An Assessment of Status and Antibacterial Properties of *Dactylorhiza hatagirea* in Annapurna Conservation Area

(A case study of Paplekharka, Lete VDC, Mustang)

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A Thesis Submitted for Partial Fulfillment of Requirement of the Bachelor's Degree in Forestry of Tribhuvan University, Institute of Forestry, Nepal

December 2009

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Citation: Ranpal, S. 2009. An Assessment of Status and Antibacterial Properties of *Dactylorhiza hatagirea* in Annapurna Conservation Area (A case study of Paplekharka, Lete VDC, Mustang). B. Sc. Forestry Research Thesis Submitted to Tribhuvan University, Institute of Forestry, Pokhara, Nepal.

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CERTIFICATE OF ACCEPTANCE

It is hereby certified that Mr. Surendra Ranpal, a B. Sc. Forestry final year student at Tribhuvan University, Institute of Forestry, Pokhara Campus has carried out research work entitled **"An Assessment of Status and Antibacterial Properties of *Dactylorhiza hatagirea in* Annapurna Conservation Area (A case study of Paplekharka, Lete VDC, Mustang)"** under my supervision and guidance. This entire work is based on the field and laboratory work performed by the candidate and the work brings out useful findings in the field of medicinal plants of Nepal.

This project paper has been accepted as a partial fulfillment of the requirement of Bachelor's Degree in Forestry at Institute of Forestry, Pokhara Campus, Tribhuvan University.

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DECLARATION

I hereby declare that this project paper, **"An Assessment of Status and Antibacterial Properties of *Dactylorhiza hatagirea in* Annapurna Conservation Area" (A case study of Paplekharka, Lete VDC, Mustang**) is my own work except otherwise acknowledged. I have not submitted it or any of its part to any other academic institutions for any degree.

……

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ACKNOWLEDGEMENT

I owe so many debts and gratitude, both intellectual and personal, too many people and institutions that have contributes in this research endeavor. It is almost impossible to acknowledge my gratitude and debt to each of them because of limited space. Thank you all, but I owe special thanks to some people who deserve the acknowledgement.

First and foremost, I extend my sincere thanks to my advisor Dr. Krishna Prasad Devkota, for his excellence guidance, encouragement and kind efforts throughout my study at Institute of Forestry, Tribhuvan University. I deeply respect his ways to instruct student how to be a dedicated in academic profession. I feel a deep sense of gratitude to him for his unconditional support and I am honored to have him as my advisor.

I am tremendously grateful to my co-advisor Narendra Rasaily for his sharp insights, creative and constructive comments and tireless guidance.

My heartfelt gratefulness goes to Madam Linda "Jay" Jackson who rendered my dream come true with financial support, inspiration, encouragement and valuable advice for directing me to the right path of the life. Without her support, I couldn't have been able to walk to the long way forward in my life. I would also like to acknowledge all my scholarship contributors for making all possible to bring me where I am now, by generating one and one rupees for my scholarship to complete B. Sc. Forestry who are the constant source of inspiration for my success. This research was also carried out under their financial support.

I am indebted to respected teachers especially Prof. Dr. I. C. Dutta, Mr. Yajna Prasad Timilsina, Mr. Bharat Mahato, Mr. Achyut Raj Gyawali for their valuable suggestions in different aspects of this research and my academic career.

My heartfelt thank to Mr. Rudra Shing Serchan, local healer, who cheerfully accompanied me throughout the field study and shared many of his insights and information regarding the medicinal and aromatic plants and their uses in the study area. Likewise, I am grateful to my

research assistant Mr. Thakur Prasad Magrati who assisted me right through the field activities and data compilation.

I wish to thank my lab mates Mr. Kagendra Raj Baral and Miss. Meena Suyal Chettri for their kind cooperation, hospitality, honorable support and friendship. Mr. Jagat Khadka, microbiologist, deserve special credit for continuous laboratory support and guidelines in performing the antibacterial activities at Western Regional Hospital Laboratory.

My special appreciation goes to Mr. Prakash Bhattarai and Mr. Anil Sharma, botanists, who helped in species identification.

I must also mention the name of my best brother Ganesh K. Chaudhari, who always takes care of me in every fortune and misfortune throughout my collage life. I perpetually grateful to my friends Sagun, Ajaya, Ram, Prativa, Shiva, Shankar, C. P. Sedai, Prajaya and all my colleagues for their encouragement, interactions, suggestions, continuous help and good company throughout my study period and thesis writing.

My most sincere gratitude goes to my sister Sakuntala and bother- in- law Mr. Om Adhikari for their encouragement and unconditional love. I am deeply grateful to my sister Pushpa who gave me a homely environment and hospitality to make my stay pleasure in Pokhara. Thanks for opening your heart and home to me.

Love and affection of my family always encouraged and inspired me to perform any work intensively. I am indebted to their inspiration and support in every turn of life but here I am unable to express my felling in words. I owe all my success to them.

Surendra Ranpal

Pokhara

December, 2009

DEDICATION

My Parents

&

Linda "Jay" Jackson

ABSTRACT

The Himalayan region of Nepal is biologically rich with a wealth of high value medicinal and aromatic plants (MAPs). The research entitled "**An Assessment of Status and Antibacterial Properties of *Dactylorhiza hatagirea* in Annapurna Conservation Area (A case study of Paplekharka, Lete VDC, Mustang)"** was conducted to assess status of *Dactylorhiza hatagirea* in Paplekharka, high altitudinal open grassland of lower Mustang, Annapurna Conservation Area, Nepal and to evaluate its antibacterial activity.

The status of *D. hatagirea* was estimated through medicinal herbs inventory data whereas its antibacterial activity was determined based on laboratory analysis. The frequency and relative frequency of *D. hatagirea* were found to be 71% and 17% respectively. The number of *D. hatagirea* was found to be 1671 per hectare whereas, the density was found to be 0.17 individuals per square meter. In comparison to other species, the relative density of *D. hatagirea* was found to be 9%.

The study also focuses on the exploration of antibacterial properties of extracts of *D. hatagirea* rhizome and aerial part prepared separately with petrol ether, chloroform, methanol and water against five bacteria for the determination of Zone of Inhibition (ZOI) and Minimum Inhibitory Concentration (MIC). The ZOI of chloroform extract of aerial part of *D. hatagirea* against *Escherichia coli* was found to be 14 mm, whereas that of water extract of rhizome against *Shigella flexinerai* was found to be 13 mm. Comparing the ZOIs between the two parts of *D. hatagirea*, the rhizome part was found to be more effective than the aerial part against all tested organisms except *E. coli*. Similarly, the MIC value of chloroform extract of aerial part of the plant was found to be $\leq$ 125 mg/ml against *E. coli*, whereas that of aqueous extract of rhizome against *Sh. flexinerai* was found to be $\leq$ 62.5 mg/ml. This plant can be a potential source for evolving newer antimicrobial compounds for treating dysentery caused by *E. coli*.

Key words: Status, Antibacterial activity, Orchidaceae, *Dactylorhiza hatagirea*, Zone of Inhibition, Minimum Inhibitory Concentration

Abbreviation

ACA	Annapurna Conservation Area
ACAP	Annapurna Conservation Area Project
CITIES	Convention on International Trade in Endangered Species
CAMP	Conservation Assessment and Management Plan
CBOs	Community Based Organisations
D.	*Dactylorhiza*
IOF	Institute of Forestry
MAPs	Medicinal and Aromatic Plants
MBC	Minimum Bactericidal Concentration
MHA	Muller Hinton Agar
MIC	Minimum Inhibitory Concentration
MFSC	Ministry of Forest and Soil Conservation
NB	Nutrient Broth
NTFP	Non Timber Forest Product
NTNC	National Trust for Nature Conservation
UCO	Unit Conservation Office
VDC	Village Development Committee
WRHL	Western Regional Hospital Laboratory
ZOI	Zone of Inhibition

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CHAPTER ONE

INTRODUCTION

1.1 Background

"*A medicinal plant is any plant which, in one or more of its organ, contains substance that can be used for therapeutic purpose or which is a precursor for synthesis of useful drugs.*" (Sofowora, 1982). This definition of medicinal plant has been formulated by WHO (World Health Organization). The plants that possess therapeutic properties or exert beneficial pharmacological effects on the animal body are generally designated as "Medicinal Plants". It has now been established that the plants which naturally synthesis and accumulate some secondary metabolites, like alkaloids, glycosides, tannins, volatiles oils and contain minerals and vitamins, possess medicinal properties

Nepal constitutes a unique and enormous diversity of flora and fauna within a relatively small geographical area due to variations in topography, altitude and climate. In spite of being a small country, it possesses around 7000 species of vascular plants having 2000 species of medicinal plants (Shrestha and Shrestha, 1999). Baral and Kurmi (2006) have compiled and described 1792 medicinal plants. According to Bhattarai and Ghimire (2006), 49% of the trade medicinal plants are herbs, 29% tress, 14% shrubs and 8% climbers. So, Nepal is veritable treasure trove of medicinal plants (Phoboo et al., 2008).

Most of the wild floras of Nepal are rich in medicinal and aromatic properties like antibacterial, antiviral, antihelminitic, anticancer, sedative, laxative, cardiotonic, diuretic and others. They are important sources of bio-molecules, with application for the manufacture of pharmaceuticals and cosmeceuticals (Heinrich and Gibbons, 2001).

People have used medicinal plants in health care since the time of earliest human evolution. These are the major sources of medication for a wide range of ailments for the rural people of Nepal. More than 75% Nepalese still depends on the herbal plants as a local source of medicine. Local healers use various medicinal plants for primary health care (Devkota, 2001). Different type of bacterial infections such as dysentery, diarrhea, fever, cough, bleeding, burning etc are

treated by traditional medicine in various forms especially under Ayurvedic, Homeopathic, Unami, Naturopathy etc. The medicines obtained from plants boost our natural recovery power. This is because of the better cultural acceptability, better compatibility with the human body and fewer side effects.

Besides their importance in health care, MAPs, have high socio cultural and socio economic values, providing off-farm income and employment opportunities to local people. The incorporation of medicinal herbs into health foods, dietary supplements, herbal teas, cosmetics, massage oils, fragrances and dyeing agents have dramatically increased the international demand of medicinal plants.

During the last ten years, an interest in NTFPs has taken the world by the storm. Huge sums have been invested in exploring the potential of NTFPs (Wollenberg, 1999). Nepal is also not far from this condition. The Master Plan for Forestry Sector (1988) and the tenth five year plan (2003-2008) has emphasis the development of MAPs as a priority programme for poverty alleviation. These show the concernment of the government for conservation and management of medicinal plants. Rare and high priced medicinal herbs are on the top priority for domestication, research and cultivation, processing and marketing.

Medicinal and aromatic plants of high altitude region are an invaluable resource not only to local communities and the nation, but also to the global community at large. They have high ecological values as well as poor rural communities are highly dependent on them for their health and economic benefit derived from harvesting for trade.

Nowadays multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. The resistance of antibacterial is a worldwide public health problem. This is responsible for number of infections that are becoming untreatable in both hospital and community settings. Use of herbal basis antibacterial may contribute the solutions of the worldwide public health problem. So, the exploration of medical properties of crude medicinal plants is needed.

There are several studies have been conducted concerning the antibacterial activity of different herbal extracts in different regions of the world. Recently, much attention has been paid to extracts and biologically active compounds isolated from medicinal plants. The reasons behind this are the side effects and the resistance that pathogenic microorganisms build against antibiotics. Plant-based antimicrobials represent a vast untapped source of medicines. So, further exploration of plant antimicrobials needs to occur. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu, 1999).

