STUDY OF ANTIMICROBIAL PROPERTIES OF HIMALAYAN FLORA AGAINST PATHOGENIC BACTERIAL SPECIES

Dissertation submitted in partial fulfillment of the requirement for the degree of

BACHELOR OF TECHNOLOGY

IN

BIOTECHNOLOGY

By

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UNDER THE GUIDANCE OF

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the B.Tech thesis entitled **"Study of antimicrobial properties of Himalayan flora against pathogenic bacterial species"** submitted at **Jaypee University of Information Technology, Waknaghat, India**, is an authentic record of work done by me (Sophia Puri-151847) and my project partner Saesha Verma (151851) for the odd semester (July 2018-December 2018) carried out under the supervision of **Dr. Rahul Shrivastava** (Associate Professor) Department of Biotechnology and Bioinformatics. I have not submitted this work elsewhere for any other degree or diploma.

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Date:

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the B.Tech thesis entitled **"Study of antimicrobial properties of Himalayan flora against pathogenic bacterial species",** submitted by **Sophia Puri (151847) at Jaypee University of Information Technology, Waknaghat, India,** is a bonafide record of her original work carried out under my supervision. Saesha Verma (151851) was also part of the project for odd semester i.e. from July 2018 till December 2018. This work has not been submitted elsewhere for any other degree or diploma.

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I bow my head before the **Almighty God** whose blessing gave me the strength to make this successful venture and I dedicate my work and achievement in his lotus feet.

Sophia Puri (151847)

LIST OF SYMBOLS AND ACRONYMS

LB	Luria-Bertani Broth
NAT	Nutrient Agar Tween
LBGT	Luria-Bertani Broth Glycerol Tween
MB7H9	Middlebrook 7H9
µl	Micro-litre
µg/ml	Microgram per Millilitre
mg/ml	Milligram per Millilitre
rpm	Revolutions per minute
ºC	Degree Celsius

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ABSTRACT

The development of antibiotic resistant bacteria stems from a number of factors, including inappropriate use of antibiotics in human and animal health and their prolonged use as growth promoters at sub-clinical doses in poultry and livestock production. Bacteria has become a pathogen of interest because of increasing cases of human infections caused by them. As bacterial strains are now becoming resistant to most of the antibiotics, it is very important to develop alternative methods in order to stop them. Recently, a lot of attention has been focused on production plant extracts that can serve as antimicrobial and antimycobacterial agents.

Objective of our work was to scrutinize herbal plants that could be convenient in safeguarding humans or animals against *Mycobacterium smegmatis* and *Escherichia coli.* Objective of our work was to explore activity of vivid flora found in Himalayan region for screening against pathogenic bacteria and to prepare an alternative for increased mycobacterial and pathogenic bacterial resistance along with the determination of plant species which could serve as potential anti-mycobacterial and anti-diarrheagenic agents.

We decided to work on extracts of leaves and roots of *Valariana jatamansi* and leaves of *Urtica dioica* plant species with good antimicrobial and antioxidant properties. Extracts of leaves and roots of *V. jatamansi* and *U. dioica* were extracted with mixture of hexane, chloroform and methanol in 1:1:1 ratio and 95% ethyl acetate respectively and aqueous extracts were prepared afterwards and their minimal inhibitory concentration (MIC) values were determined using a microtitre plate serial dilution technique and macrobroth dilution technique against *E. coli* and *M. smegmatis*.

CHAPTER 1
INTRODUCTION

The Himalayan region represents an attractive source of potential therapeutic agents derived from a number of species of medicinal herbs and plants found in this region. Advent of newer technologies, changing lifestyle and related health risks have helped the drug and medicine industries to grow exponentially. In numerous scientific investigation, the maximum therapeutic and minimum side effects of natural remedies have been verified. The Himalayan region harbors a good number of medicinal plants that are repeatedly used in traditional food, Unani system, Ayurveda and herbal industries. Himalayan region consists of many rare and high medicinal plants like *Aconitum heterophyllum, Picorrhiza kurroa, Costus speciosus* and *Valeriana sp.*

Plant materials continue to play a major role in primary health care as therapeutic remedies including anti-oxidant, anti-inflammatory, and other biological properties in many developing countries. Nowadays, a lot of attention has been focused on screening the herbal plants against various diseases to find their respective cure. Since naturally derived compounds from plants are good sources for the treatment of chronic diseases, various companies and industries work on these Himalayan herbs in large scale production of the extracted compound to use them for drug screening and also now various methods like extraction are used by the students in their minor and major projects to study the different pharmacological properties of plants that can be of any medicinal use. In this research determination of minimum inhibitory concentration is used as a procedure for drug screening.

A greek physician once stated “let food be your medicine” and this was the concept behind bringing nutraceuticals into limelight. Nutraceutical is defined as any food source derived product with extra health benefits, it consists of dietary supplements, herbal products, probiotics, prebiotics that avoid side effects and helps us live longer, and help us to avoid particular medical conditions. Nowadays, probiotics are also used widely which are the live microbes that includes some selected strains of lactic acid bacteria and Bifidobacteria spp., which when consumed may provide a health benefit to the person. Regular consumption of probiotics may promote the immune system, decline serum cholesterol, lessen diarrheal

incidences and may control infections caused by pathogens. Prebiotics are the food for probiotics and are defined as the food ingredients that are comprised of oligosaccharides which are not digested by the consumer. In this study extracts were prepared and the concentrations were made and the particular concentration was used to check the activity of the extracts against organisms like *Mycobacterium smegmatis* and *Escherichia coli.*

Diseases are the major cause of death in the developing countries and bacterial resistance poses a serious threat to health of people. As bacterial strains are now becoming resistant to most of the antibiotics, it is very important to come up with alternative methods in order to stop them. Recently, a lot of attention has been focused on production plant extracts that could serve as various antimicrobial and antimycobacterial agents. Nature has been a source of restorative agents for thousands of years and a wide range of modern drugs have been isolated from natural sources. [22]. Many extracts have been prepared to check their activity against various microorganisms causing diseases.

