phenolic compounds. Bands are shown in figure 8.

Figure 8. TLC sheets showing bands

4.4. UV-VIS Analysis

UV-VIS analysis was performed for wavelength range of 200-600nm. Both the extracts showed absorbance at 250nm and 500nm which indicates that flavonoids, phenols, tannins, and other phytochemicals are present. Ethanolic extract showed maximum absorbance at 300nm while pure showed at 200-400nm as shown in figure 9 and 10.

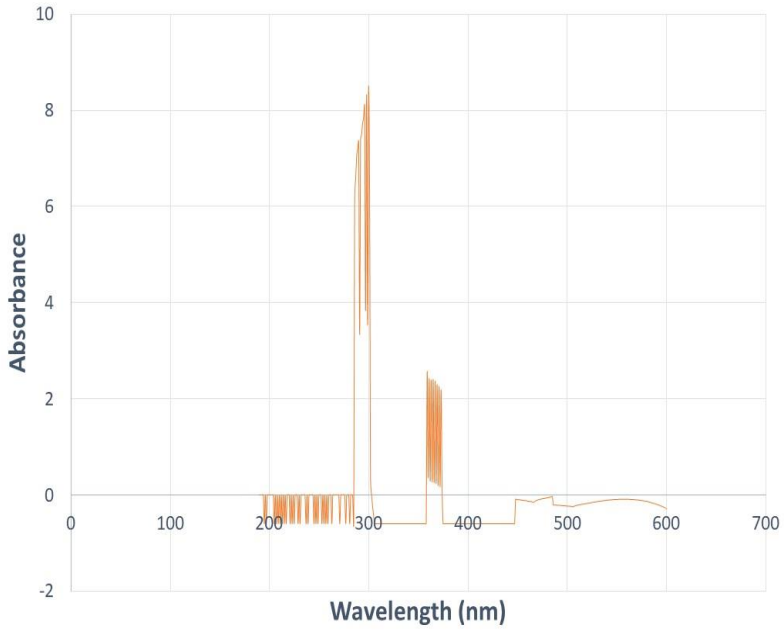

Figure 9. UV-VIS analysis of ethanolic extract

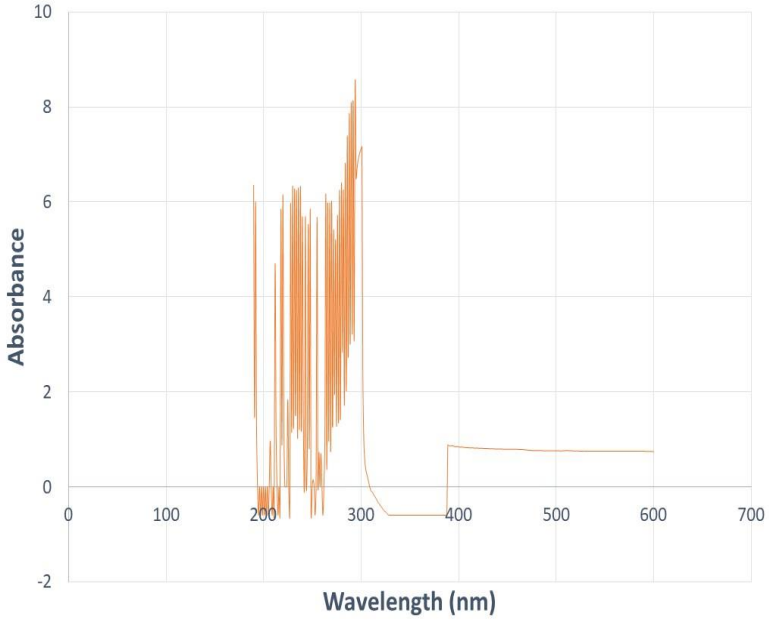

Figure 10. UV-VIS analysis of pure extract

4.5. GC-MS Analysis

A total of 26 compounds were recognized in ethanolic extract of *Rumex acetosella* as shown in figure 11. The molecular name, molecular formula, and molecular weight are shown in table 2. GC-MS analysis confirmed the existence of compounds with pharmacological activities.