Out of many MAPs, *D. hatagirea* has been identified as the endangered species listed by CITIES and vulnerable species listed by CAMP. The government of Nepal has prioritized 30 important medicinal plants for research and management. Among these, 12 plants have been selected for agro-technology. *D. hatagirea* is one of them (DPR, 2006). According to Forest act 1993 and Forest Regulation 1995, Nepal Government has banned the rhizome of *D. hatagirea* to collect, trade and process.

The next fact, there are very fewer studies have been conducted especially regarding *D. hatagirea*. There is lack of management and conservation plan from the government side. Similarly, lack of awareness of importance regarding *D. hatagirea* among rural villagers is leading towards the extinction of this valuable species. Although this is banned species, its unwise harvesting, unscientific use and illegal trading is being in practice. This is resulting improper use of *D. hatagirea* and also reducing the net income of the primary collectors and reducing national income.

The present study, thus, aims to assess the status of *D. hatagirea* in a prominent area: Paplekharka of Lete VDC, lower Mustang. It has also explored the antibacterial activity against five bacteria namely *Staphylococcus aureus, Escherichia coli, Shigella dysenteriae, Pseudomonas aureginosa* and *Bacillus subtilis*. Hence, this research is considered to be milestone in the conservation and scientific use of *D. hatagirea*.

1.2 Objectives

1.2.1 General Objective

To assess the status of *D. hatagirea* in Paplekharka, Lete VDC, lower Mustang of ACAP region and to explore its antibacterial properties.

1.2.2 Specific Objectives

- ✓ To find out the ethnobotanical uses of *D. hatagirea* and other locally available medicinal plants.
- ✓ To assess status and habitat distribution of *D. hatagirea* in the study area.
- ✓ To identify the factors causing dwindling of *D. hatagirea*.
- ✓ To find out the antibacterial activity of *D. hatagirea*.

1.3 Limitations of the Study

- ✓ The adverse climatic condition and steepest geography of the study site.
- ✓ Difficulties were experienced due to unavailability of all the laboratory and chemical facilities and some equipment.

CHAPTER TWO
LITERATURE REVIEW

This chapter attempts to review the relevant and available literatures related to the current study. Although *D. hatagirea* has been recognized well, no more specific research has been done yet in Nepal.

2.1 Ecology of *Dactylorhiza hatagirea* (D. Don) Soo

Syn. *Orchis latifolia* var. *indica*

Taxonomy

Kingdom: Plantae
(Unranked): Angiosperms
(Unranked): Moncots
Order: Asparagales
Family: Orchidaceae
Subfamily: Orchidoideae
Tribe: Orchideae
Sub tribe: Orchidinae
Genus: *Dactylorhiza*
Species: *hatagirea*

It is also known as *Panch aunle, Hatajadi* (Nepali), *Aralu, Salap* (Sanskrit), *Ongu lakpa* (Sherpa) and *Lob* (Gurung).

Distribution and Occurrence:

D. hatagirea is a Himalayan endemic medicinal orchid which is found in Hindu Kush Himalaya range. Its occurrence is sub-alpine and alpine zones from 2800-4200 m above from sea level (IUCN, 2004). Other than Nepal Himalayas, it occurs in the same altitudinal ranges of India, Pakistan, Bhutan and China also.

Flowering Period: June-July

Fruiting Period: August-September (Dutta, 2007)

Description:

It is a terrestrial, erect herb, up to 60 cm high, with palmately divided tuberoids. Leaves are broadly lanceolate or oblong-lingulate or elliptic. Flowers purplish-lilac, rose or rarely white, in many-flowered densely cylindric inflorescence (Baral and Kurmi, 2006). The special character of this plant is that, it remains erect in excessive snowfall.

Uses:

Tubers are sweet, cooling, emollient, astringent, aphrodisiac, demulcent, rejuvenating and nervine tonic. They are useful in diabetes, hemiplegia, dysentery, phthisis, chronic diarrhea, seminal weakness, neurasthenia, cerebropathy, emaciation and general debility. A decoction of tuber is given in colic pain. Powder is used to relieve fever; it is sprinkled over wounds to check bleeding. Root is also used in urinary troubles; also used as farinaceous food (Baral and Kurmi, 2006).

Chemical Constituents:

Tubers contain a glucoside, a bitter substance, starch, mucilage, albumen, a trace of volatile oil and ash (Dutta, 2007). Chemically, dactylorhins A - E, dactyloses A and B and lipids etc are found as major constituents.

Conservation Status:

According Forest Act 1993, and Forest Regulation 1995, the rhizome of *D. hatagirea* is banned to collect, trade and process. If the collection is done with the government authority, the government royalty is NRs. 500 per piece according to Forest Regulation 1995 and its amendment 2005. MFSC, Department of Plant Resources, Kathmandu has listed the plant under national priority species of medicinal herbs for cultivation and conservation. Convention on International Trade in Endangered Species (CITIES) and Conservation Assessment and Management Plan (CMAP) have listed the plant under endangered and vulnerable species respectively (Kunwar, 2006).

Ex-situ Conservation:

It is propagated by seed and rhizomes. A gentle slope, open moist areas, humus soil, well drained sandy soil with high organic matters is suitable. The light humid, moist climate of alpine and subalpine is suitable. Collected seed are shown in nursery bed during April- May at the spacing of 40-60 cm for each seedling. The dried yield in natural condition is found to be 250-300 kg from a hectare (Shrestha and Shrestha, 2004).

5 kg of seed is required for one hectare of land for cultivation. 15-20 tonnes/ ha compost fertilizer is required for manuring. During cultivation 3 to 4 hoeing and weeding is necessary. The germination percentage is 85-90% (Kunwar, 2006).

In-situ Conservation:

For sustainable harvesting, collection of rhizome is done only after flowering of plants (GoN, 2006). Collection of mother plant takes place by leaving 1 immature tubers by filling with layer of soil with the help of sharp *kuto* (a small spade like hand tool). Harvesting period is September to November after seed ripening and fall. Proper care of the surrounding vegetation should be taken while rooting out the tubers of the *D. hatagirea*. Collection of plant should be done by applying rotating system. The rotation of the plant is 4-5 years for harvesting (Kunwar, 2006). Sustainable harvestable amount is 80% (Shrestha and Shrestha, 2004).

2.2 Antibacterial Activities and Bioactive compounds of Medicinal Plants

Ali et al. (1990) studied antibacterial activity screening of some species of Caesalpiniaceae family and found that, the methanol extracts of all the examined sixteen plants showed stronger growth inhibitions against both bacteria and fungi as compared to hexane extracts. *Cassia* species were found to be biologically more active plants. Methanolic extracts of *C. alata* and *C. angustifolia* showed 100 percent growth inhibitors of *S. pyogenes* and *Corynebacterium diphtheriae.*

Hussai and Deeni (1991) tested the alcoholic extracts of 34 species, including many used in the Kano region to treat microbial diseases, for in vitro activity against 13 human and plant pathogens and for the presence of alkaloids. The results found out were that 30 of the species showed antimicrobial activity and 19 of these contained alkaloids. Eight species showed

antifungal activity. *Leptadenia hastate, Cassia occidentalis, Adansonia digitata, Suzygium guineense* and *Striga hermonthica* were both antibacterial and antifungal.

Karn (2003) tested the antibacterial activity of crude ethanolic extracts of some medicinal plants of Makawanpur district, Nepal against four bacteria and found out the strong activity of *Parmelia species*, medium activity of *Microsorum membranaceum* and *Oleandra wallichii* and low activity of *Euphorbia fusiformis.* He also concluded that the presence of steroidal glycoside in *Parmelia* seems to be responsible for its strong antibacterial activity.

Devkota et al. (2000) studied antibacterial activities of some herbal plants used in traditional medicine in Nepal. Cold extraction method was used. The ethanolic extracts of 9 medicinal plants viz., *Glycyrrhiza glabra, Azadirachta indica, Swertia chirayitia, Acorus calamus, Withania somnifera, Terminalia chebula, Berberis aristate, Parnassia nubicola* and *Curcuma angustifolia* were tested against *Pseudomonas autegiinosa* showed antimicrobial activities. The antimicrobial activities of *G. glabra*, at different dilution against *P. aureginosa, S. aureus, E. coli, Methicilin resistant S. aureus, V. cholera, S. typhi* and *Sh. dysenteriae* were also studied. The antimicrobial activities were found to be decreasing on increasing dilution of extract.

Devkota and Dutta (2001) studied seven medicinal plants (*Acontum spicatum, Sapindus mukorosii, Asparagus recemosus, Berginia ciliata, Valeriana jatamansi, Acorus calamus,* and *Sida cordata*) for their antibacterial activity against six bacteria (*Escherichai coli, Shigella dysenteriae, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonela typhi,* and *Vibrio cholerae*) and found all seven plants showing the antibacterial activity. *Berginia ciliata, Acorus calamus, Asparagus racemosus,* and *Valeriana jatamansi* showed comparatively better antibacterial activity.

Dutta and Karn (2007) prepared 33 plant extracts of 17 medicinal plants by solvent extraction method and investigated their antibacterial activity against 6 bacteria. Four most active extracts were assayed for the minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) using two fold serial dilution method. They were further subjected to phytochemical screening.

Giri (2000) studied antibacterial activity of *Lygodium japonicum.* The methanolic extract was found to be active against *S. aureus, B. subtilis, Micrococcus sps, E. coli, P. aureginosa* and *S. typhi*. But the aqueous extract was found to be inactive against all organisms.

Hewage et al. (1998) prepared 101 plant extracts from 55 medicinal plants and studied their antibacterial activites against *S.aureus, E. coli,* and *Mycobacterium fortuitum. Hortonai angustifolia* plant species showed the best activity.

Janovska et al. (2003) tested the antimicrobial activity of crude ethanolic extracts of 10 medicinal plants used in traditional Chinese medicine was tested against five species of microorganisms: *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans*. Among the 10 plants tested, 5 showed antimicrobial activity against one or more species of microorganisms.

Rahman et al. (1998) isolated two new alkaloids from *Bonzardia dhrysogonum* and found their antibacterial properties against *Proteus mirabilis, Proteus vulgaris klebsilla pneumoniae, E.coli* and *Sh. dysenteriae*.

Sasidharas (1997) tested the water and alcoholic extracts of some plants for antibacterial and antifungal activity. Alcoholic extract of all plants were found to be better than aqueous extracts.

Shanmugavalli et al. (2009) studied the Anitmicrobial activity of *Vanilla planifolia* against *E. coli* and its mutant K12, *Proteus vulgaris, Enterobacter aerogens, Bacillus cereus, Streptococcus faecalis, Klebsiella Pneumoniae, Salmonella typhi, Serratia marcescens* and *Pseudomonas aeruginosa*. Extracts were prepared using soxhelt apparatus.

Thomas (1999) studied antibacterial activity of certain medicinal plants in methanol extracts and some species showed maximum antibacterial activity.

2.3 Screening of Antibacterial Activity

The screening of antibacterial activity of plant species can be done by many techniques. Charpinella et al. (1999) recommended the methods for screening of antibacterial activity by determination of Zone of Inhibition (ZOI), Minimum Inhibitory Concentration (MIC) and/or

Minimum Bacterial Concentration (MBC). MIC is defined as the lowest concentration of anti-microbial that prevents growth of organism after over-night incubation (WHO, 1991).

2.4 General Description of Bacteria of this study

Bacillus subtilis: This is a gram positive, known as the hay bacillus or grass bacillus, commonly found in soil. *B. subtilis* is rod-shaped, and has the ability to form a tough, protective endospore, allowing the organism to tolerate extreme environmental conditions.