CHAPTER 2
REVIEW OF LITERATURE

Mycobacterium

Mycobacterium is a genus of order Actinobacteria belonging to the family Mycobacteriaceae. The genus consists of more than 190 species [1]. It gets the name myco meaning fungus due to its mold like growth. Due to the presence of mycolic acid in its cell wall and the distinctive staining characteristics under the microscope, these are called acid fast bacteria. The genus mycobacteria can be predominantly classified into *M. tuberculosis* complex (MTC) and a vast group of Nontuberculous mycobacteria (NTM).

MTC comprises of pathogenic mycobacteria like *M. tuberculosis, M. orygis,* and *M. africanum.* These are chiefly liable for causing tuberculosis in humans including other living beings. Non-tuberculous mycobacteria (NTM) are atypical mycobacteria that are non-motile slender bacilli that are inadequate to form spores or cause tuberculosis. They are expedient aerobic free living microorganisms exhibiting symbiotic, commensally and saprophytic behavior and are found ubiquitously in water sources, dust, soil, food, air particles, and animals [2].

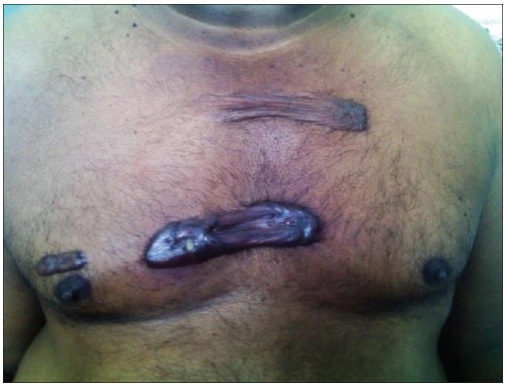
NTM consists of environmental micro-organisms that include around 186 distinctive mycobacterium species [3]. These are extensively arranged into rapidly and slow growing mycobacteria. “Fast growing mycobacteria produces mature growth on media plates within 7 days and comprises of approximately 50% of the approved mycobacterial species that are further divided into six major groups viz. *M. chelonae/M. abscessus* complex, *Mycobacterium fortuitum* group, *M. mucogenicum* group, *M. smegmatis* group, *M. mageritense/M. wolinskyi*, and the pigmented fast growing mycobacteria” [4]. For maximum growth slow growing mycobacteria require more than a week. The group comprises of *M. xenopi, M. marinum, M. avium, M. kansasi and M. intracellulare* [5].

Mycobacterium fortuitum

Mycobacterium fortuitum (formerly known as *Mycobacterium ranae*), belonging to the fast growing NTM was originally isolated from frogs in 1905. It is an opportunistic pathogen generally influencing immunocompetenttpatients and individuals receiving glucocorticoid therapy or having impaired cellular immunity[5]. Resistance of the mycobacterium towards disinfectants like chlorine, ozone and ultraviolet make it more difficult to kill [6].

Infections caused by *Mycobacterium fortuitum*

194.2% increase in occurrence of *M. fortuitum* has been reported in USA from the year 1994 to 2014 [3]. These includes Keratitis, Granulomatous Lobular Mastitis, Nail Salon Footbath-Associated Folliculitis, Anti-TNF-α Therapy-Associated Infections, IFN-γ/IL-12-Associated Infections, Localized Post traumatic Wound Infections, Surgical Wound Infections, Catheter Related Infections, Disseminated Cutaneous Infections, Chronic Pulmonary Infections, CNS Infections.



Fi. 2.1: Clinical photograph of multiple keloid lesions on the chest [18]

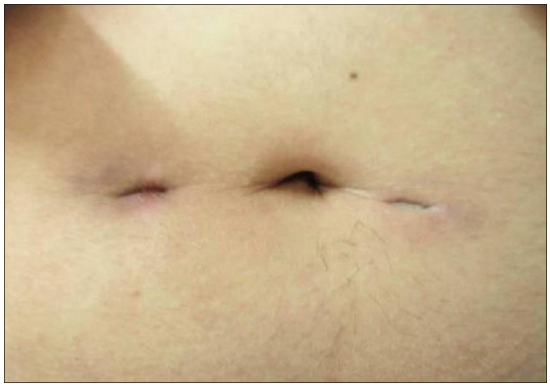


Fig. 2.2: Discharging sinus in right due to *M. fortuitum* and the healing incision wound, [21]

Treatment

The therapeutic regime for treatment of *M. fortuitum* infections includes use of trimethoprim-sulfamethoxazole, imipenem, amikacin, moxifloxacin, levofloxacin, cefoxitin, ciprofloxacin, tigecycline, , doxycycline (50%), clarithromycin (80%) and linezolid (86%) [4].

M. fortuitum also shows increased inherent aversion towards large number of antimicrobials. Some of new drugs having better potency than older drugs are tabulated below [7]:

Table 2.1: Advanced therapeutic drugs for *M. fortuitum* treatment

Drug	Mode of action	Minimum Inhibitory Concentration (MIC) (ug/ml)
DC- 159a	Inhibits supercoiling of DNA gyrase.	0.25
Pretomanid (PA-824)	Inhibits cell wall formation and leads to respiratory poisoning.	>100
Delamanid	Inhibits synthesis of mycolic acid.	>100
Bedaquiline	Targets ATP synthase.	0.13-0.25
Tigecycline	Protein synthesis inhibitor.	=<0.03–0.5
TP-271	Inhibits transcription/translation.	0.06

Mycobacterium smegmatis

M. smegmatis is generally considered non-pathogenic, although it does have finite propensity to outlast and multiply inside macrophages. It is a suitable model system for studying the intracellular survival of mycobacteria because of its ability of delaying phagosomal acidification [10]. Promptly cultivated in most synthetic or convoluted laboratory media and a fast grower, it is utilized as an alluring model organism and a substitute host for hereditary study of the universal pathogen *Mycobacterium tuberculosis*. It was originally discovered and isolated in 1884 by Lustgarten. It seldom causes diseases and does not rely on living in an animal unlike other species of its family. The bacterium is very useful for research purposes

as it can be used to test for checking the efficacy of antibiotics, extracts and therapeutic agents before opting for experimentations on the pathogenic species.