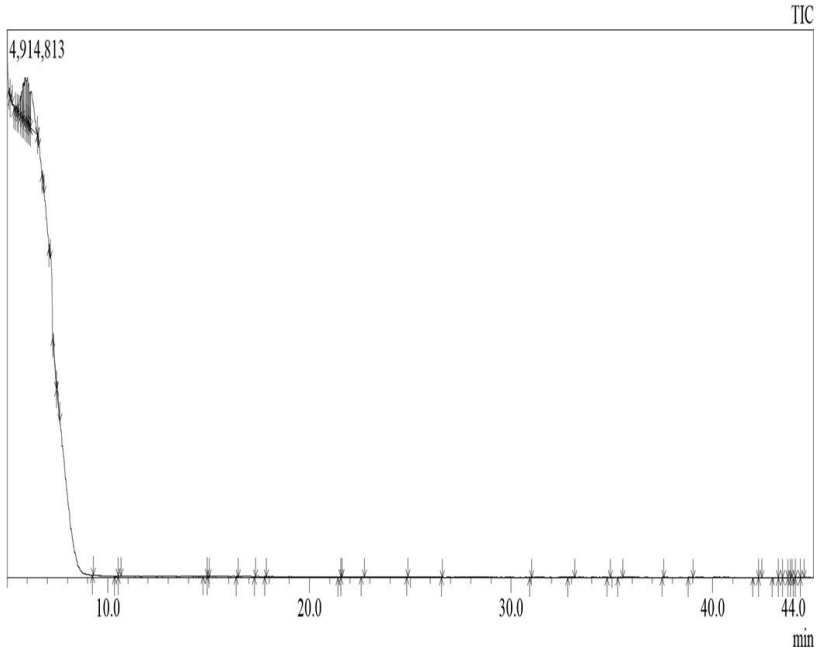

Figure 11. GC-MS chromatogram of ethanolic extract

Table 2.Compunds identified in ethanolic extract through GCMS

Sr.	Name of compound	Molecular formula	Molecular weight
1	1,2-Propadiene-1,3-dione	C3O2	68
2	1,2,5-Oxadiazole	C2H2N2O	70
3	3-Butyn-1-ol	C4H6O	70
4	Propyne	C3H4	40
5	Borane carbonyl	CH3BO	42
6	Allene	C3H4	40
7	3-Butynoic acid	C4H4O2	84
8	Iodoacetonitrile	C2H2IN	167
9	2-Cyanosuccinonitrile	C5H3N3	105

10	4-Spirohexanone, 5,5-dichloro	C6H6Cl2O	164
11	1,3,5-Triazine, 2,4,6-tris(cyanomethoxy)-	C9H6N6O3	246
12	Undecanoic acid, 10-bromo	C11H21BrO2	264
13	Benzamide, 4-butoxy-N-[2-(2-thienyl) ethyl]-	C17H21NO2S	303
14	Benzaldehyde, 3-methoxy-4-[(trimethylsilyl)oxy]-, O-methyloxime	C12H19NO3Si	253
15	D-Glucuronic acid, gamma.-lactone	C6H8O6	176
16	D-Glucopyranuronic acid	C6H10O7	194
17	4-[4-Aminopentylamino]-2-methoxy-9-methylacridine	C20H25N3O	323
18	4-Methoxy-6-morpholin-4-yl- [1,3,5]triazine-2-carboxylic acid amide	C9H13N5O3	239
19	3-Acetyl-9-isopropylcarbazole	C17H17NO	251
20	(. +/-.)-Uleine	C18H22N2	266
21	1,2,3,4-Tetrahydro-1-methyl-6-phenyl-1,5-benzodiazocin-2-one	C17H16N2O	264
22	3-Isoxazolecarboperoxoic acid, 4,5-dihydro-5-phenyl-, 1,1-dimethylethyl ester	C14H17NO4	263
23	5-Hydroxymethyluracil, 3TMS derivative	C14H30N2O3Si3	358

24	Levorphanol, TMS derivative	C20H31NOSi	329
25	Ethyl homovanillate, TMS derivative	C14H22O4Si	282
26	2-Amino-5-oxo-6,7-dihydro-5H-cyclopenta[b]pyran-3,4,4-tricarbonitrile	C11H6N4O2	226

4.6. Antimicrobial susceptibility testing results

Both ethanolic and pure extract has demonstrated antibacterial sensitivity against *E. coli* and *S. aureus*. Activity was different at different concentrations of the extracts as I had conducted dose dependent analysis and it also varies with bacterial strains. Below are the results of dose dependent analysis through well diffusion method.