Pathogenicity: It causes,

- ✓ Contamination of food but rarely causes food poisoning.
- ✓ Production of the proteolytic enzyme subtilisin. *B. subtilis* spores can survive the extreme heating that is often used to cook food, and it is responsible for causing *ropiness* - a sticky, stringy consistency caused by bacterial production of long-chain polysaccharides- in spoiled bread dough.

(http://en.wikipedia.org/wiki/*Bacillus_subtilis*)

Escherchia coli: They are gram-negative mobile rods belong to the family Enterobacteriaceae. Their normal habitat is intestinal tract of humans and animals. They can also be found in water, soil and vegetation.

Pathogenicity: It causes,

- ✓ Urinary tract infections including cystitis, pyelitis and pyelonephiritis. This is commonest pathogen isolated from patients with cystitis. Recurrent infections are common in women.
- ✓ Wound infections, appendicitis, peritonitis and infection of the gall bladder.
- ✓ Bacteraemia and meningitis especially of the newborn.
- ✓ Diarrheal disease especially in infants but also in adults.

(Cheesbrough, 1993)

Pseudomonas aureginosa: This is a gram negative, unipolar motile, slightly cured rod. They can be found in water, sewage and vegetation and also in intestinal tract. They are frequently present in hospital environments, especially in moist, cleaning buckets and humidifiers.

Pathogenicity: It causes,

- ✓ Skin infections especially at burn sites, wounds, pressure sores and ulcers.

- ✓ Urinary infection, usually following catheterization or associated with chronic urinary infection.
- ✓ Respiratory infection especially in patients with cystic fibrosis or conditions that cause immuno-suppression.
- ✓ External ear infection and eye infection.
- ✓ Septicemia especially in persons already in poor health.

(Cheesbrough, 1993)

Shigella flexneri: This is a gram negative, non spore forming rod shape bacteria belong to the family Enterobacteriaceae. It is only naturally found in humans and apes intestinal tract.

Pathogenicity: It causes,

- ✓ Bacillary dysentery (shigellosis) in developing countries, shigellosis has a high death rate especially among young children.
- ✓ The infection includes toxaemia, sometimes bacteraemia and severe dysentery leading to marked dehydration and protein loss, inflammation and ulceration of the large intestine, hemorrhage, abdominal pain and high fever. Death can occur from circulatory collapse or kidney failure.

(Cheesbrough, 1993)

Staphylococcus aureus: This is gram positive, non-motile cocci occur singly and in pairs. They are widely distributed in the environment. They form part of the normal microbial flora of the skin, upper respiratory tract and interstitial tract.

Pathogenicity: It causes,

- ✓ Abscesses, boils, styes and impetigo. It may also causes secondary infections of insect bites, ulcers, burns, wounds and skin disorders.
- ✓ Conjunctivitis, especially of the newborn.
- ✓ Septicemia, endocarditic and osteomyelitis.
- ✓ Pneumonia and empyema.
- ✓ Mastitis (inflammation of the breast)
- ✓ Food poisoning
- ✓ Scalded skin syndrome in young children due to the toxin exfoliation.

(Cheesbrough, 1993)

CHAPTER THREE

STUDY AREA

Regarding availability of *D. hatagirea*, lower Mustang was selected as the study area on the basis of suggestions made by Annapurna Conservation Area Project (ACAP) office, Pokhara. Paplekharka of Lete VDC of Mustang district was selected by the discussion made with local healers.

3.1 Annapurna Conservation Area (ACA)

The Annapurna Conservation Area was established in 1986, surrounded by high mountains and deep valley. It is the largest undertaking of National Trust for Nature Conservation (NTNC) and also the first and largest conservation area in Nepal, covering over 7,629 sq. km. Located in north-central Nepal, the ACA comprises an extremely diverse floral and faunal kingdom in a variety of interrelated ecosystems from subtropical to alpine grass lands. So, this area consists of various high altitudinal medicinal plants. The ACA harbors 38 species of orchids out of which *D. hatagirea* is one (ACAP, 2007).

The ACA presently incorporates 57 VDCs in the districts of Kaski, Myagdi, Manang, Mustang and Lamjung. Now, the ACA has been divided into seven Unit Conservation Offices (UCOs) to govern all the programs of ACAP.

3.2 Mustang District

Mustang district lies form 28^0 24' to 29^0 20' Northern latitude and 83^0 30' to 84^0 Eastern longitude. The altitudinal range varies from 1372 to 8167 m representing sub-tropical, temperate and alpine types of climate. Out of 3573 sq km of total area, the forest coverage is only 145.85 sq km which is only 4.05% of total area. The total grassland covers 1447.03 sq km (40.49%) and total shrub covers 44.16 sq km.

3.3 Lete VDC

This research was conducted in Lete VDC, which lies in Jomsom UCO of Lower Mustang. Lower Mustang is a transition between trans-Himalaya and inner Himalaya Lete VDC receives rainfall of 1545 mm/annum and per day 8.93 mm. The VDC consists deep gorges made by the *Kaligandaki* river. Paplekharka is open grassland situated at high elevation of Lete VDC.

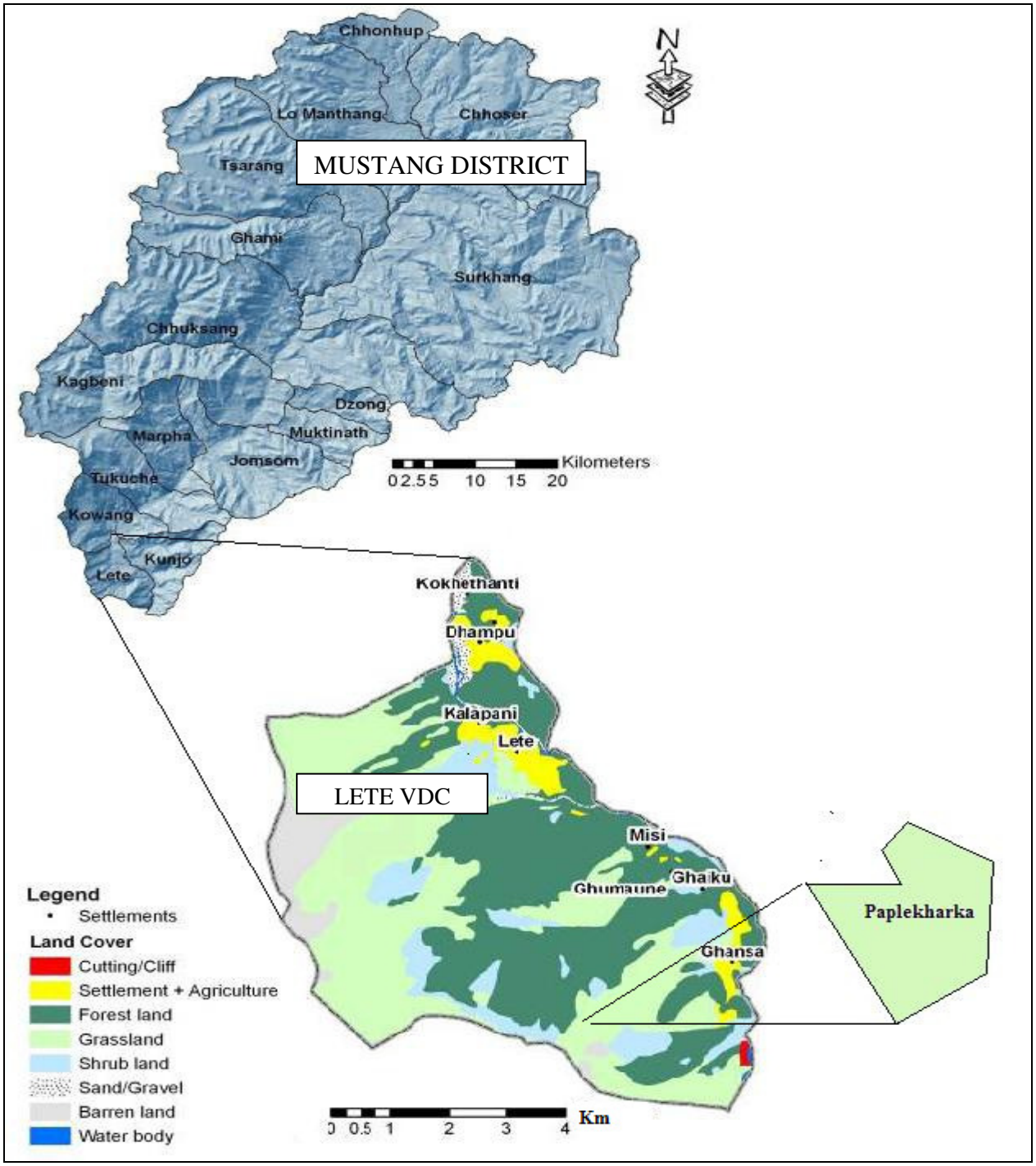


Figure 1: Map of Mustang District with Lete VDC and its Land cover and Paplekharka

CHAPTER FOUR
METHODOLOGY

Methodology includes fieldwork and laboratory works.

4.1: Fieldwork

4.1.1 Primary Data Collection

In order to collect the most reliable and useful information regarding the study, Primary data were collected by using several tools described below.

4.1.1.1 Questionnaire Survey

Face to face questionnaire was carried out to collect information concerning first and third specific objectives. The knowledgeable persons of the study area were selected for questionnaire survey. The knowledgeable persons were local healers, MAPs collectors, the head teacher of local school, staffs of local CBOs, users, etc.

4.1.1.2 Informal Discussion

Committee members, Village elders, farmers, local leaders, women, school teachers, social workers were consulted to identify and verify the facts.

4.1.1.3 Medicinal Herbs Inventory

Sampling method applied in the field was random sampling. The sampling intensity was 0.5 percent of the whole study area. Generally, 1 m × 1 m sample plot is used for inventory of herbs. As *D. hatagirea* is a low abundant herb, 5 m × 5 m sample plots were designed as recommended by Ravindranath and Premnath (1997). All the number of *D. hatagirea* and its associated species were counted within the sample plots.

Three individual plants (tall, medium and short) of *D. hatagirea* were selected in a plot and their mean height was calculated. The diameters at 5 cm above ground level of those individuals were also measured and mean diameter was calculated. Similar measurement was repeated in all plots where *D. hatagirea* were found.

4.1.1.4 Collection of plant samples and Herbarium preparation

The required amount of plant sample of *D. hatagirea* was collected to conduct antibacterial activity. Herbariums of some floras were made for further identification and documentation.

4.1.2 Secondary Data Collection

Secondary data were collected through review of literatures concerned with the research available in the form of journals, articles, thesis, publications, maps, web sites. IOF library, DNPWC, ICIMOD, ACAP library, Internet etc were used for this purpose.

Part 4.2: Laboratory Works

4.2.1 Shade drying of the plant samples

All the collected samples of *D. hatagirea* were cleaned, chopped and shade dried in the blotting paper at room temperature. The drying place was maintained dark in order to prevent the degradation of bioactive components of *D. hatagirea* by interfering through light.

4.2.2 Grinding of the plant samples

After drying all the plant parts, rhizomes and aerial parts were ground into fine powder separately.

4.2.3 Extraction of crude components

Cold extraction method was adopted for the extraction of crude components in which, the powdered form of the plant material was submerged in a suitable solvent and kept at room temperature for few days. The solvents used in this extraction process were petroleum ether (P), chloroform(C), methanol (M), and water (W). The calculated amount of the aerial and rhizome parts of the plant powders were taken in conical flasks and dissolved in calculated volume of petroleum ether first and covered with aluminum foil. It was left for 24 hours at room temperature. Then it was filtered with the help of filter paper. The plant powders were socked again in petroleum ether and left for 24 hours to obtain remaining petroleum ether extract and filtered. The residues were dissolved in chloroform and same process was repeated to obtain chloroform extract. This process was followed to obtain subsequent methanol and water extracts.