Infections caused by *Mycobacterium smegmatis*

Less than 30 cases of localized *M. smegmatis* infection in humans have been reported mostly concerning soft-tissue or skin infections following cardiac surgery or traumatic injury in immunocompetent individuals [11]. Prosthetic knee joint infection, granuloma in soft tissues, chronic cellulitis with fistula formation, catheter infections and pulmonary infections are common.

Treatment options comprise ethambutol, doxycycline, sulfamethoxazole, ciprofloxacin, imipenem and amikacin.

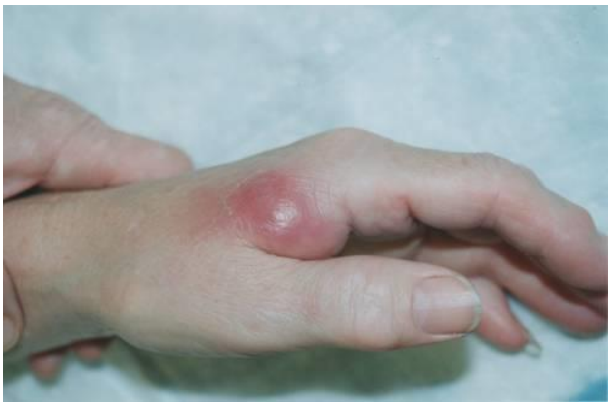


Fig. 2.3: Granuloma in soft tissue of left hand[20]

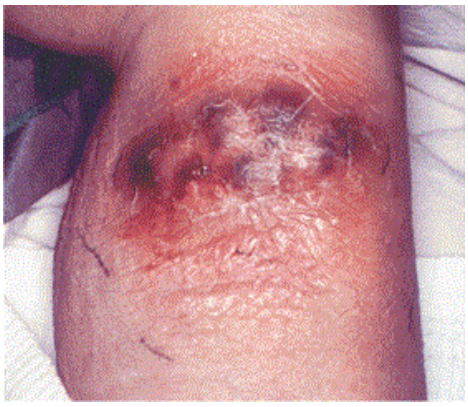


Fig. 2.4: Disseminated infection[19]

Escherichia coli

Escherichia coli is a rod shaped facultative anaerobic gram negative bacterium found in intestines and gut of certain animals. It is a widely studied prokaryotic organism especially in the field of microbiology and biotechnology because of its short doubling time of 20 minutes. It can easily be grown in luria broth and on luria agar plates. It was first discovered in 1885 by Theodor Escherich. It is normally harmless but comprises of both pathogenic as well as non pathogenic strains [12].

Infections caused by *Escherichia coli*

E. coli has been reported to cause diseases like urinary tract and intestinal infections and neonatal meningitis. Pathogenic *E. coli* is categorized based on serogroups, pathogenicity mechanisms, clinical symptoms and virulence factors. "Certain pathogenic *E. coli* strains like enterohemorrhagic *E. coli* (EHEC) produce Shiga toxins (Stxs) and cause hemorrhagic colitis (HC) and the hazardous hemolytic uremic syndrome (HUS) in humans. Serotypes of EHEC are frequently associated with human diseases such as O26:H11, O91:H21, O111:H8, O157:NM, and O157:H7. *E. coli* O157:H7 is the most frequently isolated serotype of EHEC from ill persons in the United States, Japan, and the United Kingdom" [13]. Other infections include diarrhea, food poisoning, pneumonia, abdominal cramps and vomiting.

Extraction

Extraction is the crucial first step in the analysis of herbal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. Extracted part or the substance of a plant obtained through extraction of raw materials such as plants is called an extract. Preparation techniques include microwave assisted extraction (MAE), Soxhlet extraction, supercritical fluid extraction (SFE), sonication extraction and accelerated-solvent extraction (ASE). Due to the fact that plant extracts usually occur as a coalescence of various types of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge. A number of different separation techniques such as TLC, HPLC, column chromatography, sephadex chromatography and flash chromatography are generally used to obtain pure compounds. These compounds are then used for the determination of structural and biological activity. Other non-chromatographic techniques such as immunoassay, that uses monoclonal antibodies (MAbs), Fourier-transform infrared spectroscopy (FTIR) and phytochemical screening assay can also be used to obtain and facilitate the identification of the bioactive compounds[14]. Extraction method that is used in the study was cold maceration. The extracts were prepared either by adding either solvents such as chloroform, methanol, hexane or by boiling at 80ºC to obtain the aqueous extracts. Screening was done against pathogenic species in order to elucidate their therapeutic potential.

Medicinal plants of the Himalayan region

Himalayan region consists of many medicinal plants that are used by doctors, scientists, local people. The villagers or the local people used to use the herbs or the plants by crushing it and applying it over the wounded parts to get relief in the primitive era. Now-a-days the medicinal herbs are converted to tablets and other medicinal products on the basis of their antimicrobial, antimycobacterial and antiinflamatory actions. The most beneficial medicinal plants include *Aconitum ferox, Hemp agrimony, Clematis Buchananiana, Rhus semialata* and *West Indian chickweed*.