4.6.1. Antibacterial activity of ethanolic extract

Ethanolic extract has noticeably shown activity towards *Staphylococcus aureus* with maximum inhibition zone of 24mm at 1400ug/ml while minimum zone of 11mm at 800ug/ml whereas less sensitivity towards *Escherichia coli* with maximum inhibition zone of 10mm at 1400ug/ml as shown in figure 12 and 13. Zones of inhibition are shown in table 3.

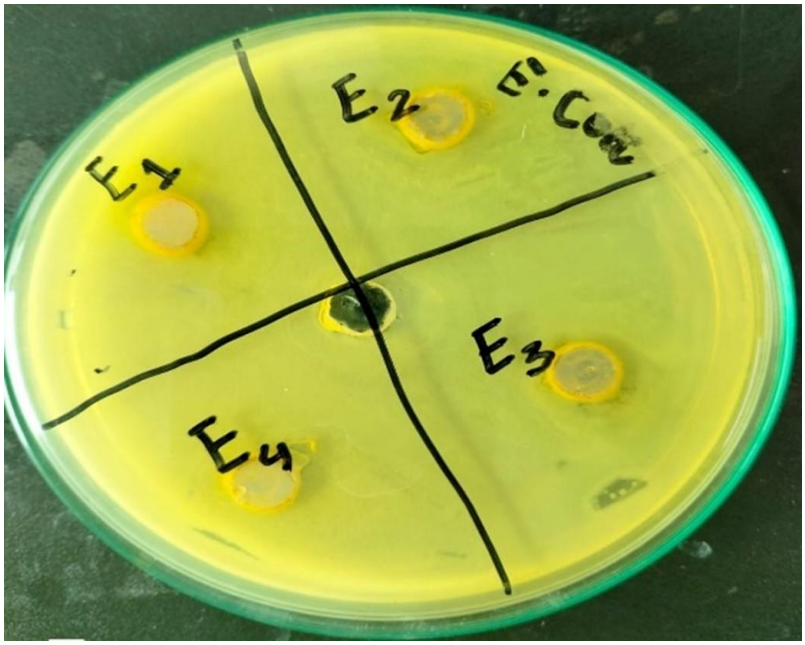

Figure 12. Well diffusion applied on *E. coli* for ethanolic extract

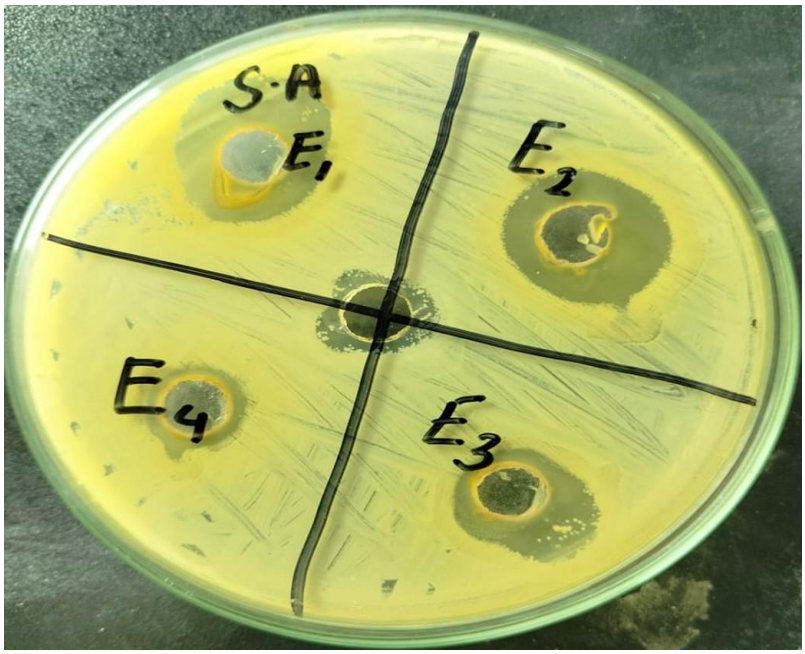

Figure 13. Well diffusion applied on *S. aureus* for ethanolic extract

4.6.2. Antibacterial activity of pure extract

Pure extract has shown sensitivity towards both bacterial strains. The maximum inhibition zones of pure extract against *S. aureus* were 23mm at 50ul and 19mm at 50ul was shown against *E. coli*. The minimum inhibition zone against *S. aureus* was 7mm at 10ul and 8mm at 20ul against *E. coli* as shown in figure 14 and 15. Zones of inhibition are shown in table 3.