4.2.4 Removal of solvent

These plant extracts were left for at least 48 hours in order to remove the solvents used (expect water). After drying, they were weighed in order to know the amount of extract of each individual plant and percentage yield.

The % yield of crude plant extract was calculated by using following formula:

$$\% \text{ yield of crude plant extract} = \frac{\text{Wt. of beaker with extract - Wt. of dry beaker}}{\text{Wt. of sample powder taken}} \times 100$$

4.2.5 Preparation of stock solution

The calculated amount of each dried plant extract obtained from different solvents were dissolved in a calculated volume of ethanol in the ratio of 500 mg/ml to make stock solution and were used for determination of ZOI of different bacteria.

4.2.6 Antibacterial test by agar well diffusion method

Antibacterial activity tests measure the ability of an antibacterial agent to inhibit bacterial growth *in vitro*. In agar well diffusion method, small cups (wells) are made by cork borer in the petri-plate containing the required medium, already inoculated by test organism. The diluted extract (test solution) is added to the well. Dingle et al. (1953) first proposed this technique for the evaluation of enzymatic activity for the degradation of pectin and other polysaccharides. But the technique is also used for evaluation of antimicrobial activities. This method is successfully used by different research groups such as Pepeljnjak et al. 1999; Devkota et al. 2000; Pokharel, 2000; Karn, 2003.

4.2.7 Two fold serial Dilution Technique

This is a technique to determine Minimum Inhibitory Concentration (MIC) of bioactive compounds. This technique was recommended by WHO (1991) and used successively by Devkota et al. 2000; Pokharel 2000 and Devkota and Dutta 2001.

4.2.8 Collection of test organism and preparation of stock culture

The test organisms (bacteria) were obtained from the WRHL, Pokhara. All the organisms were standardized by the laboratory themselves.

Table 1: Bacterial species, their code, source and type

S.N.	Bacteria species	Bacteria code	Gram stain	Source	Type
1	*S. aureus*	A	Gm +ve	WRHL	WRHL Standardized
2	*E. coli*	B	Gm –ve	WRHL	WRHL Standardized
3	*S. flexineri*	C	Gm –ve	WRHL	WRHL Standardized
4	*P. aureginosa*	D	Gm –ve	WRHL	WRHL Standardized
5	*B. subtilis*	E	Gm +ve	WRHL	WRHL Standardized

The stock culture of each organism was prepared by taking two nutrient agar slants and sub-culturing each confirmed test organism aseptically. One set slant was kept as stock culture and another as working set.

4.2.9 Equipments and Media used

4.2.9.1 Autoclave

All the necessary equipments/apparatus for the antibacterial activity testing like cotton swabs, culture media, micropipette tips, glassware etc were sterilized in an autoclave at 15 psi pressure and 121°C for 15-20 minutes.

4.2.9.2 Laminar flow

The antibacterial activity testing of plant extract was carried out inside the laminar flow hood (horizontal type) strictly by creating sterile environment through spirit lamp.

4.2.9.3 Refrigerator

The bacterial stock cultures, subcultures agar plates etc were stored in the refrigerator.

4.2.9.4 Incubator

The bacterial suspension in nutrient broth, the zone of inhibition testing plates, the MIC tubes, etc. were placed inside the incubator at 37°C for needed period.

4.2.9.5 Cotton swabs

Sterile transport viscose swab with polypropylene stick of size 150×2.5 mm diameter for bacteriological culture work was used.

4.2.9.6 Nutrient Broth (NB) solution

Nutrient broth solution was prepared on the guideline of manufacturer's (13 gm/lit). The required amount of solution was prepared by dissolving 2.6 ml of NB powder on 200 ml of distilled water and homogenized by proper shaking. Calculated volume of NB solution was put in different test tubes (e.g. 5 ml for standard working inoculums and 1 ml for serial dilution techniques etc.). The mouth of all tubes was tightly cotton plugged and sterilized on autoclave.

4.2.9.7 Standard working inoculums

One-two colonies of pure culture of different organisms from stock culture were transferred through sterile loop to the respective sterile test tubes (A, B, C, D and E organism) containing 5

ml of nutrient broth by taking out cotton plug. For transferring each organism the loop was sterilized over oxidizing flame of spirit lamp. It was then incubated for 4 hours and the turbidity of NB was observed which was matched with McFarland (turbidity) standard. The NB tubes having less or high turbidity (if) than the McFarland standard were made some by adding little organism or by adding fresh sterile nutrient broth. In such standard working inoculums, the bacterial cell density is 1.5×10^8 CFU/ml.

4.2.9.8 Muller Hinton Agar (MHA) plates

The MHA was prepared according to the manufacturer's recommendations (38 gm/lit respectively). On the basis of needs of plates, the calculated amount of media was dissolved in calculated volume of distilled water in a conical flask by warming above burner to homogenize them. The conical flasks were then tightly cotton plugged and sterilized in autoclave at 121°C (15 lbs) for 15-20 minutes. Sterilized media was then cooled to about 50°C. It was then poured to 90 mm diameter Petri-plates aseptically in the amount of about 25 ml per plate (approximately 4 mm thickness) and labeled as MHA plates. The plates were left at room temperature for 15-20 minutes for solidification and stored in refrigerator packed up in sterilized polythene.

4.2.10 Determination of Zone of Inhibition (ZOI)

The zone of inhibition was determined as follows:

4.2.10.1 Inoculation of MHA plates

Fifteen numbers of MHA plates prepared as described above were taken and dried in an incubator at 37°C for 30 minutes. The standard working inoculums of five different organisms were also taken. A sterile cotton swab was dipped into the standard working inoculums (equivalent with McFarland turbidity standard, cell density 1.5×10^8CFU/ml) of code A organism tube (e.g. *S. aureus*). The swab was then pressed to the wall of tube above liquid with rotating to remove excess inoculums (WHO, 1991). The swab was streaked all over the MHA plates in the angle of 60°c for three times with rotating the plates. All fifteen plates were inoculated by this method, but each three plates by one organism only (i.e. 3 plates for *S. aureus*, 3 plates for *E. coli* and so on). All the inoculated plates were left to dry at room temperature for about 10 minutes with closed lid. The used cotton swab was discarded in a beaker containing Lysol solution.

4.2.10.2 Preparation of wells and transfer of diluted plant extract

The cork borer having 4 mm diameter was sterilized and used to prepare wells in the MHA plates. On each plate, four wells were prepared. Each of two plates containing similar organism (e.g. *S. aureus*) have altogether eight wells. Forty such wells were made for eight different diluted plant extracts. This process is called Agar well diffusion method. Five MHA plates inoculated with five different organisms were used for standard drugs: Amikacin, Azithromycin, Ciprofloxacin, Nitrofurantoin and Norfloxacin as shown in figure 2**.** This process is called disc diffusion method.

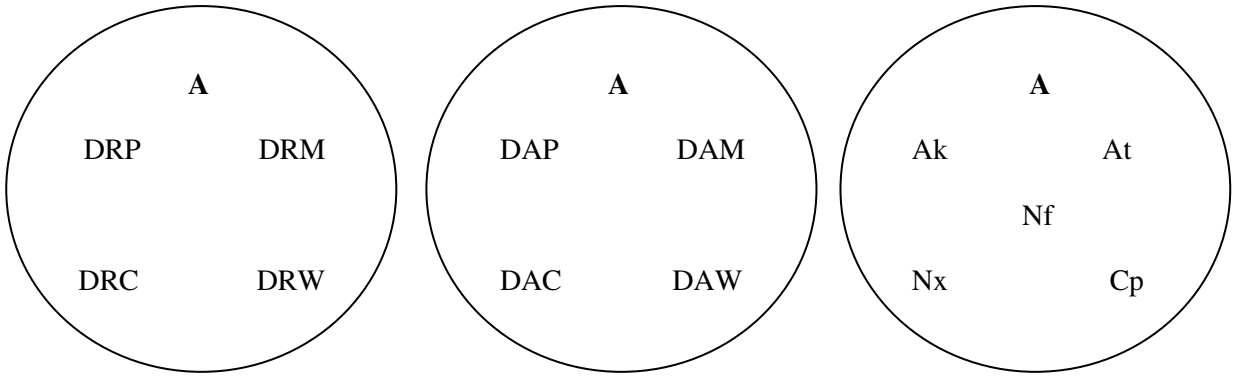


Figure 2: Agar well diffusion method.

Three plates were used for one bacterium (e.g. A). The codes DRP-DAW in first two plates represent the plant extract code as shown in table respectively. The third plate was used for standard drugs. The well diameter was 4 mm and well was also 4 mm.

4.2.10.3 Incubation of the MHA plates

All the fifteen MHA plates prepared by the above process were incubated for 18 hrs in an incubator at 37°C. After 18 hrs the result of zone of inhibition was measured with the help of a ruler and tabulated.

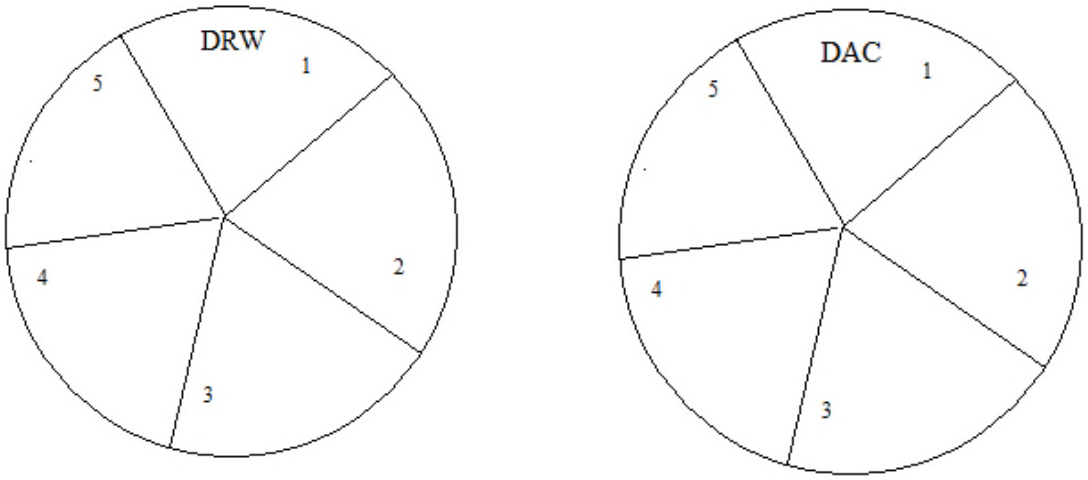
4.2.11 Determination of MIC values of potent extracts

From the result of ZOI the plant extracts having code DRW (against *Sh. flexinerai*) and DAC (against *E. coli*) showed excellent antimicrobial activity. So, these extracts were chosen for determination of MIC up to maximum dilution by two-fold serial dilution method. Ten numbers of tubes were taken and 1 ml of such NB solution was aseptically transferred to each tube having codes and placed properly in test tube racks. Five test tubes for each bacterium were labeled as no. 1^{st} to 5^{th}. In the same way, other two test tubes were taken for positive and negative control.

The mouth of each test tube was tightly plugged by cotton and sterilized for 15-20 minutes at 121°C (15 lbs). After sterilization, each tube was left to cool for some time. To the positive control (+ve) test tubes 1 ml of nutrient broth and 5 µl of bacteria suspension were added. In the same way 1 ml of nutrient broth and diluted plant extract were added to the negative control (-ve) tube. To the remaining test tubes containing 1 ml of nutrient broth, the diluted plant extracts were added on the basis of two fold serial dilution techniques.