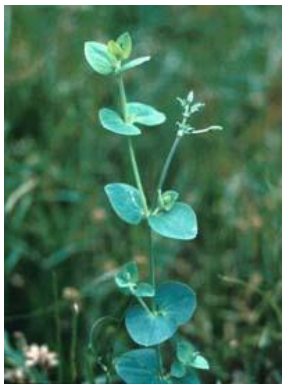
Medicinal plants are increasingly used in ayurveda, traditional unani systems and in plant based pharmaceutical companies. Like other states of the Indian Himalayan Region, Himachal Pradesh has a representative, essential, instinctive and socio-economically important biodiversity having large altitudinal range (200–7109 m), with diverse habitats, species, populations, communities and ecosystems. "Tropical vegetation is distributed in lower parts (Shiwalik Hills) of the state and includes broadleaf deciduous and evergreen forests of *Shorea robusta, Dalbergia sissoo* and *Tectona grandis* Subtropical vegetation ranges from 500 to 1800 m and is dominated by broadleaf deciduous and evergreen forests and evergreen coniferous forests of *S. robusta,* mixed *Pinus roxburghii–Quercus leucotrichophora,* mixed *S. robusta–Pinus roxburghii* and *Q. leucotrichophora.* Temperate vegetation comprises *Cedrus deodara, Aesculus indica, Alnus nitida, Picea smithiana, Pinus wallichiana, Q. leuco-trichophora* and *Q. floribunda forests* and ranges from 1801 to 2800 m. Subalpine vegetation ranges from 2801 to 3800 m and is dominated by *Q. semecarpifolia Betula utilis, Picea smithiana* and *Abies pindrow* forests. Alpine vegetation is usually found above 3800 m but goes down to 3300 m in the valleys. While the region mainly incorporates herbaceous species, scattered patches of shrubs such as *Rhododendron campanulatum Rh. antho-pogon, Juniperus indica, Rosa macrophylla, R. sericea* and *Salix lindleyana* are widespread" [15].

Conventional old stories information is a fortune of India, assumes an imperative job in rustic Population. Conventional drugs are utilized by our progenitors since time long for their prosperity. It gives orderly information about custom, culture and different perspectives in public activity. Western Himalaya is a store of numerous characteristic assets, of which

vegetational angle is prevalent. Today about 65% of Indian populace rely upon the conventional arrangement of prescription. They analyze and fix diverse ailments through their own customary information. Shimla has a rich assorted variety of plants that include several medicinal plants like *Ocimum tenuiflorum, Rubia cordifolia, Solanum nigrum* and *Habenaria intermedia*.

Aconitum ferox

Clematis buchananiana



Drymaria cordata

Rhus semialata

Hemp agrimony

Fig. 2.5 Top 5 medicinal plants of the Himalayan region [23]

Plants studied

The plants used in this study include ***Valeriana jatamansi*** and ***Urtica dioica***. The genus *Valeriana* (Family-Valerianaceae) comprises over 250 species, distributed throughout the world out of which 12 species are found in India. The genus is known for its popular name 'Valerian'. *Valeriana jatamansi* Jones is commonly known as Indian

Valerian, *Muskbala*, *Sugandhbala* (Hindi) and *Tagar* (Sanskrit) [8]. The plant was collected from the green house which was *invitro* grown having herbarium number 13568. Roots of the plant are used to treat insomnia, blood and circulatory disorders, leprosy, asthma, jaundiceand skin diseases and shares antimicrobial and anti-inflammatory properties. *Urtica dioica* also called as stinging nettle belongs to the family Urticaceae. It was collected from the mughals garden of JUIT campus. It has anti-inflammatory and antimicrobial properties. Although its leaves are stingy, people consume it as a part of their meal. The study is carried out based on the prior knowledge about the plant and the activities already been reported against it that is, anti mycobacterial studies were carried out in these plants to check the activity so that it can be used as a drug or as a therapeutic agent.

Figure 2.6: *Valeriana jatamansi*

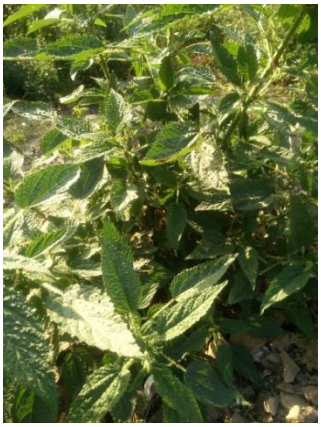


Figure 2.7:*Urtica dioica*

Pharmacological properties

Valeriana jatamansi possesses tremendous pharmacological properties including antioxidant and antimicrobial property, anti inflammatory, anti depressant properties. It cures insomnia by intensifying sleep duration and improves depression. It also has analgesic activity that relates to the inhibition of prostaglandins and deals with migraine, nervous tension, neuralgia and neuroasthemia. Anti tumor activity against breast cancer, ovarian cancer, colon cancer has also been reported. It also improves cardiovascular disorders like nervous dyspepsia and a well known antidiarrhoeal component that helps in improving irritable bowl, abdominal spasm and diarrhea. It is considered useful in diseases of eye, blood and liver and is used as a remedy for hysteria, nervous unrest and emotional stress. It is also useful in clearing voices

and acts as a stimulant in fever and nervous disorder.

Urtica dioica is a herbaceous perennial plant. For a long time *U. dioica* has been used as an alternative medicine and a constituent of paint, fiber, manure, food and cosmetics. Recently *U. dioica* has been shown to have antibacterial, antioxidant, analgesic, anti-inflammatory, antiviral, anti-colitis, anticancer and anti-alzheimer activities. Flavonoids, tanins, scopoletin, fatty acids, polysaccharides, isolectins and sterols are the major phytochemicals reported from this plant. Due to the remarkable biological activities and easy collection, the plant is being used both as a medicine and food supplement in many countries of the Mediterranean region [9]. It is also used in aquaculture as a dietary supplement to make fishes anti bacterial and fight against bacteria.

CHAPTER-3

MATERIAL AND METHODS

Collection of plant materials

Fresh leaves of *Urtica dioica* and and leaves and roots of *Valeriana jatamansi* were collected from JUIT campus. Leaves and root samples were then brought to the laboratory and washed with tap water. These were air dried and pulverized into fine powder using a grinder along with mortar pestle. The grinded material was then stored at 4˚C until use.