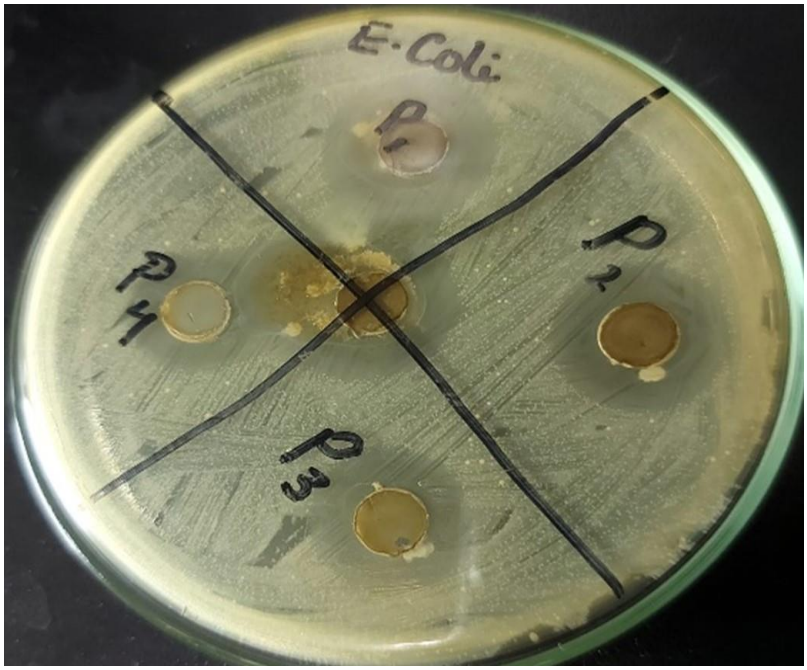

Figure 14. Well diffusion applied on *E. coli* for pure extract

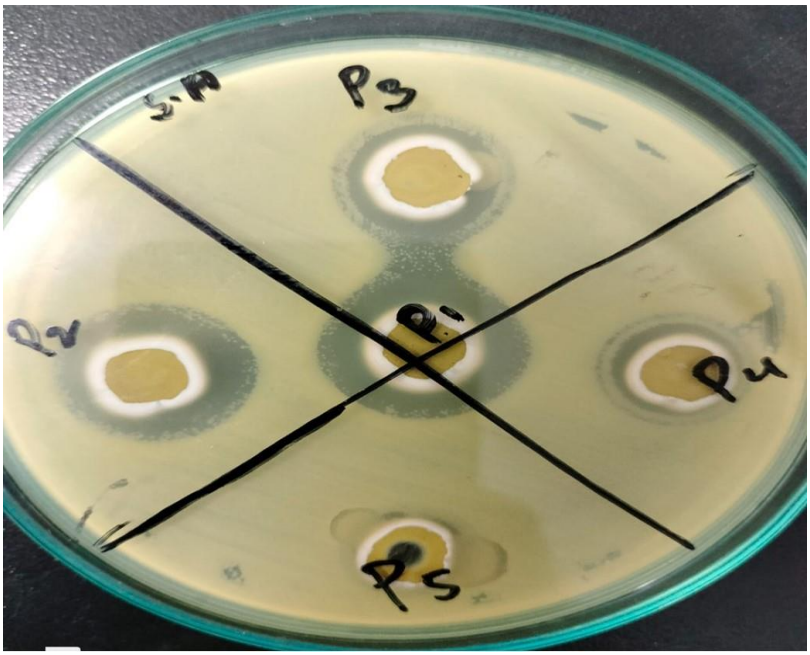

Figure 15. Well diffusion applied on *S. aureus* for pure extract

Table 3. Inhibition zones of ethanolic and pure extract against *S. aureus* and *E. coli*