After the two fold dilution techniques, 50 µL of standard working inoculums of each bacterium were added aseptically to each test tube except in negative control for all set. All these test tubes of seven sets were incubated at 37°C for 18 hours. One of the principles is that, after 18 hours incubation, some of serial number of tubes will not show the turbidity due to inhibition of bacteria by plant extract and such last tube having less concentration gives the reading of MIC value.

Two fresh and dry MHA media plates were taken. Each plate was divided into 5 fractions for five diluted plant extracts. All tubes of one set (1st, 2nd, 3rd, 4th and 5th) were sub-cultured in MHA medium plates on the basis of respective labeling on the plates as shown in the figure 3. Similarly, other sets were also sub-cultured in respective plates and incubated at 37°C for 18 hours. After that, the growth of subculture was noted to determine MIC values.



C (*Sh. flexinerai)* B (*E. coli*)

Figure 3: Determination of MIC

The MIC values of potent extracts were determined by sub-culturing two-fold serial dilution method. The codes 1, 2, 3, 4 and 5 are used for 1st, 2nd, 3rd, 4th and 5th two fold serially diluted plant extracts test tubes.

4.3 Research Framework:

The research framework was as follows:

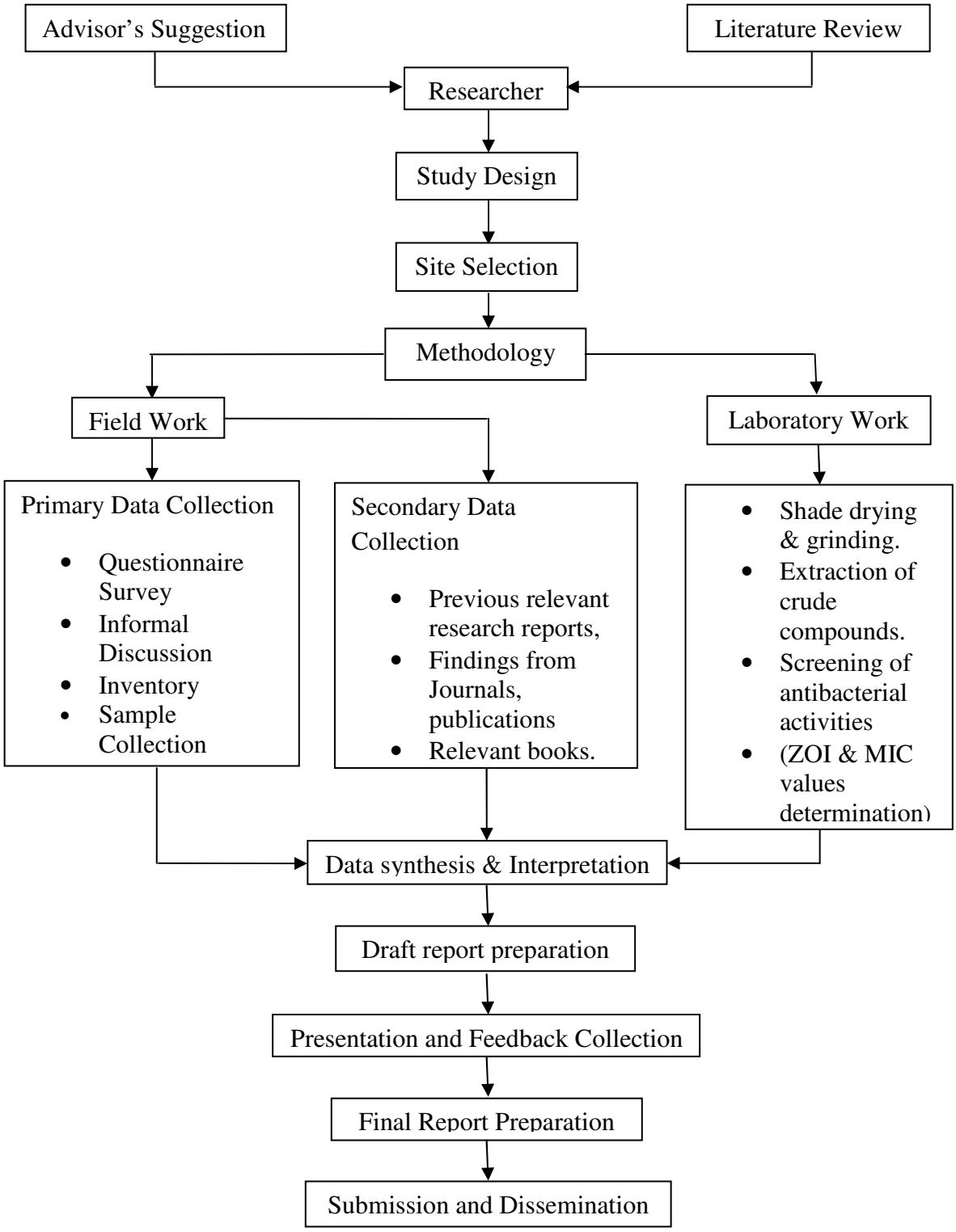


Figure 4: Research Framework.

4.4 Data analysis of D. hatagirea

MS- Excel computer program was used extensively**.** The quantitative data has been analyzed as follows:

A) Frequency = $\frac{\text{No. of plots where } \textit{D. hatagirea} \text{ occurs} \times 100}{\text{Total no. of plots}}$

B) Relative Frequency = $\frac{\text{Frequency of } \textit{D. hatagirea} \times 100}{\text{Sum of all frequency}}$

C) Density = $\frac{\text{No. of } \textit{D. hatagirea} \text{ in all plots} \times 10{,}000\ m^2}{\text{Total no. of plots} \times \text{area of plot}}$

D) Relative Density = $\frac{\text{No. of } \textit{D. hatagirea} \text{ in all plots} \times 100}{\text{Total no. of individuals of all species}}$

E) Abundance = $\frac{\text{No. of } \textit{D. hatagirea} \text{ in all plots} \times 10{,}000\ m^2}{\text{No. of plots in which } \textit{D. hatagirea} \text{ found} \times \text{area of plot}}$

Source: Ravindranath and Premnath (1997)

The qualitative data were analyzed descriptively.

CHAPTER FIVE

RESULT AND DISCUSSION

5.1 Ethnomedicinal uses of the medicinal plants:

According to the responses of local households of Ghasa, Lete VDC, following medicinal plants were used in the village. These medicinal plants were collected from vicinity forests and grasslands.

1. *D. hatagirea* (Panch aunle): - The rhizome paste of *D. hatagirea* is used in cut, burn, infectious wound, all type of skin allergies and skin diseases for speedy healing. Locally, it is processed by cleaning and drying. The dried rhizome is made power and is used in wounds. The powder either can be drink with milk as tonic or can be used as spice.

2. *Neopicrorhiza scrophulariiflora* (Kutki):- The rhizome of this plant is used in skin diseases, fever, cold cough, asthma, bronchitis, diabetes, jaundice etc. It is also useful in scorpion sting. It is locally famous as appetizer. It is cleaned, dried and grinded for local use.

3. *Paris polyph*ylla (Satuwa):- The root paste is used in fast healing from cut and powder of rhizome is used against fever. It is prominently used as antidote to the bite of poisonous insects and snake bite.

4. *Delphinium denudatum* (Nirmasi): - Its rhizome is used in fever and headache. It is used as antidote and also in purifying urination of mules.

5. *Periploca calophyllum* (Sikaari lahara):- Whole plant is applied in form of paste to set dislocated bone. It is antibiotic in nature.

6. *Coccinia grandis* (Golkaankri) and *Phytolacca acinosa* (Jaringo saag):- They are abortifacient. Rhizomes of these two medicinal plants are jointly used for abortion.

7. *Nardostachys jatamansi* (Jataamasi):- Its root is aromatic so used in making local incense sticks (*Dhup*). It is also antiseptic and its essential oil is used to color hair.

8. *Acorus calamus* (Bojho):- Its rhizome is used to treat throat infection. Rhizome is chewed to get relief from cold and cough.

9. *Rheum australe* (Padamchal):- Its leaf-stalks are eaten raw or boiled, sprinkled with salt and pepper. Cooked stalks act as a powerful purgative.

10. *Aconitum spicatum* (Bhik):- The roots are used for the treatment of nervous disorder and rheumatism fever.

5.2 Distribution of *D. hatagirea*

A total of seven herbs species were encountered sparsely dispersed across the study sites with dominant coverage of Babero Ghans*. Out of seventeen study sites, only twelve sites showed presence of *D. hatagirea.* The altitudinal range of habitat distribution of *D. hatagirea* in the study site was 3200 to 3600 meter above sea level. The aspect of habitat distribution of *D. hatagirea* in the study site was north-east and the slope was ranged from 30˚ to 60˚.

*****Babero Ghans is local name which was scientifically unidentified.

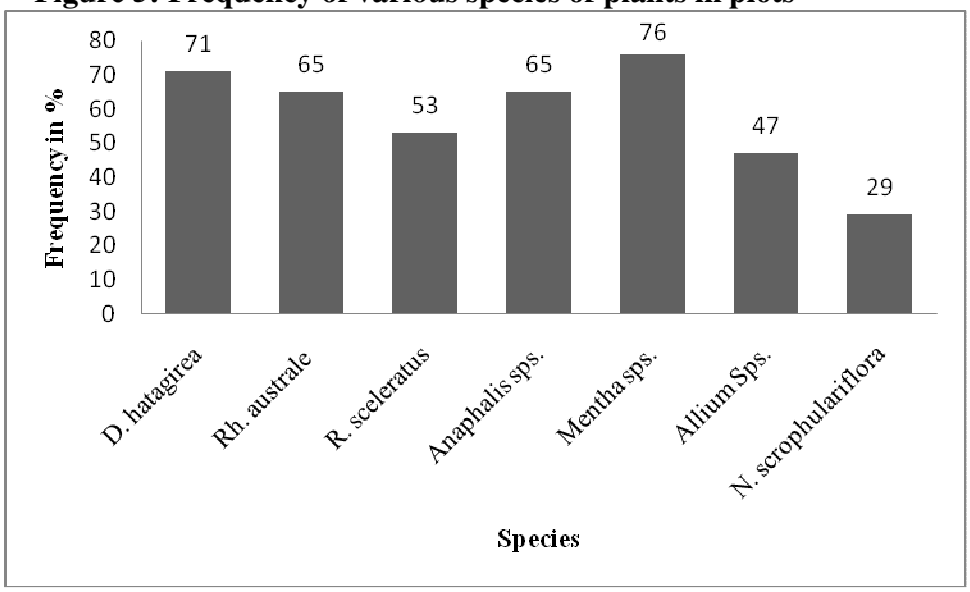
5.3 Mean height and mean diameter of *D. hatagirea*

The mean height of D. hatagirea at the study area was 41.97 cm which is less than reported mean height of D. hatagirea in Nepal i.e. 60 cm (Dutta, 2007). It may be due to age factor, topographic factor, soil factor and/or climatic factor. Likewise, the mean diameter of D. hatagirea at 5 cm above the ground was 0.77 cm.

5.4 Frequency, Relative frequency and associates of *D. hatagirea*

Frequency is the number of sampling units in which the particular species occur, thus express the dispersion of various species in a community. It refers to the degree of dispersion in terms of percentage occurrence. In the study area, occurrences of species according to plot, *D. hatagirea* was found 71%. Mainly other species were *Rheum australe* 65%, *Mentha sps.*76% etc. Detail frequency of species was given below through chart (figure: 5).