Fig. 3.1 Collection and washing of plants

Fig. 3.2 Drying of plants

Fig. 3.3 Extracted powder

Preparation of crude extract

1. Air dried leaves and roots of *Valeriana jatamansi* (20 gm) were soaked in Chloroform: Methanol: Hexane in 1:1:1 ratio (100 ml each).
2. The extract of *Urtica dioica* was prepared in 100 ml of 95% ethyl acetate. Cold maceration was done in flask which was kept for shaking at 150 rpm for 24 hr. at ambient temperature [16].
3. Extracts were then filtered using filter paper, (Whatman No.1) evaporated under reduced pressure using a rotary vacuum evaporator at 50°C. The extracts were then lyophilized using acetonitrile in order to get the concentrated extract.
4. These were further diluted to 10 mg/ml with 8% dimethyl sulfoxide (DMSO) solution for *Valeriana* extract and 5% DMSO for *Urtica dioica.* Purified extracts were then stored at 4°C until use.
5. After this, aqueous extracts were prepared in which 20g of powdered plant materials were dissolved in 200ml of distilled water. The flasks were heated at 80ºC over a hot plate
6. Finally, the extracts were filtered and kept at -80ºC for 24hrs [17]. These were lyophilized for 3 days and then the prepared extract powder was stored at 4ºC.

Fig. 3.4 Preparation and filtration of methanolic extracts

Fig. 3.5 Preparation and filtration of aqueous extracts

Fig, 3.6 Rota evaporation and lyophilization of the extracts

Microorganisms and media used

1. *Mycobacterium smegmatis* and *Escherichia coli* were chosen as the test microorganisms. These microorganisms were obtained from the University microorganism repository.
2. *Mycobacterium smegmatis* was cultured in Middlebrook7H9 media supplemented with 0.15% Tween80 and 0.5% Glycerol. The bacteria was plated on NAT media (Nutrient agar Tween80) containing 0.15% Tween 80.

Culture preparation

1. Revival of *Mycobacterium smegmatis* was done by inoculating the culture in a test tube containing MB7H9 media. The culture was allowed to grow for 4-5 days.
2. Purity of the culture was determined by acid fast staining.
    - For acid fast staining smear was prepared and air dried.
    - It was kept over hot plate. Carbol fuchsin was added for 5 mins.
    - The stain was washed off by tap water.
    - The slide was then covered with 3% acid alcohol and then washed with clean water.
    - Malachite green was added and kept for 1-2 mins.
    - The stain was further washed-off and observed under a microscope.
3. Streaking was done on NAT media and then kept for incubation. The primary culture was setup in 50ml flask.
4. Revival of *Escherichia coli* was done by inoculating the culture in a test tube containing LB media and allowed to grow for 1 day.
5. Purity of culture was determined by gram staining. Streaking was done on LB agar plate and was kept for incubation.
    - For gram staining first of all a smear was prepared and heat fixed.
    - Crystal violet stain was added for 1 min. and rinsed by tap water.
    - Gram's iodine was added for 1 min and washed-off by tap water and decolorized using 95 % ethyl alcohol and again rinsed with water.
    - Safranin as a counter stain was then added for 45 seconds and rinsed by tap water and observed under microscop

Determination of minimum inhibitory concentration

1. Two fold dilution of 10mg/ml of extract was done up to 19.5µl/mg. Similar dilution of 10mg/ml of drugs (Amikacin & Kanamycin) was done. (Optical density at 600nm) was adjusted to 0.2 for *M. smegmatis* and *E.coli*.
2. 20µl extract (leaves & roots of *V. jatamansi* and leaves of *U. dioica*) and 20µl drug was added to wells along with 180µl of cells.
3. For positive control 20µl distilled water & 180µl cell, for negative control 20µl distilled water & 180µl media and for solvent control 20µl DMSO and 180µl cells were added and incubated at 37˚C for 3-4 days.
4. Macrodilution broth method was thereafter performed with stocks of 10mg/ml, 20mg/ml & 40mg/ml that were added to 2ml culture of *M. smegmatis* and *E. coli* respectively and then plated to check the activity.
5. For aqueous extracts 10mg/ml, 20mg/ml, 40mg/ml stocks were prepared of all the 3 plant materials and MIC was carried out in triplicates using kanamycin as the standard drug. In each well 180 µl of cells and 20 µl of extract was added and in some wells media along with kanamycin is added.
6. For positive control 20µl autoclaved distilled water & 180µl cells and for negative control 20µl distilled water & 180µl media was added and kept at incubator at 37ºC for 1 day.
7. Cells in this case were used by comparing it with Mc Farland standard with turbidity standard no. 0.5. that is in 9 ml of media 1 ml of cells were added and the turbidity was adjusted by comparing its turbidity with Mc farland standard solution.

CHAPTER 4

RESULTS

4.1 Gram Staining Results

To check the purity of the culture, gram staining was performed for *E. coli*

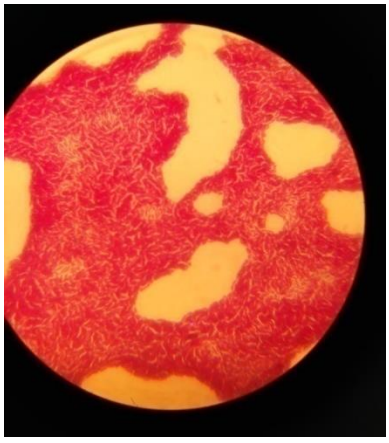


Fig. 4.1 Gram staining of *E. coli* showing pure culture of gram negative bacteria *E.coli*

4.2 Acid Fast Staining Results

Acid fast staining was performed to check the purity of the culture of *M. smegmatis*

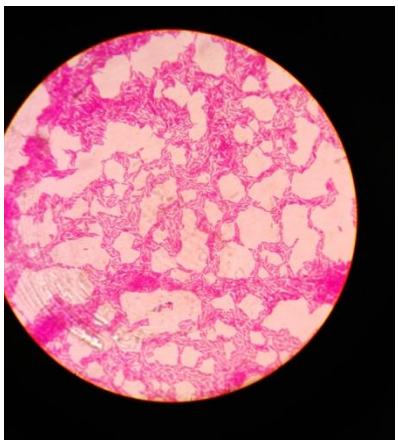


Fig. 4.2 Acid fast staining of *M. smegmatis* showing rod shaped Mycobacteria.