Extracts	Concentrations	Zones against *S. AUREUS* (mm)	Zones against *E. COLI* (mm)
Ethanolic extract (ug/ml)	800ug/ml	11±0.2	0±0.0
	1000ug/ml	18±0.23	6±0.40
	1200ug/ml	20±0.3	9±0.51
	1400ug/ml	24±0.43	10±0.63
Pure extract (ul)	10ul	7±0.36	0±0.0
	20ul	15±0.51	8±0.47
	30ul	18±0.54	10±0.38
	40ul	20±0.62	14±0.57
	50ul	23±0.67	19±0.68

4.6.3. Dose dependent analysis of antibacterial activity

Dose dependent analysis for ethanolic extract and pure extract is shown in figures 16 and 17 respectively.

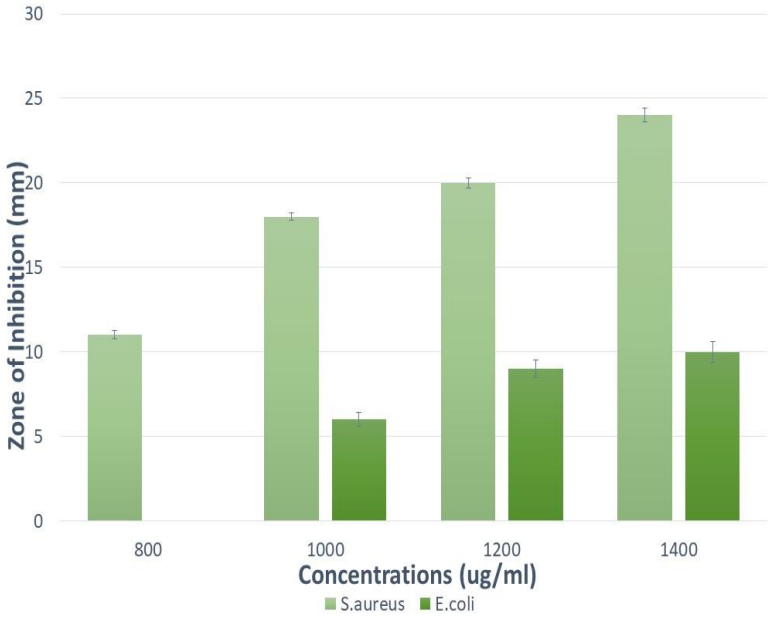

Figure 16. Graph showing inhibition zones of ethanolic extract against S.*aureus* and *E.coli*

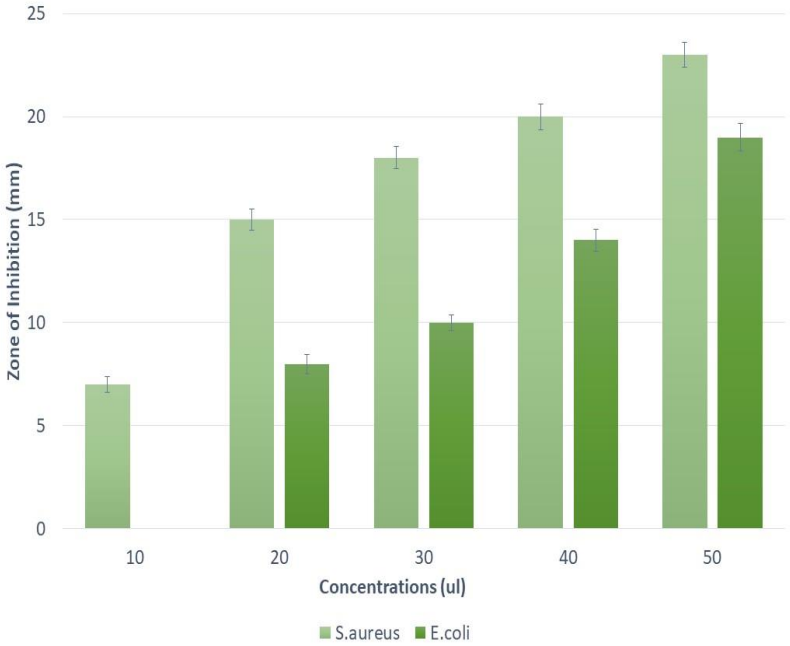

Figure 17. Graph showing inhibition zones of pure extract against *S. aureus* and *E.coli*