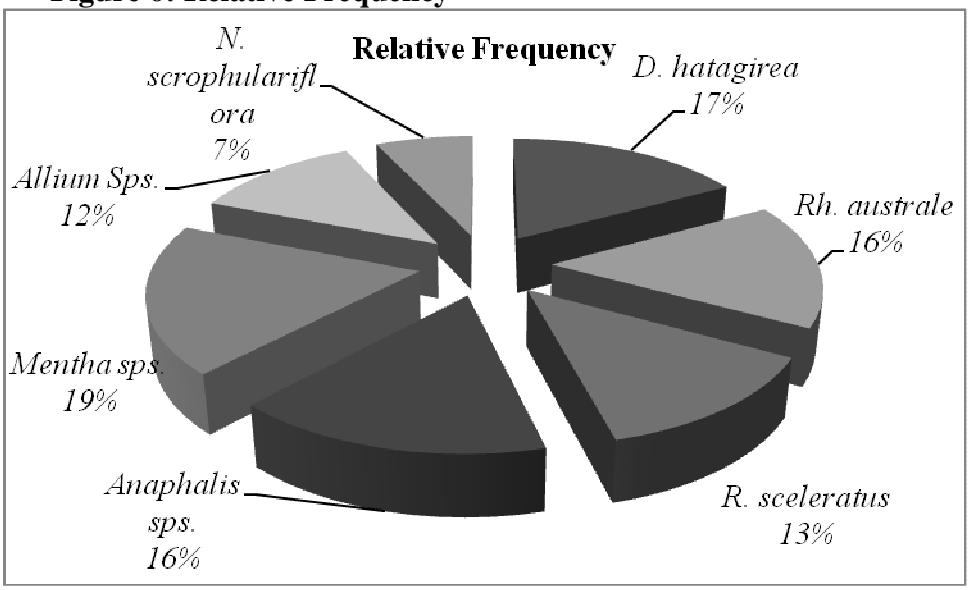
Figure 5: Frequency of various species of plants in plots



(Source: Field survey, 2009)

The frequency of *N. scrophulariflora* is the lowest at the study site. It is due to the higher altitudinal distribution of this species as compare to the other medicinal herbs.

Figure 6: Relative Frequency



(Source: Field survey, 2009)

Relative frequency is frequency of a species in relation to other species. Figure 6, shows that *Mentha sps.* has highest relative frequency followed by *D. hatagirea*, *Rh. australe*, *Anaphalis sps., Ranunculus sceleratus* , *Allium sps.* and *N. scrophulariiflora.*

Associated species

D. hatagirea was observed to be associated with number of species in the study area. Main associative species were recorded in the study areas are given below the table 2.

Table 2: Associated species of *D. hatagirea*

S.N	Local Name	Scientific Name	Family	Nature of Plant
1	Panch aunle	*D. hatagirea*	Orchidaceae	Herb
2	Padamchal	*Rh. Austral*	Polygonaceae	Herb
3	Nak Phorne jhar	*R. sceleratus*	Ranunculaceae	Herb
4	Gastric jhar	*Anaphalis sps.*	Asteraceae	Herb
5	Lekh pudina	*Mentha sps.*	Labiateae	Herb
6	Ban lasun	*Allium sps.*	Amaryllidaceae	Herb
7	Kutki	*N. scrophulariiflora*	Scrophulariaceae	Herb

5.5 Density and Relative Density

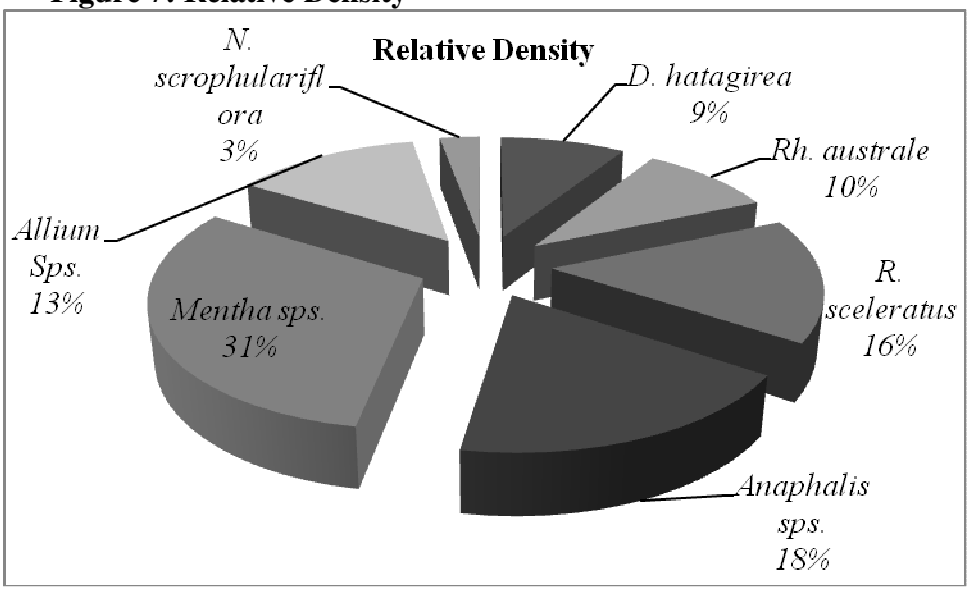
Density expresses the numerical strength of the presence of species in a community. It is the number of individuals per unit area and is expressed as number per hectare. Based on the 25 m^2 rectangular plots, analysis of density of different species per hectare, *Mentha sps.* was found higher density then other species, where as plant number of *Mentha sps.* was found 5812/ha. Number of *D.hatagirea* was found 1671/ha. In comparison to other species, the relative density of *D. hatagirea* was found 9%. Detail densities of other species are given below by table 3.

Table 3: Density/ha of species in study area

S.N	**Species**	**Density per hectare**
1	*D. hatagirea*	1671
2	*Rh. austral*	1788
3	*R. sceleratus*	2965
4	*Anaphalis sps.*	3459
5	*Mentha sps.*	5812
6	*Allium Sps.*	2494
7	*N. scrophulariiflora*	541

On the basis of field visit and observable grazing pressure, the study site is fallen within the category of unprotected area. The density of *D. hatagirea* was 0.17 ind/m^2 in this site which was comparatively less from the reported density of *D. hatagirea i.e.*0.2 ind/m^2 in Samar Lek, Upper Mustang (Chhetri and Gupta, 2006). The next reported density of *D. hatagirea* is 2.66 ind/m^2 in grazed sites and 3.2 ind/m^2 in ungrazed sites at Tungnath, India (Nautiyal et al. 2004). Bhatt et al (2005) also reported 2.02-2.19 ind/m^2 density in protected area and 1.13-1.64 ind/m^2 in unprotected area in west Himalaya for *D. hatagirea*. The low density in unprotected areas may be due to heavy grazing pressure.

Figure 7: Relative Density



Relative density is the density of a species with respect to the total density of all species. Fig. 7 represents relative density of *D. hatagirea* and associated herbs. As relative density depends upon the occurrence of individual species. *Mentha sps.* shows highest value due to its high availability and *Neopicrorhiza scrophulariiflora* shows lowest value. *D. hatagirea* has relative density of 9%.

5.6 Abundance

Abundance refers to the number of individuals of each species present in the total population. It is a component of species diversity.

Table 4: Abundance/ha of species in plots

S.N	Species	Abundance/hectare
1	*D. hatagirea*	2367
2	*Rh. austral*	2764
3	*R. sceleratus*	5600
4	*Anaphalis sps.*	5345
5	*Mentha sps.*	7600
6	*Allium Sps.*	5300
7	*N. scrophulariiflora*	1840

As shown in table 4, *Mentha sps.* shows the highest abundance and *N. scrophulariiflora* shows the lowest. *D. hatagirea* represents medium abundance as 2367 per hectare.

5.7 Status of *D. hatagirea* and its associates

Table 5: Status of MAPs in the study site

Species	Frequency (%)	Relative Frequency (%)	Population Density/ha	Relative Density (%)	Abundance/ha
D. hatagirea	71	17	1671	9	2367
Rh. Austral	65	16	1788	10	2764
R. sceleratus	53	1	2965	16	5600
Anaphalis sps.	65	16	3459	18	5345
Mentha sps.	76	19	5812	31	7600
Allium Sps.	47	12	2494	13	5300
N. scrophulariflora	29	7	541	3	1840

The table above reveals the status of *D. hatagirea* and its associates.

5.8 Factors causing the dwindling of *D. hatagirea*

According to questionnaire survey and field observation the following points are the main factors those causing the dwindling of *D. hatagirea*.

- *D. hatagirea* are naturally distributed over remote grasslands far from human settlement. So, human labor is being unable to use for conservation endeavors.

- Unprotected grasslands are free for periodic grazing. Heavy grazing and trampling by livestock adversely affect the above-ground plant parts and disturb the life cycle of the species.

- Grazing indirectly promotes plant growth and enhances vegetative reproduction as well as seed production of unpalatable species. So weed proliferation suppresses the growth of *D. hatagirea*.

- Illegal collection and trading by outsiders results heavily decrease in the density of *D. hatagirea*. There is no action against it.

- There is lack of effective management and conservation plan for MAPs in the national level.

- There is no initiation of in-situ and ex-situ conservation practice of *D. hatagirea* in Nepal yet.

- According to local people the Himalayan Monal, (*Lophophorus impenjans*) destroy its underground parts i.e. tuber for food. Consequently, damaged mother tubers become unable to reproduce daughter tubers. It was also observed during field visit.

5.9 Percentage yield of crude extracts

The following table shows that rhizome part of *D. hatagirea* has the highest percentage yield in water extract and the lowest one in petroleum ether extract. Likewise, aerial part of *D. hatagirea* has the highest percentage yield in methanol extract and the lowest in petroleum ether extract.

Greater the percentage yield, greater will be the quantity of soluble compounds in *D. hatagirea* and vice versa.

Table 6: Percentage yield of crude extracts

S.N	Parts of *D. hatagiera*	Solvent	Extract code	Volume of Solvent (ml)	Percentage yields (%)
1	Aerial	Petroleum ether	DAP	150	5.46
2	Aerial	Chloroform	DAC	150	5.46
3	Aerial	Methanol	DAM	100	17.73
4	Aerial	Water	DAW	25	10.91
5	Rhizome	Petroleum ether	DRP	100	2.42
6	Rhizome	Chloroform	DRC	100	3.64
7	Rhizome	Methanol	DRM	50	6.67
8	Rhizome	Water	DRW	25	12.12

5.10 Screening and evaluation of antibacterial activity

The screening and evaluation of antibacterial activity were carried out by agar well diffusion method and determination of MIC values was carried out by two-fold serial dilution method. The test organisms were *Staphlococcus aureus* (A), *Escherichia coli* (B), *Shigella flexineri* (C), *Pseudomonas aureginosa* (D) and *Bacillus subtilis* (E).

5.11 Zone of Inhibition (ZOI)

The ZOI of petroleum ether, chloroform, methanol and water extracts of 500 mg/ml concentrations on the test organisms are shown in Table 6. The table provides the measured ZOI of the extracts of aerial and rhizome parts of *D. hatagirea* with different solvents against five bacteria.

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Table 7: ZOI of plant extracts against the tested bacteria

S.N	Extract code / Bacteria	Zone of Inhibition (in mm)				
		A	B	C	D	E

1	DAP	0	**6**	**6**	0	0
2	DAC	**7**	**14**	**6**	0	0
3	DAM	**9**	0	0	0	0
4	DAW	**11**	0	0	0	0
5	DRP	**8**	0	**6**	0	**10**
6	DRC	**8**	0	0	0	0
7	DRM	**11**	**11**	**6**	0	0
8	DRW	**8**	0	**13**	**7**	0

N.B. Dilution of plant extract = 500 mg/ml, Diameter of well = 4 mm, Depth of well = 4 mm.
ZOI more than or equal to 12 mm was considered the best i.e., most active; from 9-11 mm to be better; and from 7-8 to be good (Nostra, et. al., 2000)

All the diluted plant extracts showed antibacterial activity against *S. aureus*, except petroleum ether extract of aerial part of *D. hatagirea.* Methanol extract of rhizome part and aqueous extract of aerial part showed better ZOIs among other.