4.3 MIC Results Against Methanolic Extracts

MIC was performed in order to check the significant property of extracts prepared against *M. smegamtis* and *E. coli*

	1 (+ve control)	2 (-ve control)	3	4	5	6	7	8	9	10	11	12
Conc. of drugs (mg/ml)	180 µl cells + 20µl water	180 µl media + 20µl water	10	5	2.5	1.25	0.625	0.312	0.156	0.0781	0.0390	0.0195
Visible growth	+	-	++	++	+	+	+	+	+	+	+	+
Dilution	-	-	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}

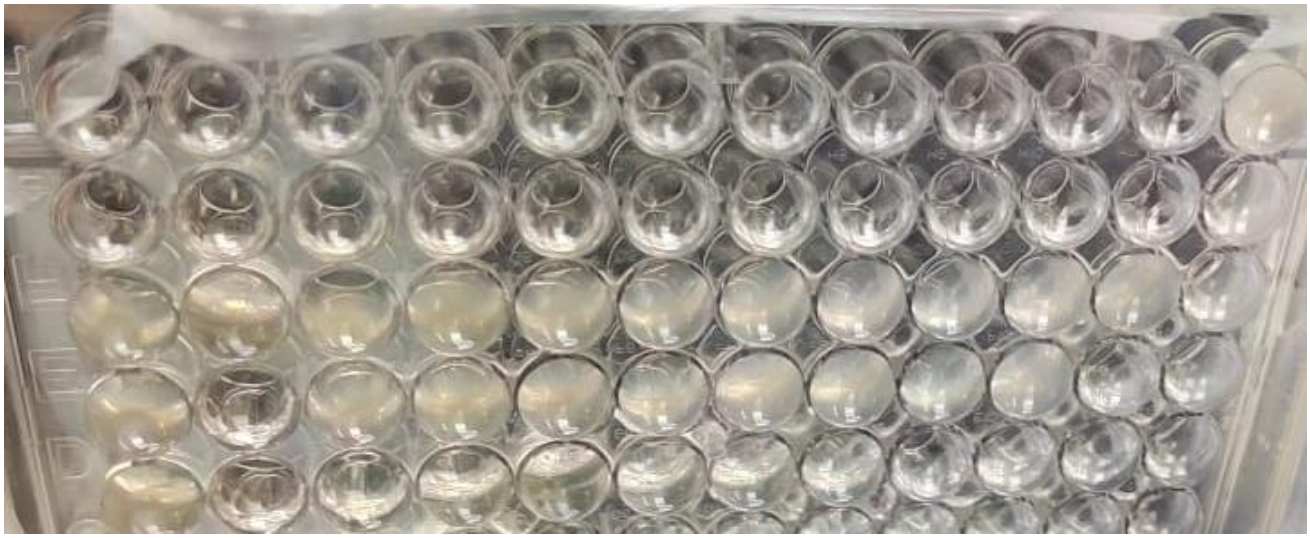
Fig. 4.3 MIC results of *M. smegmatis* against leaves and roots of *Valeriana jatamansi*

	1 (+ve control)	2 (-ve control)	3	4	5	6	7	8	9	10	11	12
Conc. of drugs (mg/ml)	180 µl cells + 20µl water	180 µl media + 20µl water	10	5	2.5	1.25	0.625	0.312	0.156	0.0781	0.0390	0.0195
Visible growth	+	-	++	++	++	+	+	+	+	+	+	+
Dilution	-	-	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}

Fig. 4.4 MIC results of *M. smegmatis* against leaves of *Urtica dioica*

	1 (+ve control)	2 (-ve control)	3	4	5	6	7	8	9	10	11	12
Conc. of drugs (mg/ml)	180 µl cells + 20µl water	180 µl media + 20µl water	10	5	2.5	1.25	0.625	0.312	0.156	0.0781	0.0390	0.0195
Visible growth	+	-	++	++	+	+	+	+	+	+	+	+
Dilution	-	-	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}

Fig. 4.5 MIC results of *E.coli* against leaves and roots of *Valeriana jatamansi*



	1 (+ve control)	2 (-ve control)	3	4	5	6	7	8	9	10	11	12
Conc. of drugs (mg/ml)	180 µl cells + 20µl water	180 µl media + 20µl water	10	5	2.5	1.25	0.625	0.312	0.156	0.0781	0.0390	0.0195
Visible growth	+	-	+	+	+	+	+	+	+	+	+	+
Dilution	-	-	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}