4.7. MIC and MBC determination

MIC and MBC determination was done through broth dilution assay. MIC of ethanolic extract against *S. aureus* was observed as 800ug/ml whereas MBC was 1400ug/ml. Ethanolic extract was less sensitive towards *E. coli.* MIC of pure extract against *S. aureus* was 10ul while 20ul for *E. coli* and MBC was shown as 30ul for *S. aureus* and 40ul for *E. coli*. The analysis of MICs and MBCs proposed the effectiveness of extracts to prevent and cure infections.

4.8. RBC Hemolysis results

The same concentrations of the extracts were used for the hemolysis assay. These are the following, 800ug/ml, 1000ug/ml, 1200ug/ml, 1400ug/ml for ethanolic extract and 10ul, 20ul, 30ul, 40ul, 50ul for pure extract. Ethanolic extract has less toxic effect even at higher concentrations while pure extract showed increased toxicity at higher concentrations. A compound with 50% or greater toxicity is considered toxic. Figure 18 to 20 shows hemolysis by control and extracts while figure 21 and 22 shows present hemolytic activity of ethanolic and pure extracts.

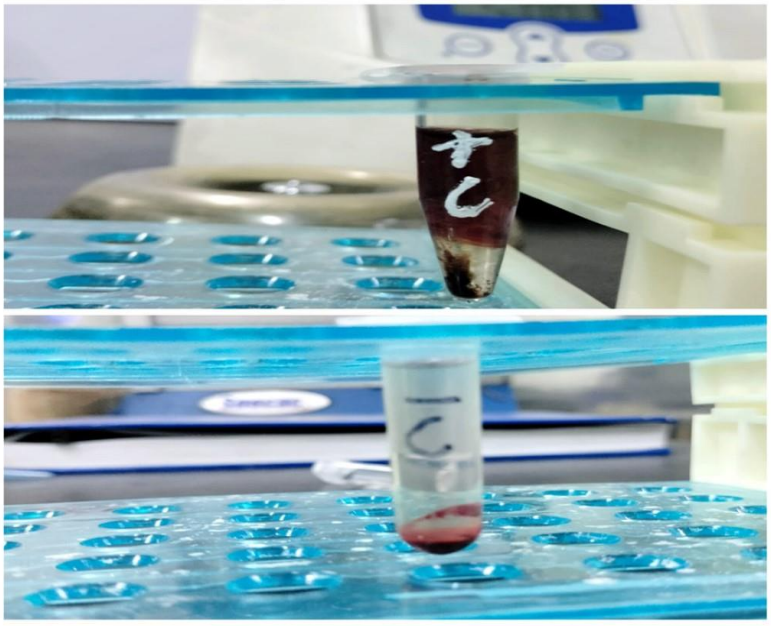

Figure 19. +ive and -ive controls for RBC hemolysis

Figure 18. RBC hemolysis by ethanolic extract

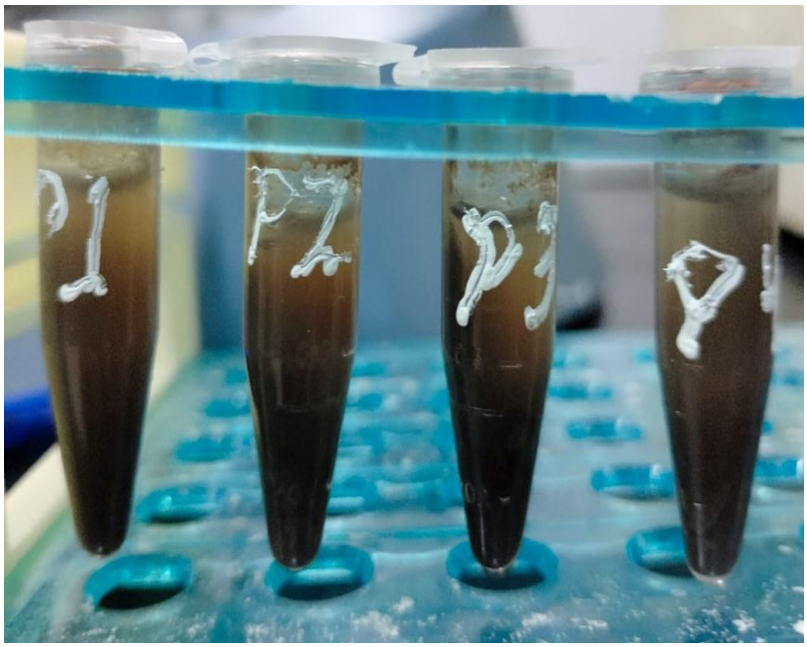

Figure 20. RBC hemolysis by pure extract

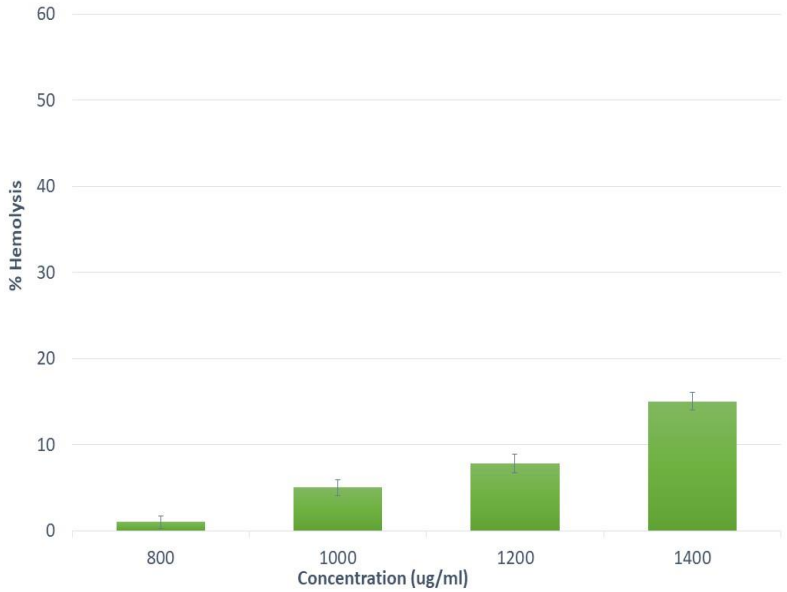

Figure 21. Graph showing % hemolysis by ethanolic extract

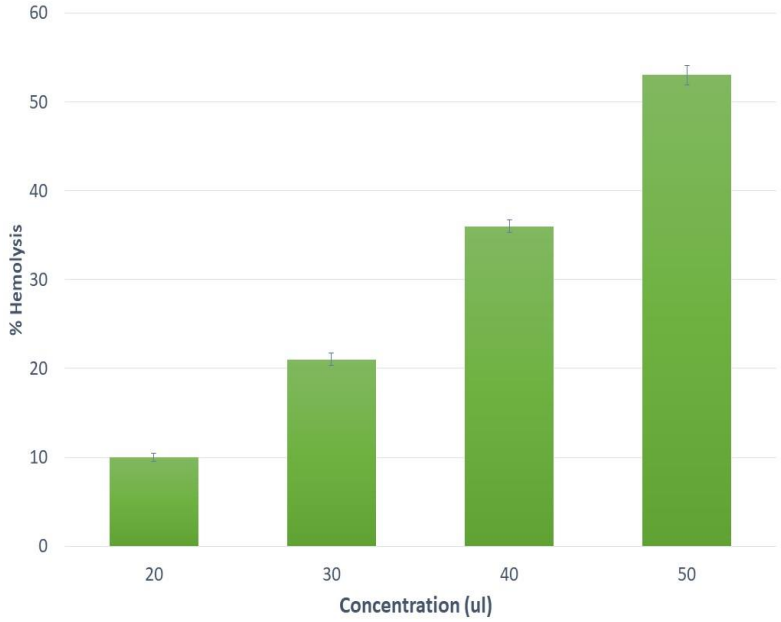

Figure 22. Graph showing % hemolysis by pure extract

CHAPTER 5

5.0. Discussion

A human pathogen is a microbe that causes diseases in human beings. Pathogen could be a virus, bacteria, fungi, or parasites. The history of human health has been greatly compromised by infectious diseases caused by bacterial infections [3]. Along with the antibiotics there are the various ways to treat the infectious diseases which includes, non-antibiotic drugs, killing factors, phages, antibacterial activities, quorum quenching, and bacteriocins [38]. In many parts of the world, *Rumex spp.* have been used as folk medicine for centuries. *Rumex acetosella* is used as an antibacterial in different types of infections in regions of AJK.