Petroleum ether extract and chloroform extract of aerial part and methanol extract of rhizome of *D. hatagirea* showed antibacterial activity against *E. coli*. Other showed no activity. The chloroform extract of aerial part showed the best ZOI i.e. 14 mm against *E. coli* among all tests conducted in the laboratory.

The antibacterial activities of petroleum ether extract and chloroform extract of aerial parts and methanol extract of rhizome were found same against *S. flexineri*. They showed 6 mm of ZOI. Against *S. flexineri,* aqueous extract of rhizome of *D. hatagirea* showed the best activity having 13 mm of ZOI.

The above table showed that the antibacterial activity of *D. hatagirea* against *P. aureginosa* and *B. subtilis* is very low. The underlined values of ZOI were taken for further MIC test.

5.12 Determination of MIC Value

After evaluating the ZOI values of various extracts, two prominent extracts were taken for MIC test by two fold serial dilution method. MIC of chloroform extract of aerial parts was tested against *E. coli* and that of aqueous extract of rhizome part was tested against *S. flexineri.* The

test organisms were inoculated in various concentrations of plant extracts i.e. 250mg/ml, 125mg/ml, 62.5mg/ml, 31.25mg/ml and 15.625mg/ml, which are presented in Table 7.

Table 8: MIC value determination of extracts

Extract code	Bacteria code	Dilution of extracts (mg/ml)				
		250	125	62.5	31.25	15.625
DRW	C	Nil	√	√	√	√
DAC	B	Nil	Nil	√	√	√

N.B: nil = No growth of bacteria, √ = Growth of bacteria

There was growth of bacteria in the solutions of aqueous extract of rhizome part in all concentrations except 250 mg/ml. Likewise, there was growth of bacteria in the solutions of chloroform extract of aerial part except 250 mg/ml and 125 mg/ml.

Table 9: MIC of *D. hatagirea* against tested bacteria

S.N.	Bacteria	Medicinal plant	MIC value (mg/ml)
1	*Shigella flexinerai*	Rhizome part of *D. hatagirea*	>250
2	*Escherichia coli*	Aerial part of *D. hatagirea*	>125

The exact MIC value couldn't be determined, due to the turbidity of nutrient broth and plant extract in the two-fold dilution process. Though, the MIC value is not exact, this gives approximate idea about MIC value. So, MIC value is also included in the result and discussion.

The growth of *Sh. flexinerai* in the rhizome extract was seen below the concentrations of 250 mg/ml. So, 250 mg/ml can be said the MIC of the rhizome of *D. hatagirea*. Similarly, the growth of *E. coli* was seen in the aerial part extract was seen below the concentrations of 125 mg/ml. So, 125 mg/ml can be considered the MIC of the aerial part of *D. hatagirea*.

5.13 Comparison of ZOIs of Plant Extracts with Standards

Five antibiotics, namely, Azithromycin (At), Amikacin (Ak), Ciprofloxacin (Cp), Norfloxacin (Nx) and Nitrofurantoin (Nf), were tested for all five bacteria. The highest ZOI values of different extracts of rhizome and aerial part of *D. hatagirea* against five bacteria were compared

with ZOI of the antibiotics (Table 10). This was conducted to find out the strength of antibacterial activity in relation to the antibiotics.

Among the five antibiotics, the Ciprofloxacin and Nitrofurantoin was broad-spectrum bacteria, having no any resistant variety developed against it. However, the Azithromycin, Amikacin and Norfloxacin do not affect the *E. coli*, i.e., the *D. hatagirea* has become resistant to these antibiotics.

Table 10: Comparison between ZOI of plant extracts and that of antibiotics

S.N.	Antibiotics & plant extracts	Zone of Inhibition (in mm)				
		S. aureus	*E. coli*	*Sh. flexinerai*	*P. aureginosa*	*B. subtilis*
1	Azithromycin	14	0	28	28	30
2	Amikacin	27	0	23	10	25
3	Ciprofloxacin	8	15	14	24	10
4	Norfloxacin	11	0	32	26	24
5	Nitrofurantoin	16	20	22	20	11
6	Rhizome part of *D.hatagirea*	11	11	13	7	10
7	Aerial part of *D. hatagirea*	11	14	6	0	0

D. hatagirea has greater ZOI than Ciprofloxacin for *S. aureus* and thus, it is effective than Ciprofloxacin in this case. Similarly, the ZOIs of rhizome and aerial parts of *D. hatagirea* against *S. aureus* are equal to that of Norfloxacin. So, they are also equally effective against *S. aureus.*

For *E. coli,* aerial part of *D. hatagirea* is more effective than its rhizome. In this case, effectiveness of aerial part of *D. hatagirea* is near to Ciprofloxacin.

For *Sh. flexinerai,* effectiveness of rhizome is greater than aerial parts of *D. hatagirea* and close to Ciprofloxacin, but low than other standards.

For *P. aureginosa,* aerial part of *D. hatagirea* has no resistance but its rhizome has low resistance as compared to the standards.

For *B. subtilis,* aerial part of *D. hatagirea* has no effectiveness. But the ZOI values of rhizome of *D. hatagirea* and Ciprofloxacin are same against *B. subtilis*, as a result they are equally effective against this bacteria.

Thus the rhizome part of *D. hatagirea* has resistance against all Gram-positive and Gram-negative bacteria but its aerial part has limited resistance against some bacteria. In this way, comparing the ZOIs between the two parts of *D. hatagirea*, the rhizome part is more effective than the aerial part against all tested organisms except *E. coli*.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

- There are numerous medicinal plants used by villagers of Lete. They have good knowledge of ethnobotanical uses of those medicinal plants.

- *D. hatagirea* are sparsely distributed over the study area. Its frequency, relative frequency, density, relative density and abundance are 71%, 17%, 1671 per hectare, 9% and 2367 per hectare respectively. So, the density of *D. hatagirea* was 0.17 individuals per square meter in this site which was comparatively less from the reported density of *D. hatagirea.*

- The altitudinal range of distribution of *D. hatagirea* in the study site was 3200 to 3600 m above sea level. The aspect of habitat distribution of *D. hatagirea* was the north-east and slope was ranged from 30˚ to 60˚.

- *D. hatagirea* is an endangered medicinal plant whose status is dwindling day by day. Overgrazing, illegal collection, lack of conservation, lack of awareness, etc are the main factors of dwindling.

- *D. hatagirea* appears to have different spectrums of antibacterial action as evidenced from this study. The result of the various screening tests also indicates that the rhizome part of *D. hatagirea* has resistance against all Gram-positive and Gram-negative bacteria but its aerial part has limited resistance against some bacteria. In this way, comparing the ZOIs between the two parts of *D. hatagirea*, the rhizome part is more effective than the aerial part against all tested organisms except *E. coli*.

- It is of interest to note that *E. coli*, one of the very resistant bacteria to synthetic drugs, was found to be very susceptible to the extract of this plant. This finding is distinctive

from the folkloric uses *D. hatagirea*. Hence, this plant can be a potential source for evolving newer antimicrobial compounds for treating dysentery caused by *E. coli*.

- This study has revealed the unique importance of *D. hatagirea*. So, this result may be useful for the pharmaceutical utilization and conservation of this endangered medicinal plant.

- This results form a good basis for further phytochemical and pharmacological investigations on *D. hatagirea*.

6.2 Recommendation

- Antimicrobial screening of the other medicinal plants should be done against the common pathogens and thus a database should be established based on findings.

- In-situ and ex-situ conservation of *D. hatagirea* should be encouraged in initiation of the government and ACAP.

- Interaction and awareness programs should be conduct regarding importance, management, conservation and sustainable harvesting of *D. hatagirea* among villagers, local healers, collectors, traders, grazers etc.

- Control over illegal collection and trading by concern authorities is required.

- Control graze lands should be separated from the habitat of high value medicinal plants.

- There is a need to recognize the important role of traditional medicine and to create a separate department of Nepalese System of Medicines from the state level.

REFERENCES

Ali, M.S., Azhar, I., Amtul, Z., Ahmad, V.U. and Usnmanghani, K. 1999. Antibacterial Screening of some Casesalpeniaceae, Fitoterapia, 703 (3): 299-304.

Ministry of Forest and Soil Conservation, Government of Nepal, 2007. *Cultivation of Medicinal plants. Prasar Pustika.* Department of Forest, Babarmahal Kathmandu (In Nepali).

Bhattarai, K.R. and Ghimire, M.D. 2006. Commercially important medicinal and aromatic plants of Nepal and their distribution pattern and conservation measure along the elevation gradient of the Himalayas. *Banko Jankari*. 16: 3-13.

Baral, S.R. and Kurmi, P.P. 2006. *A Compendium of Medicinal Plants in Nepal.* Mrs Rachana Sharma, Kathmandu, Nepal.

Bauer, A.W., Kirby W. M. M., Sherris, J.C. and Turck, M. 1996. Antibiotic suspecttibility testing by a standardized single disk method. *American Journal of clinical pathology,y* 45: 493-496.

Bhatt A., Joshi S.K. and Garola S. 2005. *Dactylorhiza hatagirea* (D.Don) soo- a west Himalayan Orchid in peril, curr.sci. 89: 610-612.

Charpinella M.C., Herrero G.G., Alonso, R.A. and Palacios, S.M. 1999. *Antifungal Activity of Melia azedarch Fruit Extract.* Fitterapia 70: 296-298.

Cheesbroug, M. 1993. *Medicinal Laboratoury Manual for Tropical countries. ELBS Reprinted Edition.*

Chettri, H.B. and Gupta, V.N.P. 2006. *NTFP Potential of Upper Mustang, a trans-himalayan region in wester Nepal,* Scientific World, 4(4): 38-43.

Devkota, K.P., Acharya, R., Baral, M.P. and Pokherel, R.P. 2000 Antimicrobial Activity of Some Herbal Plants Used in Traditional Medicine in Nepal. Proceedings, Third National Conference on Science & Technology, RONAST, II : 1311-1317.

Devkota, K.P. and Dutta, I.C. 2001. Antibacterial activities of commercially traded herbs used in traditional medicines in communities of Doti district, Nepal, Report submitted to IUCN, Kathmandu, Nepal.

Dingle, J., Reed, W.W. and Solomons, G.I. 1953. *The Enzymatic degradation of Pectin and other polysaccharide II. Application of the Cup Plate Assay to the Estimation of enzymes.* J. Sci. Food. Agric. 4: 149-153

DPR. 2006. *Prioritized Medicinal Plants for Economics Development of Nepal.* Department of Plant Resources, MFSC, Gonernment of Nepal, Kathmandu, Nepal (in Nepali).

DoF. 2004. *Community Forestry Inventory Guideline*, published by Department of Forest, Kathmandu.

Dutta, I.C. 2007. Non Timber Forest Products of Nepal: *identification, classification, ethnic uses & cultivation.* HillSide Press, Kathmandu, Nepal. 98p.

Dutta, I.C. & Karn, A.K. 2007. *Antibacterial Activities of Some Traditionally Used Medicinal Plants of Daman, Nepal.* TU, IOF & ComForm, Pokhara, Nepal.