Fig. 4.6 MIC results of *E. coli* against leaves of *Urtica dioica*

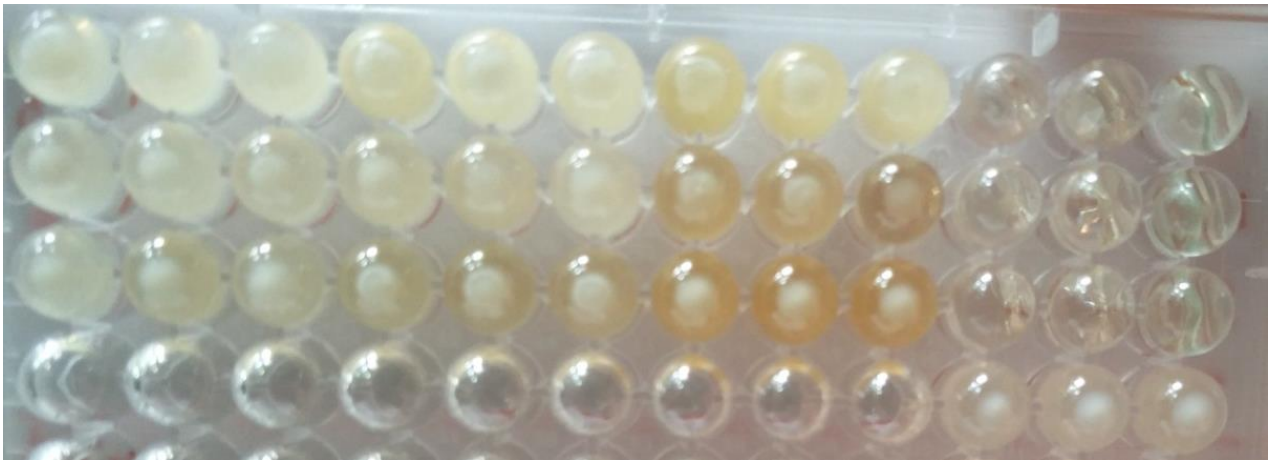
4.4 Macrobroth dilution results

Since the results obtained in microtitre plate were not properly visible, macrobroth dilution method was performed in which the organisms were cultured in test tubes with the extracts prepared and then were streaked in plates to get the end result.

Fig. 4.7 Macrobroth diltution results of culture grown with extracts and then streaked on the plates

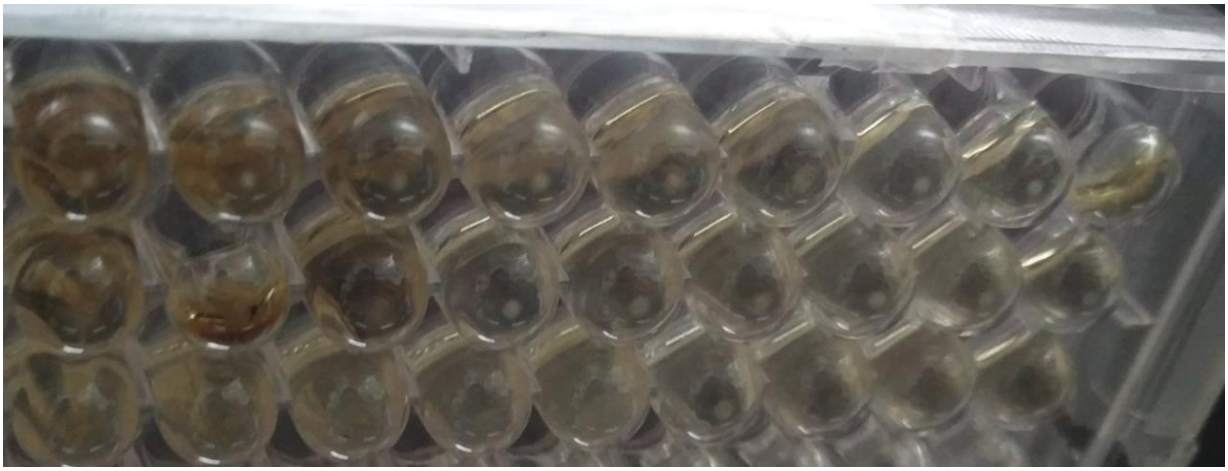
4.5 MIC Results of aqueous extracts

MIC was performed for water extracts in order to check significant activity against *M. smegmatis* and *E. coli*



	1	2	3	4	5	6	7	8	9	10	11	12
Conc. of drugs (mg/ml)	10	10	10	20	20	20	40	40	40	10	10	10
Visible growth of leaves (*V. jatamansi)*	++	++	++	++	++	++	++	++	++	-	-	-
Visible growth of roots (*V. jatamansi)*	+	+	+	+	+	++	+	+	+	-	-	-
Visible growth (U. dioica)	+	+	+	+	+	+	+	+	+	-	-	-

Fig. 4.8 MIC of aqueous extracts against *E. coli*



	1	2	3	4	5	6	7	8	9	10	11	12
Conc. of drugs (mg/ml)	10	10	10	20	20	20	40	40	40	10	10	10
Visible growth of leaves (*V. jatamansi)*	-	-	-	++	++	++	++	++	++	-	-	-
Visible growth of roots (*V. jatamansi)*	+	+	+	+	+	++	+	+	+	-	-	-
Visible growth (U. dioica)	-	+	+	+	+	+	+	+	+	-	-	-

Fig. 4.9 MIC results of aqueous extracts against *M. smegmatis*

CHAPTER 5
DISCUSSION AND CONCLUSION

Valeriana jatamansi have many medicinal properties like antimicrobial, antipyretic and diuretic properties, antioxidant activity and also used as hepatic and nervine tonic. *Urtica dioica* is also a potent plant and have many medicinal properties like reducing inflammation, lowering blood pressure, antimicrobial and antioxidant properties. Ethyl acetate extract of the plant did not show significant results in case of *Mycobacterium*.

Through our study we found that methanolic extract of *V. jatamansi* can reduce growth of *E.coli* but do not have much effect on *Mycobacterium* whereas in case of aqueous extracts at concentration 10 mg/ml, 88.81% and 55.99% inhibition was observed against leaves of *V.* jatamansi and *U. dioica* respectively.

So from our experiment it is concluded that the aqueous extract of leaves of *Valeriana jatamansi* shows significant property against *M. smegmatis* and is able to inhibit the bacterial growth.

CHAPTER 6
APPENDIX

6.1 Bacteriological media:

All the media mentioned below were dissolved in distilled water and prepared by autoclaving at 15 psi for 20 mins.