In the current scenario, ethanolic and pure extract was prepared by maceration technique using fresh leaves of *R. acetosella.* Extracts were further fractionated by thin layer chromatography (TLC) using hexane, ethyl acetate, chloroform, and methanol solvents. Ethanolic extract and pure extract of *R. acetosella* was tested for antibacterial testing against *S. aureus* and *E. coli* along with phytochemical analysis. The qualitative tests were performed to confirm the presence of flavonoids, quinones, saponins, tannins and terpenoids. All these constituents were present in ethanolic extract except for terpenoids which were absent in pure extract. UV-VIS spectroscopy was carried out at wavelength range of 200-600nm, and both the extracts absorbed wavelengths at 250-500nm. Ethanolic extract showed maximum absorbance at 300nm while pure extract had maximum absorbance at 200-400nm. Flavonoids, phenols, tannins and other secondary metabolites are reported to absorb wavelength between 250-500nm [39].

GCMS analysis of ethanolic fraction showed presence of 27 different compounds, 12 of which have pharmacological activities. 1,2-Propadiene-1,3-dione, 1,2,5-Oxadiazole, 4-Spirohexanone, 5,5-dichloro, 1,3,5-Triazine, 2,4,6-tris(cyanomethoxy)-, 4-[4-Aminopentylamino]-2-methoxy-9-methylacridine, 3-Acetyl-9-isopropylcarbazole and are the phytochemicals present in ethanolic extract which are reported to have antimicrobial, anti-inflammatory and other pharmacological activities.

Antimicrobial susceptibility testing (AST) was applied on *S. aureus* and *E. coli* bacterial strains. Ethanolic extract has depicted stronger antibacterial activity towards *S. aureus* but less sensitivity towards *E. coli.* while pure extract was sensitive towards both bacterial strains (*S. aureus* and *E. coli)* at higher concentrations as compared to ethanolic extract. MIC for ethanolic extract was 800ug/ml and 10ul for pure extract against *S. aureus*.

Mostly active plants are safe at lower concentration but these become toxic at higher concentration [36]. According to the hemolysis assay any compound which has toxicity 50% or greater than this is considered toxic. Cytotoxicity testing was performed using hemolysis assay for different concentrations of ethanolic and pure extract as used for AST. RBC hemolysis showed less toxicity by ethanolic extract even at higher concentration while pure extract showed increased toxicity with increase in concentration.

As a result of this study, it can be concluded that both the extracts screened here presented inhibitory potential towards bacterial strains in dose dependent analysis and ethanolic extract has less toxicity as compared to pure extract. Hence, they can be investigated further in the management of infectious diseases.

Conclusion

Pakistani flora has exhibited to be a rich resource for antibacterial agents. Infections are commonly caused by different bacterial strains. The extracts of *Rumex acetosella* were proved to have a great potential as antibiotic. Qualitative analysis confirmed the presence of pharmacologically active constituents in ethanolic and pure extracts of *Rumex acetosella* leaves. Ethanolic and pure extract both have shown antibacterial potential and can be employed as natural alternative to antibiotics in preventing infections, mostly wound infections. RBC hemolysis further affirmed that ethanolic extract has less toxicity as compared to pure extract.

Future prospects

For antibacterial activity, a huge number of species of plants from various parts of the world have been investigated. There is still a necessity to explore the nature for newer effective antibacterial agents with minimum side effects. Rumex acetosella plant extracts have shown antibacterial potential towards bacterial strains. Further studies can be formulated for its application in the treatment of infections. As it was traditionally used in wound infections, further invitro and in vivo investigations are required to evaluate its wound healing potential. New formulations with other antibiotics can also be made to further boost its effect. As the extracts have less toxic effect on blood at lower concentration it might be used as dietary medicine after further research.

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