Ghimire, S.K., Sah, J.P., Shrestha, K.K and Bajracharya, D. 1999. "Ecological Study of Some High Altitude Medicinal and Aromatic Plants in the Gyasumdo Valley, Manang, Nepal", Ecoprint, 16(1): 17-25.

Giri, R. 2000. Antibacterial activity of *Lygodium japonicum*, M. Sc. Thesis, Central Department of Chemistry, T. U., Nepal.

GoN. 2006. *Nepalko Antarik Bikaskalagi Prathamikata Prapta Jadibutiharu.* Ministry of Forest and Soil Conservation, Thapathali, Kathmandu Nepal. (In Nepali).

Heinrich, M. and Gibbons. 2001. Ethonopharmacology in drug discovery: an analysis of its role and potential countribution. Journal of Pharmacy and Pharmacology. 53: 425-432.

Hewage, C.M., Bandara, B.M.R., Karunaratna, V., Wannigama G.P., Pinto M.R.M and Wijesundara, D.S.A. 1998. Antibacterial Activity of some Medicinal plants of Sri Lanka, Journal of National Science Council of Sri Lanka, 26 (1): 27-34

http://en.wikipedia.org/wiki/Bacillus_subtilis, 2009/Aug/31

http://www.eson.org.np/mapsnetnepal.htm, 2009/ Sep/10

Hussain, H. S. N. and Deeni, Y. Y. 1999. *Plants in Kano ethnomedicine; screening for antimicrobial activity and alkaloids.* International Journal of Pharmacology: Department of Biological Sciences, Bayero University, Kano, Nigeria.

IUCN. 2004. National Register of Medicinal & Aromatic Plants, IUCN Nepal, May 2004.

Iwu, M.W., Duncan A.R. and Okunji, C.O. 1999. New antimicrobials of plant origin In: Janick, J. (Ed), Perspectives on New Crops and New Uses. ASHS Press, Alexandria, VA. 457-462p.

Janovska D., Kuvikoba, K. and Kokoska, L. 2003. *Screening for antimicrobial activity of some medicinal plants species of traditional Chinese medicine*. Czech J. Food Sci., 21: 107–110.

Karn, A.K. 2003. *Relationship Between contribution to Local Livelihood and Antibacterial Activities of Selected Medicinal Plants: a case study from Makawanpur District, Nepal*, A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Forestry. Tribhuvan University, Institute of Forestry, Pokhara, Nepal.

Kunwar, R.M. 2006. Non timber forest product of Nepal: *A sustainable management approach.* Centre for Biological Conservation International Tropical Timber Organization Nepal –Japan, 423p.

Master Plan for Forestry Sector Nepal, 1988. *Master Plan for Forestry Sector Nepal.* Master Plan for the Forestry Sector Project: HMGN/ ADB/ FINNIDA.

Miles, R.S. and Amyes, S.G.B. 1999. Machie and Mc cartney *Practical medicinal microbiology,* International student edition 4th ed. Churchill Livingstone, London. 151-177p.

Nepal, R.C. 2006. "Status, Use and Management of *Jimbu* (*Allium* spp.): A Case Study from Upper Mustang, Nepal". A master dissertation submitted to the Norwegian University of Life Sciences.

Nautiyal, M.C., Nautiyal, B.P. and Prakash, V. 2004. Effect of Grazing and Climatic Changes on Alpine Vegetation of Tungnath, Garhwal Himalaya, India. The Environmentalist. 24: 125–134.

Nostro, A., Germano, M. P., D' Angelo, V., Marino, A., and Cannatelli, M. A., 2000. Extration methods and bioautographyt for evaluation of medicinal plant antimicrobial activity. *Letter in Applied Microbiology*, Vol. 30, 379-84: The Society for Applied Microbiology.

NSCFP/SDC, 2001. *Participatory inventory guidelines for non timber forest product* Nepal Swiss Community forestry project, Kathmandu Nepal.

Pepeljnjak, S., Stanik, G. and Potochi, P. 1999. *Antimaicrobial Activity of the Ethanolic Extract of Saturej Montana spp. Acta Pharmaceutica,* 49 (1): 65-69.

Phoboo S., Devkota, A. and Jha, P.K. 2008. "Medicinal Plants in Nepal- an overview". *Medicinal Plants in Nepal, an anthology of contemporary research.* Ecological Society, Kathmandu, Nepal. 1-24p.

Pokharel, N.R. 2000. *Screening and Evaluation of Antibacterial Activity of some Medicinal Plants of Nepal and Isolation of Pure Antibacterial compound from Bauhinia variegate linn*, M. Sc. Theses, Central Dept. of Microbiology, and T.U. Nepal.

Rahman, A., Shahwar, D., Parveen, Z., Choudhary, M.I., Sener, B., Toker, G. and Baseer, K.H.C. 1998. *New Alkaloids from Bangardia chrysogonum*, Natural product letters 12 (3): 161-173.

Retrospective Report 2002/02 to 2006/07, 2007. National Trust for Nature Conservation, ACAP.

Sasidharas, V.K. 1997. *Search for Antimicrobial and Antifungal Activity of some plants of Kerala.* Acta. Pharmaceutical 47(1): 47-52.

Shanmugavalli, N., Umashankar, V. and Raheem 2009. Antimicrobial activity of *Vanilla*. Indian Journal of Science and Technology 2(3): 37-40p (ISSN: 0974- 6846).

Shrestha, G.L. and Shrestha, B. 1999. An overview of cultivated plants in Nepal; Proceedings of National Conference, the Green Energy Mission/Nepal, Kathmandu.

Shrestha, U.B. and Shrestha, S. 2004. Major Non-Timber Forest Products of Nepal. Bhundipuran Parkashan, Kathmandu , Nepal. 411p.

Sofowara, A. 1982. *Medicinal plants and traditional medicine in Africa.* Wiley(Chichester, West Sussex, New York).

World Health Organisation, 1991. *Basic Laboratory Procedure in Chinical Bacteriology.* World Health Organization, Geneva.

ANNEXES

Annex-A: Questionnaire Survey

Name of respondent: - ……..
Age: …… Cast: ……. Sex: (M\F)
Occupation: …….. Education: ……..
Address of the respondent:
VDC…….. Ward No……. Village……. District: …….

1. What types of medicinal plants are found here?

Spp. name	**Parts used**	**For what Purpose?**	**How? (Procedure)**

2. Do you have any knowledge about *D. hatagirea* (Panch aunle)?
   Yes No
   If yes,
3. Where is it found?
   ……
4. Do you use it?
   Yes…… No…….
5. If yes, which part …… And for what purpose……...
6. Which month does it germinate?
   ……
7. Which month does it sprout?
   …….
8. Which month does the flowering and fruiting occur?
   …….
9. What are the means of propagation of *D. hatagirea?*
   a) Seed b) Rhizome c) Vegetative parts d) others
10. When did you harvest it?
    ……
11. What is the rotation age of *D. hatagirea* for collection?
    ……
12. What types of tool are used for harvesting?
    ……..
13. What are the general uses of *D. hatagirea* in your locality except medicinal purpose?
    ……
14. Did you do any processing?
    Yes……. No……..

15. If yes, what did you do?
……
16. Do you know any policy, regulation and legislations related to *D. hatagirea*?
…….
17. The Gov of Nepal has banned to collect, process and trade the rhizome of *D. hatagirea*. Are you happy with this?
Yes No
If yes, why? ……...
If no, why? ……

18. In your experience and opinion; what would be the relatively availability of the *D.hatagirea* in the forest areas as compare to before and after ban.
a) Highly decreasing
b) Decreasing
c) Constant
d) Increasing
e) Highly Increasing
19. Do you know if there is illegal collection of *D. hatagirea* by brokers?
Yes No
If yes,
a) Are villagers support them?
b) Are villagers getting some rewards?
c) Are government and other authorities letting them free?

20. In your opinion what are the factors, those causing dwindling of *D. hatagirea*?
……
21. Do you think that conservation of *D. hatageria* is necessary?
Yes No

24. What type of activities is being performed to conserve *D. hatageria?*
a. Any NGO or INGO organized meeting to aware the conservation.
b. The community formed a team to conserve it.
c. The area is banned for grazing.
d. Any operation such as weeding to preserve it.

25. What are the problems for conservation of D. hategaria?
……

26. Your last words for conserving this orchid.
……

Annex-B: Inventory Sheet for Medicinal Herbs.

Inventory sheet. Date:- GPS record: Lat:- Altitude:- Researcher:-			Plot no.:- Long:- Forest name:- Assistant:-		
S. N	Species name	No. of plants	Mean ht.(cm)	Mean Dai(cm)	Comments

Annex-C: Some snaps of field and lab works.

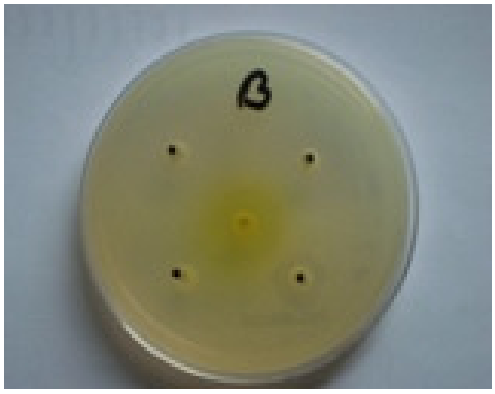
Researcher conducting questionnaire survey with local healers.

Measurement of *Dactylorhiza hatagirea* in the study area.

Laboratory work for antibacterial assay of *D. hatagirea*.

Herbarium plate of *Dactylorhiza hatagirea*

ZOI of standards against *S. aureus*



ZOI of standards against *E. coli*

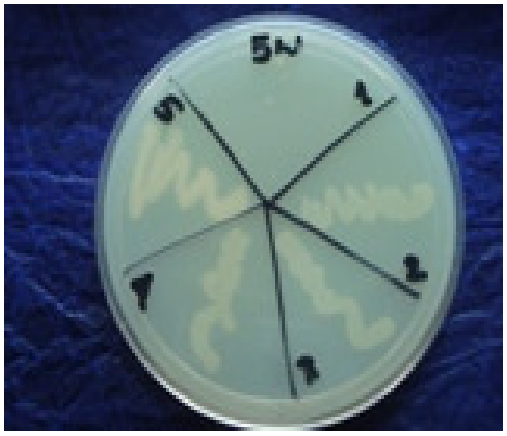
ZOI of standards against *Sh. flexinerai*

ZOI of standards against *P. aureginosa*

ZOI of standards against *B. subtilis*

ZOI of rhizome of *Dactylorhiza hatagirea* against *Sh. flexinerai*

ZOI of aerial part of *Dactylorhiza hatagirea* against *E. coli.*



Determination of MIC of aqueous extract of rhizome of *D. hatagirea* against *Sh. flexinerai,* 1, …… 5 two fold serial dilution of plant extract

Determination of MIC of chloroform extract of aerial part of *D. hatagirea* against *Sh. flexinerai,* 1, …… 5 two fold serial dilution of plant extract

Annex-D: Formula of Sterile Media

Formula of Nutrient Agar

Ingredients	Gram per liter
Beef extract	3.0
Peptone	5.0
Sodium chloride	8.0
Agar	15.0

Final pH (at 25^{o}C): 7.3 ± 0.2

Formula for Nutrient Broth

Ingredients	Gram per liter
Beef extract	10.0
Peptic digest of animal tissue	10.0
Sodium chloride	50.0

Final pH (at 25^{o}C): 7.4 ± 0.2

Formula for Muller-Hilton Agar

Ingredients	Gram per liter
Beef extract	300.0
Casein acid hydrolyzate	17.0
Starch	1.5
Agar	17.0

Final pH (at 25^{o}C): 7.4 ± 0.2