- **Components of Middlebrook 7H9**
  - Ingredients (g/L)

Ammonium sulphate	0.50
Disodium phosphate	0.50
Monopotassium phosphate	1.00
Sodium citrate	0.10
Magnesium sulphate	0.05
Calcium chloride	0.0005
Zinc sulphate	0.001
Copper sulphate	0.001
Ferric ammonium citrate,	0.04
L-Glutamic acid	0.50
Pyridoxine	0.001
Biotin	0.0005

- **Preparation of media:-**

MB7H9	2.34 g in 450 ml
Glycerol	0.5%
Tween 80	0.15%

- **Preparation of NAT**

1000ml→ 28g media

0.05% → Tween80

Components of Luria Bertini

Tryptone	1g
Yeast extract	0.5g
NaCl	1g
Bacto agar (optional)	1.5g

Directions:-

1000ml→ 20g media

6.2 **Preparation of dyes** :

1. Carbol fushion (1%):
   Phenol crystals → 5g/5ml + 100%ethanol→ 10ml
   Distilled water→ 90ml
   Filter

2. Basic Fushsin:
   1g dye→ 100ml water
   Filter

3. Malachite green:
   1g dye→ 100ml water
   Filter

6.3 Preparation of Mc Farland

1% BaCl2 and 1% H2SO4

1gm in 100 ml of baCl2 and 1ml of H2SO4 in 99ml of water

0.05ml BaCl2 in 9.95ml of H2SO4.

i.e., 1×1^{8} CFU/ml

turbidity standard no. = 0.5

6.4 Stock solutions:

10mg/ml stock was prepared by dissolving

10mg-extract of *Valeriana jatamansi* in 8% DMSO (80µl DMSO in 920µl water)

10mg- extract of *Urtica dioica* in 5% DMSO (50µl DMSO in 950µl water)

6.5 Preparation of Dilutions :

10mg/ml 2 fold dilutions were done up to 19.5µg/ml

Using formula $\mathbf{C_1V_1=C_2V_2}$

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Poster

Therapeutic activity of *Valeriana jatamansi* plant extract against diarrheagenic infections

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Abstract

Diarrhea is a gastrointestinal illness of multietiology and multifactorial disease associated with high morbidity and mortality incidences. A vast array of diarrhea associated pathogens includes bacterial, viral and parasitic agents of which rotavirus and diarrheagenic *Escherichia coli* have been reported as main diarrheal pathogens. Diarrhea is a major health concern in developing countries with enteropathogenic *E. coli* being a leading cause of infantile diarrhea. Other bacteria like Salmonella, Shigella spp. have also been reported as diarrheagenic infection causing pathogens. Nutraceuticals are products which are derived from plants, which other than nutrition are also used as medicine and provide protection against chronic diseases. Nutraceuticals have received significant importance for their role in promotion of human health and disease prevention, along with negligible toxicity. The Himalayan region has rich flora which can be exploited to procure compounds which can be used as neutraceuticals. There are several plants in himalayan region which have been reported for their antibacterial properties out of which *Valeriana jatamansi* is one. *V. jatamansi,* a rhizomatous herb is regarded as a tranquilizer, antiseptic, nerve tonic, ophthalmic, sedative, and tonic useful in hysteria, snakebite, scorpion sting, asthma, and eurosis. The therapeutic properties of the plant are attributed to a class of compounds called valepotriates. Aqueous, methanolic and chloroform based extracts were prepared from this plant using soxhlet and microwave assisted extraction. Minimum Inhibitory Concentration was determined against diarrhea causing infections *E. coli, Shigella and Salmonella* sps. to evaluate its antibacterial property against such diarrheagenic infections in comparison to known standard drugs. The extracts of *V. jatamansi* may serve as potential neutraceutical or may be used as a supplement with already available drugs in the market leading to innate health benefits.

Introduction

- *Valeriana jatamansi* belongs to the family *Valerianaceae* is a rhizomatous herb known as valerian[1].
- It is reported for its antimicrobial, anti-inflammatory activities and other medicinal properties[2].
- The roots of the plant are used for ulcers, convulsions, jaundice, cardiac debility, dry cough, asthma, seminal weakness, skin diseases, leprosy, general debility and for sleep enhancement[2].
- Diarrhea is a gastrointestinal disorder in which there is an increase in flow rate of feces with or without the presence of blood and mucus, accompanied by increased secretion and decreased absorption of fluid, leading to loss of water and electrolytes[3].
- Diarrhea is a major health concern in developing countries with enteropathogenic *E. coli* being a leading cause of infantile diarrhea[3].

Materials and Methods

Collection of leaves and roots of Valeriana jatamansi
↓
Preparation of extract using cold maceration
↓
Hexane:Chloroform:Methanol were added in the ratio 1:1:1
↓
Kept for 24 hours in shaker incubator
↓
Filtration of extract using whatman filter paper
↓
Filtered extract were concentrated using rota evaporator
↓
Sticky semi solid substance was obtained
↓
Revival and purification of Escherichia coli
↓
MIC by microbroth dilution method to be performed using CLSI guidelines
↓
Phenolic content to be checked using ABTS and DPPH method

Results

- After cold maceration, followed by filtration and rotary evaporation, 3g of purified plant extract was obtained.
- Extract will be further processed for Minimum inhibitory concentration (MIC) against *Escherichia coli*.
- After this the phenolic content is to be estimated using ABTS and DPPH methods.






Discussion

Anti-diarrheal activities of *Valeriana jatamansi* have been reported in earlier studies, through our study we will investigate the property of Valeriana's roots and leaves extract against *Escherichia coli*. If any anti-diarrheal activity is found in any of the leave or root extract of this plant then it will help in further study against other diarrhea causing species. Also these extracts may serve as potential neutraceutical or may be used as a supplement with already available drugs in the market leading to innate health benefits.

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